United States Department of Agriculture
Agricultural Marketing Service | National Organic Program
Document Cover Sheet
https://www.ams.usda.gov/rules-regulations/organic/petitioned-substances

Document Type:

☒ **National List Petition or Petition Update**

A petition is a request to amend the USDA National Organic Program's National List of Allowed and Prohibited Substances (National List).

Any person may submit a petition to have a substance evaluated by the National Organic Standards Board (7 CFR 205.607(a)).

Guidelines for submitting a petition are available in the NOP Handbook as NOP 3011, National List Petition Guidelines.

Petitions are posted for the public on the NOP website for Petitioned Substances.

☐ **Technical Report**

A technical report is developed in response to a petition to amend the National List. Reports are also developed to assist in the review of substances that are already on the National List.

Technical reports are completed by third-party contractors and are available to the public on the NOP website for Petitioned Substances.

Contractor names and dates completed are available in the report.

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December 23rd, 2024

National List Manager

USDA/AMS/NOP, Standards Division

Attention: National List Manager

1400 Independence Ave, SW

Room 2642-So., Ag Stop 0268

Washington, DC 20250-0268

Dear National List Manager,

We, Enartis USA Inc., are submitting this petition to request the inclusion of Poly-D-Glucosamine derived from *Aspergillus niger* and more commonly known as chitosan, on the National List under Section 205.605 (b) as a nonagricultural (nonorganic) substance permitted in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s))."

Chitosan, derived from *A. niger*, plays a pivotal role in modern organic winemaking by improving product stability and safety without the use of synthetic chemical additions. The inclusion of chitosan on the National List supports the principles of organic agriculture, which aim to promote ecological balance, enhance soil and plant quality, and minimize the use of synthetic substances. By emphasizing chitosan's role in reducing reliance on synthetic chemicals and its biodegradability and non-toxicity, the petition aligns with the broader goals of the OFPA to foster environmentally friendly farming and handling practices.

To the best of my knowledge this petition is complete, accurate, and meets the requirements as laid out in NOP 3011 Procedure: National List Petition Guidelines, and CFR § 205.600 Evaluation criteria for allowed and prohibited substances, methods, and ingredients.

Thank you for taking the time to review this petition, and please contact me if you have any questions.

Best Regards,

Enartis USA Inc.

Nick Sherry

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(707) 508-6786

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ITEM A

Item A.1 – National List Section: Section 205.605 (b): Nonagricultural (nonorganic) substances allowed in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group(s))".

Item A.2 – OFPA Category: N/A

Item A.3 – Inert Ingredients: N/A

ITEM B

Item B.1 – Substance Name: Poly-D-Glucosamine derived from *Aspergillus niger*. More commonly known as chitosan.

Item B.2 – Petitioner and Manufacturer Information:

USA: Nick Sherry
Enartis USA Inc.
7795 Bell Road,
Windsor, CA 95492

Italy: Enartis Srl.
Via San Cassiano 99,
28069 San Martino NO
Italy

Item B.3 – Intended or Current Use:

"Numerous industries have taken interest in chitosan over the last few decades because of the ability to fine-tune its physicochemical properties for specific purposes (Bellich et al. 2016). Chitosan is a versatile and promising material for developing bioplastics (including films), healthcare products, food additives, pesticides, fruit coatings, seed treatments, wastewater treatments, and other products. The substance's versatility is due to its properties as a structural polymer as well as its ability to form cations, to chelate, and to be chemically modified in a number of ways. These properties, combined with its biocompatibility, biodegradability, and antimicrobial effect, has made it an attractive molecule for product development. Bellich et al. (2016) notes that more than 1,100 papers were published about chitosan in the 1980s, 5,700

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in the 1990s, and more than 23,000 in the 2000s." – National Organic Standards Board (NOSB) Chitosan Technical Report (2020)

Chitosan is a positively charged copolymer composed of two different chemical subunits that repeat in particular order: glucosamine and N-acetyl-glucosamine. It is derived from chitin which is structurally similar to cellulose. It is the second most abundant polysaccharide in nature, present in fungus, crustaceans, and other life forms.

In wine production, chitosan from *A. niger* is a processing aid used to improve clarification and filterability, to prevent and treat volatile off aromas (such as those caused by the spoilage yeast *Brettanomyces bruxellensis)* and to improve microbial stability in wine by decreasing the microbial load. Chitosan is positively charged and acts on the negatively charged cell walls of a wide spectrum of microorganisms (*Brettanomyces, Zygosaccharomyces, Acetobacter, Oenoccus, Pediococcus, and Lactobacillus)*, inhibiting cell growth and leading to cell death, followed by precipitation. Furthermore, its affinity to metal cations facilitates removal of prooxidant metals from the wine while also destabilizing the structure of spoilage microbe cell walls by removing structural cations.

Usage regulations:

- Chitosan is permitted by the TTB as an authorized 'wine treating material'.
- The amount used must not exceed 500 g/hL according to the Alcohol and Tobacco Tax and Trade Bureau (TTB) and the European Union (EU) regulations 2019/934.
- Chitosan is recognized by the Food & Drug Administration (FDA) as 'Generally Recognized as Safe' (GRAS): Agency Response Letter GRN 000397
- The specifications for enological chitosan are described in detail by the International Oenological Codex, compiled by the International Organisation of Vine and Wine (OIV).
- Recognized as 'Bio' by The International Organization of Vine and Wine and the European Union, the use of fungal chitosan is permitted for organic wine elaboration (Regulation EU 1584/2018, amending the EU 889/2008 Annexes)
- Chitosan is already permitted for use in section § 205.601(m) of the National List regulations, albeit as an inert ingredient that is not specifically mentioned by name.

EnartisStab MICRO M is a preparation of pre-activated chitosan from *Aspergillus niger* and yeast hulls. It was designed for the treatment of grapes, juice, or must as an allergen-free, vegan alternative to lysozyme and SO2. Chitosan acts quickly and settles out rapidly, facilitating easy removal without leaving residues that might require additional processing or affect the wine's clarity and quality.

Additionally, chitosan is a very effective fining agent that, by itself, or combined with other clarification agents (such as pea protein, silica, bentonite), can improve the clarity and sensory characteristics of juice and wine.

In summary, chitosan:

Enhances Clarification and Filterability: Improves the clarity and filtration efficiency of wine.

Combats Volatile Off-Aromas: Effective against spoilage organisms like Brettanomyces bruxellensis, which are known for producing undesirable flavors in wine.

Boosts Microbial Stability: Reduces the overall microbial load, thereby enhancing the stability and safety of the wine.

Item B.4 – Intended Activities and Application Rate

Chitosan can help replace or reduce the use of SO_2. It has antimicrobial, antioxidant, and antioxidasic activity, and unlike SO_2, its efficacy is not pH dependent. Using EnartisStab MICRO M (chitosan product produced by Enartis Srl) is a very effective way of preventing microbial contamination and oxidation, while keeping SO_2 levels low.While EnartisStab MICRO M is approved for eliminating spoilage microorganisms, it has many beneficial side activities as well. It exhibits robust antimicrobial, antioxidant, and antioxidasic properties, which are essential for:

- **Preventing microbial contamination** and **oxidation**, thereby maintaining lower levels of SO_2.
- **Eliminating spoilage microorganisms** such as *Brettanomyces bruxellensis*, known for causing off-flavors.
- **Removing oxidative precursors** like catechins and inhibiting enzymes such as laccase that contribute to wine spoilage.
- **Chelating pro-oxidant metals** like copper and iron, thus reducing the potential for oxidation.

Chitosan is among one of the most prominent materials investigated in the search for alternatives to sulfur dioxide (SO_2) in winemaking, which have included the use of chemical substances (sorbic acid, ascorbic acid, lysozyme, dimethyl dicarbonate etc.), physical processes (microfiltration, ultraviolet radiation, electrical pulses etc), and biological strategies, such as non-*saccharomyces* yeast strains which inhibit the growth of spoilage yeast and bacteria.

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Used in conjunction with other enological products - such as technical tannins - that are permitted for wine "made with organic grapes", chitosan could serve to reduce, and potentially, eliminate the necessity for SO_2 in organic winemaking.

"Pre-activated chitosan derived from *Aspergillus niger* can be an alternative to SO2 during all stages of the winemaking process due to its antioxidant, antioxidasic, and antimicrobial effect. It has demonstrated strong results in controlling a wide spectrum of spoilage microorganisms such as *Botrytis cinerea, Acetobacter, Lactobacillus, Pediococcus* even at high pH (pH 3.9). Furthermore, it can limit oxidation reactions by chelating metals such as copper and iron, which are catalysts of enzymatic and non-enzymatic oxidation reactions. This is useful as climate chaos and organic farming necessitate increased copper use in vineyards." (Allen et al., 2023)

Please see Appendix A for a scientific poster titled "Mitigating the Effects of Climate Change on Wine Production Using Activated Chitosan and Technical Tannin". This scientific poster highlights the role of chitosan in addressing climate-related challenges such as increased wine pH and oxidative spoilage.

Application rates vary and depend on the stage of winemaking and reason for using the product.

Application Rates:

Must Treatment: 5 - 10 g/hL

Chitosan reduces microbial contamination in must by binding to negatively charged cell walls of spoilage organisms, such as *Lactobacillus* and *Acetobacter*, causing cell death. This process stabilizes the must early, minimizing the risk of fermentation disruptions.

Wine Treatment: 5 - 20 g/hL

In wine, chitosan continues to decrease microbial loads by targeting spoilage bacteria and yeast like *Brettanomyces*. Its action ensures microbial stability during aging, reducing the need for sulfur dioxide.

Brettanomyces bruxellensis Control: 5 - 12 g/hL

Chitosan disrupts the cell walls of *Brettanomyces bruxellensis* by chelating essential structural cations, leading to cell death. This effectively mitigates spoilage aromas without affecting the sensory profile of the wine

Fermentation Issues: 5 - 10 g/hL

Chitosan absorbs inhibitory compounds that are toxic to yeast while reducing populations of spoilage microbes like lactic acid bacteria. This creates a cleaner environment, helping to resolve sluggish or stuck fermentations organically.

Metal Reduction: 10 - 50 g/hL

Chitosan's chelating properties bind and precipitate pro-oxidant metals such as copper and iron, which catalyze oxidation reactions. This reduces oxidative spoilage risks and maintains wine freshness.

Ochratoxin A Reduction: 20 - 100 g/hL

Chitosan binds and removes ochratoxin A, a harmful mycotoxin produced by certain molds, during wine clarification processes. This ensures compliance with safety standards while preserving wine quality.

These application rates are adaptable based on wine pH, turbidity, microbial load, and production goals, ensuring versatility and alignment with organic winemaking standards.

For a "No SO2 Winemaking Protocol", please see Appendix B. This document details step-by-step guidelines for reducing or eliminating sulfur dioxide in wine production using chitosan-based practices.

Item B.5 – Manufacturing Process

Chitosan Production from Crustaceans

The production of chitosan from crustacean sources has environmental concerns due to the harsh chemical processes involved. According to the NOP Guidance 5033-1, the conversion of chitin to chitosan is classified as synthetic due to these processes.

"Following NOP Guidance 5033-1 Guidance Decision Tree for Classification of Materials as Synthetic or Nonsynthetic (NOP, 2016) leads to a determination that the chitosan is synthetic." Chitosan Technical Report NOSB, 2020

It should be noted that chitosan derived from crustaceans is not permitted in winemaking.

Advancements in Chitosan Production

Recognizing the need for more sustainable practices, recent research has focused on developing greener methods for extracting chitosan. These methods aim to reduce the environmental impact traditionally associated with chitosan production (Crognale et al. 2022; Huq et al. 2022; Pellis et al. 2022).

Fungal-Derived Chitosan

Enological chitosan, however, is derived from *Aspergillus niger* which offers several advantages over traditional crustacean-derived chitosan. This fungal method avoids the need for demineralization and decolorization—steps that are intensive in chemical use and are required for approximately 70-80% of crustacean-based extractions.

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The fungal approach allows for the production of chitosan with varied properties, adaptable to specific applications: "Among the advantages of the fungal approach, there is the possibility of obtaining chitosan's with different properties by varying species and culture conditions. For instance, the chitosan derived from shellfish wastes has a high molecular mass (around 1.5×10^6 Da), while the MW of chitosan from fungal sources widely ranges from 6.4×10^3 to 1.4×10^6 Da. High MW chitosans are sparingly soluble in neutral pH aqueous solutions and yield high viscous solutions that limit their exploitation in the food, health, and agricultural sectors. Fungal-derived chitosan with medium-low MW can be used as hypocholesterolemic agents in healthcare products and as a thread or membrane in a variety of biomedical applications. Moreover, chitosan extraction from fungal sources is more environmentally benign than that from shellfish wastes since the latter source requires highly concentrated acid and alkaline solutions for demineralization and chemical deacetylation that have to be disposed of. Another advantage associated with fungal chitosan encompasses the absence of allergenic proteins, such as tropomyosin. Chitin and chitosan extraction from fungi can lower disposal costs of fungal-based waste materials in association with the production of value-added products, which may offer a lucrative opportunity to the biotechnological industries." (Crognale et al., 2022)

Enartis Srl. sources chitosan from *Aspergillus niger*, to meet the specifications set by the OIV Codex on Chitosan. Only chitosan with a purity equal to 95% or higher may be used. Chitosan comes as a white, odorless, and flavorless powder, that is almost completely insoluble in aqueous or organic medium (OIV, 2022).

Fungal-Derived Chitosan Manufacturing Process:

1. **Cultivation of *Aspergillus niger*:** The fungus is grown in controlled fermentation conditions, utilizing substrates approved for organic production.

2. **Chitin Extraction:** Chitin is extracted from the fungal cell walls through a gentle process using minimal chemical inputs. Unlike crustacean-derived chitosan, this does not require harsh demineralization or deproteinization steps.

3. **Deacetylation:** The extracted chitin undergoes partial deacetylation, converting it into chitosan. This step uses controlled pH adjustments with biodegradable reagents.

4. **Purification:** The resulting chitosan is washed and filtered to achieve a purity level of 95% or higher, as required by the International Organisation of Vine and Wine (OIV).

5. **Drying and Packaging:** Chitosan is dried, milled into a fine powder, and packaged in compliance with food-grade and organic production standards.

Advantages of Fungal Derived Chitosan

Variability in Molecular Weight: Fungal chitosan offers a range of molecular weights (from 6.4 × 10^3 to 1.4 × 10^6 Da), which enhances its solubility and functionality across different applications.

Environmental Benefits: The fungal extraction process is less reliant on harmful chemicals, reducing environmental impact compared to crustacean-derived chitosan.

Absence of Allergenic Proteins: Unlike crustacean-derived chitosan, fungal chitosan does not contain allergenic proteins such as tropomyosin, making it safer for use in various industries.

Cost-Effectiveness and Waste Reduction: Utilizing fungal sources can reduce the disposal costs associated with waste materials and supports the production of value-added products, presenting opportunities for biotechnological innovations.

Item B.6 Ancillary Substances

Inactivated Yeast – Yeast is a non-agricultural substance on the National List, §205.605(a), and is used to maximize chitosan's effect in turbid wines and must by preventing interactions with suspended solids. It is derived from yeast fermentation processes.

Ascorbic Acid (E 300) – Added to the National List, §205.605(b), effective April 21, 2001. Ascorbic acid is an organic acid produced via fermentation of glucose using bacteria such as *Ketoacidophilus.* Used as a preservative (antioxidant) and as a component of the pre-activation process for active chitosan products.

Lactic Acid (E270) – An organic acid produced through bacterial fermentation of carbohydrates that is Included in the National List §205.605(b). It enhances product stability and microbial control.

These ancillary substances improve the functionality of chitosan without compromising its compliance with organic production standards.

Item B.7 Previous Reviews

Since the publication of the Chitosan Technical Report (2020) there have been no additional reviews by state, private certification programs, or other organizations, that the author could find.

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Item B.8 Regulatory Authority

The Food & Drug Administration (FDA):

In response to GRN No. 937 Chitosan from *Aspergillus niger*, filed August 8, 2011, the FDA had no questions for the petitioner regarding their conclusion that chitosan is GRAS for its intended use as a **secondary direct food ingredient** in alcoholic beverage production at levels 10 and 500 grams per hectoliter (100 L).

21 CFR 173: A secondary direct food additive has a technical effect in food during processing but not in the finished food (e.g., processing aid). The technical use of chitosan in the FDA's GRAS Notice GRN 000397 is for microbiological stabilization, removal of contaminants, and/or clarification of the alcoholic beverage.

GRN No, 997 'Chitosan and *beta*-1,3- glucans from white button mushrooms (*Agaricus bisporus*), submitted by Chinova Bioworks inc, was closed February 28, 2022 with no questions from the FDA, although it was clearly stated that all manufacturers and food producers be compliant with all applicable legal and regulatory requirements (FDA, 2022)

The Alcohol and Tobacco Tax and Trade Bureau (TTB):

The TBB recognizes chitosan as a 'material authorized for the treatment of wine and juice', as specified under the CFR §24.246.

Chitosan from *Aspergillus niger:* To remove spoilage organisms such as *Brettanomyces* from wine	The amount used must not exceed 0.04 pounds per 1 gallon (500 g/100 L) of wine	GRAS Notice No. GRN 000397.

Source: eCFR :: 27 CFR 24.246 -- Materials authorized for the treatment of wine and juice.

On 08/22/2022 following a rulemaking and comment period, the TTB amended the maximum addition rate of chitosan in winemaking to 500 g/hL, from the 100 g/hL that was initially allowed for removing spoilage microorganisms (Federal Register, 2022).

The TTB has also made a preliminary conclusion to allow chitosan to remove off flavors from wine, and it is currently listed as a 'wine and juice treating material that has been administratively approved for continual use'.

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Chitosan: To remove spoilage organisms such as Brettanomyces from wine, and for clarification, fining, and removing off flavors from wine and juice.	The Chitosan must be derived from Aspergillus niger. The amount used must not exceed 500 grams per 100 liters of wine. GRAS Notice No. GRN 000397.	TTB regulations authorize the use of chitosan to remove spoilage organisms such as Brettanomyces at a rate not to exceed 0.04 pounds per 1 gallon (500 g/100 L) of wine. TTB has also made the preliminary conclusion, as of March 17, 2022, of allowing the use of chitosan to remove off flavors.

Source: TTBGov - Treating Materials

Organic Foods Production Act. USDA Final Rule:

Under the USDA's Organic Foods Production Act, chitosan is indirectly included through its listing on the EPA's List 4 (2004), which is referenced in sections §205.601(m) and §205.603(e) of the National List. This inclusion supports the use of chitosan as a pesticide and processing aid, conforming to organic production standards.

United States Environmental Protection Agency (EPA):

On November 8, 2022, the EPA recognized the safety and efficacy of chitosan by adding chitosan (also known by its chemical name: poly-D-glucosamine) (CAS No. 9012–76–4) to the list of active ingredients eligible for use in minimum risk pesticide products **exempt** from registration and other requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). In doing so, the EPA is specifying that the listing also includes those chitosan salts that can be formed when chitosan is mixed with the acids that are listed as active or inert ingredients eligible for use in minimum risk pesticide products.

"The purpose of the exemption list is to eliminate the need for the Agency to expend significant resources to regulate products deemed to be of minimum risk to human health and the environment." (Federal Register, 2022)

Prior to that, the EPA had released, on May 6, 2022, data on the aquatic toxicity of chitosan salts, allowing for public comment before the final rulemaking (EPA, April 21, 2023).

This final ruling went into effect on January 9th, 2023 (Federal Register, 2022)

Chitosan is on the EPA's Safer Chemicals Ingredients List with a green circle indicating that *"The chemical has been verified to be of low concern based on experimental and modeled data."* (EPA, 2023)

EPA Tolerance Exemption:

CFR 40 180.1072 exempts chitosan from the requirement of a tolerance limit for residues when used as a seed treatment in or on barley, beans oats, peas, rice, and wheat, and when used as a pesticide in the production of any raw agricultural activity.

CFR 40 180.950 states that: "residues resulting from the use of the following substances as either an inert or an active ingredient in a pesticide chemical formulation, including antimicrobial pesticide chemicals, are exempted from the requirement of a tolerance under FFDCA section 408, if such use is in accordance with good agricultural or manufacturing practices."

EU & OIV:

The International Organization of Vine and Wine (OIV/OENO 336A/2009, 337A/2009, 338A/2009) and the European Union (Regulation EU 53/2011) authorized the use of fungal chitosan including for organic wine elaboration (Regulation EU 1584/2018, amending the EU 889/2008 Annexes) (EUR-Lex, 2023)

Item B.9 Chemical Abstracts Service (CAS) Number and Product Labels

Chemical Abstracts Services Registry Number: *Chitosan CA No. 9012-76-4*

Product Label:

Please refer to Appendix C

Item B.10 Physical and Chemical Properties

Overview of Chitosan: Chitosan is a versatile biopolymer obtained by deacetylating chitin, which is found in crustacean shells and fungal cell structures, such as those of *Aspergillus niger* and *Agaricus bisporus*. This process transforms the naturally occurring chitin, composed of N-acetyl-D-glucosamine units, into chitosan—a polymer primarily made up of β-(1-4)-linked D-glucosamine units.

Physicochemical Attributes: The distinct physicochemical properties of chitosan, particularly its molecular weight (MW) and degree of acetylation, are critical to its functionality. These characteristics influence its solubility, viscosity, and bioactivity, making chitosan a highly adaptable material for various applications, including winemaking.

Mechanistic Insights and Research Progress: While the exact mechanisms by which chitosan exerts its beneficial properties are still under investigation, several theories suggest its interaction with microbial cell walls and metal ions as key factors. Ongoing research continues to uncover new benefits of chitosan in winemaking, enhancing our understanding and application of this biopolymer. Recent studies, such as those by Medeiros et al. (2023), highlight its potential in improving the stability and quality of wine.

- Chelating activity enables chitosan to bind with metal ions, which can be crucial in removing unwanted metallic tastes and stabilizing wine.

- Adsorption capacity and film-forming ability contribute to chitosan's effectiveness in clarifying wines and improving their filtration.

- Antimicrobial activities provide natural preservation by inhibiting the growth of spoilage organisms in wine.

The physical and chemical properties of the chitosan Enartis uses in winemaking are specified by the OIV (OIV, 2009). Table 1. provides the specifications available on the 'Certificate of Analysis' (CoA) for EnartisStab Micro M, Enartis USA's most popular chitosan-based product.

Parameter	Unit	Specification	Type
Appearance	na	Microgranules	Actual Value
Colour of product	na	Beige	Actual Value
pH 5% solution (20 °C)	na	3,0 – 5,0	Actual Value
Dry Matter	%	>/= 94,0	Actual Value
Loss on drying	%	</= 6,0	Typical Value
Insolubles in H20	%	56,00 - 75,00	Typical Value
Medium size	µ	</= 250	Typical Value
Heavy Metal (Pb)	mg/kg	</= 10	Typical Value

Table 1. Values shown on a typical EnartisStab MICRO M 'Certificate of Analyses'.

Unique Properties and Applications of Chitosan: Chitosan stands out within the polysaccharide family due to its distinctive and beneficial properties, which have led to its wide adoption across various industries, including winemaking. These properties include:

- Biocompatibility and biodegradability ensure that chitosan is safe for use in food and beverage applications, aligning with environmental sustainability goals.

- Non-toxicity and non-allergenicity makes chitosan a safe choice for consumers, particularly important in products intended for human consumption.

- Chelating activity enables chitosan to bind with metal ions, which can be crucial in removing unwanted metallic tastes and stabilizing wine.

- Adsorption capacity and film-forming ability contribute to chitosan's effectiveness in clarifying wines and improving their filtration.

- Antimicrobial activities provide natural preservation by inhibiting the growth of spoilage organisms in wine.

a) Chemical Interactions with other substances, especially substances used in organic production.

Chitosan's interaction with other approved substances used in organic winemaking has shown no adverse effects. In fact, EnartisStab Micro M, a chitosan-based product formulated with inactive yeast and organic acids, enhances its stabilizing and clarifying actions through synergistic interactions between the components.

Additionally, synergy with other substances, such as sulfur dioxide and various fining agents, typically results in a reduction in the quantities required of these additives. This not only preserves the natural quality of the wine but also aligns with the principles of organic winemaking by minimizing chemical inputs.

(b) Toxicity and environmental persistence

The Formal Recommendation from the National Organic Standards Board (NOSB) to the National Organic Program (NOP) stated that disposing of, or misusing chitosan, is not expected to cause concerning levels of environmental contamination, due to its low toxicity (USDA, 2021).

c) Environmental impacts from its use and/or manufacture

Commercial chitosan production is still limited to use of harsh alkaline treatment conditions, although other more environmentally friendly processes have been demonstrated using hot water, mechanochemical, and glycerol treatments for crustacean-based chitosan (Huq et al, 2022). No research was found using these novel methods for fungal chitosan.

Production of fungal chitosan is more environmentally friendly because the production process is simpler and does not require the demineralization and decolorization steps needed for crustacean sources, therefore lowering the amounts of harsh chemicals used and reducing the amount of waste effluent produced. However, to date, there are no greener solutions available to commercial chitosan producers, although current research shows a commitment to producing chitosan more sustainably (Pellis et al, 2022; Crognale et al, 2022; Huq et al, 2022).

A recent bibliometric analysis focusing on the use of chitosan for sustainable development uncovers an upward trend in the use of chitosan for this purpose, and highlights chitosan's use

as an environmentally friendly absorbent, and as a flocculant for water treatment (Lam et al, 2023).

d) Effects on human health

A scientific opinion on the list of health claims associated with taking chitosan supplements and associated 'weight management' benefits established there was no cause and effect related to the claim (EFSA, 2011). A final rule by the United States Environmental Protection Agency (Federal Register, 2022) suggests that a lack of reports relating to chitosan and adverse human health effects, implies that there are minimal risks to humans and the environment. Chitosan is classified in Toxicity Category IV for acute oral toxicity, acute dermal toxicity, acute eye irritation, and acute dermal irritation, or practically non-toxic.

The National Toxicology Program published a report on the toxicity of chitosan administered in feed to Sprague Dawley rats concluding that chitosan was nearly non-toxic to animals and humans (National Toxicology Program, 2017).

The NOSB Technical Report summarizes the available information on the effects upon human health and concludes that there are no known negative effects on humans from chitosan use.

e) Effect on soil organisms, crops, or livestock.

Since the NOSB Technical Report (2020), the EPA added chitosan to its list of active ingredients eligible for use in minimum risk pesticide products **exempt** from registration and other requirements of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) in 2022.

A recent review summarizes the applications of chitosan, and its derivatives, as an elicitor, a plant protector, and a biostimulator which can lead to improved plant growth, stress tolerance, and overall plant quality, as well as inducing the plants defenses, protecting against pests and pathogens, and increasing production of secondary metabolites (Stasińska-Jakubas & Hawrylak-Nowak, 2022).

According to a recent review on the application of low molecular weight chitosan in animal nutrition, husbandry, and health, there is: "…. sufficient research to demonstrate its usefulness in animal nutrition and health, such as feed additives, wound healing, bone regeneration, analgesic, and antimicrobial effect. Low molecular weight chitosan is a potential alternative antibiotic in livestock production to address the rising global cost of veterinary drugs. However, there a need for more literature on its utilization, especially in livestock production. Hence, more in-depth studies should be carried out." (Boamah et al, 2023)

Studies on the impact of chitosan on the mechanical stability, water stability, and wettability of soils, continue to demonstrate that the effect on soils is not detrimental to the soil environment, with positive benefits being demonstrated with the addition of chitosan (Adamczuk et al, 2021; Adamczuk & Jozefaciuk, 2023)

Item B.11 Safety Information

To our knowledge, a specific NIEHS substance report on chitosan does not exist. However, chitosan's safety is well-documented in peer-reviewed studies and regulatory approvals, including:

- The National Toxicology Program's report on the low toxicity of chitosan when administered to Sprague Dawley rats.
- EPA's classification of chitosan in Toxicity Category IV (practically non-toxic).

Please refer to Appendix D for the Safety Data Sheet (SDS) of EnartisStab Micro M and Appendix E for the National Toxicology Program's report on the toxicity of chitosan (CASRN 9012-76-4) administered in feed to Sprague Dawley rats. These findings confirm that chitosan is safe for human and environmental health, aligning with organic standards.

Item B.12 Research Information

In researching this petition to have chitosan included on the National List, the focus was on searching for research and publications dated since the publication NOSB Chitosan Technical Report (2020). Research papers used are all specific to the requirements of the petition.

Since the NOSB Chitosan Technical Report (2020), extensive research has underscored the multifaceted applications and benefits of chitosan, particularly fungal-derived, in alignment with organic agricultural practices. Our petition is supported by recent studies that focus on addressing the challenges in chitosan production and advocate for sustainable manufacturing methods, as documented by Lam et al. (2023), Crognale et al. (2022), Huq et al. (2022), and Pellis et al. (2022).

Significant research into chitosan nanoparticles highlights its potential in developing biodegradable products for sectors including packaging (Yanat & Schroen 2021), medicine (Jha & Mayanovic 2023), and agriculture (Ingle et al. 2022), along with assessing its environmental and safety impacts (Khan et al. 2024). Reviews on the effects of chitosan on crops, soils, and in animal husbandry determine that chitosan has many beneficial

applications (Stasińska-Jakubas & Hawrylak-Nowak, 2022; Boamah et al, 2023; Adamczuk et al, 2021; Adamczuk & Jozefaciuk, 2023).

Fungal chitosan in relation to winemaking was the primary area of research in determining that chitosan should be included on the National list as a tool for winemakers to reduce the level of sulfites used in organic winemaking. Studies by Marchante et al (2020), Picariello et al (2020), Tedesco et al, (2022), Baris et al (2023), Medeiros et al, 2023, and Velasquez, C. (2023), continue to highlight chitosan's positive attributes in winemaking as a complementary product or alternative to SO_2. It should be noted that compared to the volume of research papers on chitosan in other industries, there are substantially fewer for its use in winemaking.

Research and reviews of alternative strategies to using SO_2 in winemaking were evaluated and indicates a willingness by the industry to move away from using SO_2 as the sole means of preserving wines quality and attributes (Barril et al, 2016; Frascassetti et al (2019); Yildirim, H.K (2020); Picariello et al (2020); Silva & Wyk, 2021; Canonico et al, 2023; Fia et al, 2023). Research into non-thermal technologies further demonstrate an effort to find an alternative to preserving wines qualities without the use of SO_2 (Falgera et al, 2013; Morata et al, 2014; Fracasseti et al, 2019; Silva &Wyk, 2021; Delso et al, 2023).

Regulatory and Safety information was established from the websites of the Food & Drug Administration (FDA), The Agricultural Marketing Service (AMS), The National Organic Standards Board (NOSB), United States Environmental Protection Agency (USEPA), The Federal Register, The International Organization of Vine and Wine (OIV), The Alcohol and Tobacco, Tax and Trade Bureau (TTB), and European Union (EU).

In researching the negative attributes of chitosan from *A. niger* and its potential to be used in wine "made from organic grapes", it is clear that the manufacturing process may pose a significant hurdle in justifying it's use due to the nature of the chemicals used. In a review of the sources, production, and commercial application of fungal chitosan, Huq et al. (2022) suggest that future research and development activities should focus on improving the production process to be more eco-friendly. However, no research papers or evidence could be found to present a case for not including chitosan from *A. niger* on the National List. Chitosan's status as "bio" in the European Union provides a strong case for its inclusion, as this would facilitate trade and provide US wine producers with an opportunity to compete in the global organic wine market, without sacrificing quality.

Item B.13 Petition Justification Statement

E. Inclusion of a Synthetic on the National List (7 C.F.R. §§ 205.601, 205.603, 205.605(b))

Explain why the substance is necessary for use in organic handling.

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Chitosan is proposed for use as either a complimentary tool to other materials already on the National List (e.g., tannins such as HIDEKI (TDS in Appendix F) or sulfur dioxide), or an alternative to SO_2 for use in the production of wine "made with organic grapes".

Recent studies highlight chitosan's role in enhancing wine quality without the adverse effects associated with SO2. For instance, a 2020 study found chitosan, as a total or partial substitute for SO_2, produced quality red wines without changing important considerations in the wines chemical profile, when used as a pre-fermentative addition (Marchante et al., 2020). Chitosan can be used throughout the winemaking process, in much the same way as SO_2. Picariello et al (2020) determined that post-fermentative additions of chitosan reduced the amount of SO_2 bound acetaldehyde, coupled with a higher polymeric pigment content and lower tannin reactivity with proteins. Highlights from a recent study on the oxidative browning of model white wine using two fungoid chitosan's include an 85% reduction of oxidative browning, and a noticeable impact on the wine's antioxidant behavior due to its ability to chelate iron (Baris et al, 2023).Tedesco et al, 2022 provides a review of chitosan in oenology, concluding that chitosan will continue to be used in winemaking, with the possibilities of its use widening more and more as research continues to be carried out.

"In the wine industry it is essential to reduce or even eliminate SO_2 especially in the production of organic wines." (Yildirim H.K 2020) Sulfur dioxide, though widely used for its preservative qualities, has been associated with adverse health reactions, including respiratory issues and organoleptic changes in wines. Chitosan, derived from fungal sources like Aspergillus niger, is free from common allergens found in shellfish-based chitosans and does not persist in the environment, making it a safer, more sustainable choice.

Research has shown that fungal chitosan has been found to be free of the allergenic proteins – tropomyosin, myosin light chain, and arginine kinase – which are found in shellfish (Li et al, 2012). Sulfites, on the other hand, have been named 'Allergen of the Year 2024' by the American Contact Dermatitis Society in a bid to raise awareness of this significant allergen that are commonly used in food/beverages, personal care products, and pharmaceuticals (Ekstein & Warshaw, 2024). Not known to be toxic to humans or animals, not persistent, and highly biodegradable, chitosan is less harmful to the environment than SO_2. It should be noted that the levels of SO_2 used in winemaking are not considered to be harmful to the environment, however sulfites must be declared in winemaking at levels over 10 ppm to protect the consumer, according to the FDA.

The environmental and health advantages of chitosan are significant, especially in the context of climate change, which has caused an increase in wine pH (decreased acidity) (Venios et al, 2020) and increased copper use in vineyards (Allen et, 2023). A wine with a pH of 4.0 requires up to four times the dose of SO_2 compared to a wine with a pH of 3.2 to achieve the same molecular SO_2 and therefore microbial control. Droz et al. (2021) suggest that with a predicted 10% increase in soil organic matter by 2100, copper transport in soil may decrease

therefore increasing the amount of copper accumulated in vineyard soil. An increase in the pro-oxidant metal copper in the vineyard would see a concomitant increase in wine, leading to a potential increase in both enzymatic and non-enzymatic oxidation reactions. Chitosan's ability to chelate copper provides a useful tool to counter this without increasing SO_2 additions.

A "Review of practices for the reduction of so2 doses used in winemaking" (REFERENCE OIV-OENO 63102020) identifies Points of Intervention throughout the winemaking process where SO_2 could be limited or reduced for reasons that ultimately affect wine quality. SO_2 additions prior to fermentation can bind to carbonyl compounds, such as acetaldehyde, leading to reduced color stability. During fermentation, SO_2 has a proven risk of producing volatile sulfur compounds through direct metabolization by fermentative yeast. SO_2 additions can also mute wine aromatics at high doses and are responsible for bleaching, or turning pigments colorless, when it binds to anthocyanins, as well as preventing the formation of more stable polymeric pigments. Castro & Chinnici (2020) studied the effect of chitosan on tannin removal, concluding that at the doses used chitosan's impact on wine quality was marginal.

Of the alternatives discussed in this petition below, chitosan from *Aspergillus niger* provides a wide spectrum of benefits and can be used to lower or eliminate SO_2 additions, is non-toxic, biodegradable, biocompatible, and non-allergenic. By granting this petition, the NOSB would provide organic winemakers with a proven, effective, and sustainable solution to meet modern winemaking challenges while maintaining the integrity of organic standards.

Describe nonsynthetic or synthetic substances on the National List or alternative cultural methods that could be used in place of the petitioned synthetic substance.

1. Non-Thermal Technologies

Pulsed Electric Field (PEF) technology has been shown to achieve adequate levels of microbial inactivation (3-log10) in must and wine under industrial applicable processing parameters, making it a suitable alternative to SO2 or sterilizing filtration for microbial control in winemaking. (Delso et al, 2023). An increase in color intensity of treated wines was observed while the organoleptic wine qualities remained unaltered (Yildirim, H.K, 2020).

Morata et al, (2014) found that **High Hydrostatic Pressure (HHP**) reduced wild yeast populations on *Vitis vinifera* (var. Tempranillo) significantly, although bacterial populations had a higher resistance leaving a residual load at the highest treatment (550 MPa). Other studies have determined that HHP has a strong microbial effect on mold, yeasts, acetic and lactic acid bacteria (Yidrim, H.K., 2020). The technology is not widely adopted in the wine industry to my knowledge.

Ultraviolet (UV)-vis (Falgera et al, 2013) and **UV-C** (Agnolucci et al, 2019) have both been shown to reduce the microbial populations of in wines and musts without affecting the other important considerations. Both have been proposed to reduce SO_2 use in food processing.

Low Electric Current (LEC) has been demonstrated to be effective against yeast and microorganisms in grape musts and wines, although no research beyond 2006 was found for LEC.

A critical review on **Ultrasound** in winemaking by Zhang et al (2023) determined that ultrasound can be used effectively to reduce SO_2 consumption, amongst other benefits, but that further research is needed before ultrasound can be scaled up to commercial application due to current limitations.

"Despite the encouraging results demonstrating less or no SO_2 addition to wine by using non-thermal technologies such as HPP and PEF, more research is needed to determine the extent to which the use of SO_2 can be reduced or eliminated in the production/stabilization of different types of wine." (Silva & Wyk, 2021)

While these non-thermal technologies have been shown to be effective, adoption has been slow due to cost, infrastructure and scalability issues.

2. Chemical Alternatives

Sulfur dioxide: Section 205.605 (b) permits the use of sulfur dioxide for use only in wine labeled "made with organic grapes', provided that the total sulfite concentration does not exceed 100 ppm. The TTB states that finished wine must not exceed the limitations described in 27 CFR 4.22(b)(1), which is 350 parts per million (ppm) total.

Acorbic Acid: Permitted under Section 7 CFR 205.605(b)(6) of the National List, since April 21, 2001 (USDA, AMS, 2022). The antioxidant capacity of ascorbic acid is well-known, however its use in winemaking can also produce detrimental effects, such as pro-oxidant color development, pro-oxidant action, and reductive off-aroma development (Barril et al, 2016).

Sorbic Acid: Not recommended for rulemaking by the NOSB (USDA, AMS, 2022)

Lysozyme: A protein derived from egg albumin that is active at wine pH. It is allergenic and generally not favored by winemakers due to procedures required to remove lysozyme (clarification and fining procedure). It is permitted for winemaking in the USA and EU, although it was removed from the National List on September 12, 2016 (Federal Register, 2016).

"…the use of lysozyme might lead to a reduction of SO_2, but it cannot be used for total replacement of sulfites alone, since it has only antibacterial activity, but it is not able to control

the proliferation of contaminating yeasts and gram-negative bacteria, nor oxidation phenomena in musts and wines." (Tedesco et al, 2022)

Dimethyl Dicarbonate (DMDC): Not permitted in organic winemaking due to potential toxicity concerns.

3. Biological Alternatives

Yeast (§ 205.605 (a)(30)) on the National List has the following limitations in place:

'When used as food or a fermentation agent in products labeled as "organic," yeast must be organic if its end use is for human consumption; nonorganic yeast may be used when organic yeast is not commercially available. Growth on petrochemical substrate and sulfite waste liquor is prohibited. For smoked yeast, nonsynthetic smoke flavoring process must be documented." (Federal Register)

Selected non-*saccharomyces* yeast have been demonstrated to inhibit indigenous flora on grapes:

"One of the main objectives for a sustainable winemaking process is the reduction of the use of sulfur dioxide. In this regard, non-Saccharomyces wine yeasts are proposed as biocontrol agent in different steps of wine production chain. Here, a selected strain of Metschnikowia pulcherrima (DiSVA 269) and a native Saccharomyces cerevisiae low sulfite producer strain (DiSVA 708) were investigated…The overall results indicate that the combined use of M. pulcherrima DiSVA 269 and native S. cerevisiae DiSVA 708 led a biocontrol action and an improvement of aromatic and sensorial profile of wine with low SO2 content." (Canonico et al, 2023)

Unripe Grape Extract (UGE) was used in combination with chitosan in a recent study evaluating the potential of UGE as an alternative to SO_2. Fia et al. (2023) found that microbiological stabilization was comparable to SO_2, when used at 200 mg/L in combination with chitosan. Earlier and better color stabilization was also observed and attributed to UGE. Although this method has a synergistic effect with chitosan, it may be valuable as an alternative as well. A market study presented in the study also confirmed a positive consumer response to UGE as an alternative to SO_2.

Describe any beneficial effects on the environment, or human health from the use of the substance that support its use instead of the use of non-synthetic or synthetic substances on the National List or alternative cultural methods.

Only chitosan derived from *Aspergillus niger*, with a purity equal to 95% or higher is permitted for use in oenology in the EU. It is compliant with the stringent standards set by the European

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Union and aligns with the International Organisation of Vine and Wine (OIV) specifications, ensuring its safe use in enology. Since many companies selling chitosan for enological use trade globally, this is the typical purity level on the market.

Environmental Impact

Chitosan is recognized for its minimal environmental impact compared to SO2, which is categorized under Section 313 of the Emergency Planning and Community Right-to-Know Act (EPCRA) as a toxic substance requiring careful disposal and reporting due to its potential environmental hazards. In contrast, chitosan is biodegradable, non-toxic, and does not persist in the environment, reducing the ecological footprint associated with its use.

Human Health

Unlike SO2, which is known to cause allergic reactions and other health issues such as respiratory problems and skin irritation, chitosan is non-allergenic and safe for human contact. Its use in winemaking contributes to a healthier product by avoiding the addition of potentially harmful chemicals.

Contribution to Sustainable Practices

Research has highlighted chitosan's role in promoting sustainable agricultural practices, including its ability to bind and remove heavy metals and toxins from soils and water, thereby playing a crucial role in environmental remediation. This attribute of chitosan not only improves the quality of the wine but also supports broader environmental sustainability goals.

Regulatory and Safety Compliance

The U.S. Environmental Protection Agency (EPA) has exempted chitosan from tolerance limits due to its low toxicity, underscoring its safety and environmental benefits. This exemption indicates a recognition of chitosan's benign nature and supports its inclusion as a preferred substance in organic agriculture and winemaking.

The evidence clearly supports the use of chitosan not only as a technically effective alternative but also as a means to enhance environmental and human health outcomes compared to more traditional methods. This makes it a valuable addition to the tools available for organic winemakers seeking to produce high-quality, sustainable, safe products in a healthy environment.

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Appendix

Appendix A

Mitigating-effects-of-climate-change-using-activated-chitosan-technical-tannin.pdf

This scientific poster highlights the role of chitosan in addressing climate-related challenges such as increased wine pH and oxidative spoilage.

Appendix B

Enartis_SO2_flyer_USA.pdf

This document details step-by-step guidelines for reducing or eliminating sulfur dioxide in wine production using chitosan-based practices.

MITIGATING THE EFFECTS OF CLIMATE CHANGE ON WINE PRODUCTION USING ACTIVATED CHITOSAN AND TECHNICAL TANNIN

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INTRODUCTION

Some of the biggest challenges for the wine industry result from increased temperatures and long periods of water stress due to climate change. These conditions favor accelerated ripening of fruit which leads to higher sugar content, delayed phenolic ripeness, increased pH, and decreased acidity.

This consistent rise in pH comes with a lower effectiveness of sulfur dioxide (SO_2), requiring higher doses to provide the desired microbiological and antioxidant protection. A wine with a pH of 4.0 requires up to four times the dose of SO_2 compared to a wine with a pH of 3.2 to achieve the same molecular SO_2 and therefore microbial control.

After many years of research and experience, Enartis has observed that quality, stable wine can be obtained even when reducing or removing SO_2 additions. This was achieved using various allergen-free and vegan-friendly products with the same antioxidant, antioxidasic, and antimicrobial properties as SO_2, regardless of wine pH.

Pre-activated chitosan derived from *Aspergillus niger* can be an alternative to SO_2 during all stages of the winemaking process due to its antioxidant, antioxidasic, and antimicrobial effect. It has demonstrated strong results in controlling a wide spectrum of spoilage microorganisms such as *Botrytis cinerea*, *Acetobacter, Lactobacillus, Pediococcus* even at high pH (pH 3.9). Furthermore, it can limit oxidation reactions by chelating metals such as copper and iron, which are catalysts of enzymatic and non-enzymatic oxidation reactions. This is useful as climate chaos and organic farming necessitate increased copper use in vineyards.

Specifically formulated **technical tannins** have been selected and purified to provide strong antioxidant protection, as well as inhibit bacteria growth over time. This blend is also particularly beneficial in situations where wines contain a low amount of free SO_2, preventing oxidation and increasing the shelf life of the finished product.

MATERIALS & METHODS

MATERIALS

- **EnartisStab MICRO M** is a preparation of pre-activated chitosan. It is designed to disrupt the cellular functions of a wide spectrum of microorganisms, inhibiting their activity and growth while leaving *Saccharomyces cerevisiae* populations unaffected.
- **HIDEKI** is a technical tannin composed of molecular fractions obtained through the selection and purification of gallic, ellagic, and condensed tannins, which were selected to be the most efficient in terms of antioxidant and microbiostatic action.

ANALYSES

- **Lactic acid and malic acid**: Malolactic fermentation progress was tracked by periodically analyzing malic acid consumption and lactic acid formation using the enzymatic multiparameter Dionysos 150-SinaTech.
- **pH**: A pH-meter was used to track pH throughout the experiments.
- **Molecular SO_2:** Analyses with Dionysos 150-SinaTech using a colorimetric kit were used to determine free SO_2. Consequently, molecular SO_2 was calculated using free SO_2, temperature, alcohol, and pH data.
- **Petri dish:** Culture plating with selected media for *Lactobacillus, Brettanomyces,* and *Acetobacter* were used to track bacterial development.
- **Absorbance 420 nm:** Optical density was measured by spectrophotometer to track the amount of yellow and brown color in wine.

FIGURES

Figure 1
Standard chitosan vs Enartis pre-activated chitosan.
Images taken with a scanning electron microscope

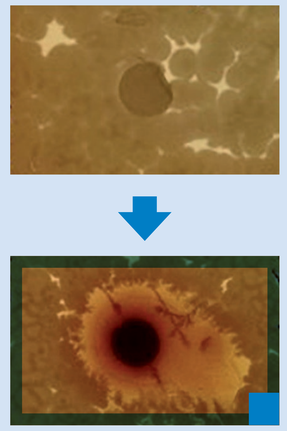


Figure 2
HIDEKI's inhibiting effect
on lactic acid bacteria growth.

TABLES

Antimicrobial effect

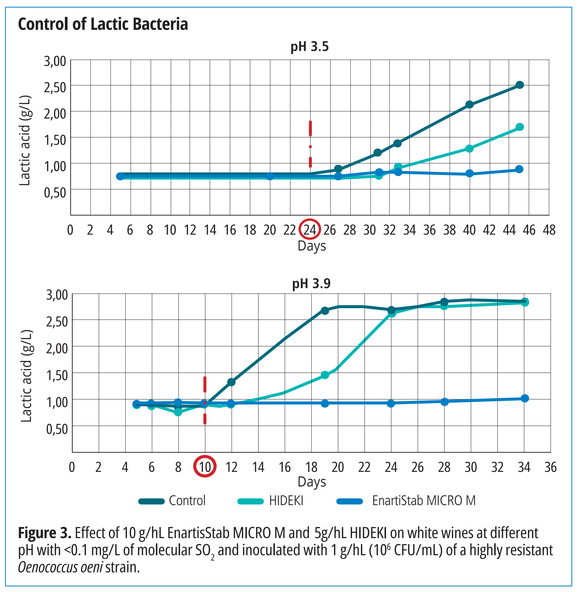


Figure 3. Effect of 10 g/hL EnartisStab MICRO M and 5g/hL HIDEKI on white wines at different pH with <0.1 mg/L of molecular SO_2 and inoculated with 1 g/hL (10^6 CFU/mL) of a highly resistant *Oenococcus oeni* strain.

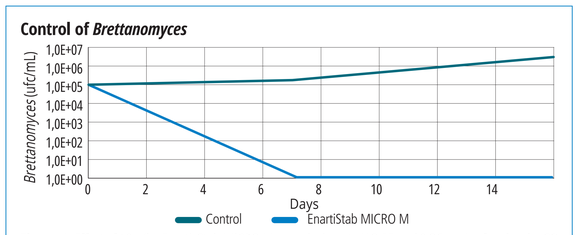


Figure 4. Effect of 10 g/hL EnartisStab MICRO M on red wine at pH 3.55 and 0.36 mg/L of molecular SO_2. The wine was inoculated with 10^5 CFU/mL of *Brettanomyces*.

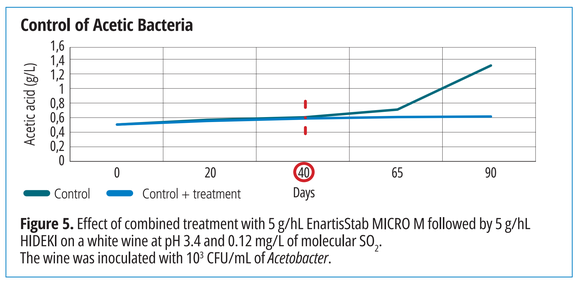


Figure 5. Effect of combined treatment with 5 g/hL EnartisStab MICRO M followed by 5 g/hL HIDEKI on a white wine at pH 3.4 and 0.12 mg/L of molecular SO_2.
The wine was inoculated with 10^3 CFU/mL of *Acetobacter*.

Antioxidant effect

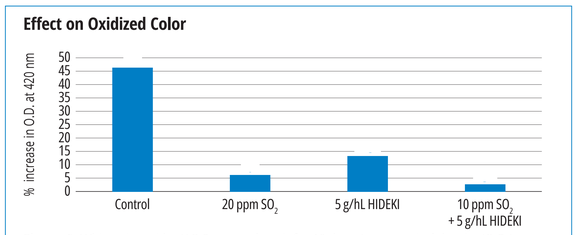


Figure 6. White wine with pH 3.5 exposed to air for 10 days. Lower optical density (O.D.) at 420 nm shows that HIDEKI aids SO_2 management.

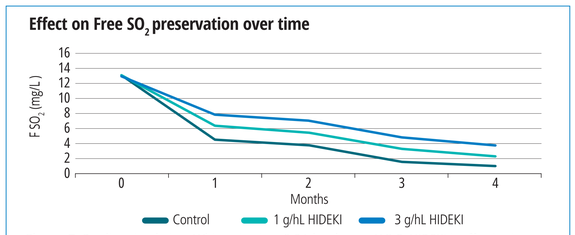


Figure 7. Bottling simulation: White wine into 0.5 L bottles at pH 3.5. HIDEKI is effective in protecting Free SO_2 (FSO_2) concentration over time.

CONCLUSIONS

The use of EnartisStab MICRO M, a preparation of pre-activated chitosan of fungal origin, in conjunction with HIDEKI, a blend of selected technical tannins, offers a promising solution for controlling bacteria, yeast, fungus, VA, and VSCs in wine production. Due to their effectiveness, allergen-free and vegan-friendly status, and the improvement of the sensory qualities of the final product, they provide sustainable and effective alternatives to traditional winemaking techniques. This is especially crucial in light of the challenges posed by climate change to wine production.

LOW OR NO SO_2 WINEMAKING

Facing climate challenges and market demand with allergen-free solutions

Sulfur dioxide (SO_2) is the additive most currently used to preserve, protect, and stabilize wine due to its antioxidant, antioxidasic, and antimicrobial activities.
Increasing demand for allergen-free wines and the challenges of climate change are leading winemakers to seek more sustainable alternatives to achieve the same wine quality and shelf-life. In addition, the increase in pH in must and wine makes SO_2 less effective, which requires much higher doses to obtain the same result, sometimes even to inconceivable levels.

EFFECTIVE ALTERNATIVES TO SO_2, REGARDLESS OF WINE pH

1 ACTIVATED CHITOSAN

Prevents and removes spoilage microorganisms, limits chemical and enzymatic oxidation reactions, among other benefits.

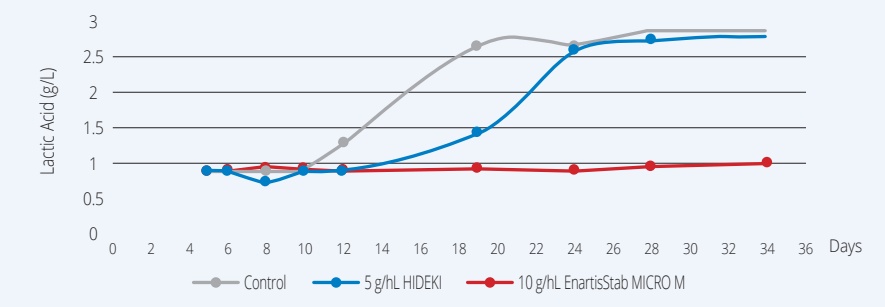
2 TECHNICAL TANNINS

Prevent oxidation of phenolic compounds and, consequently, browning and loss of aromatics. Increase microbial protection by using a bacteriostatic tannin to inhibit microorganism growth.

3 SELECTIVE FINING AGENTS

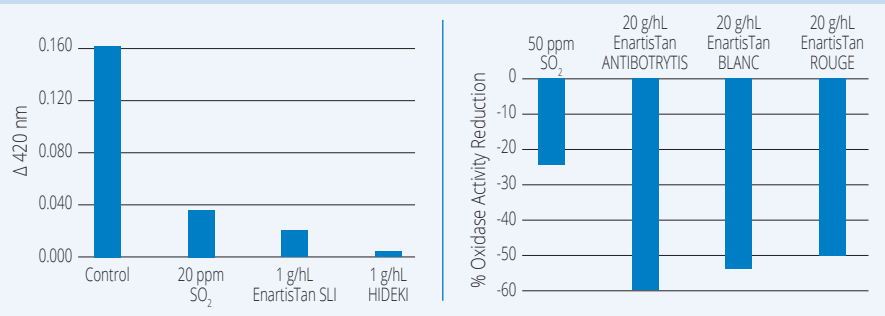
Reduce the catalysts (metals such as copper and iron) and substrates of oxidation reactions (oxidizable polyphenols), preventing and treating possible changes in the bottle.

ANTIMICROBIAL & BACTERIOSTATIC EFFECT

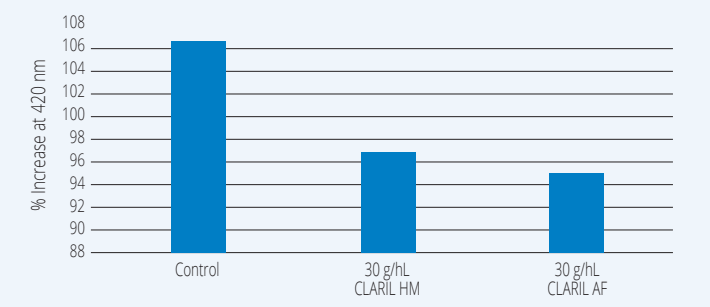


The use of ACTIVATED CHITOSAN (EnartisStab MICRO M) and a blend of selected technical tannins (HIDEKI) offers a solution to control microorganisms. Whether the wine has high pH, low molecular SO_2, or a highly resistant strain of *Oenococcus oeni*, this strategy is highly effective for microbial suppression.

ANTIOXIDANT & ANTIOXIDASIC EFFECT



The use of TECHNICAL TANNINS with high antioxidant activity prevents color degradation in wines with high oxidation potential due to high catechin content, low SO_2 and/or excessive exposure to oxygen. To obtain the same laccase inhibitory effect of technical tannins, large amounts of SO_2 are necessary.



SELECTIVE FINING AGENTS contribute to the antioxidant protection of wine that has a high content of heavy metals, potentially oxidizable polyphenols, and/or excessive exposure to air.

LOW OR NO SO_2 WINEMAKING

LOW OR NO SO_2 WINE PRODUCTION PROTOCOL

Enartis SO_2-free winemaking protocol showed good wine quality results post-fermentation, increased aromatic intensity and complexity, good mouthfeel and structure, and no faults.

WINEMAKING PHASE		RECOMMENDED DOSAGE	WHITE & ROSÉ WINE	RED WINE
GRAPE RECEPTION/CRUSHER		10-20 g/100 kg	AST	
		15 g/100 kg	EnartisTan BLANC or EnartisTan AROM	EnartisTan ROUGE or EnartisTan COLOR
		5-10 g/100 kg	EnartisStab MICRO M (Recommendation if performing a coinoculation: add activated chitosan only after completion of MLF)	
PRESS/MACERATION		2 g/100 kg	EnartisZym AROM MP	EnartisZym COLOR PLUS
JUICE CLARIFICATION		2 g/hL	EnartisZym RS	
		15-20 g/hL	PLANTIS AF or PLANTIS AF-Q	
		20-40 g/hL	Metal removal: CLARIL HM	
		40-80 g/hL	Polyphenol removal: CLARIL AF	
TANK FILLING		5 g/hL	INCANTO NC SLI	
YEAST (Select yeast with low SO_2 production)		20 g/hL	EnartisFerm ES181or EnartisFerm Q9	EnartisFerm ES454 or EnartisFerm ES488
NUTRITION	YEAST INOCULATION	20 g/hL	Enhance aroma: NUTRIFERM AROM PLUS Respect varietal aroma: NUTRIFERM ULTRA	
	1/3 AF	20 g/hL	NUTRIFERM ADVANCE	
	2/3 AF	20 g/hL	NUTRIFERM NO STOP	
POST AF		Rack off gross lees		
		1-2 g/hL	EnartisTan SLI	
		10-20 g/hL	EnartisStab MICRO M	
			Adjust SO_2 content 15 days after completing alcoholic fermentation, to avoid H_2S and acetaldehyde formation.	
		1-3 g/hL	HIDEKI	
PRE-BOTTLING		20-50 g/hL	CITROSTAB rH	

Protocol suitable for ZERO SO_2 wine production (red text is for LOW SO_2).

ANTIOXIDASIC ACTIVITY	ANTIOXIDANT ACTIVITY	ANTIMICROBIAL ACTIVITY
	• CLARIL HM • CLARIL AF • EnartisTan SLI • INCANTO NC SLI	
• EnartisTan ANTIBOTRYTIS • EnartisTan ROUGE • EnartisTan BLANC		
	• HIDEKI • CITROSTAB rH	
• EnartisStab MICRO M		

LEARN MORE

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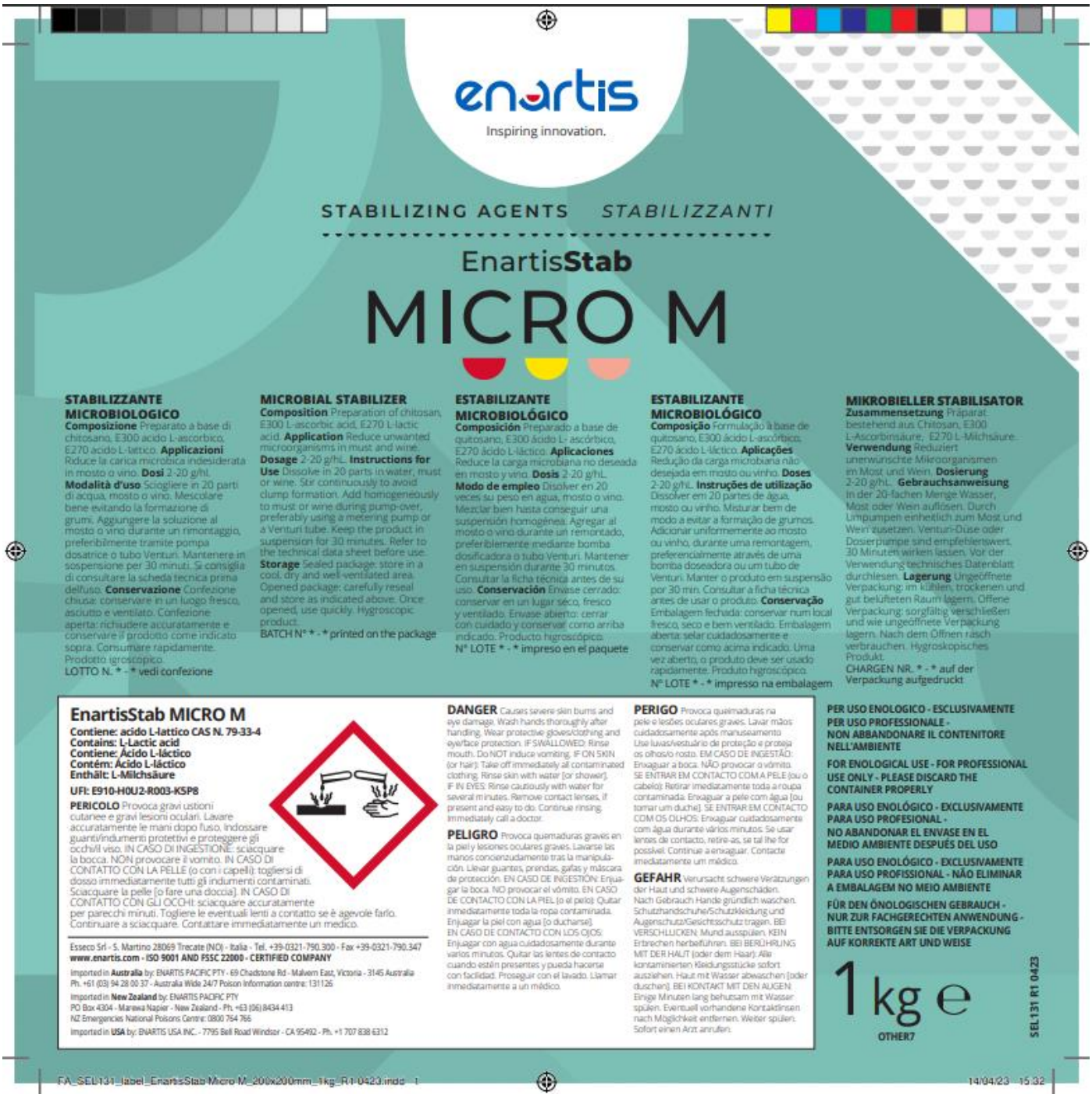
Appendix C – Click on image for PDF



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STABILIZING AGENTS *STABILIZZANTI*

EnartisStab

MICRO M

STABILIZZANTE MICROBIOLOGICO

Composizione Preparato a base di chitosano, E300 acido L-ascorbico, E270 acido L-lattico. **Applicazioni** Riduce la carica microbica indesiderata in mosto o vino. **Dosi** 2-20 g/hl. **Modalità d'uso** Sciogliere in 20 parti di acqua, mosto o vino. Mescolare bene evitando la formazione di grumi. Aggiungere la soluzione al mosto o vino durante un rimontaggio, preferibilmente tramite pompa dosatrice o tubo Venturi. Mantenere in sospensione per 30 minuti. Si consiglia di consultare la scheda tecnica prima dell'uso. **Conservazione** Confezione chiusa: conservare in un luogo fresco, asciutto e ventilato. Confezione aperta: richiudere accuratamente e conservare il prodotto come indicato sopra. Consumare rapidamente. Prodotto igroscopico.
LOTTO N. * - * vedi confezione

MICROBIAL STABILIZER

Composition Preparation of chitosan, E300 L-ascorbic acid, E270 L-lactic acid. **Application** Reduce unwanted microorganisms in must and wine. **Dosage** 2-20 g/hL. **Instructions for Use** Dissolve in 20 parts in water, must or wine. Stir continuously to avoid clump formation. Add homogeneously to must or wine during pump-over, preferably using a metering pump or a Venturi tube. Keep the product in suspension for 30 minutes. Refer to the technical data sheet before use. **Storage** Sealed package: store in a cool, dry and well-ventilated area. Opened package: carefully reseal and store as indicated above. Once opened, use quickly. Hygroscopic product.
BATCH N° * - * printed on the package

ESTABILIZANTE MICROBIOLÓGICO

Composición Preparado a base de quitosano, E300 ácido L- ascórbico, E270 ácido L-láctico. **Aplicaciones** Reduce la carga microbiana no deseada en mosto y vino. **Dosis** 2-20 g/hL. **Modo de empleo** Disolver en 20 veces su peso en agua, mosto o vino. Mezclar bien hasta conseguir una suspensión homogénea. Agregar al mosto o vino durante un remontado, preferiblemente mediante bomba dosificadora o tubo Venturi. Mantener en suspensión durante 30 minutos. Consultar la ficha técnica antes de su uso. **Conservación** Envase cerrado: conservar en un lugar seco, fresco y ventilado. Envase abierto: cerrar con cuidado y conservar como arriba indicado. Producto higroscópico.
N° LOTE * - * impreso en el paquete

ESTABILIZANTE MICROBIOLÓGICO

Composição Formulação à base de quitosano, E300 ácido L-ascórbico, E270 ácido L-láctico. **Aplicações** Redução da carga microbiana não desejada em mosto ou vinho. **Doses** 2-20 g/hL. **Instruções de utilização** Dissolver em 20 partes de água, mosto ou vinho. Misturar bem de modo a evitar a formação de grumos. Adicionar uniformemente ao mosto ou vinho, durante uma remontagem, preferencialmente através de uma bomba doseadora ou um tubo de Venturi. Manter o produto em suspensão por 30 min. Consultar a ficha técnica antes de usar o produto. **Conservação** Embalagem fechada: conservar num local fresco, seco e bem ventilado. Embalagem aberta: selar cuidadosamente e conservar como acima indicado. Uma vez aberto, o produto deve ser usado rapidamente. Produto higroscópico.
N° LOTE * - * impresso na embalagem

MIKROBIELLER STABILISATOR

Zusammensetzung Präparat bestehend aus Chitosan, E300 L-Ascorbinsäure, E270 L-Milchsäure. **Verwendung** Reduziert unerwünschte Mikroorganismen im Most und Wein. **Dosierung** 2-20 g/hL. **Gebrauchsanweisung** In der 20-fachen Menge Wasser, Most oder Wein auflösen. Durch Umpumpen einheitlich zum Most und Wein zusetzen. Venturi-Düse oder Dosierpumpe sind empfehlenswert. 30 Minuten wirken lassen. Vor der Verwendung technisches Datenblatt durchlesen. **Lagerung** Ungeöffnete Verpackung: im kühlen, trockenen und gut belüfteten Raum lagern. Offene Verpackung: sorgfältig verschließen und wie ungeöffnete Verpackung lagern. Nach dem Öffnen rasch verbrauchen. Hygroskopisches Produkt.
CHARGEN NR. * - * auf der Verpackung aufgedruckt

EnartisStab MICRO M

Contiene: acido L-lattico CAS N. 79-33-4
Contains: L-Lactic acid
Contiene: Ácido L-láctico
Contém: Ácido L-láctico
Enthält: L-Milchsäure

UFI: E910-H0U2-R003-K5P8

PERICOLO Provoca gravi ustioni cutanee e gravi lesioni oculari. Lavare accuratamente le mani dopo l'uso. Indossare guanti/indumenti protettivi e proteggere gli occhi/il viso. IN CASO DI INGESTIONE: sciacquare la bocca. NON provocare il vomito. IN CASO DI CONTATTO CON LA PELLE (o con i capelli): togliersi di dosso immediatamente tutti gli indumenti contaminati. Sciacquare la pelle [o fare una doccia]. IN CASO DI CONTATTO CON GLI OCCHI: sciacquare accuratamente per parecchi minuti. Togliere le eventuali lenti a contatto se è agevole farlo. Continuare a sciacquare. Contattare immediatamente un medico.

Esseco Srl - S. Martino 28069 Trecate (NO) - Italia - Tel. +39-0321-790.300 - Fax +39-0321-790.347
www.enartis.com - ISO 9001 AND FSSC 22000 - CERTIFIED COMPANY

Imported in **Australia** by: ENARTIS PACIFIC PTY - 69 Chadstone Rd - Malvern East, Victoria - 3145 Australia
Ph. +61 (03) 94 28 00 37 - Australia Wide 24/7 Poison Information centre: 131126

Imported in **New Zealand** by: ENARTIS PACIFIC PTY
PO Box 4304 - Marewa Napier - New Zealand - Ph. +63 (06) 8434 413
NZ Emergencies National Poisons Centre: 0800 764 766

Imported in **USA** by: ENARTIS USA INC. - 7795 Bell Road Windsor - CA 95492 - Ph. +1 707 838 6312

DANGER Causes severe skin burns and eye damage. Wash hands thoroughly after handling. Wear protective gloves/clothing and eye/face protection. IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water [or shower]. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a doctor.

PELIGRO Provoca quemaduras graves en la piel y lesiones oculares graves. Lavarse las manos concienzudamente tras la manipulación. Llevar guantes, prendas, gafas y máscara de protección. EN CASO DE INGESTIÓN: Enjuagar la boca. NO provocar el vómito. EN CASO DE CONTACTO CON LA PIEL (o el pelo): Quitar inmediatamente toda la ropa contaminada. Enjuagar la piel con agua [o ducharse]. EN CASO DE CONTACTO CON LOS OJOS: Enjuagar con agua cuidadosamente durante varios minutos. Quitar las lentes de contacto cuando estén presentes y pueda hacerse con facilidad. Proseguir con el lavado. Llamar inmediatamente a un médico.

PERIGO Provoca queimaduras na pele e lesões oculares graves. Lavar mãos cuidadosamente após manuseamento Use luvas/vestuário de proteção e proteja os olhos/o rosto. EM CASO DE INGESTÃO: Enxaguar a boca. NÃO provocar o vómito. SE ENTRAR EM CONTACTO COM A PELE (ou o cabelo): Retirar imediatamente toda a roupa contaminada. Enxaguar a pele com água [ou tomar um duche]. SE ENTRAR EM CONTACTO COM OS OLHOS: Enxaguar cuidadosamente com água durante vários minutos. Se usar lentes de contacto, retire-as, se tal lhe for possível. Continue a enxaguar. Contacte imediatamente um médico.

GEFAHR Verursacht schwere Verätzungen der Haut und schwere Augenschäden. Nach Gebrauch Hande gründlich waschen. Schutzhandschuhe/Schutzkleidung und Augenschutz/Gesichtsschutz tragen. BEI VERSCHLUCKEN: Mund ausspülen. KEIN Erbrechen herbeiführen. BEI BERÜHRUNG MIT DER HAUT (oder dem Haar): Alle kontaminierten Kleidungsstücke sofort ausziehen. Haut mit Wasser abwaschen [oder duschen]. BEI KONTAKT MIT DEN AUGEN: Einige Minuten lang behutsam mit Wasser spülen. Eventuell vorhandene Kontaktlinsen nach Möglichkeit entfernen. Weiter spülen. Sofort einen Arzt anrufen.

PER USO ENOLOGICO - ESCLUSIVAMENTE PER USO PROFESSIONALE - NON ABBANDONARE IL CONTENITORE NELL'AMBIENTE

FOR ENOLOGICAL USE - FOR PROFESSIONAL USE ONLY - PLEASE DISCARD THE CONTAINER PROPERLY

PARA USO ENOLÓGICO - EXCLUSIVAMENTE PARA USO PROFESIONAL - NO ABANDONAR EL ENVASE EN EL MEDIO AMBIENTE DESPUÉS DEL USO

PARA USO ENOLÓGICO - EXCLUSIVAMENTE PARA USO PROFISSIONAL - NÃO ELIMINAR A EMBALAGEM NO MEIO AMBIENTE

FÜR DEN ÖNOLOGISCHEN GEBRAUCH - NUR ZUR FACHGERECHTEN ANWENDUNG - BITTE ENTSORGEN SIE DIE VERPACKUNG AUF KORREKTE ART UND WEISE

1 kg e

OTHER7

SEL131 R1 0423

Appendix D

Safety Data Sheet
ENARTIS STAB MICRO M

Safety Data Sheet dated 5/20/2016, version 0

1. IDENTIFICATION

Product identifier
- Mixture identification:
- Trade name: ENARTIS STAB MICRO M
- Other means of identification:
- Product code: 35-762-0001

Recommended use of the chemical and restrictions on use
- Recommended use:
- FOR PROFESSIONAL USE
- Restrictions on use:

Name, address, and telephone number of the chemical manufacturer, importer, or other responsible party
- Company:
- Enartis USA Inc.
- 7795 Bell Road
- Windsor
- CA 95492

Competent person responsible for the safety data sheet:
- compliance@enartisvinquiry.com

Emergency phone number
- Phone: +1 (707) 838 6312
- Fax: +1 (707) 838 1765

2. HAZARD(S) IDENTIFICATION

Classification of the chemical
- Danger, Eye Dam. 1, Causes serious eye damage.

Label elements

Hazard pictograms:

- Danger

Hazard statements:
- H318 Causes serious eye damage.

Precautionary statements:
- P280 Wear eye/face protection: wear eye glasses with side protection.
- P305+P351+P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
- P310 Immediately call a doctor.

Special Provisions:
- None

Hazards not otherwise classified identified during the classification process:
- None

Ingredient(s) with unknown acute toxicity:
- None.

3. COMPOSITION/INFORMATION ON INGREDIENTS

NRSMM/0
Page n. 1 of 7

Substances
N.A.
Mixtures
Hazardous components within the meaning of 29 CFR 1910.1200 and related classification:

>= 7% - < 10% LACTIC ACID
REACH No.: 01-2119548400-48-XXXX, CAS: 79-33-4, EC: 201-196-2
A.2/2 Skin Irrit. 2 H315
A.3/1 Eye Dam. 1 H318

4. FIRST-AID MEASURES

Description of necessary measures
In case of skin contact:
Immediately take off all contaminated clothing.
Areas of the body that have - or are only even suspected of having - come into contact with the product must be rinsed immediately with plenty of running water and possibly with soap.
OBTAIN IMMEDIATE MEDICAL ATTENTION.
Wash thoroughly the body (shower or bath).
Remove contaminated clothing immediately and dispose off safely.
In case of eyes contact:
After contact with the eyes, rinse with water with the eyelids open for a sufficient length of time, then consult an opthalmologist immediately.
Protect uninjured eye.
In case of Ingestion:
Induce vomiting. SEEK A MEDICAL EXAMINATION IMMEDIATELY and present the safety-data sheet.
In case of Inhalation:
Remove casualty to fresh air and keep warm and at rest.
Most important symptoms/effects, acute and delayed
None
Indication of immediate medical attention and special treatment needed
In case of accident or unwellness, seek medical advice immediately (show directions for use or safety data sheet if possible).
Treatment:
None

5. FIRE-FIGHTING MEASURES

Suitable extinguishing media:
Water.
Carbon dioxide (CO2).
Unsuitable extinguishing media:
None in particular.
Specific hazards arising from the chemical
Do not inhale explosion and combustion gases.
Hazardous combustion products:
None
Explosive properties: N.A.
Oxidizing properties: N.A.
Special protective equipment and precautions for fire-fighters
Use suitable breathing apparatus .
Collect contaminated fire extinguishing water separately. This must not be discharged into drains.
Move undamaged containers from immediate hazard area if it can be done safely.

6. ACCIDENTAL RELEASE MEASURES

Personal precautions, protective equipment, and emergency procedures
Wear personal protection equipment.
Remove all sources of ignition.
Wear breathing apparatus if exposed to vapours/dusts/aerosols.
Provide adequate ventilation.
Remove persons to safety.
Use appropriate respiratory protection.
See protective measures under point 7 and 8.
Methods and materials for containment and cleaning up
Wash with plenty of water.

7. HANDLING AND STORAGE

Precautions for safe handling
Avoid contact with skin and eyes, inhalation of vapours and mists.
Do not use on extensive surface areas in premises where there are occupants.
Don't use empty container before they have been cleaned.
Before making transfer operations, assure that there aren't any incompatible material residuals in the containers.
Contamined clothing should be changed before entering eating areas.
Do not eat or drink while working.
See also section 8 for recommended protective equipment.
Conditions for safe storage, including any incompatibilities
Keep away from food, drink and feed.
Incompatible materials:
None in particular.
Instructions as regards storage premises:
Adequately ventilated premises.
Storage temperature:
Store at ambient temperature.

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

Control parameters
No occupational exposure limit available
Appropriate engineering controls:
None
Individual protection measures
Eye protection:
Eye glasses with side protection.
Protection for skin:
Use clothing that provides comprehensive protection to the skin, e.g. cotton, rubber, PVC or viton.
Protection for hands:
Use protective gloves that provides comprehensive protection.
Suitable material:
UNI EN 420/UNI EN 374
Respiratory protection:
Not needed for normal use.
Thermal Hazards:
None

9. PHYSICAL AND CHEMICAL PROPERTIES

Appearance and colour:	Solid
Odour:	Characteristic
Odour threshold:	N.A.
pH:	5.5
Melting point / freezing point:	N.A.

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Initial boiling point and boiling range:	N.A.	
Solid/gas flammability:	N.A.	
Upper/lower flammability or explosive limits:		N.A.
Vapour density:	N.A.	
Flash point:	N.A. °F	
Evaporation rate:	N.A.	
Vapour pressure:	N.A.	
Relative density:	N.A.	
Solubility in water:	N.A.	
Solubility in oil:	N.A.	
Partition coefficient (n-octanol/water):	N.A.	
Auto-ignition temperature:	N.A.	
Decomposition temperature:	N.A.	
Viscosity:	N.A.	
Miscibility:	N.A.	
Fat Solubility:	N.A.	
Conductivity:	N.A.	
Substance Groups relevant properties	N.A.	

10. STABILITY AND REACTIVITY

Reactivity
Stable under normal conditions
Chemical stability
Stable under normal conditions
Possibility of hazardous reactions
None
Conditions to avoid
Stable under normal conditions.
Incompatible materials
None in particular.
Hazardous decomposition products
None.

11. TOXICOLOGICAL INFORMATION

Information on toxicological effects
Toxicological information of the mixture:
N.A.
Toxicological information of the main substances found in the mixture:
LACTIC ACID - CAS: 79-33-4
a) acute toxicity:
Test: LD50 - Route: Skin - Species: Rabbit > 2000 mg/kg
Test: LC50 - Route: Inhalation - Species: Rat = 7.94 mg/l - Duration: 4h
Test: LD50 - Route: Oral - Species: Rat = 4875 mg/kg
Test: LD50 - Route: Oral - Species: Rat = 3730 mg/kg
b) skin corrosion/irritation:
Test: Skin Irritant - Route: Skin Positive

Substance(s) listed on the NTP report on Carcinogens:
None.
Substance(s) listed on the IARC Monographs:
None.
Substance(s) listed as OSHA Carcinogen(s):
None.
Substance(s) listed as NIOSH Carcinogen(s):
None.

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12. ECOLOGICAL INFORMATION

Ecotoxicity

Adopt good working practices, so that the product is not released into the environment.

LACTIC ACID - CAS: 79-33-4

a) Aquatic acute toxicity:

Endpoint: EC50 - Species: Daphnia = 240 mg/l - Duration h: 48
Endpoint: LC50 - Species: Fish = 320 mg/l - Duration h: 48
Endpoint: EC50 - Species: Algae = 3500 mg/l

Persistence and degradability

N.A.

Bioaccumulative potential

N.A.

Mobility in soil

N.A.

Other adverse effects

None

13. DISPOSAL CONSIDERATIONS

Waste treatment and disposal methods

Recover, if possible. Send to authorised disposal plants or for incineration under controlled conditions. In so doing, comply with the local and national regulations currently in force.

14. TRANSPORT INFORMATION

UN number

Not classified as dangerous in the meaning of transport regulations.

UN proper shipping name

N.A.

Transport hazard class(es)

N.A.

Packing group

N.A.

Environmental hazards

N.A.

Transport in bulk (according to Annex II of MARPOL 73/78 and the IBC Code)

N.A.

Special precautions

N.A.

15. REGULATORY INFORMATION

USA - Federal regulations

TSCA - Toxic Substances Control Act

TSCA inventory: all the components are listed on the TSCA inventory.
TSCA listed substances:
None.

SARA - Superfund Amendments and Reauthorization Act

Section 302 – Extremely Hazardous Substances: no substances listed.
Section 304 – Hazardous substances: no substances listed.
Section 313 – Toxic chemical list: no substances listed.

CERCLA - Comprehensive Environmental Response, Compensation, and Liability Act

No substances listed.

CAA - Clean Air Act

CAA listed substances:
None.

CWA - Clean Water Act

CWA listed substances:
None.

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USA - State specific regulations
California Proposition 65
Substance(s) listed under California Proposition 65:
None.
Massachusetts Right to know
Substance(s) listed under Massachusetts Right to know:
No substances listed.
New Jersey Right to know
Substance(s) listed under New Jersey Right to know:
LACTIC ACID.
Pennsylvania Right to know
Substance(s) listed under Pennsylvania Right to know:
LACTIC ACID.

16. OTHER INFORMATION

Full text of phrases referred to in Section 3:
H315 Causes skin irritation.
H318 Causes serious eye damage.

Safety Data Sheet dated 5/20/2016, version 0
Disclaimer:
The information contained herein is based on our state of knowledge at the above-specified date. It refers solely to the product indicated and constitutes no guarantee of particular quality. The information relates only to the specific material and may not be valid for such material used in combination with any other material or in any process.
This Safety Data Sheet cancels and replaces any preceding release.

ADR:	European Agreement concerning the International Carriage of Dangerous Goods by Road.
CAS:	Chemical Abstracts Service (division of the American Chemical Society).
CLP:	Classification, Labeling, Packaging.
DNEL:	Derived No Effect Level.
EINECS:	European Inventory of Existing Commercial Chemical Substances.
GHS:	Globally Harmonized System of Classification and Labeling of Chemicals.
HMIS:	Hazardous Materials Identification System
IARC:	International Agency for Research on Cancer
IATA:	International Air Transport Association.
IATA-DGR:	Dangerous Goods Regulation by the "International Air Transport Association" (IATA).
ICAO:	International Civil Aviation Organization.
ICAO-TI:	Technical Instructions by the "International Civil Aviation Organization" (ICAO).
IMDG:	International Maritime Code for Dangerous Goods.
INCI:	International Nomenclature of Cosmetic Ingredients.
KSt:	Explosion coefficient.
LC50:	Lethal concentration, for 50 percent of test population.
LD50:	Lethal dose, for 50 percent of test population.
LTE:	Long-term exposure.
NFPA:	National Fire Protection Association
NIOSH:	National Institute for Occupational Safety and Health
NTP:	National Toxicology Program
OSHA:	Occupational Safety and Health Administration
PNEC:	Predicted No Effect Concentration.

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RID:	Regulation Concerning the International Transport of Dangerous Goods by Rail.
STE:	Short-term exposure.
STEL:	Short Term Exposure limit.
STOT:	Specific Target Organ Toxicity.
TLV:	Threshold Limiting Value.
TWATLV:	Threshold Limit Value for the Time Weighted Average 8 hour day. (ACGIH Standard).

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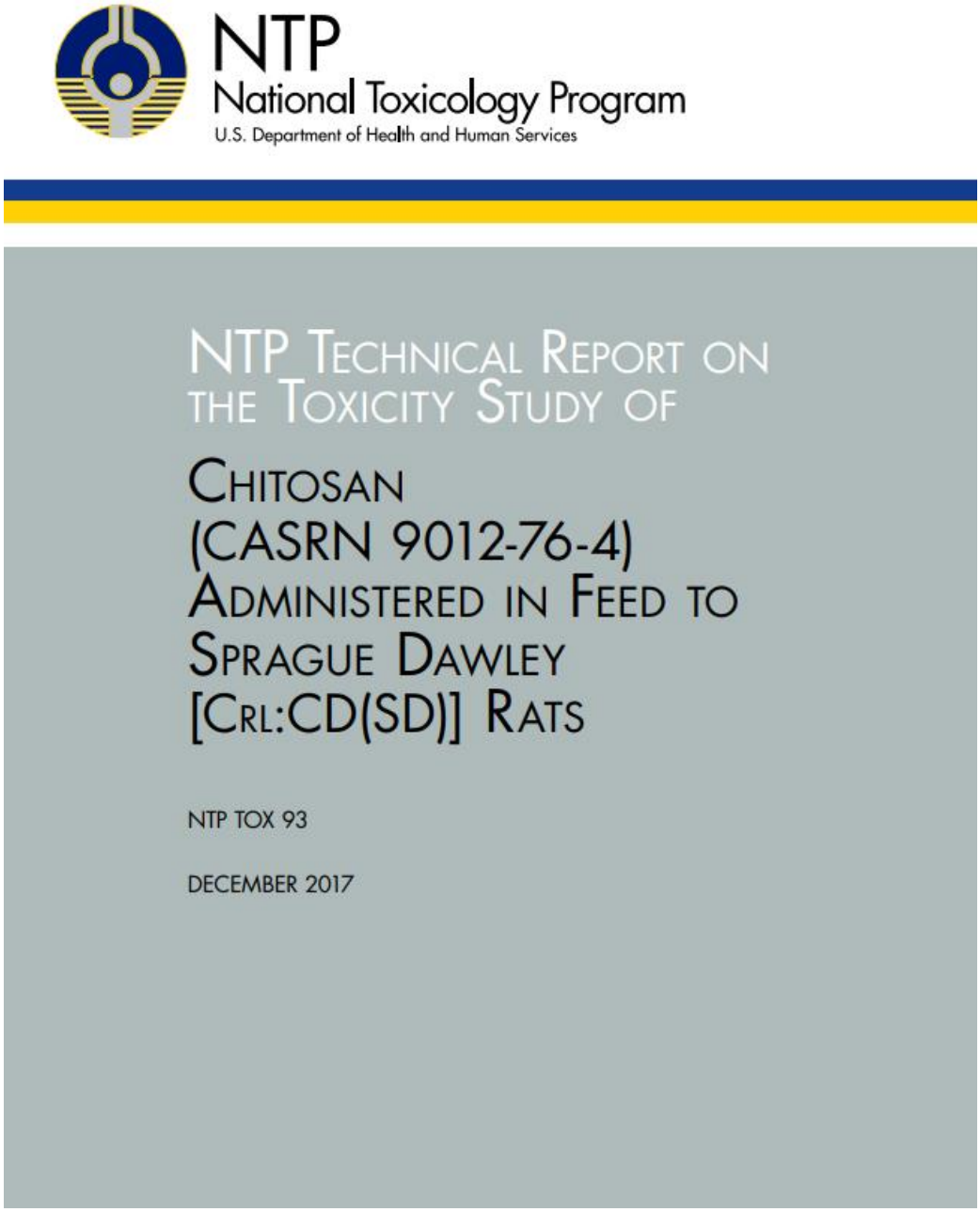
Appendix E – ***Click on image for link to NTP Technical Report on the Toxicity Study of Chitosan Administered in Sprague Dawley Rats***



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Appendix F

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TECHNICAL DATA SHEET

TANNINS

HIDEKI

Tannin providing wine antioxidant and antimicrobial protection.

COMPOSITION
Blend of gallic, ellagic and condensed tannins.

GENERAL CHARACTERISTICS
HIDEKI is a wine protection tool composed of tannins with an excellent antioxidant effect and a high ability to interfere with the functional proteins of microorganism cells.

In nature, plants produce polyphenolic substances in response to injuries caused by external agents: viruses, fungi, bacteria, insects, etc. as well as vertebrates. In cases of viral, bacterial and fungal infections, tannins play a protective role in plant tissue by reducing the activity of enzymes produced by the pathogen entering the plant cell and by blocking the transport proteins present in the cell membrane of the pathogen to prevent its exchange with the growth substrate. At the same time, due to their ability to chelate metals and block free radicals, tannins limit the deterioration of plant tissues caused by oxidation triggered by the pathogen attack.

The best gallic, ellagic and condensed tannins were selected for HIDEKI due to their ability to interfere with the action of transport proteins of microorganisms, chelating capacity and antiradical activity. After selection, a purification process concentrated the most active polyphenolic fractions and amplified the desired protective effect.
The result is a blend of tannins able to protect wine from chemical oxidation, enzymatic oxidation (laccase) and to limit the growth of undesired microorganisms, particularly bacteria, for long periods of time and in wine with pH close to 4.

APPLICATIONS
During wine preparation for bottling:
- As an alternative to the antioxidant and antimicrobial action of sulfur dioxide.
- In wine previously treated with EnartisStab Micro/Micro M (activated chitosan) to prolong protection after the removal of the antimicrobial agent.
- To prevent the development of unwanted microorganisms that can alter the composition and sensory quality of bottled wine (increase in volatile acidity, appearance of anomalous odors, loss of acidity, etc.).

DOSAGE
As an antioxidant: 1 - 3 g/hL (0.08-0.25 lb/1,000 gal)
As microbiostatic: 5 - 10 g/hL (0.4-0.8 lb/1000 gal)

INSTRUCTIONS FOR USE
Dissolve HIDEKI at a 1:10 ratio in water or wine while mixing continuously to avoid clumps. Add to wine during pump-over with a dosage pump or Venturi tube.
Performing laboratory trials is recommended to evaluate the sensory impact of the tannin and in order to determine the best dosage. When added close to bottling, it is important to evaluate the effect on filterability and protein and colloidal stability by completing preliminary laboratory trials.
In the case of bacteriostatic application, it is recommended to perform microbiological analysis regularly.

PACKAGING AND STORAGE CONDITIONS
1 kg

Sealed package: store in a cool, dry, well-ventilated area.
Opened package: carefully reseal and store as indicated above.

The indications given here correspond to the current state of our knowledge and experience, however they do not relieve the user from compliance with safety and protection regulations or from improper use of the product.

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materials

MDPI

Article

Impact of Chitosan on Water Stability and Wettability of Soils

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Abstract: Chitosan has become increasingly applied in agriculture worldwide, thus entering the soil environment. We hypothesized that chitosan should affect the water stability of soil. Since this problem has not been studied to date, we examined, for the first time, the influence of chitosan on the water stability and wettability of soil aggregates. The aggregates were prepared from four soils with various properties amended with different amounts of two kinds of powdered chitosan, and subjected to 1 and/or 10 wetting–drying cycles. The water stability was measured by monitoring air bubbling after aggregate immersion in water, and the wettability was measured by a water drop penetration test. The biopolymer with a lower molecular mass, lower viscosity, and higher degree of deacetylation was more effective in increasing the water stability of the soil than the biopolymer with a higher molecular mass, higher viscosity, and lower deacetylation degree. After a single wetting-drying cycle, the water stability of the soil aggregates containing chitosan with a higher molecular mass was generally lower than that of the soil; after ten wetting–drying cycles, the water stability increased 1.5 to 20 times depending on the soil. The addition of low-molecular-mass chitosan after a single wetting-drying cycle caused the water stability to become one to two hundred times higher than that of the soil. A trial to find out which soil properties (pH, C and N content, bulk density, porosity, and particle size distribution) are responsible for the effectiveness of chitosan action was not successful, and this will be the objective of further studies.

Keywords: aggregate; chitin derivatives; soil reaction; water repellency; destruction kinetics; food wastes

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1. Introduction

Due to the increasing food demand of the growing human population, the recycling and utilization of food industry wastes has become an urgent task worldwide. The main waste product of the food industry is chitin, an N-acetylglucosamine polysaccharide present in crustaceans (shrimps, lobsters, and crabs), mollusks (oysters and squids), insects, and fungi [1]. Currently, the main commercial source of chitin comprises waste streams from the marine food industry—mainly the exoskeletons of crustaceans. The annual world production of eight million tons of crustaceans for human consumption was estimated in 2016, 40% of which comprised waste exoskeletons with a chitin content of 15–40% [2], which equates to about 1600 tons of chitin produced yearly [3]. For comparison, one should mention that chitin comprises the second largest renewable carbon source after lignocelluloses coming from wood and agricultural wastes, such as straw of various kinds and sugar cane bagasse [4]. Starting from the mid-eighties, chitin-originated materials became broadly applied in wastewater treatment, pharmacy, medicine, biotechnology, the textile and paper industry, and many others [5]. Due to their high availability, biodegradability, phosphorus and nitrogen content, non-toxicity, bacteriostatic properties, and low

cost, chitin-derived substances have become, more recently, increasingly applied in agriculture [6–8]. For the vast majority of the above applications, solid chitin is transformed to chitosan by decalcification (acid treatment) and removal of the acetyl residues (alkali treatment). The term "chitosan" is not uniquely related to a defined compound, but to a group of commercially available copolymers that are heterogeneous for the deacetylation degree, molecular mass, polymerization degree, surface charge, and acid dissociation constant [9]. These different characteristics, the degree of deacetylation and the molecular weight in particular, differentiate the physicochemical properties of the substance and, in consequence, the mode of its applications.

Up to date, the main interest of agriculture-related studies has been mostly concentrated on the effects of chitosan on plants and pests. It was proved that chitosan exerts significant effects on plant development and the survival of abiotic stresses [9–12]. A stimulating effect of chitosan on plant growth, yield, and macronutrient (nitrogen and phosphorus) uptake was observed by Boukhlifi et al. [13] for wheat and potatoes, Silva et al. [14] for melon, Chen et al. [15] for begonia, and Chookhongkha et al. [16] for chili fruits and seeds. Seeds coated with chitosan have a better germination capacity [17]. Chitosan is used to mitigate the following soil and plant pathogens: bacteria [18,19], viruses [20,21], fungi [22,23], or nematodes [24]. Chitosan is a promising coating material for slow-release fertilizers [25–28]. Studies related to the soil environment are mainly directed to the elaboration of new composite or copolymer systems containing chitosan for improvement of the soil water-holding capacity [29–35] and for soil stabilization [36]. Chitosan is also applied for the removal of various types of contaminants from soils [7,37–40].

As was shown above, chitosan may be introduced into a soil in various ways. Despite the fact that it is hypothesized to impact the physical and physicochemical properties of soil, studies on the above problem are very rare. Particularly, we could not find any papers reporting an effect of chitosan on the water stability and wettability of soil, which are crucial to understand the vast majority of soil agricultural, geotechnical and environmental functions, and properties important for tillage, erosion, compaction, aeration, slaking, water and solute transport, root penetration, road and building construction, and many others [41–44]. Therefore the objective of this study was to evaluate the influence of chitosan on the water stability of soil. To do this, we selected two different chitosans and four different soils. At first, the physicochemical properties of the chitosans and of the soils were characterized extensively, and soil–chitosan aggregates were prepared. The effects of biopolymer concentration and soil–biopolymer contact time on the water stability and wettability of the aggregates were investigated.

The water stability was measured by monitoring air bubbling after aggregate immersion in water, which is probably the only method that allows the kinetic parameters of the destruction process for rapidly destroyed large aggregates to be estimated.

The wettability was assessed by a water drop penetration test (WDPT), which reflected the rate of water infiltration into the aggregate.

2. Materials and Methods

2.1. Soils

Soil samples were taken from upper 5–15 cm layers of four soils localized in East Poland, air dried and screened by a 1 mm sieve. The characteristics of the soil samples are presented in Table 1. These data include the following:

- pH measured in 1:2.5 soil:water suspension after 5 min continuous stirring;
- Particle size distribution determined for organic matter-depleted soil (H_2O_2) by sieving and the pipette method after chemical dispersion of soil sample in sodium pyrophosphate;
- Particle density, PD, measured by helium pycnometry using Quantum Crome Ultrapycnometer 1000 (Quantachrome, Boynton Beach, FL, USA);
- Total organic carbon content determined by dichromate digestion [45,46] and nitrogen content determined by Kjeldahl method.

Table 1. Characteristics of the studied soils.

Abbreviation	POD	ARE	FLU	UMB
Soil type	Podzol	Arenosol	Fluvisol	Umbrisol
Locality	Trawniki	Strzyzewice	Dorohucza	Prusy
Longitude E	22°58′41″	22°26′6″	22°59′38″	21°41′59″
Latitude N	51°9′14″	51°2′9″	51°9′43″	50°49′25″
pH	4.1	5.5	6.5	7.7
PD, (g cm^{-3})	2.54	2.62	2.62	2.68
Nitrogen (%)	0.16	0.13	0.46	0.14
Total organic carbon (%)	0.65	1.55	3.04	0.9
Sand (0.063–2 mm) (%)	72.4	47.1	20.2	10.4
Silt (0.002–0.063 mm) (%)	25.9	46.2	52.2	72.4
Clay (<0.002 mm) (%)	1.7	6.7	27.6	17.2

2.2. Chitosans

Two different kinds of chitosan were used. The first, abbreviated as CS1, was provided by Sigma Aldrich (St. Louis, MO, USA) and the second (CS2) was provided by Beijing Be-Better Technology Co., Ltd. (Beijing, China). The basic properties of both chitosans are presented in Table 2. The data presented include the following:

- Total carbon and nitrogen content and particle density determined similarly to soil analysis;
- Degree of deacetylation (DD) calculated from the carbon/nitrogen ratio (C/N) using the following equation from Xu et al. [47]:

$$DD = 1 - (C/N - 5.14)/1.72 \quad (1)$$

Table 2. Properties of the studied chitosans.

	N [%]	TOC (%)	PD (g cm^{-3})	DD	M (kDa)	η (1% Solution) (cP)	x (at pH = 4, μ = 0.01)	θ (deg)
CS1	7.51	41.59	1.51	0.77	699	111.0	5.75	106.0
CS2	7.79	41.27	1.54	0.91	280	12.3	2.25	95.4

- Average molecular weight (M) determined from viscometric measurements performed for series of CS1 and CS2 solutions of decreasing concentrations in 0.02 Mol dm^{-3} acetic acid/0.02 Mol dm^{-3} NaCl, at 24 °C, using Hoppler rheo-viscometer. The intrinsic viscosity (η_{int}) was determined as follows:

$$\eta_{int} = \lim_{c \to 0} [(\eta(c) - \eta_s)/(\eta_s \times f)] \quad (2)$$

where $\eta(c)$ is the viscosity of the chitosan solution at a given concentration c, η_s is the viscosity of the solvent and f is the w/w fraction of the chitosan in the solution.

The average molecular weight was calculated from Mark–Houwink equation [48], as follows:

$$\eta = KM^{\alpha'} \quad (3)$$

where η is the intrinsic viscosity, and K and α' are constants for a given solute–solvent system.

The following K and α' values evaluated by Varum and Smidsrod [49] were used:

$$K = 8.43 \times 10^{-3}; \alpha' = 0.93 \quad (4)$$

- Chitosan chain stiffness parameter (x) introduced by Kasaai [50], calculated as follows:

$$x = DA/(pH \times \mu) \quad (5)$$

where DA = 1−DD is the acetylation degree [51] of the chitosan and μ is the ionic strength of the chitosan solution of a given pH.

- Contact angle (θ) measured on the pressed chitosan pellets using a DSO 100 automatic drop shape analyzer (KRUSS, Hamburg, Germany).

The biopolymer with lower DD is characterized by lower content of nitrogen due to the lower number of NH_2 groups. The molecular mass of CS2 (with higher DD) is lower than for CS1, which is in line with Kofuji et al. [52] who indicated that progress in deacetylation process decreases the molecular weight. They also noticed that solutions of chitosan with higher molecular weight tended to have higher viscosity as was observed in this study. Chitosan with lower DD (higher DA) has stiffer chain conformation. The measured density of both chitosans is close to a value of 1.5 g cm^{-3} reported by Gierszewska-Druzynska et al. [53]. The water contact angle of CS1 is higher than that of CS2. The contact angle decreases in time due to wetting of the chitosan surface and soaking of the droplet into the pellet body, as illustrated in Figure 1.

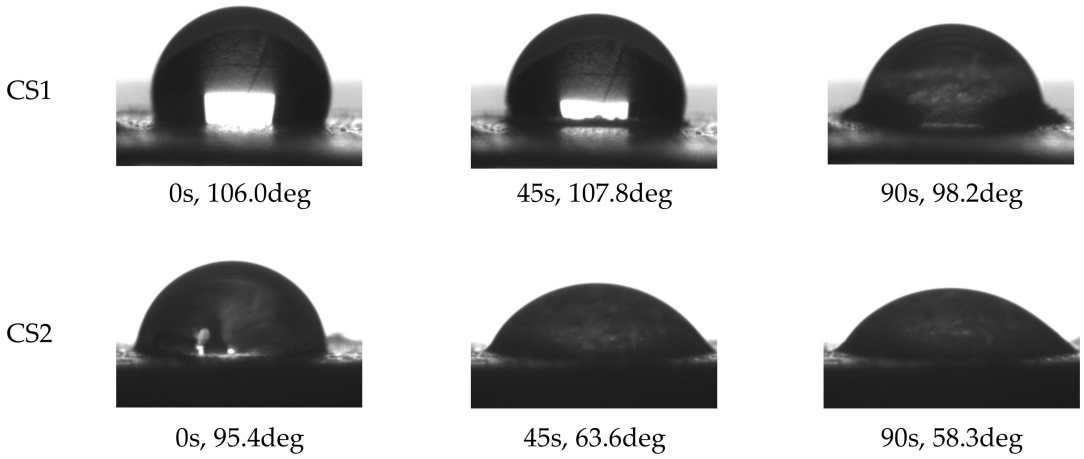

Figure 1. Time behavior of water drop settled on pellets prepared from CS1 and CS2. Below each photograph, time (seconds) and contact angle (degrees) are written.

The wetting pathways seem to differ for both materials. The CS1 appears to swell much more intensively than CS2. The contact zone of water drop and CS1 "grows up" and finally an embankment of the swollen chitosan forms around the droplet. Such occurrences are hardly recognizable for CS2.

2.3. Preparation of Soil–Chitosan Aggregates

The soil samples were air-dried and screened by 1 mm sieve (mesh 18). To minimize eventual effects of chitosan granulometric composition on soil properties when studied further, both CS1 and CS2 were screened by the following set of sieves: 0.177 mm (mesh 80), 0.105 mm (mesh 140) and 0.053 mm (mesh 270), and the final materials were composed from equal weights of 0.177–0.105 mm and 0.105–0.053 mm fractions. Carefully homogenized water-saturated pastes were prepared from mixtures of the soils and the chitosans. Distilled water was used for paste preparation. The content of CS1 and/or CS2 in the mixtures was 0 (control), 0.5, 1, 2, 4, and 8%. Spherical aggregates with 20 mm diameter were formed from the pastes using ordinary silicon forms sold in fishing stores to prepare fish bait. The first set of aggregates was prepared just after the paste preparation and then air-dried (one cycle of wetting–drying), and the second set from the pastes subjected to 10 wetting–drying cycles (6 days per cycle). All aggregates were then dried until constant mass in laboratory conditions (relative humidity around 60% and temperature around 25 °C). The aggregates are abbreviated further using the abbreviation of a given soil (see Table 1) followed by the number of wetting–drying cycles (e.g., POD1 and POD10 denote aggregates formed from podzol preconditioned with one and ten wetting–drying cycles, respectively).

2.4. Studies of Soil–Chitosan Aggregates

Bulk density (BD) of the aggregates was estimated for laboratory-dried specimens. The aggregate weight minus the moisture content was divided by the volume of the aggregate. The aggregate volume was established based on Archimedes' principle. The aggregate was totally immersed in the mercury liquid by forcing the aggregate down to a constant depth using an iron wire formed into a conical spiral and the increase in the system weight after immersion was measured. Knowing the mercury density we calculated the aggregate volume (the volume of the spiral was of course subtracted).

Water stability of the aggregates was estimated from air bubbling after immersion using a method described by Jozefaciuk et al. [54], which is briefly outlined below. The aggregate was thrown into a vessel submerged in water and hanging on a scale pan, and time-dependent increase in the weight of the aggregate, Δw, due to the evolution of entrapped air from the interior of the destructed aggregate (decreasing in buoyancy), was registered. Next, one calculated the dependence of the extent of destruction (α) defined as follows:

$$\alpha = \Delta w \div (w_{final} - w_{initial}) \quad (6)$$

where w_{final} is the weight of the submerged aggregate after termination of the destruction and $w_{initial}$ is its initial weight registered just after immersion.

The dependence of α on time gives a characteristic sigmoidal curve reaching a plateau at the moment when the aggregate is totally destroyed. The above water destruction curve is interpreted in terms of a shrinking sphere model with the following equation:

$$1 - (1 - \alpha)^{1/3} = 1/t_d \times t \quad (7)$$

where t is the time of the process and t_d is the time needed to terminate the destruction of the aggregate (destruction time).

The data plotted in coordinates of Equation (7) give a straight line reaching the value of $1 - (1 - \alpha)^{1/3} = 1$ when $t = t_d$ (when $\alpha = 1$). The value of the destruction time depends both on the characteristics of the aggregated material and the size of the aggregate. In the shrinking sphere model, the destruction time is proportional to the initial surface of the aggregate (S_0). Therefore the ratio of t_d/S_0 [s m^{-2}], which can be read as time necessary to destroy the unit surface of the aggregate, characterizing the aggregated material regardless of the size of the aggregate, is used as water stability parameter. For measurements of the destruction curves of the studied aggregates we used EXPLORER® ANALYTICAL EX324M balance provided by OHAUS (Parsippany, NY, USA) with time resolution equal to 1 s. The final curves, averaged from at least 6 most similar destruction curves selected from 10 replicates for each aggregate, are considered further. Such selection was performed to minimize effects of structural artifacts influencing the destruction. The value of S_0 was estimated for each aggregate from its mass divided by bulk density (assumed to be the same for each aggregate).

It is worth mentioning here that we also attempted to test water stability of aggregates using wet sieving method (measuring the size distribution of aggregates and their mean weight diameter (MWD), before and after the action of water). Three to five millimeter fraction sieved out from the crushed aggregates was studied; however, in most cases the destruction was very fast and the final MWD reflected the granulometric composition of soil–chitosan mixtures. In the wet sieving method some external energy (mixing) is given to the aggregates, which markedly increases their destruction rate as compared to undisturbed process conditions.

Water repellency (hydrophobicity) was measured by a water drop penetration test [55], modified to achieve more precise results. Four microliters of distilled water was settled onto a surface of the aggregate (flattened with fine sandpaper and dust removed with a blower) and the time of the whole drop soaking was read from a video registering the process. The measurements were performed in four replicates using a DSO 100 automatic drop shape analyzer (KRUSS, Hamburg, Germany). It is worth mentioning that our first

idea was to measure contact angles, CA, of the soil and soil–chitosan mixture; however, due to very fast water infiltration into the aggregates this was not possible. The WDPT selection was a matter of choice, since CA and WDPT frequently do not correlate.

3. Results and Discussion

Changes in the bulk density of the soil aggregates, amended with various doses of the studied chitosans, are illustrated in Figure 2.

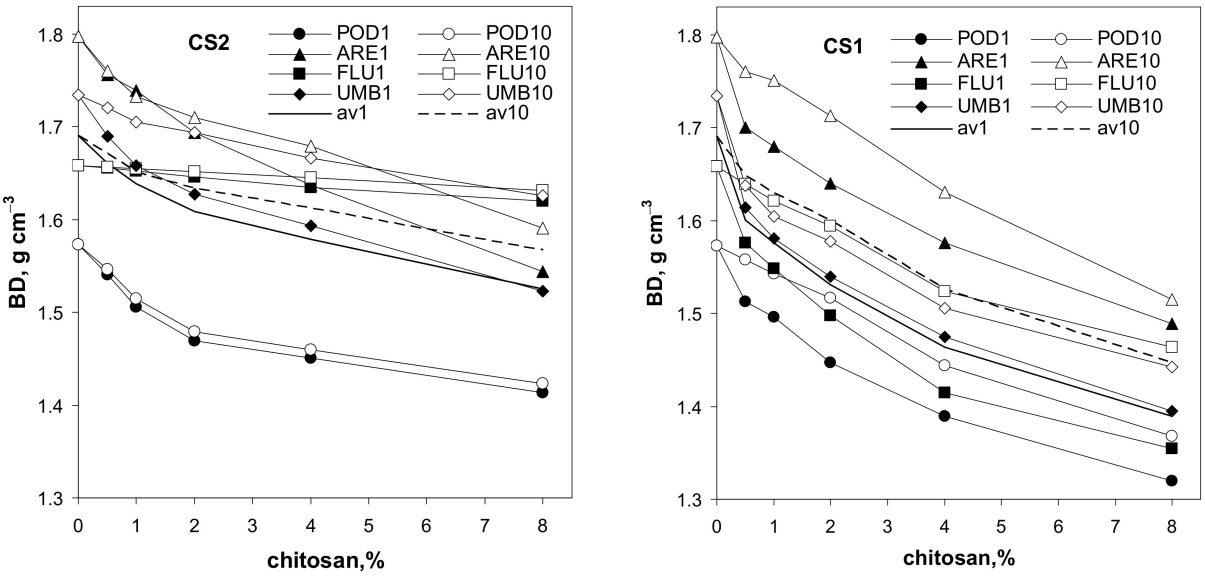

Figure 2. Dependence of bulk density of soil aggregates on amendment of the studied chitosans (CS1 and CS2). The soils are abbreviated as follows: POD—podzol, ARE—arenosol, FLU—fluvisol, UMB—umbrisol. The number after the soil abbreviation shows the number of wetting–drying cycles applied to soil aggregates. The curves denoted av1 and av10 show average data for all soils preconditioned with one and ten wetting–drying cycles, respectively. The error bars are covered by the labels of the points.

The addition of both chitosans causes a marked decrease in the soil bulk density, which may be a direct consequence of the low particle density of the chitosans in respect to the solid phases of the four soils (Tables 1 and 2). However, loosening of the soil structure, due to the addition of coarse chitosan particles, is also possible. The wetting–drying cycles consolidate the structure of the soils containing chitosans. The average effect is similar for both kinds of chitosan. The gelling/solubilization of chitosan is possibly responsible for the above effect. Consolidation of the structure was also observed by Hataf et al. [36] after the addition of chitosan dissolved in acetic acid to a sandy soil. They stated that chitosan increases interparticle interactions, concluding that this mechanism depends on the water content. Under wet conditions, the biopolymer enhances the bonds between soil particles, and during dry conditions, the chitosan gel converts to fibers with very low mechanical strength.

Exemplary water destruction curves of the soil aggregates, showing changes in the extent of destruction of the aggregates over time, as well as the above data plotted in coordinates of Equation (7), are shown in Figure 3.

Similar curves to those in Figure 4 were obtained for the other soils containing both CS1 and CS2, which indicates that the shrinking sphere model can be applied to identify the water destruction of the studied aggregates. Taking the t_d values calculated from the slopes of the linear fits of the destruction data of Equation (7), plotted in coordinates (see Figure 3), and dividing them by the initial surface of each aggregate, S_0, the values of the time necessary to destroy the unit surface of the aggregate, t_d/S_0 [s m^{-2}], were calculated.

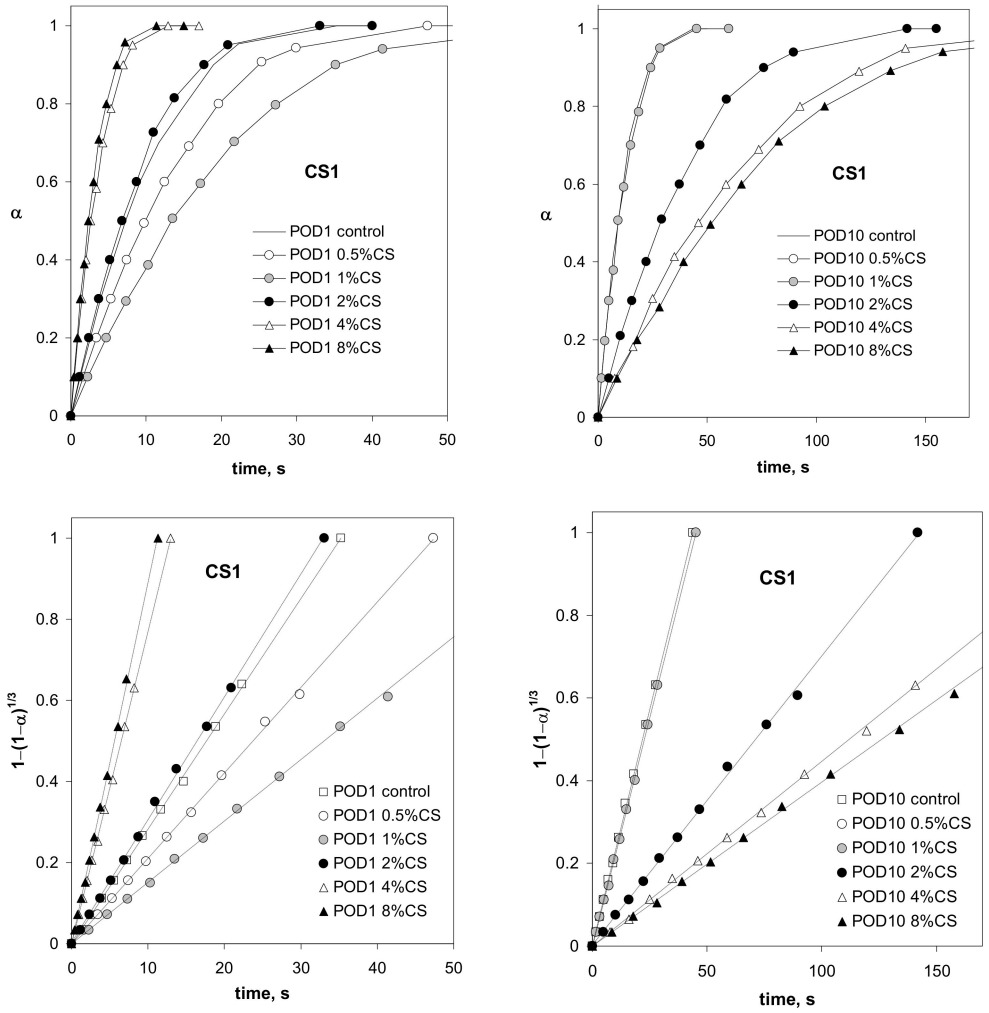

Figure 3. Time dependence of the extent of destruction of the aggregates of podzol (POD) amended with different amounts of chitosan CS1, preconditioned with one (left) or ten (right) cycles of wetting–drying (above) and the respective data, plotted in coordinates, of the shrinking sphere model according to Equation (7) (below).

The water stability of the aggregates containing CS1, preconditioned with a single cycle of wetting–drying, is generally lower than for the aggregates of the control soils. After ten wetting–drying cycles, these aggregates became more water resistant, with the exception of the aggregates of fluvisol and umbrisol, which contained the maximum dose of CS1. The latter aggregates are more water resistant than their counterparts when subjected to the single wetting–drying cycle, but they are still less water resistant than the control soils. In contrast to CS1, the water stability of the CS2 containing aggregates reaches high water resistance after the first wetting–drying cycle, and it increases only slightly after the next nine wetting–drying cycles. The impact of high doses of CS2 on the water stability of fluvisol and umbrisol is extremely high. The t_d/S_0 values reach up to three thousand seconds per square centimeter, which means that the time of destruction of the aggregate with a 20 mm diameter is over 10 h, while the control aggregates need a few minutes to be destroyed.

At least a few mechanisms can be responsible for the above effects. The addition of coarse chitosan particles may lead to loosening of the soil structure and breaking of the distance-dependent interparticle bonds; thus, the soil becomes more susceptible to water destruction. On the other hand, the improvement in water resistance may be connected with the solubilization/gelling of chitosan and the gluing action of its colloidal particles on soil grains. The first mechanism seems to dominate in CS1-containing aggregates, particularly after a single wetting–drying cycle, and for the maximum CS1 doses in neutral

and alkaline soils. The effect of the second mechanism should increase over time, since solubilization and gelling are time dependent. The increase in water stability over time was observed for all the aggregates. The gluing of soil particles by the jellified chitosan should also affect the mechanical stability of the aggregates. If the gluing action overcomes the soil material dilution by the chitosan, the mechanical stability should increase, which may also be connected with water stability changes. We intend to study this problem in the near future.

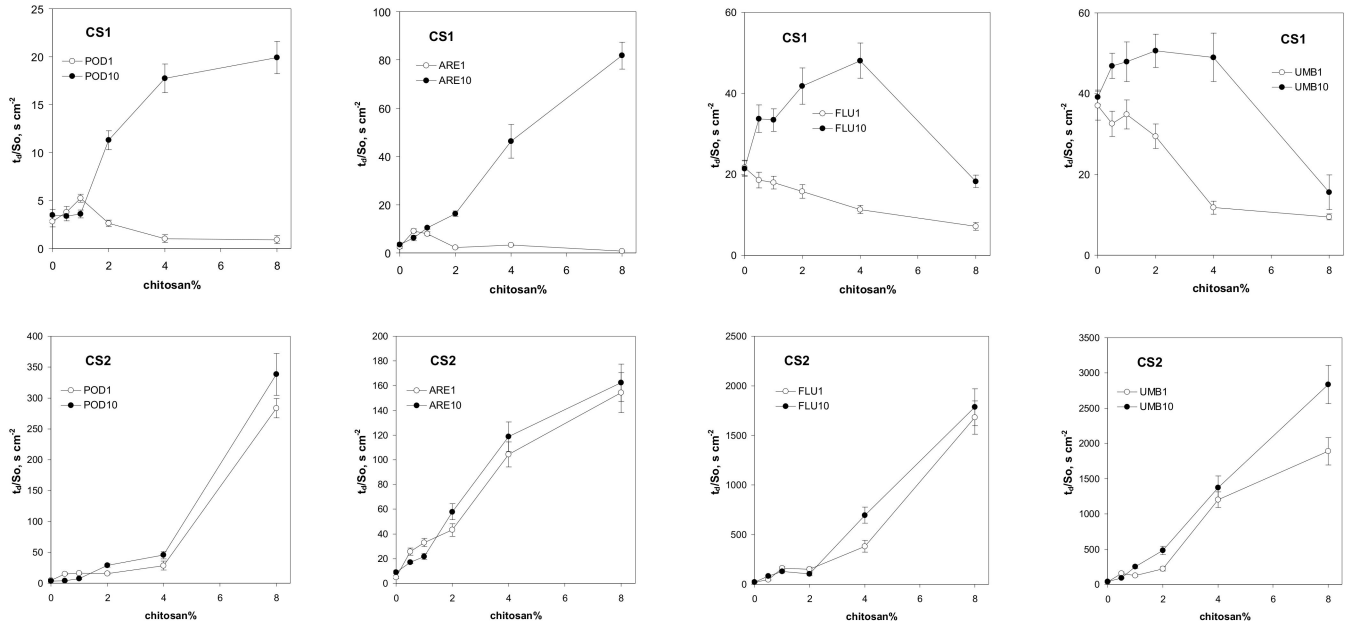

Figure 4. Dependence of time necessary to destroy the unit surface of the aggregate on the percentage of the added chitosan CS1 (above) and CS2 (below). The soils are abbreviated as follows: POD—podzol, ARE—arenosol, FLU—fluvisol, UMB—umbrisol. The number after the soil abbreviation shows the number of wetting–drying cycles applied to soil aggregates.

The differences in the effects of both kinds of chitosan on aggregate stability may be connected with their molecular characteristics. CS1, with a greater molecular mass, may dissolve slower, so the time effect of CS1 on water stability is lower than that of CS2, which has a lower molecular mass. The rapid solubilization of CS2 may also be a reason why, just after the first wetting, the soils admixed with CS2 reach almost maximum water stability. The differences in the swelling properties of CS1 and CS2 (CS1 swells markedly faster than CS2; see Materials and Methods section) may also influence the lower stability of CS1-containing soils.

The water drop penetration time for the studied soils, amended with different amounts of the studied chitosans, is shown in Figure 5.

As a consequence of their high contact angles, both chitosans make the soils more water repellent. The time needed for droplet penetration for the biopolymer with a lower DD (CS1) was shorter than for the material with a higher DD (CS2). These results are in agreement with Mucha et al. [56], who reported that the water sorptivity of chitosan films decreased with increasing DD. In general, water repellency increases with an increasing number of wetting–drying cycles. Similar mechanisms to those governing water stability may be responsible for water repellency. The highest effect of chitosan on increasing water repellency was observed in podzol. We think that this soil has the lowest surface area (due to the smallest content of clay and organic matter), and the molecules of the dissolved chitosan may cover the surface to the greatest extent, forming hydrophobic patches. We thought that the water stability of the aggregates may be governed by their wettability; however, no correlation between the aggregate destruction time and WDPT was found.

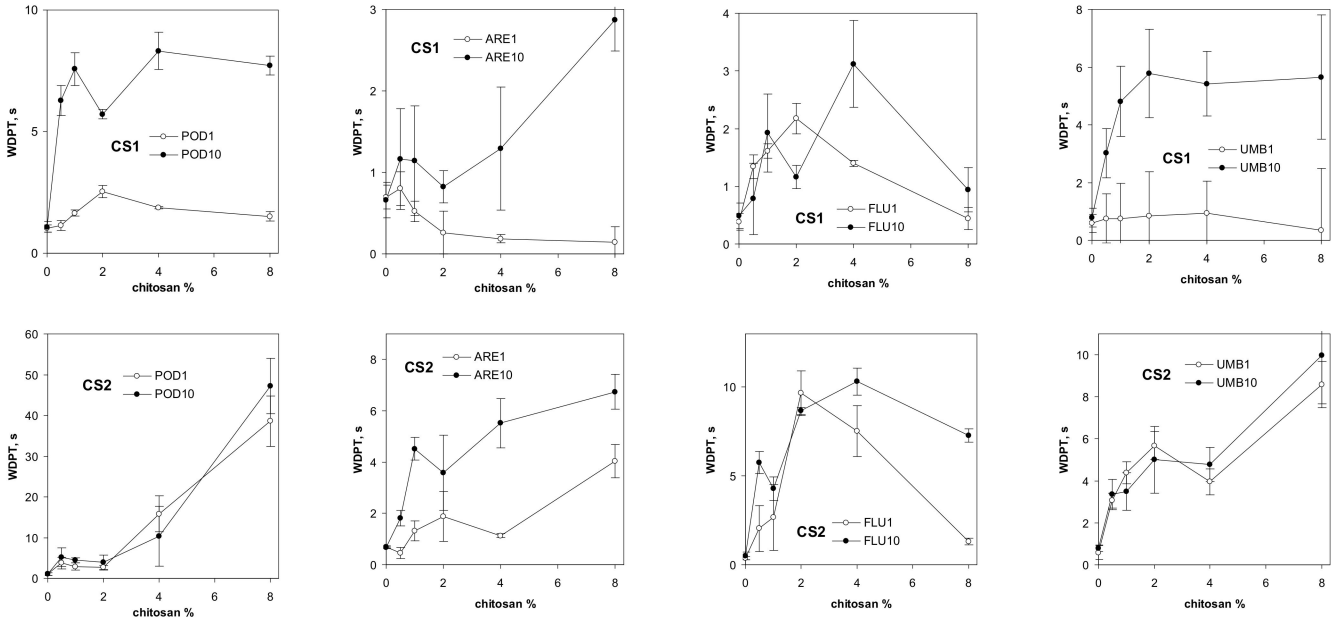

Figure 5. Dependence of water drop penetration time for aggregates of different soils on percentage of chitosan CS1 (above) and chitosan CS2 (below). The soils are abbreviated as follows: POD—podzol, ARE—arenosol, FLU—fluvisol, UMB—umbrisol. The number after the soil abbreviation shows the number of wetting–drying cycles applied to soil aggregates.

We tried to establish which properties of the soils correlated with their reaction with chitosan. To do this, we used a parameter expressing the maximum impact of chitosan on the water stability of the soil, which was taken as a ratio of the maximum value of t_d/S_0 for the chitosan-containing aggregate to the same value for the control soil. For CS1, this parameter was 5.7 (for podzol), 23.9 (for arenosol), 2.2 (for fluvisol), and 1.3 (for umbrisol). The same values for CS2 were 97.3,17.8, 83.2, and 72.4, respectively. The first candidate to govern the water stability of the soil–chitosan aggregates was the pH of the soil, since it affects the chitosan solubility and, according to Kaasai [50] (see Equation (5)), an increase in pH reduces the stiffness of chitosan chains. However, no correlation was found between soil pH and the water stability of chitosan-containing soils. The next candidates, the amount of clay fraction and/or organic carbon content, also did not correlate well with the water stability of soil–chitosan aggregates. It seems that the properties of chitosan are more important in governing the water stability of soil aggregates than the properties of the soil; however, studying more soils may clarify the above problem.

In summary, the effect of chitosan on the water stability and wettability of soils increased over time. The water stability and wettability of soil depended on the properties of the added chitosan. Stronger and faster action was noted for chitosan with a lower molecular mass, lower viscosity, and higher deacetylation degree. The above material improved the stability of the soil aggregates by 100 to 200 times after just one cycle of wetting–drying, whereas chitosan with a higher molecular mass, higher viscosity, and lower deacetylation degree reduced the water stability of the soil aggregates after a single wetting–drying cycle, and caused it to increase 1.5- to 20-fold after 10 wetting–drying cycles. The water stability of the soil aggregates (time of aggregate destruction in water) did not correlate with the wettability of the soil (water drop penetration time). Wetting–drying cycles consolidated the structure (increased the soil bulk density) of the soils containing chitosans. The effect of chitosan on the water stability and wettability of soils depended on the physicochemical properties of the chitosan. No correlations were found between soil pH, organic matter, or clay content and the water stability of soils containing chitosan.

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G.J.; project administration G.J.; funding acquisition G.J. All authors have read and agreed to the published version of the manuscript.

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Impact of Chitosan on the Mechanical Stability of Soils

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Abstract: Chitosan is becoming increasingly applied in agriculture, mostly as a powder, however little is known about its effect on soil mechanical properties. Uniaxial compression test was performed for cylindrical soil aggregates prepared from four soils of various properties (very acidic Podzol, acidic Arenosol, neutral Fluvisol and alkaline Umbrisol) containing different proportions of two kinds of chitosan (CS1 of higher molecular mass and lower deacetylation degree, and CS2 of lower molecular mass and higher deacetylation degree), pretreated with 1 and 10 wetting–drying cycles. In most cases increasing chitosan rates successively decreased the mechanical stability of soils that was accompanied by a tendential increase in soil porosity. In one case (Fluvisol treated with CS2) the porosity decreased and mechanical stability increased with increasing chitosan dose. The behavior of acidic soils (Podzol and Arenosol) treated with CS2, differed from the other soils: after an initial decrease, the strength of aggregates increased with increasing chitosan amendment, despite the porosity consequently decreasing. After 10 wetting–drying cycles, the strength of the aggregates of acidic soils appeared to increase while it decreased for neutral and alkaline soils. Possible mechanisms of soil–chitosan interactions affecting mechanical strength are discussed and linked with soil water stability and wettability.

Keywords: aggregate; chitin derivatives; food wastes; stress; strain; Young's modulus

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1. Introduction

Due to its non-toxicity, bacteriostatic properties, and polycationic nature, chitosan has found many industrial, medical, pharmaceutical, and environmental applications [1–3]. The availability of industrial quantities of chitosan in the late 1980s enabled it to be tested in agriculture [4]. Up to date, chitosan has been increasingly applied for the improvement of the degradative capacity of contaminated soils by introducing specific consortia of microorganisms (bioaugmentation), providing nutrients to the chemical degrading indigenous microorganisms present at the contaminated sites (biostimulation), biochemical degradation of xenobiotic compounds. Chitosan is used as a carrier for microbial communities that can be encapsulated for further use and for providing resistance to the enzymes from harsh environmental conditions like pH and temperature. It is applied as an antiviral, antibacterial, antifungal and antinematode agent, for seeds coatings, and plant protection by controlling the spreading of diseases [5,6]. Chitosan has been proven to stimulate plant growth and yield, and to induce abiotic and biotic stresses tolerance in various commodities [7,8]. Besides that, chitosan has been employed in soil as a plant nutrient and has shown great efficacy in combination with other industrial fertilizers without affecting the soil's beneficial microbes. Furthermore, it is helpful in reducing fertilizer losses thus reducing the overuse of synthetic fertilizers during crop production, which is important in keeping environmental pollution under check. More recently chitosan is applied as a valuable delivery system for fertilizers, herbicides, pesticides, and micronutrients for crop growth promotion by maintaining balanced and sustained nutrition [1,4,9,10]. Various composite or copolymer systems containing chitosan are used to improve the soil's water-holding

capacity [11,12]. Chitosan is also applied for the removal of various types of contaminants from soils [13,14].

Chitosan introduced into a soil in one or more above ways can, as any other biopolymer, interact with soil components in different ways, including adsorption of polymer molecules on surfaces of soil components, covering soil particles with a thin polymer film, formation of polymer ties connecting neighboring particles, adhesion, hydrogen bonding or bridging of soil particles via polyvalent counterions [15,16]. Direct binding of a charged polymer to negatively charged soil components (clay minerals, silica, feldspars, organic matter) with electrostatic forces seems to be a rare feature of chitosan, because, contrary to the majority of natural biopolymers applied for soil treatment (tragacanth, arabic, karaya, gellan, carrageenan, locust bean, xanthan, guar and/or tamarind gums, agar, pectin, alginate, arabinans, amylase, lipids, kasein, cellulose) having anionic or non-ionic character [17–19], it carries a positive charge in a broad range of neutral and acidic pH values [20]. So strong interactions of chitosan with soil components imply that the addition of chitosan should increase soil mechanical resistance leading to soil stabilization, consolidation and hardening. However, among various biopolymers implemented in recent years for soil stabilization, erosion mitigation, and dust control, chitosan has been applied rarely. Huang et al. [17], in their comprehensive review of the application of various polymers for soil stabilization, mentioned only a single application of chitosan. Most probably the application of chitosan for soil stabilization is limited by its very poor solubility in water which changes to some extent depending on molecular weight, deacetylation degree and crystal structure [21–23] and/or by its high biodegradation rate (studies of Mostafa et al. [24] showed the fastest biodegradation of chitosan as compared to the other studied biopolymers). Apart from the successful application of various interpolymer complexes, nanoparticles or composites including chitosan [25–31], a positive effect on soil stabilization by pure chitosan has been achieved only for its highly dispersed jellified forms (chitosan dissolved usually in organic acids). Orts et al. [28] applied chitosan gel for hardening eroded furrows. They observed a strong effect of soil stabilization in laboratory conditions, however, under natural conditions, the effect was observed only in the initial part of the furrow, which was explained by the very strong binding of chitosan to soil components in the area close to the zone of its application. Shariatmadari et al. [32] studied the effects of chitosan dissolved in acetic acid on sandy soil stabilization by using unconfined compression tests. The unconfined compression tests conducted after 7, 14 and 28 days showed the strength of samples treated in dry conditions was higher than for samples cured in saturated water conditions. In contrast, Hataf et al. [27] who studied uniaxial compression of sand–chitosan gel mixtures, observed that chitosan improved soil strength, but its effect decreased with the reduction of water. They concluded that the additional strength that could be achieved with the addition of chitosan to the soil fades as the soil becomes dry. Aguilar et al. [33] investigated the feasibility of using chitosan dissolved in acetic acid as an admixture, or as an external coating, for earthen constructions to improve their resistance in terms of both their key mechanical properties and, more specifically, during the occurrence of water-induced degradation. They showed that in both instances the use of low concentrations of chitosan can significantly improve performance.

However, for agricultural purposes, the application of chitosan gels containing huge amounts of acid may be dangerous for the soil environment because of the potential for inducing aluminum toxicity, mineral destruction, nutrient leaching or heavy metal mobilization [34–36]. In agricultural applications, chitosan is usually introduced into the soil mostly as powders (pure components and admixtures), and also as membranes (chitosan coatings) or particulates (suspensions) [1,2,10,37–41]. Despite this, the literature brings extremely small information on the mechanical properties of soils amended with chitosan, not in a gel but in a powder form. After an extensive search, the authors found only a single paper on this topic by Soldo et al. [42], who studied the mechanical properties of Piedmont well-graded sand with silt, amended with various biopolymers, including chitosan. They mentioned that chitosan was applied in a form as it was supplied by the

manufacturer (probably as the powder). After five days of curing, chitosan did not lead to any further increase in the compressive strength even though the specimens with chitosan had the highest compressive strength right after the mixing. Therefore they excluded chitosan from further research of this type.

To fill this knowledge gap, this paper studies the mechanical stability of a few soils amended with different doses of chitosan powder by a uniaxial compression test. Because various chitosan–soil binding mechanisms contribute differently depending on polymer solubilization, concentration, charge, conformation, molecular weight and molecule size, that in turn depend on the ambient solution pH and ionic strength [43–45], four soils of different pH values and two types of chitosan, differing in molecular masses and deacetylation degrees were selected for the testing. Since cyclic wetting–drying affects biopolymer treated soils [46–48] and is used as a measure of the durability of biopolymers [46], the studied soils were subjected also to one and ten wetting–drying cycles.

2. Results

The addition of chitosan of high molecular mass and low deacetylation degree (CS1, see M&M section) in the rates of 0.5, 1, 2, 4 and 8% decreased, in general, the mechanical strength of four studied soils: very acidic Podzol, acidic Arenosol, neutral Fluvisol and alkaline Umbrisol. The intensity of this decrease depended on chitosan dose and the number of wetting–drying cycles with which the soil–chitosan mixtures were pretreated, as is seen from exemplary stress–strain curves of soil cylindrical aggregates shown in Figure 1.

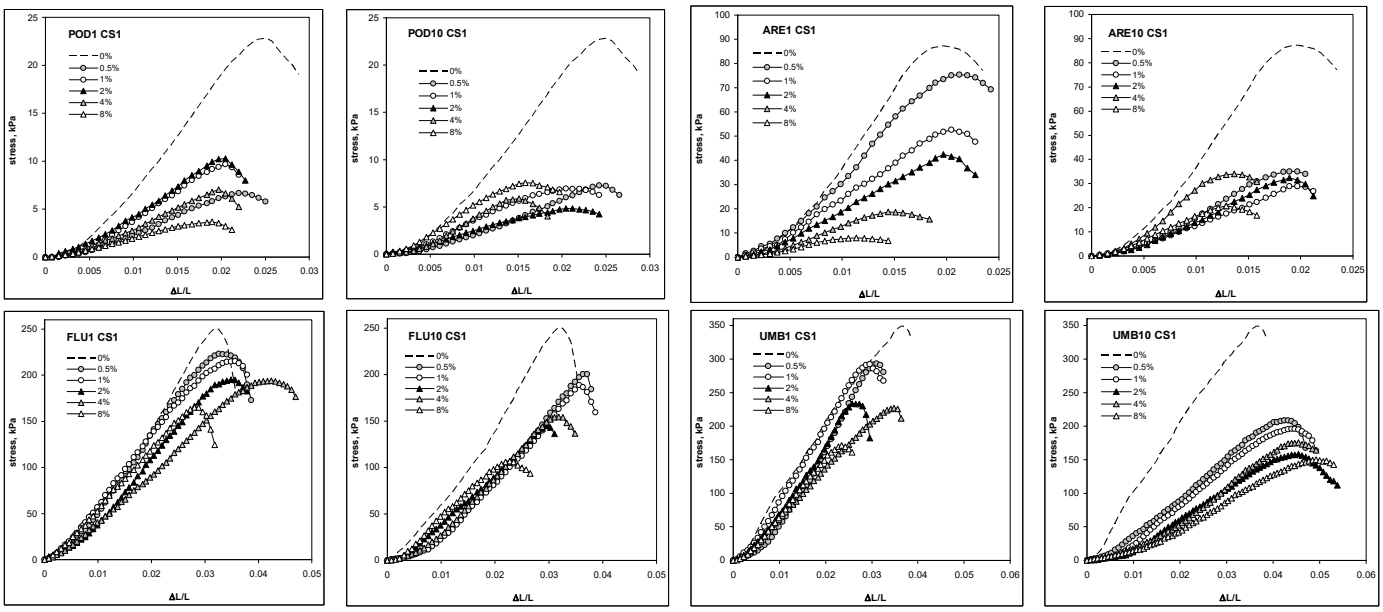

Figure 1. Exemplary compression stress-dilatation dependence for the aggregates of Podzol (POD), Fluvisol (FLU), Umbrisol (UMB) and Arenosol (ARE) amended with different amounts of chitosan CS1, preconditioned with 1 or 10 cycles of wetting–drying. The number of the cycles is written after the abbreviation of each soil.

The addition of chitosan of lower molecular mass and higher deacetylation degree (CS2, see M&M section) in the same rates of 0.5, 1, 2, 4 and 8% decreased the mechanical strength of Podzol, Arenosol and Umbrisol to the lower extent than CS1, and increased the mechanical strength of Fluvisol. Similarly, as for CS1, the intensity of these changes depended on chitosan dose and on the number of wetting–drying cycles with which the soil–chitosan mixtures were pretreated. Exemplary stress–strain curves of soil cylindrical aggregates containing CS2 are shown in Figure 2.

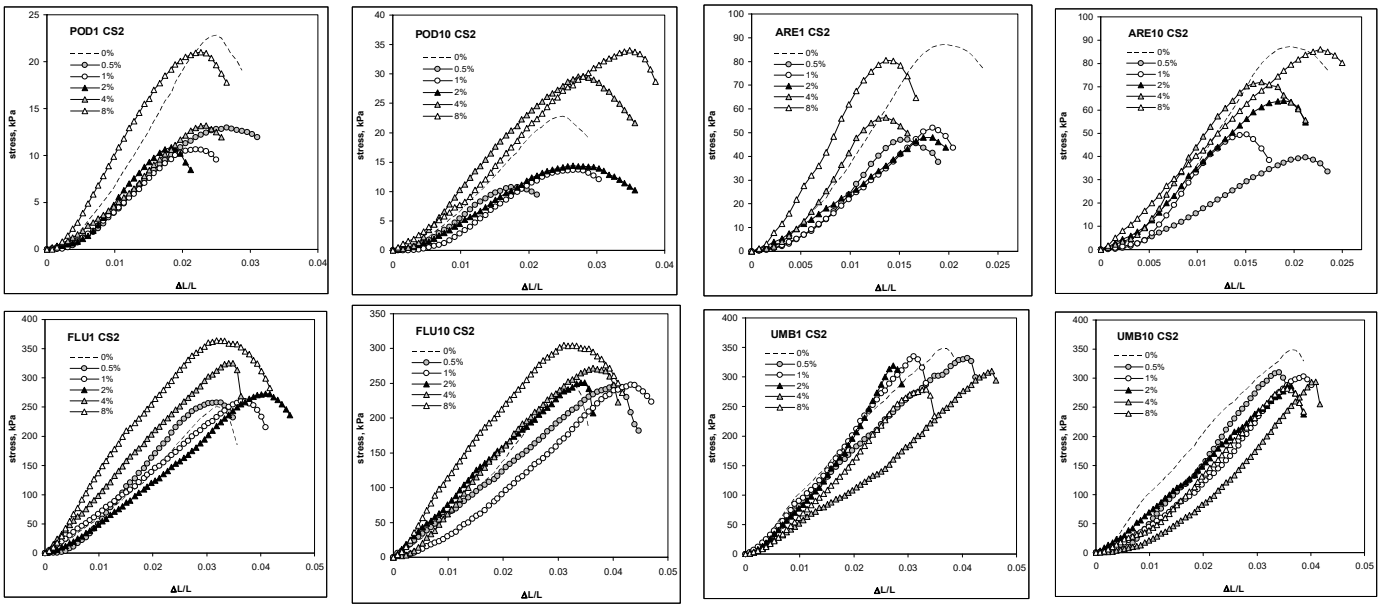

Figure 2. Exemplary compression stress-dilatation dependence for the aggregates of Podzol (POD), Fluvisol (FLU), Umbrisol (UMB) and Arenosol (ARE) amended with different amounts of chitosan CS2, preconditioned with 1 or 10 cycles of wetting–drying. The number of the cycles is written after the abbreviation of each soil.

The maxima of the dependencies shown in Figures 1 and 2, expressing a load per unit average of the cross-sectional area at which the cylindrical specimen of soil fails under compression during the Unconfined Compression Strength, UCS [Pa], are presented in Figure 3.

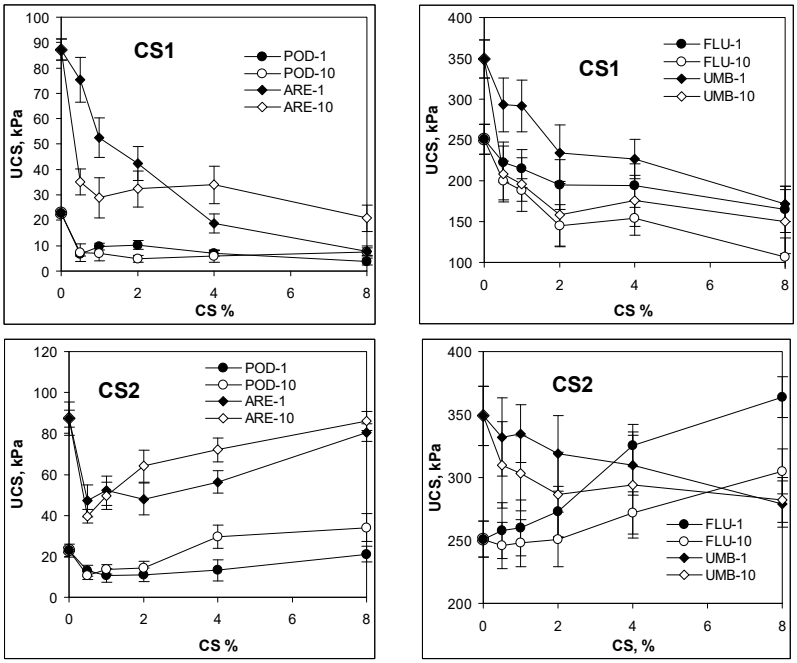

Figure 3. Average values of unconfined compressive strength of the studied aggregates of Podzol (POD), Fluvisol (FLU), Umbrisol (UMB) and Arenosol (ARE) amended with different amounts of chitosan (CS1 and CS2), preconditioned with one or ten cycles of wetting–drying. The number of the cycles is written after the abbreviation of soils. Error bars depict standard deviations.

The UCS of the aggregates containing CS1 generally decreases with the increase in chitosan percentage. A similar effect is observed for CS2 amended Umbrisol. Both acidic soils (Podzol and Arenosol) amended with CS2 behave in a different way: after the initial drop, the UCS increases with increasing chitosan dose, finally approaching the UCS value for the nontreated soil. Quite opposite behavior is noted for CS2 amended Fluvisol, for which the UCS seems to tendentially increase with an increase in the chitosan rate.

The UCS of neutral and alkaline soils (Fluvisol and Umbrisol, respectively) after 10 wetting/drying cycles is lower than the UCS of these soils subjected to a single cycle, whereas the increase in the number of wetting–drying cycles increases the UCS of acidic soils, Podzol and Arenosol, containing higher doses of both kinds of chitosan.

Similar dependencies were observed for Young's modulus and chitosan percentage, since, as commonly observed, UCS and Young's modulus are roughly proportional to each other, as it is presented in Figure 4 for the studied soils.

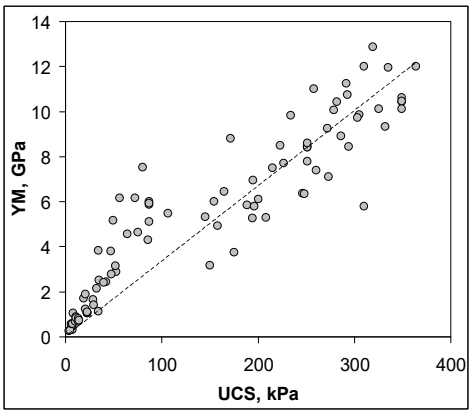

Figure 4. Dependence of Young's (YM) modulus and unconfined compressive strength (UCS) for all studied aggregates.

Structural porosity is considered to be the best measure of the susceptibility of soils and other granular materials to mechanical damage: more porous aggregates would break easier. The porosities of the studied soil/chitosan aggregates, preconditioned with one or ten wetting–drying cycles, are presented in Figure 5.

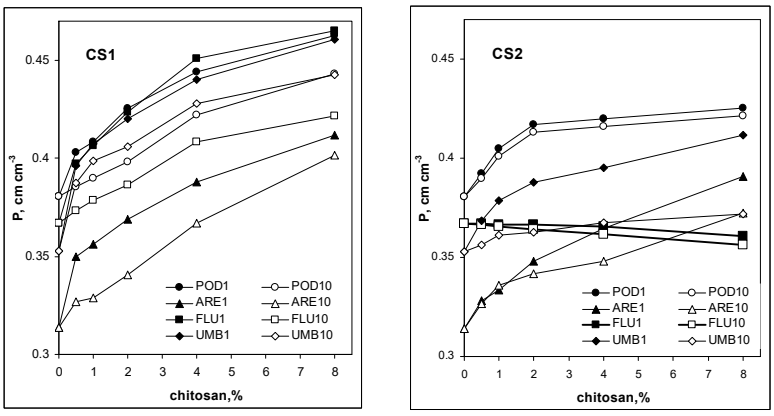

Figure 5. Dependencies of the porosity (P) of soil aggregates on the amendment of the studied chitosan (CS1 and CS2). The soils are abbreviated as: POD—Podzol, ARE—Arenosol, FLU—Fluvisol, UMB—Umbrisol. The number after the soil abbreviation shows the number of wetting–drying cycles applied to soil aggregates. Error bars are covered by the labels of the points.

In general, the addition of both types of chitosan causes a loosening of the soil structure (increasing porosity) with an exception of Fluvisol amended with CS2, for which chitosan

addition decreased soil porosity and made the soil more compact. It was observed that CS1 increased soil porosity more than CS2 and the structure became less porous with an increase in the number of wetting–drying cycles.

3. Discussion

Changes in UCS due to the addition of CS1 accompany the opposite changes in porosity for all soils. This trend is valid also for CS2 amended Fluvisol and Umbrisol. However, for very acidic Podzol and acidic Arenosol despite the porosity tendentially increasing with increasing chitosan dose, after the initial drop, the UCS increases also. The increase in porosity observed in the vast majority of the studied soil/chitosan aggregates is most probably caused by the loosening of the soil structure by coarser chitosan particles. However, Kubavat et al. [49] mentioned about soil porosity increase also after the addition of nanoparticles of chitosan copolymerized with methacrylic acid. The higher effect of CS1 on the porosity increase may indicate that CS1 is composed of larger particles than CS2. The exceptional decrease in porosity and a simultaneous increase in UCS for Fluvisol amended with CS2 can be probably connected with the highest content of clay in this soil. In the control soil, the total volume of porous conglomerates of clay particles may exceed the total volume of the soil skeletal pores (free spaces between coarser soil particles) and the coarser particles are "suspended" in the clay phase. In such a case, the addition of nonporous chitosan particles replaces porous clay conglomerates and so the volumetric porosity decreases. In the "suspended" state, rigid skeletal particles rarely contact each other and the mechanical stability of such a system approaches that of the pure clay itself. Additional skeletal porosity created by coarser chitosan particles may be consecutively filled by clay and more contacts between coarser particles occur, and so, the mechanical strength increases. As reported by Jozefaciuk et al. [50] and Horabik and Jozefaciuk [51] there exists a maximum of UCS at a point where all free spaces between coarser particles are totally filled by clay conglomerates and no "free" clay excess is present. Such be the case, increasing the volume of clay makes the UCS smaller. The fact that a similar phenomenon does not occur for CS1 may be because CS1 particles are larger than those of CS2, and, only with the smallest CS1 additions does the volume of newly created free skeletal spaces exceed the volume of the excess of the clay which is available for their total filling.

The other exceptional behavior which needs more detailed examination is that in soils of low pH (Podzol and Arenosol) higher CS2 doses induced the increase in UCS. Electrostatic bonds of positively charged chitosan in a low pH range (PZC of chitosan is usually reported to occur at pH values around 6.5–7.6 [52–54]) with negatively charged soil particles may be responsible for this effect. In soils of higher pH, chitosan around its PZC develops either a small amount of positive charge or a small amount of negative charge on a small surface area, and the electrostatic forces that are generated with negatively charged soil components can range from weakly attractive to weakly repulsive. Additionally, different dissolution/jellification patterns of both studied types of chitosan may exert a significant effect on their interactions with soil components because chitosan gel of highly extended surface can form much more electrostatic bonds than chitosan particles of much smaller surface area. Because CS2 of lower molecular mass dissolves better and faster in soil organic acids (fulvic and humic acids) than CS1 having a higher molecular mass, the effect on UCS increase was observed only in the case of CS2. In a jellified form, chitosan can also glue soil particles together by adhesive forces or by the formation of polymer ties connecting neighboring soil particles which are not in direct contact [55]. Differences between the action of both kinds of chitosan on soils are apparently connected to their molecular properties. As it has been frequently reported, molecular weight and molecule size of polymers play a key role in defining the nature of interactions with various soils and soil minerals. Moen and Richardson [56] found that small-sized polymers distribute more evenly in the microaggregate fraction of soils because of their greater ability to penetrate the fine pores. Richardson et al. [57] also found that high molecular weight polymers may maximize soil–polymer interactions; however, the effectiveness could be affected by

limited polymer penetration of the soil surface and failure to attain uniform aggregation adsorption. On the other hand, they found that small-sized polymers could create a more homogeneous soil stabilizing polymer network.

The effect of wetting–drying cycles on soil mechanical resistance depended both on the soil reaction and the kind of chitosan. Ambient solution pH and concentration affect the surface charge and conformation of charged polymers and therefore influence polymer adsorption onto soil particles. A higher concentration of the polymer solutions enables sufficient active functional and structural groups in the polymer to be available for interaction with soil particles and therefore could increase the efficiency of polymer stabilization [45]. Since solubilization and gelling are time-dependent, an increase in the mechanical stability of soil–chitosan mixtures in time occurs in acidic soils. In contrast, for neutral Fluvisol and alkaline Umbrisol, wherein chitosan around its PZC is hardly soluble [58], increasing the number of wetting/drying cycles leads to a decrease in UCS. A similar decrease in the strength of biopolymer treated soils with increasing wetting–drying cycles has been frequently reported [48,59].

As was observed by Adamczuk et al. [60] the water stability of aggregates containing CS1 preconditioned with a single cycle of wetting–drying was generally lower than for aggregates of the control soils. After ten wetting–drying cycles, these aggregates became more water-resistant. In contrast to CS1, the water stability of CS2-containing aggregates reached high water resistance just after the first drying–wetting cycle and it increased only slightly after the next nine wetting–drying cycles. Both types of chitosan increased soil water repellency, which increased further after 10 wetting–drying cycles. The wettability for CS1 amended soils was higher than for CS2. Both water stability and wettability may increase because of the high wetting angles of chitosan, according to Cassie's law [61]. The effect of chitosan on increasing water stability and water repellency might have been caused also by adsorption of the dissolved chitosan molecules on soil components. Even very small amounts of large chitosan molecules can cover the surface of soil particles to a great extent, forming hydrophobic patches. It is possible that this mechanism may decrease the mechanical stability of the aggregates, as well. If, as is likely, the surface covered by chitosan molecules becomes flatter (the surface roughnesses are levelled), the internal friction between smoother soil particles is reduced, and, in consequence, the UCS becomes smaller. It is probable that such an occurrence may be proven by studies of the fractal dimension of chitosan adsorbed soil (a decrease in fractal dimension would indicate surface flattening), which will be a problem in further studies.

Summarizing: coexistence of several antagonistic and synergic mechanisms can explain the observed impact of chitosan on the mechanical and water stability, and wettability of soils. The first is the loosening of soil structure (increase in porosity) due to the addition of coarse chitosan particles that can decrease both mechanical and water stability. However, in clayey soils addition of coarser chitosan particles can make the soil more mechanically resistant. The second mechanism is the formation of electrostatic bonds between positively charged chitosan particles (or jellified/dissolved chitosan molecules) and negatively charged soil components leading to an increase of both mechanical and water stability. This phenomenon would increase with a decrease in soil pH due to better charging and solubilization of chitosan at low pHs. The third mechanism is the formation of adhesive bonds between chitosan and soil components, which, similarly to the previous mechanism, should depend on the amount of the dissolved/jellified chitosan. The fourth mechanism is the adsorption of chitosan molecules on surfaces of soil components causing an increase in soil water stability and hydrophobicity of the soil material along with a possible reduction of the mechanical stability of the soil due to decreasing surface roughness and internal friction. The intensity of all aforementioned mechanisms depending on chitosan jellification, solubilization, surface charge and chain stiffness are governed by the ambient pH [44] and on physicochemical properties of a given kind of chitosan.

4. Materials and Methods

Two kinds of chitosan selected to differ in molecular mass and deacetylation degree were used. The first, abbreviated as CS1, was provided by Sigma Aldrich (St. Louis, MO, USA) and the second (CS2) was provided by Beijing Be-Better Technology Co., Ltd. (Beijing, China). Both forms of chitosan were composed of equal parts of the 0.105–0.053 and 0.177–0.105 mm dry sieved fractions. Soil samples were taken from upper 5–15 cm layers of four soils localized in East Poland, air-dried and screened by a 1 mm sieve. The basic properties of the studied soils and types of chitosan are presented in Table 1. This table recalls data reported by Adamczuk et al. [60] who studied water stability and wettability of the same materials as used in the present paper.

Table 1. Characteristics of the studied soils and chitosan.

Abbreviation	POD	ARE	FLU	UMB	CS1	CS2
Material	Podzol	Arenosol	Fluvisol	Umbrisol	Chitosan1	Chitosan2
Locality E	22°58′41″	22°26′6″	22°59′38″	21°41′59″		
Locality N	51°9′14″	51°2′9″	51°9′43″	50°49′25″		
pH	4.1	5.5	6.5	7.7		
Particle density [g cm^{-3}]	2.54	2.62	2.62	2.68	1.51	1.54
Nitrogen [%]	0.16	0.13	0.46	0.14	7.51	7.79
Total organic carbon [%]	0.65	1.55	3.04	0.9	41.59	41.27
Sand (0.063–2 mm) [%]	72.4	47.1	20.2	10.4		
Silt (0.002–0.063 mm) [%]	25.9	46.2	52.2	72.4		
Clay (<0.002 mm) [%]	1.7	6.7	27.6	17.2		
Grain fraction 0.177–0.105 mm [%]					50	50
Grain fraction 0.105–0.053 mm [%]					50	50
Degree of Deacetylation (DD)					0.77	0.91
Average molecular mass (M), [kDa]					699	280

4.1. Preparation of Soil/Chitosan Aggregates

The soil samples were air-dried and screened by a 1 mm sieve (mesh 18). Carefully homogenized water-saturated pastes were prepared from mixtures of the soils and the chitosan. Distilled water was used for pastes preparation. The content of CS1 and/or CS2 in the mixtures was 0 (control), 0.5, 1, 2, 4, and 8%. Cylindrical aggregates of 20 mm height and 10 mm diameter were formed from the pastes using plastic forms. The first set of aggregates was prepared just after the preparation of the paste and then air-dried (one cycle of wetting–drying) and the second set from the pastes was subjected to 10 wetting–drying cycles (6 days per cycle). All aggregates were then dried until constant mass at laboratory conditions (relative humidity around 60% and temperature around 25 °C). The aggregates are abbreviated further using the abbreviation of a given soil (see Table 1) followed by the number of wetting/drying cycles (e.g., POD1 and POD10 denote aggregates formed from Podzol preconditioned with one and ten wetting/drying cycles, respectively).

4.2. Studies of Soil/Chitosan Aggregates

Unconfined compression tests were performed for ten replicates of each aggregate using the Lloyd LRX material testing machine (Bognor Regis, UK). An aggregate placed vertically on the machine basement was pressed by a piston. The load measured with the accuracy of 0.05 N against displacement of the piston moved with the lowest apparatus speed of 10^{-5} m·s^{-1} was registered. The dependence of the compression stress [Pa], (load divided by the aggregate cross-sectional area) versus strain, $\Delta L/L$ (relative aggregate deformation, equal to piston displacement divided by the aggregate height) was calculated. From these dependencies values of the Unconfined Compression Strength UCS [Pa] (maximum load per unit average cross-sectional area at which the cylindrical specimen of soil falls in compression) and Young's modulus, E [Pa] (slope of the linear parts of the stress–strain dependencies) were estimated.

Volumetric porosity (volumetric fraction of pores on the total volume), P, [cm^3 cm^{-3}] of the aggregates were estimated as follows: at first, the volumes of the solids (soil + chitosan)

present in 1 cm^3 of the aggregates, V_s [cm^3], were calculated based on the chitosan percentage in the soil–chitosan mixtures (CS%), the respective particle densities (PD_{soil} and PD_{CS1} or PD_{CS2}, presented in Table 1) and the aggregate bulk densities, BD [g cm^{-3}] reported in [60]:

$$V_s = 1 - [BD \cdot (1 - CS\%/100)/PD_{soil} + BD \cdot (CS\%/100)/PD_{CS}] \quad (1)$$

and next the porosity was calculated as:

$$P = 1 - V_s. \quad (2)$$

4.3. Statistical Analysis

Average values ± standard deviations were calculated.

Author Contributions: Conceptualization, A.A. and G.J.; methodology G.J. and A.A.; experiments: A.A.; software G.J.; data elaboration: G.J.; writing of the original draft: A.A., G.J.; review and editing: G.J. and A.A.; visualization G.J.; supervision G.J.; project administration G.J.; funding acquisition G.J. All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples of the soils and chitosan are available from the authors.

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Review

Chitosan: An Overview of Its Properties and Applications

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Abstract: Chitosan has garnered much interest due to its properties and possible applications. Every year the number of publications and patents based on this polymer increase. Chitosan exhibits poor solubility in neutral and basic media, limiting its use in such conditions. Another serious obstacle is directly related to its natural origin. Chitosan is not a single polymer with a defined structure but a family of molecules with differences in their composition, size, and monomer distribution. These properties have a fundamental effect on the biological and technological performance of the polymer. Moreover, some of the biological properties claimed are discrete. In this review, we discuss how chitosan chemistry can solve the problems related to its poor solubility and can boost the polymer properties. We focus on some of the main biological properties of chitosan and the relationship with the physicochemical properties of the polymer. Then, we review two polymer applications related to green processes: the use of chitosan in the green synthesis of metallic nanoparticles and its use as support for biocatalysts. Finally, we briefly describe how making use of the technological properties of chitosan makes it possible to develop a variety of systems for drug delivery.

Keywords: chitosan; chitin; biological activity; drug delivery; antioxidant; antimicrobial; metallic nanoparticles; biocatalysis

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1. Introduction

Chitin and its deacetylated derivative, chitosan, are a family of linear polysaccharides composed of varying amounts of (β1→4) linked residues of *N*-acetyl-2 amino-2-deoxy-D-glucose (glucosamine, GlcN) and 2-amino-2-deoxy-D-glucose (*N*-acetyl-glucosamine, GlcNAc) residues. Chitosan is soluble in aqueous acidic media via primary amine protonation. In contrast, in chitin, the number of acetylated residues is high enough to prevent the polymer for dissolving in aqueous acidic media.

Chitin is a very abundant biopolymer that can be found in the exoskeleton of crustacea, insect's cuticles, algae and in the cell wall of fungi. Chitosan is less frequent in nature occurring in some fungi (*Mucoraceae*). Historically, commercial chitosan samples were mainly produced from chemical deacetylation of chitin from crustacean sources. More recently, chitosan from fungi is gaining interest in the market, driven by vegan demands. Moreover, these samples are better controlled in terms of low viscosity and exhibit a very high deacetylation degree [1]. Production from insect cuticles is also gaining interest, driven by the increased interest in protein production from these sources.

The interest in chitin and chitosan relies on the myriad biological and technological properties exhibited by these polymers (Table 1). However, these properties are tightly related to the physicochemical properties of the polymers (mainly molecular weight and acetylation degree) [2]. Therefore, when working with chitin and chitosan a good and completed polymer characterization is mandatory. Several methodologies have been described to characterize chitin, chitosan and chitooligosaccharides, a description of which is far from the objective of this paper—but for interested readers, we recommend publications [3,4].

Table 1. The main properties of chitin and chitosan.

Property/Activity	Reference
Mucoadhesive	[5,6]
Anti-inflammatory	[7]
Antioxidant	[8]
Antimicrobial	[9]
Antifungal	[10]
Antihyperglycemic	[11]
Antitumoral	[7–12]
Wound healing	[13]

Chitosan is the only polycation in nature and its charge density depends on the degree of acetylation and pH of the media. The solubility of the polymer depends on the acetylation degree and molecular weight. Chitosan oligomers are soluble over a wide pH range, from acidic to basic ones (i.e., physiological pH 7.4). On the contrary, chitosan samples with higher Mw are only soluble in acidic aqueous media even at high deacetylation degrees. This lack of solubility at neutral and basic pH has hindered the use of chitosan in some applications under neutral physiological conditions (i.e., pH 7.4). This is the reason why a great number of chitosan derivatives with enhanced solubility have been synthetized.

In 2019, the global chitosan market size was valued at USD 6.8 billion, and it is expected to expand at a revenue based CAGR of 24.7% between 2020 and 2027. The drivers for the market's growth are the increasing application of the polymer in water treatment and several high-value industries such as the pharmaceutical, biomedical, cosmetics and food industries [14]. Some of the interest areas identified include the modification of the polymers to extend their applicability; knowledge of the mechanisms involved in the biological activity of chitosan, chitosan derivatives and chitooligosaccharides; and the in-depth study of chitosanolytic and chitinolytic enzymes presented in different microorganisms [15].

This review aims to provide readers with a general overview of the state of the art of chitosan science, covering different aspects such as polymer chemistry, biological and technological properties and applications in drug delivery and as a biocatalyst.

2. Technological Chitosan Properties

2.1. Solubility

Chitosan is produced by deacetylation of chitin; in this process, some *N*-acetylglucosamine moieties are converted into glucosamine units. The presence of large amounts of protonated $-NH_2$ groups on the chitosan structure accounts for its solubility in acid aqueous media since its pKa value is approximately 6.5 [16]. When around 50% of all amino groups are protonated, chitosan becomes soluble [17].

Chitosan solubility depends on different factors such as polymer molecular weight, degree of acetylation, pH, temperature, and polymer crystallinity. Homogeneous deacetylation (alkali treatment, 0 °C) of chitin permits the production of polymers soluble in aqueous acetic acid solutions with DD as low as 28%, with this value never being reached under heterogeneous deacetylation (alkali treatment, high temperatures). Moreover, with a DD of 49%, the samples are soluble in water. This behaviour is explained by the fact that homogeneous deacetylation leads to an increase in the number of glucosamine units and a modification in the crystalline structure of the polymer. Depending on polymer DD, these modifications range from a reduction in crystal size and crystal perfection to the presence of a new crystal structure close to β-chitin [18]. Sogias et al. [19] studied the role of crystallinity and inter- or intramolecular forces on chitosan solubility; in this work, a parent chitosan sample was half re-acetylated with anhydride acetic or fully *N*-deacetylated under homogeneous conditions. After reacetylation, the solubility of the polymer was expanded until pH 7.4, while a slight reduction in the solubility range of the fully deacetylated chitosan was determined. The lower solubility was explained due to the increase in the polymer crystallinity after deacetylation, which offsets the effect of the increase in

glucosamine moieties. On the contrary, a reduction in the crystallinity was observed in the half-acetylated sample. The use of hydrogen bond disruptors such as urea or guanidine hydrochloride also alters the solubility window of chitosan. In fact, by a combination of chemical and physical disruption of the hydrogen bonds, broad solubility is achieved.

2.2. Viscosity

The viscosity of polymers is a parametre of great interest from the technological point of view since highly viscous solutions are difficult to manage. Moreover, viscometry is a powerful tool for determining chitosan's molecular weight, as it is a simple and rapid method even though it is not an absolute method, therefore requiring the determination of constants that are specific to the solvent. The average molecular weight is determined by the Mark–Houwink–Sakurada equation, which relates this parametre with the intrinsic viscosity:

$$\eta = KM_v{}^{\alpha} \quad (1)$$

where K and α are constants that must be determined experimentally. Several values of K and α have been reported depending on the solvent composition, pH, and ionic strength [20]. Chitosan viscosity depends on the molecular weight of the polymer and deacetylation degree and decreases as the molecular weight of chitosan is reduced. In fact, viscosity can be used to determine the stability of the polymer in solution, as a reduction is observed during polymer storage due to polymer degradation [21]. Shear viscosity increases with chitosan deacetylation degree. The shear viscosity at the same rate was studied in two samples with different deacetylation degrees (91% vs. 75%) and represented versus intrinsic viscosity [22]; it was reported that shear viscosity was larger for those samples with the highest deacetylation degree; when the curves were evaluated, straight lines were observed in both chitosan samples This is explained due to the nature of chitosan, as this polymer is a cationic polyelectrolyte because of the amine protonation in acidic media. Therefore, the higher the DD, the larger chain expansion is expected, as more glucosamine units are found in the polymer chain, leading to a greater charge density in this sample. In order to modulate chitosan viscosity, the addition of different co-solvents has been evaluated; in this sense, Kassai et al. [20] studied the effect of the addition of isopropanol and ethanol to a chitosan solution in 1% acetic acid, reporting that the presence of the cosolvents decreased the intrinsic viscosity of the polymer.

3. Chemistry of Chitosan

As seen in Figure 1, the reactive groups found in chitosan are a primary amino group (C2) and primary and secondary hydroxyl groups (C6, C3). Glycosidic bonds and the acetamide group can also be considered functional groups. These functional groups allow for a great number of modifications, producing polymers with new properties and behaviours.

Chitosan derivatives have been produced, aiming to improve chitosan's properties, such as solubility or biodegradability, or to introduce new functions or properties. For instance, solubility has been improved in water aqueous media by deacetylation, depolymerization, or quaternisation among other processes [23]. New chitosan activities have been reported after its modification, for example, 6-O-sulphated chitosan promotes neuronal differentiation while phosphorylated chitosan inhibits corrosion [24,25].

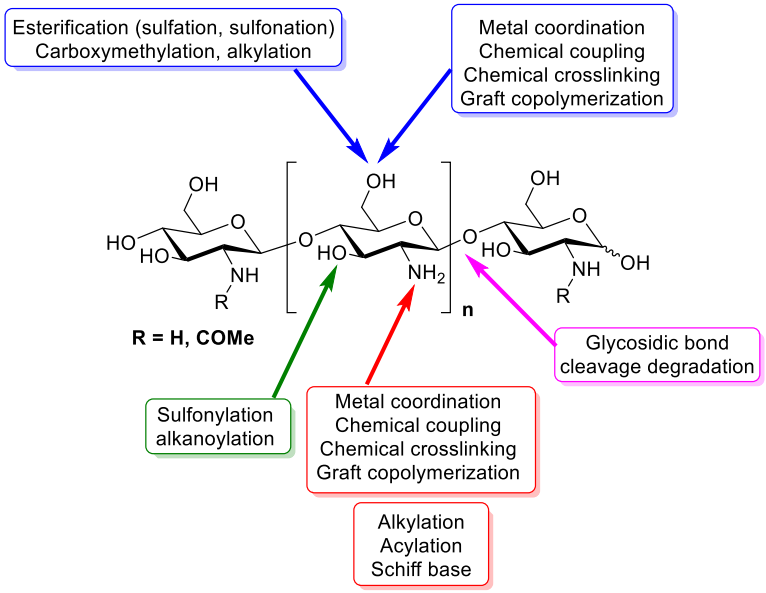

Figure 1. Functional groups in chitosan's structure that are able to be chemically modified.

The field of chitosan chemistry is wide, and in this review, we want to focus on two types of processes, chitosan phosphorylation and chitosan degradation. Our group has participated in the development of a phosphorylated derivative via a simple method in which chitosan and phosphorus acid are mixed at the same ratio and formaldehyde is added at 70 °C [26] (Figure 2).

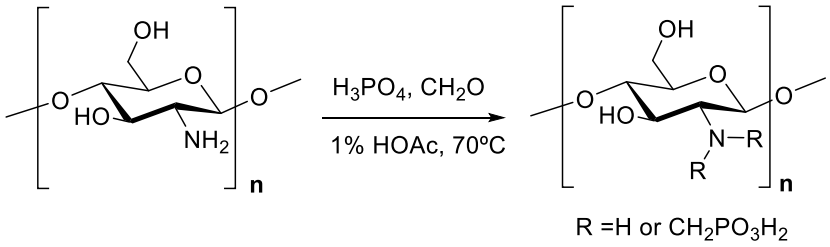

Figure 2. Scheme of phosphorylated chitosan derivative synthesis.

This *N*-methylene phosphonic chitosan is soluble in water and keeps the filmogenic properties of the parent chitosan. With a similar methodology, a soluble in water *N*-methylenephenyl phosphonic chitosan has been produced [27]. Additionally, the surfactant derivative *N*-lauryl-*N*-methylene phosphonic chitosan was produced via *N*-alkylation of *N*-methylene phosphonic chitosan [28]. This derivative has a lower solubility in aqueous media compared to *N*-methylene phosphonic chitosan but better solubility in organic media and forms micelles. *N*-methylene phosphonic *N*-methylene carboxylic chitosan has been obtained in water-soluble form using *N*-methylene phosphonic chitosan and glyoxylic acid. The polymer maintains the filmogenic properties of parent chitosan and, because of the presence of multidentate ligands, its use as a bivalent metal chelating agent is proposed [29].

Although the use of chitosan as a gene carrier has been reported, the use of this biopolymer for this application is limited due to a relatively low transgenic efficacy. Phosphorylated derivatives have shown better performance (transfection was improved 100-fold) and therefore are more suitable than chitosan to this end. Moreover, phosphorylated derivatives also exhibit and improve metal ion chelating activity when compared to the parent chitosan [30,31].

Due to the presence of cleavage glycosidic bonds, it is possible to degrade chitosan, thus reducing its molecular weight. As previously mentioned, the control of chitosan

depolymerization (polymer size) permits us to control some properties such as solubility or viscosity. Moreover, the biological and technological properties of chitosan are related to size, among other properties as previously reviewed [2]. Chitosan degradation can occur through different mechanisms such as acid hydrolysis, oxidative–reductive or nitrous acid depolymerization, ultrasonic degradation, or enzymatic degradation using specific and non-specific enzymes. Chitosan has four types of glycosidic linkages -D-D-, -A-A-, -A-D- and -D-A- (where A and D denote *N*-acetylglucosamine and glucosamine monomers, respectively). Depending on the process, there is a prevalence in the breakage of certain linkages and therefore different samples can be produced from the same parent chitosan by selecting different methodologies. Chemical and physical methods are less selective than enzymatic ones for producing specific patterns due to enzyme-specific recognition but by controlling the parametres of the process some control over the composition can be gained.

Ultrasonic degradation of chitosan does not affect the degree of acetylation or polydispersity of the recovered polymers allowing for the moderate degradation of the polymer [32]. The rate of degradation depends on the acetylation degree of the parent chitosan and not on the initial molecular weight [33].

Hydrogen peroxide produces random degradation of chitosan in a faster manner than ultrasonic methodologies, producing a significant number of monomers and chitooligosaccharides, the composition of which depends on the temperature and H_2O_2 concentration [34]. Nitrous acid depolymerization can be considered somewhat specific since HNO_2 attacks the primary amine in glucosamine and subsequently the cleavage of the glycosidic bonds occurs. That is, only the glycosidic linkage following a *D*-unit can be cleaved [35]. The chemical processes yield large amounts of monomers (*D*-glucosamine) and when the intended final products are chitooligosaccharides rather than low molecular weight chitosan, the yields are low [36]. HNO_2 provokes the formation of 2,5-anhydro-*D*-mannose at the new reducing end, which may be considered a disadvantage of this acid. When chitosan is degraded by HCl, the polymer not only suffers the hydrolysis of the *O*-glycosidic linkage between residues but also the *N*-acetyl linkage can be hydrolyzed but at a lower rate. The hydrolysis rate of D-D and D-A glycosidic linkages is lower than the hydrolysis of A-A and A-D, therefore the reducing ends are dominated by acetylated units [37]. By using a controlled precipitation method with methanol, it has been possible to obtain chitooligosaccharides with DPs up to 16 with few low molecular weight oligomers with a good yield [38].

The specific enzymatic degradation of chitosan occurs with a family of enzymes named chitosanases (EC 3.2.1.132) and chitinases (EC 3.2.1.14). Chitosanases are glycosyl hydrolases that catalyse the *endo* hydrolysis of β-1,4-glycosidic bonds of partially acetylated chitosan to release chitosan oligosaccharides (COS) with little monomer release [39]. Chitosanase specifically hydrolyses chitosan by cleavage of glycosidic bonds with a -DD·DA- pattern or a -DD·DD-pattern. Chitinases, which occur in families GH18 and GH 19, are glycosyl hydrolases that can degrade both A-A and A-D linkages and show no activity against D-D linkages. Chitinases can be classified into two major categories (endochitinases and exochitinases) according to their mode of action [40].

Non-specific enzymes, also called promiscuous enzymes, are also able to degrade chitosan. These enzymes belong to the protease, lipase, cellulase, and hemicellulase families, among others. Lysozyme is one of the most studied due to its relationship with polymer biodegradation. this enzyme is a protease that hydrolyses chitosan by cleavage of glycosidic bonds with A-A-A-A- pattern or A-A-A-D-pattern, while A-D-A-pattern or D-D-A-A are not or very slowly hydrolysed by lysozyme [14,17]. Apart from the previously mentioned lysozyme, other proteolytic enzymes such as pepsin, papain and pronase caused chitosan depolymerization, rendering low molecular chitosans (4–10 kDa) as the main products and chitooligosaccharides and monomers in smaller amounts. Results indicated that papain and pepsin had a similar action pattern. Both enzymes decreased LMWC acetylation degree when compared to the parent chitosan; DP 2–6 were detected in the supernatant monomers (D and A) and oligomers. Pronase showed different behaviour

since no glucosamine was detected. It showed selectivity through A-A and A-D, resulting in products having A monomers at the reducing end [41].

Neutral protease degraded chitosan in a manner dependent on the deacetylation degree. The higher the DD, the higher the Km and the lower the Vmax. During degradation, a reduction in the DD of the recovered LMW chitosans was observed. An analysis of the partially hydrolysed chitosan revealed that the enzyme degraded D-D and A-D β-1,4-glycosidic linkages, producing a mixture of hetero oligosaccharides carrying an A residue at the reducing end [42]. The same authors have studied the effect of the chitosan molecular weight in the enzymatic activity since this parametre affects its chain flexibility in solution, which in turn may affect its affinity for the enzyme in hydrolysis reactions. Their results showed a lower affinity of the enzyme with a slower degradation rate when high molecular weight chitosan samples were tested [43].

Hemicellulase, an enzyme related to the degradation of hemicellulose, has proven its ability to reduce chitosan molecular weight in a manner that depends on the deacetylation degree of the chitosan, rendering lower molecular weight samples when a chitosan sample with a DD of 85% was tested. Dimers, trimers, tetramers, pentamers and hexamers were observed after 4 hours of reaction, and the enzyme was considered endo-acting since no *N*-acetylglucosamine was detected [44].

Lipases have also proved their ability to hydrolysate chitosan, although the degradation rates are slower than the ones reported by other enzymes such as proteases or hemicellulose. Controlling reaction temperature, a commercial lipase rendered low molecular weight samples or chitooligosaccharides [45,46]. This lipase acted following both *exo* and *endo* cleavage mode. The presence of D end products indicates that it acted on chitosan in an *exo*-type mode while the sharp reduction in viscosity during the hydrolysis indicates that an *endo* splitting occurred in the initial hydrolysis stage. Therefore, by controlling the reaction time the final products can be led to oligomers with high DP or monomers. The polymer polydispersity depended on the used enzyme, lipase from wheat germ rendered samples with very wide molecular weight while lipase from *R. japonicus* exhibited better control over polydispersity [47].

The data previously showed that it is possible to somehow select the degradation products (LMW chitosans or oligosaccharides) by selecting the appropriate methodology (Table 2). As we can see in some sections of this review, specific biological and technological behaviour of chitosan degradation products depends not only on the method (physical, chemical, or enzymatic) selected to degrade the chitosan but also on the type of chemical or enzyme used for these processes. This effect is more related to the degraded polymer pattern rather than to the size or acetylation degree of the samples.

Table 2. The main products produced in the enzymatic degradation of chitosan.

Enzyme	Main Product
Chitosanase	Oligomers DP 2–3
Hemicellulase	Dimers, trimers, tetramers, pentamers and hexamers
Pepsine	Glucosamine, N-acetylglucosamine oligomers with DP 2–6
Pronase	4–10 kDa
Papain	Glucosamine, N-acetylglucosamine oligomers with DP 2–6
Lipase	High DP

DP: depolymerization degree.

4. Biological Properties

Chitin, chitosan, oligosaccharides, and derivatives exert many biological activities including antitumoral, antimicrobial, antioxidant, and anti-inflammatory activities, which could be used as therapeutic polymers. It is remarkable that up today chitosan and chitosan hydrochloride are only accepted as excipients by the regulatory agencies and not as a drug for the treatment of diseases.

4.1. Antimicrobial Activity

Bacterial resistance to antibiotics is a critical public health concern and, therefore, there is an urgency to find alternatives to antibiotics. Chitosan, chitosan derivatives and chitooligosaccharides exert antimicrobial activity against different microorganisms, including bacteria, filamentous fungi, and yeast [48]; some examples of the different microorganisms sensible to chitosan are shown in Table 3. Chitosan seems to have a growth-inhibitory activity since bacteria is able to grow after the polymer is removed from the media. This is of importance since resistant populations might emerge if the cells adapt to chitosan [49].

Table 3. Antimicrobial and antifungal activity of chitosan.

System	Target	Inhibition	References
Chitosan	*Aeromonas hydrophila* *Edwardsiella ictalurid* *Flavobacterium columnare* *Candida albicans*	Complete 0.4% (E I, F C) 0.8% (A. H)	[50]
Chitosan	Gram-positive bacteria (such as *Bacillus cereus*, *S. aureus*, *Bacillus megaterium*, *Lactobacillus plantarum*, *Listeria monocytogenes*, *Lactobacillus brevis*, and *Lactobacillus bulgaricus)* Gram-negative bacteria (such as *Salmonella typhimurium*, *E. coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Vibrio parahaemolyticus*, *Enterobacter aerogenes*, and *Vibrio cholera)*	Strong and safe effect	[51,52]
Chitosan hydrochloride Carboxymethyl chitosan Chitosan oligosaccharide *N*-acetyl-*D*-glucosamine	*Candida krusei*, *C. albicans*, *C. glabrata*	No effect: chitosan oligosaccharide and *N*-acetyl-*D*-glucosamine. Weak effect: Carboxymethyl chitosan. Strong effect: Chitosan hydrochlorides.	[53]
Chitosan wound dressing	*P. aeruginosa*, *B. cereus*, *L. monocytogenes*	Strong effect: wound management due to their antimicrobial nature, ability to accelerate wound contraction and healing, haemostatic and analgesic	[54–57]
Chitosan sponges	*S. aureus*, *E. coli*		[58,59]
Chitosan microparticles and nanoparticles	*E. coli*, *Vibrio cholerae*, *S. enterica*, *Streptococcus uberis*, *S. uberis*, *S. enterica*, *K. pneumonia*, *S. aureus*, *V. cholerae*, *Salmonella choleraesuis*, *S. typhimurium*	Strong effect	[60–62]

Due to chitosan's poor solubility above pH 6.5, the use of chitooligosaccharides is under consideration as polycationic biocides since they are soluble in water. Chitosan soluble derivatives such as sulphated chitosan, *N*-trimethyl chitosan, *N*-diethylmethyl chitosan or 2,6-diamino chitosan also avoid the use of acidic environments and exert antimicrobial activity [63–65]. This antimicrobial activity has applications in different fields such as the food, textile, or cosmetic industry, among others. Thus, due to the ability of chitosan to form shift bases, some new chitosan derivatives based on heterocyclic moieties have been developed, including pyrazole ring and furanyl, pyridyl, or thiophenyl moieties. Although these derivatives do not show higher solubility in aqueous media, their performance against gram-positive microorganism was improved when compared with the parent chitosan [66].

How these polymers (chitosan, chitooligosaccharides and derivatives) exert their antimicrobial activity is still under discussion. This fact can be explained by taking into account the lack of appropriate polymer characterization, purity issues, the use of different microorganisms, and the lack of methodological uniformity. Some studies point to the

reduction in cell membrane permeability due to polymer coating on the surface of the cells that blocks cell access to nutrients. This process occurs due to the interaction of -NH_2 groups from chitosan chains with -COO- groups on the external cell membranes of microorganisms. Therefore, the antimicrobial activity depends on the acetylation degree. It has also been hypothesized that chitosan can penetrate the cells and block RNA transcription as a result of adsorption with bacterial DNA [9]. Most likely, these mechanisms are not mutually exclusive, and several events are related to cell growth inhibition.

Intrinsic factors affecting the antimicrobial chitosan activity are due to the polymer characteristics such as Mw, acetylation degree, polymer viscosity, or polymer concentration. The solvent used to dissolve the polymer also affects its behaviour. We have observed that typical solvents used to dissolve chitosan such as acetic acid, citric acid, or buffers such as AcOH-NaAc exert some antimicrobial activity per se (unpublished results). Other factors with great impact on the antimicrobial activity are related to the tested microorganism, growth media, pH, temperature, ionic strength, or physiological state of the cells.

The effect of polymer size is controversial. Some studies claim that the antimicrobial activity of chitosan improves with the polymer size and have found that oligosaccharides have lower antimicrobial activity [67–69]. When comparing chitooligosaccharides, those showing higher DP exhibited higher antimicrobial activity [70]. Moreover, Tokura and co-workers reported that chemically produced chitooligosaccharides of 2200 Da not only had no antimicrobial activity but also served as growth accelerators of E. coli, while a sample with 9300 Da inhibited bacterial growth [71]. On the contrary, other studies showed better antimicrobial activity for a lower molecular weight chitosan sample (55 kDa) than a higher one (155 kDa); in the same study when a sample of 90 kDa was tested a promotion of bacterial growth was observed [72]. In another study, different tendencies were observed depending on the pH of the media. In acidic pH conditions, the antimicrobial activity increased with increasing MW. However, at neutral pH, antimicrobial activity increased as the MW decreased [73]. Even so, no trend on the effect of chitosan Mw on antimicrobial activity has been reported [74]. Regarding acetylation degree, it seems that the lower the acetylation degree, the better the antimicrobial activity [69,74,75].

After depolimerization of a chitosan sample (400 kDa, DD~85%) with hemicellulose, a set of chitosan samples with similar DD and Mw ranging from 130 to 2.8 kDa and a chitooligosacharide sample with Mw 1.4 were produced. Some of these samples were also half-acetylated, furnishing two chitosan samples with Mw of 53 and 18 kDa, and some chitoligosaccharides with Mw of 1.4 kDa. Both chitooligosacharides samples and the half-acetylated samples were water soluble, while the others were not soluble in water. All samples were tested against Staphylococcus aureus, Escherichia coli, and Candida albicans. In this study, water soluble chitosans and oligosaccharides did not exhibit antimicrobial activity; in fact, they promoted the growth of C. albicans. Insoluble chitosan samples exhibited antimicrobial activity with the most pronounced effect when medium molecular weight samples were tested (Mw 78–48 kDa) [76].

Our group has studied the antimicrobial activity of low molecular weight chitosans and oligosaccharides produced by enzymatic degradation in order to determine if the polymer pattern has some effect on this activity. Chitooligosaccharides were produced by two different processes; thus, in process P1 chitosan was enzymatically depolymerized with chitosanase, while in process P2 the sample was depolymerized in a two-step process with HNO_2 and chitosanase. The samples were tested against E coli and L. monocytogenes. COS from P1 showed a higher capability to inhibit bacterial growth than COS from P2. In both cases, COS were more effective at inhibiting E. coli (Gram-negative) than the Gram-positive L. monocytogenes. Antimicrobial activity depended on the production process and composition and structure of COS. COS produced in a one-step enzymatic procedure showed better antimicrobial activity than those produced in the two-step chemical–enzymatic process even when the samples exhibited similar DA and MW [77].

4.2. Antioxidant Activity

Antioxidants are gaining interest due to the relationship between oxidative stress and several diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and cancer. Moreover, it is related to complications in other diseases such as diabetes [78–80].

Chitosan contains an amino and several hydroxyl groups, which can react with free radicals exhibiting scavenging ability. Some chitosan derivatives such as chitosan sulphates or N-2 carboxyethyl chitosan exhibited improved antioxidant activity [81–83]. Chitooligosaccharides have also been chemically modified to improve their antioxidant activity, for instance by modification of the polymers with gallic acid [84,85] or phenolic compounds [86].

Different methodologies have been used to determine chitosan and its derivatives' antioxidant assays, which includes DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid), and FRAP (ferric antioxidant power) assays, peroxide and hydroxyl radical scavenging assays or the use of macrophage models. DPPH and ABTS assays are based on electron and H atom transfer, while the FRAP assay is based on electron transfer reaction, as depicted in Figure 3. The ORAC (oxygen radical absorbance capacity assay) is also widely used to test antioxidant activities.

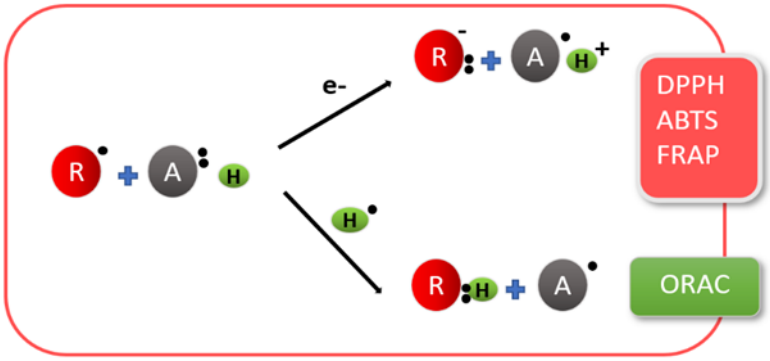

Figure 3. Methodologies used to determine antioxidant activities.

The disparity between the polymers tested and the methodologies used to test the activity produces considerable differences in the polymer concentrations that range from 50 μg/mL to 400 mg/mL [83]. Antioxidant activity is more remarkable for low molecular weight samples rather than for high molecular weight ones since shorter chains form fewer intramolecular hydrogen bonds and therefore the reactive groups are more accessible, contributing to the radical scavenging activity [87,88]. Regarding the effect of the acetylation degree, the antioxidant activity seems to decrease when this parametre increases [88].

4.3. Anti-Inflammatory Properties

The inflammatory process is an automatic physiological response of the body related to tissue damage. The main goal of the inflammatory response is to bring circulating leukocytes and plasma proteins to the site of the infection or tissue damage, to eliminate the causative agent, when possible, and to start the healing process. Although inflammation is necessary for survival, when it is very severe, unable to eradicate the causative agent, or is directed against the host, the inflammatory process may cause damage. The inflammatory process is strongly related to the generation of free radicals. Again, this activity seems to be more remarkable when the molecular weight of the chitosan is reduced and chitooligosaccharides exhibit higher activity.

After chitosan (300 kDa) depolymerization with cellulose, the activity of degraded polymers with medium molecular weight, low molecular weight and chitooligosaccharides (156, 72, 7 and 3.3 kDa) were tested in terms of NO secretion, cytokine production, and mitogen-activated protein kinase pathways in a model of lipopolysaccharide (LPS)-induced

murine RAW 264.7 macrophages. Chitosan samples (parent, medium, and low) significantly inhibited NO production. On the contrary, the opposite effect was observed with the COS. The mechanism followed by the medium and low Mw chitosan to inhibited NF-κB activation and iNOS expression differed. For medium chitosan (156 kDa) the process occurred via the binding to CR3 while for low molecular weight chitosan the process occurred via the binding to CR3 and TLR4 receptors. On the contrary, the lower molecular weight chitosans activated NF-κB and enhanced iNOS expression by binding to CD14, TLR4, and CR3 receptors to activate JNK signalling proteins [89]. In general, chitooligosaccharides are studied in more detail for this application compared to chitosan, due to their better solubility in aqueous media and better performance.

The effect of acetylation degree on the anti-inflammatory activities of COS has also been studied. Chitooligosaccharides with MW between 0.2 and 1.2 kDa were enzymatically depolymerized, depending on the enzyme, fully deacetylated (fdCOS, mainly GlcN, (GlcN)2, (GlcN)3, and (GlcN)4), partially acetylated (paCOS: a mixture of at least 11 Cos with different proportions of GlcNAc and GlcN), and fully acetylated (faCOS, mainly GlcNAc, (GlcNAc)2 and (GlcNAc)3) were produced. The anti-inflammatory activity of the three COS mixtures was studied by measuring their ability to reduce the level of TNF-α in stimulated LPS murine macrophages (RAW 264.7). Only fdCOS and faCOS were able to significantly reduce this factor [90,91]. The inhibition of NO secretion by COSs revealed that 10% acetylated COS inhibited NO secretion significantly more than those with 50% acetylation [92]. Citronellol grafted chitosan oligosaccharide derivatives have been produced to improve the anti-inflammatory activity of the oligosaccharides with degrees of substitution of 0.165, 0.199 and 0.182, respectively. In all cases, the derivatives showed better performance than the parent COS. These derivatives reduced the expression levels of TNF-α by promoting the secretion of IL-4 and IL-10 and inactivated the NF-κB signalling pathway via inhibiting the phosphorylation of p65, IKBα, and IKKβ [93].

Using the same chitosan as a starting material to produce chitooligosaccharides rendered samples with different anti-inflammatory behaviour. Chitooligosaccharides (5–10 kDa, DD: 87%) composed mainly of 42% fully deacetylated oligomers (A1-A3) plus 54% monoacetylated oligomers, produced by enzymatic degradation with chitosanase, attenuated the inflammation in lipopolysaccharide-induced mice and in RAW264.7 macrophages. On the contrary, chitooligosaccharides (5–10 kDa, DD: 89%) from a two-step preparation (chemical degradation followed by enzymatic degradation with chitosanase) were composed of 50% fully deacetylated oligomers plus 27% monoacetylated oligomers (A1-A3) promoted the inflammatory response in both in vivo and in vitro models [94]. This result shows how small differences in the COS mixture have a strong effect on the mixture behaviour.

5. Metallic Nanoparticles and Chitosan

Metallic nanoparticles are usually defined as particles of metal atoms with sizes ranging between 1 nm to a few hundred nanometres [95]. These particles exhibit optical, chemical, and electronic properties that differ from individual atoms or bulk materials. These unique properties are highly appreciated for different applications such as catalysis, photonics, or biomedicine [96].

Metallic nanoparticles can be prepared using myriad physical or chemical methods. Metal ions can be reduced using chemicals ($NaBH_4$, vitamin C and others) [97,98], plant extracts (due to their phenolic compounds) [99], using polymers such as chondroitin sulphate or heparin [100,101], or using microorganisms containing specific enzymes such as nitrate reductase [102,103]. Other authors have proposed the use of sonochemical reduction [104], radiation [105], electrochemical reduction [106] or heat evaporation [107]. Once the formed metallic nanoparticles aggregate, the addition of stabilizers is needed [108] (Figure 4).

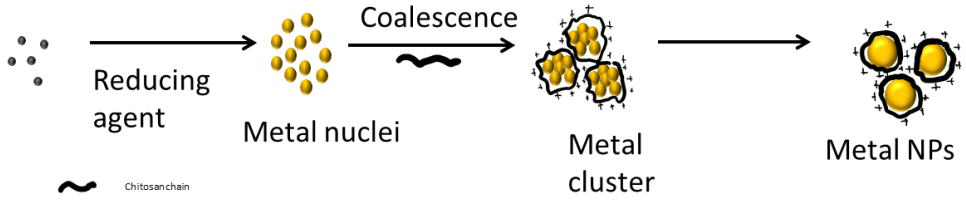

Figure 4. Scheme of metallic nanoparticle production and stabilization with chitosan.

The synthesis of metallic nanoparticles using chitosan as a reducing agent and/or stabilizing agent is well described. Some authors have also proposed that chitosan plays a role in the control of nanoparticle nucleation, thus controlling nanoparticle size to some extent since metal concentration also affects the nanoparticle size [97,109].

The reducing and stabilizing properties of chitosan seems to be related to the presence of CH_2OH, CHO, and NH_2 groups in the polymeric chain. Changes in the molecular weight or deacetylation degrees not only alter the number of these reactive groups but also modify the interactions (hydrogen bonds, electrostatic interactions, or steric interactions) present in the system.

In Table 4, some examples of the usage of chitosan in metallic nanoparticle synthesis are reviewed, including information about the molecules used as reducing agents, properties of the chitosan used when data are given, nanoparticle size, and morphology.

Table 4. Metallic nanoparticle based on chitosan.

Metal	Reducing Agent	Stabilizer Chitosan Mw and DD	NPs Size	Morphology	Ref.
Palladium	Ascorbic acid	Cs 180 kDa, 75–85% DD	5–20	Spherical	[98]
	Ascorbic acid	Cs 50 to 190 kDa, 75–85%	50–70	Flower-spherical	[110]
	Ascorbic acid	Cs, 50 to 190 kDa, 75–85%	30–150	Flower	[111]
	Ascorbic acid	TMCs 20 kDa	55–120	Spherical	[112]
	$NaBH_4$	Cs, 400 kDa DD 100%	nd	nd	[109]
	$NaBH_4$	Cs, (~400 kDa)	2	Spherical	[113]
	MeOH	Cs, (~400 kDa)	2–5	Spherical, large aggregate (Pd:MeOH 10:1)	[113]
	Hydrazine N_2H_4	Cs, (~400 kDa)	20 *	Highly aggregate	[113]
Platinum	$NaBH_4$	Cs, 400 kDa DD 100%	2–5	spherical	[109]
	$NaBH_4$	Cs, (~400 kDa)	2–3	spherical	[113]
	MeOH	Cs, (~400 kDa)	2	spherical	[113]
	Hydrazine N_2H_4	Cs, (~400 kDa)	17–25 *	aggregates	[113]
Gold	Cs, 1278 kDa	Cs, 1278 kDa	16		[114]
	Cs 817 KDa	Cs, 817 KDa	5	Spherical	[115]
	$NaBH_4$	Cs, 400 kDa DD 100%			[109]
	Cs DD > 85%; >200,000 cps	Cs, DD > 85%; >200,000	5–20	Spherical	[101]
	$NaBH_4$	Cs n.c.	6–20	Spherical; polyhedral	[97]
	COS 5 kDa	COS 5 kDa	7–15	Spherical	[116]
	Cs,	Cs, DD 53–95%, Mw 2.6–490 kDa	5–200 nm	Spherical, triangles, polyhedral	[117]
Silver	Cs	Cs 1240 kDa, DA 0.13	10–150	Spherical Triangles in long storage	[118]
	Cs	Cs, high Mw, DA 0.25	5	Spherical	[119]
	Cs DD > 85%; >200,000 cps	Cs DD > 85%; >200,000 cps	20–200	Spherical, fractal	[101]
	Ascorbic acid	Cs 180 kDa, 75–85% DD	5–20	Spherical	[98]
	$NaBH_4$	Cs 400 kDa DD 100%	30–200	Spherical clusters	[109]
	Gamma radiation	Cs n.c.	4–5	Spherical	[101]
	Cs n.c.	Cs n.c.	10–60	Spherical	[120]
	Ascorbic acid/Cs 1278 kDa	Cs 1278 kDa	8		[114]
	Cs n.c.	Cs n.c.			[121]
	Cs	Cs (50–190 kDa DD 75–85%)		Fractal patterns	[122]

Cs: chitosan; TMCs: trimethyl chitosan; n.c.: non-characterized; nd: non-determined; * aggregate size.

Data from Table 4 clearly show that the characteristics of the produced nanoparticles depend on the method used to produce the nanoparticles and the characteristics of the chitosan used to reduce and stabilize the metal ions. In general, due to the lack of a proper characterization of the chitosan samples and the variety of reaction conditions used it

is very difficult to relate chitosan properties with the characteristics of the nanoparticles. Recently, the effect of chitosan Mw and acetylation degree on the preparation of AuNPs both as reducing and stabilizing agents has been analysed in detail [117]. The authors also took into consideration the effect of polymer and gold concentration, temperature, and reaction time. Their results showed that the chitosan acetylation degree and polymer concentration are the main parameters affecting the size and shape of the nanoparticles. Polymer molecular weight is related to the reductive efficiency since the reduction of the polymer size increases the amount of reducing sugars in the media. Our group has focused its research on the production of AgNPs using low molecular weight chitosan samples. As previously described in this review, the characteristics of these low molecular weight chitosan samples depend on the enzyme used to produce the samples. We hypothesised that samples with similar Mw and acetylation degrees may exhibit different behaviour due to the monomer pattern. Our results showed that pattern is a key parameter in the stabilization of the AgNPs, corroborating this hypothesis [123] A chitosan sample (538 kDa, DD 52%) with little ability to stabilize AgNPs was depolymerized with lysozyme (fraction L) and chitosanase (fraction Q) and the resulting reaction mixture was separated into three fractions by tangential ultrafiltration (fraction F1 (Mw > 30 kDa), fraction F2 (Mw 30–10 kDa), and fraction F3 (Mw 10–5 kDa). After depolymerization, an increase in the DD was observed with values between 62–74%). All fractions were able to reduce the silver ion, but relevant differences were observed in terms of stabilization (Figure 5). AgNPs produced with chitosan samples depolymerized with chitosanase (FQ2 and FQ3) were larger, poorly stabilized, and tended to form large aggregates visible with the naked eye. On the contrary, AgNPs produced with chitosan depolymerized with lysozyme were smaller and more stable in all cases. As the Mw of the fraction was reduced, the polydispersity was also lowered. After one month, the stability of the AgNPs was evaluated and results showed that AgNPs produced with the fractions F1Q and F1L were the most appropriate for nanoparticle stabilization.

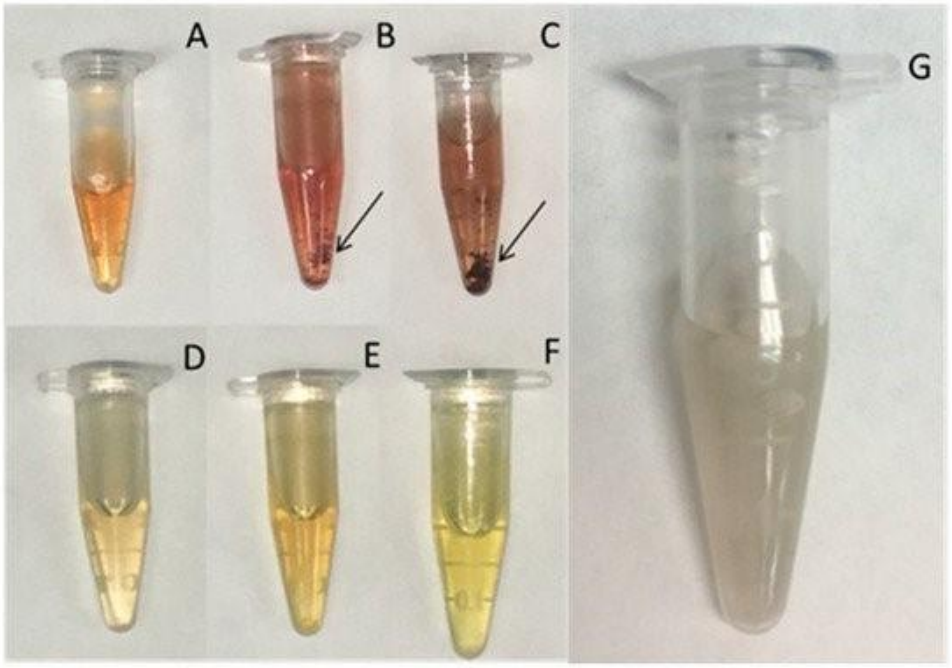

Figure 5. Visual evaluation of AgNP–polymer solutions after 5 h at 90 °C. (**A**) F1Q, (**B**) F2Q, (**C**) F3Q, (**D**) F1L, (**E**) F2L, (**F**) F3L, and (**G**) parent chitosan. Arrows indicate the presence of aggregates.

The AgNPs produced with lysozyme fractions and the higher Mw fraction of chitosanase were tested in the catalytic reduction of TBO [124]. AgNPs produced through

chitosan depolymerization with lysozyme showed better performance than the sample produced using chitosanase. Moreover, AgNPs produced with fraction F1L exhibited the best performance in the reaction. That is, the effect of the polymer pattern goes further than affecting optical properties and stability and differences in the catalytical behaviour was also observed. This difference is not due to the polymer, since control reactions showed that the polymeric fractions were not able to catalyse the reduction in TBO and therefore the effect is solely ascribed to the AgNPs.

6. Chitosan in Biocatalysis

The use of immobilized enzymes for catalysing chemo-, regio- and/or stereoselective chemical reactions is a very useful and well-known technique [125–142]. In this sense, the use of chitosan for immobilizing enzymes, either as a carrier for covalent linking or as an encapsulation vehicle, is well reported [143–149]. Our group described the production of enantiopure D-p-hydroxyphenylglycine (D-p-HPG, Figure 6) using a multi-enzyme system containing D-hydantoinase and D-carbamoylase encapsulated in chitosan-based materials [150–153].

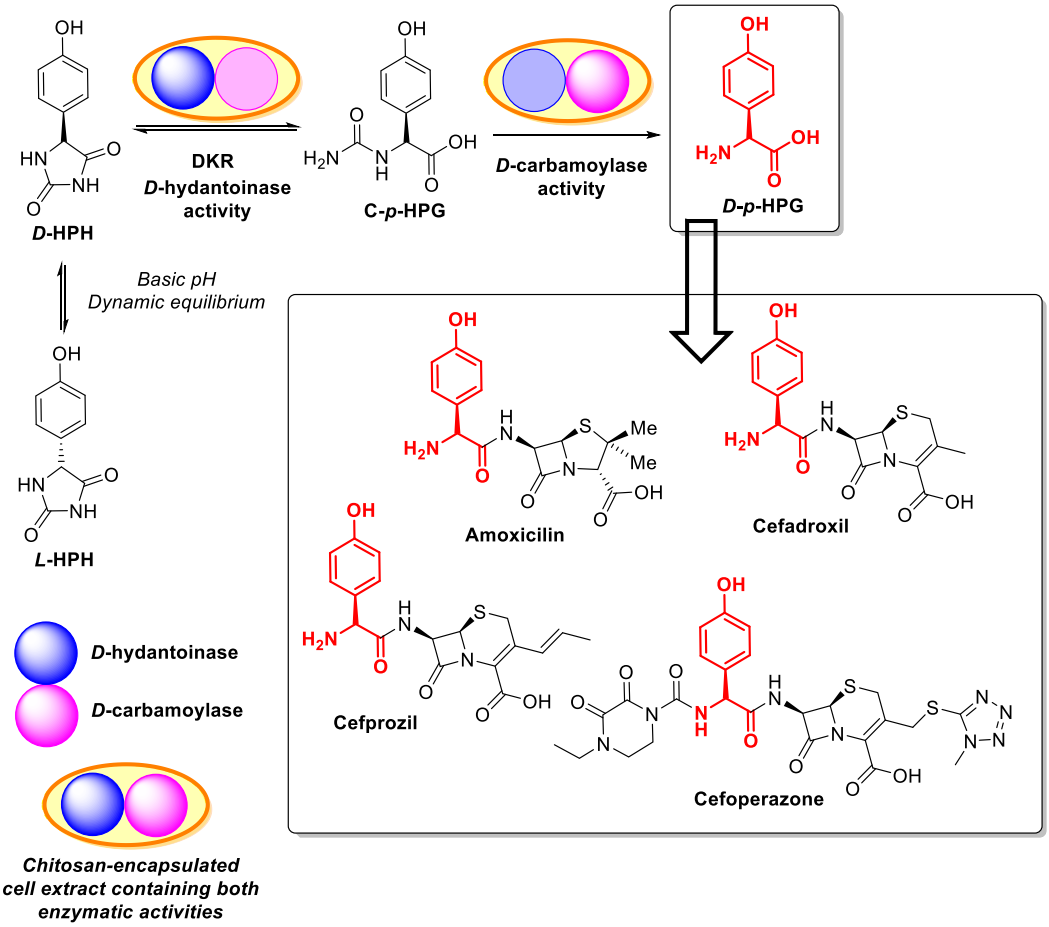

Figure 6. Schematic representation of the production of *p*-hydroxyphenylglycine (p-HPG) starting from a racemic mixture of *p*-hydroxyphenyl hydantoin (HPH) using a multi-enzyme system containing immobilized *D*-hydantoinase and *D*-carbamoylase.

D-p-HPG (or simply D-HPG, a D-amino acid) is a very useful chiral synthon, mainly used for the preparation of different semi-synthetic antibiotics, such as amoxicillin, cefadroxil, cefprozil, or cefoperazone [154–156] (Figure 6), but also anticancer drugs [157] and some heterocyclic compounds [158–161].

For preparing D-HPG, one of the most efficient processes is the so-called "hydantoinase process", depicted in Figure 6. This cascade of enzymatic reactions, aiming to produce optically pure amino acids [162,163], requires an initial step catalyzed by a D-specific hydantoinase [E.C. 3.5.2.2.] to transform D-p-hydroxyphenyl hydantoin (D-HPH) into N-carbamoyl-D-p-hydroxyphenylglycine (C-p-HPG), which should be subsequently hydrolyzed by a second enzyme, a highly enantiospecific N-carbamoyl amino acid amidohydrolase (also termed D-carbamoylase; E.C.3.5.1.77), to furnish the free amino acid. One of the main features of the hydantoinase process derives from the spontaneous racemization of D-HPH at pH values higher than pH 8, caused by the acidic hydrogen at position 5 of the imidazolidine-2,4-dione ring, which allows for oxo-enol-tautomerism. This leads to a dynamic-kinetic resolution (DKR), allowing for the use of a mixture of L-and D-HPH as the initial substrate and a theoretical 100% conversion and 100% optically pure D-amino acid production (Figure 6).

Both enzymes have been reported to be present in different microorganisms, such as Agrobacterium sp., Pseudomonas sp., Arthrobacter crystallopoites, or Sinorhizobium morelense [151], and can be used either as whole cells, crude cell extracts, or purified enzymes (see Aranaz et al. [151] and references therein). If using isolated enzymes, immobilization is an excellent strategy for stabilizing the enzymatic cocktail due to the fact that D-hydantoinases are quite stable but D-carbamoylases display low thermostability and are prone to suffer oxidative degradations. In this sense, different protocols have been described (see Aranaz et al. [151] and references therein), and our group described how a multi-enzyme extract from Agrobacterium radiobacter rich in D-hydantoinase and N-carbamoyl-D-amino acid amidohydrolase was easily immobilized via adsorption on chitin and chitosan for its application in the synthesis of p-hydroxyphenylglycine [153]. In fact, this adsorption derivative on chitin showed higher activity compared to the covalent one, and much greater pH stability compared to the soluble multi-enzymatic extract; on the other hand, the adsorption derivative exhibited greater pH-stability in the pH range under study, showing higher activity at low temperatures. Anyhow, as the immobilized derivatives could not be properly reused, we developed a new strategy based on the encapsulation of a crude cell extract from the same microorganism, containing both enzymes, in alginate beads [164]. This biocatalyst could be reused six times in the presence of solid HPH particles in a stirred batch reactor without losing any activity until the beads started to burst. Anyhow, as these alginate-based catalysts showed low stability in calcium chelating buffers (i.e. phosphate buffers) and easy microbial contamination during storage at 4 °C, another immobilization matrix, alginate–chitosan polyelectrolyte complexes, was assessed [150,152]. Thus, alginate mixed chitosan capsules were prepared in one step (by simply dropping an alginate solution containing the extract into a chitosan solution containing calcium ions) or in a two-step process (preformed calcium–alginate capsules loaded with the crude cell extract were subsequently coated with chitosan). The encapsulation yields were around 60% and independent of the characteristics of the different chitosans used. However, p-HPG production was indeed affected by chitosan acylation degree D-D (the lower D-D, the lower p-HPG) but not by chitosan molecular weight. Generally speaking, the best biocatalyst allowed for a p-HPG production yield of around 60% without any significant protein release to the reaction media. Interestingly, this encapsulation procedure improved the stability of D-carbamoylase against oxidative damage during storage, particularly after freeze-drying. In addition, the alginate coated chitosan capsules could be reused eight times without enzymatic activity loss before D-carbamoylase started losing its activity and alginate–chitosan beads suffered burst problems contaminating the reaction.

In a collaboration with the group of Dr. Fernández-Lucas, we described the covalent immobilization of a recombinant nucleoside 2′-deoxyribosyltransferase from Lactobacillus reuteri (LrNDT) on cross-linked magnetic chitosan beads via epichlorohydrin activation under alkaline conditions, and subsequent incubation with glutaraldehyde [165], as schematized in Figure 7.

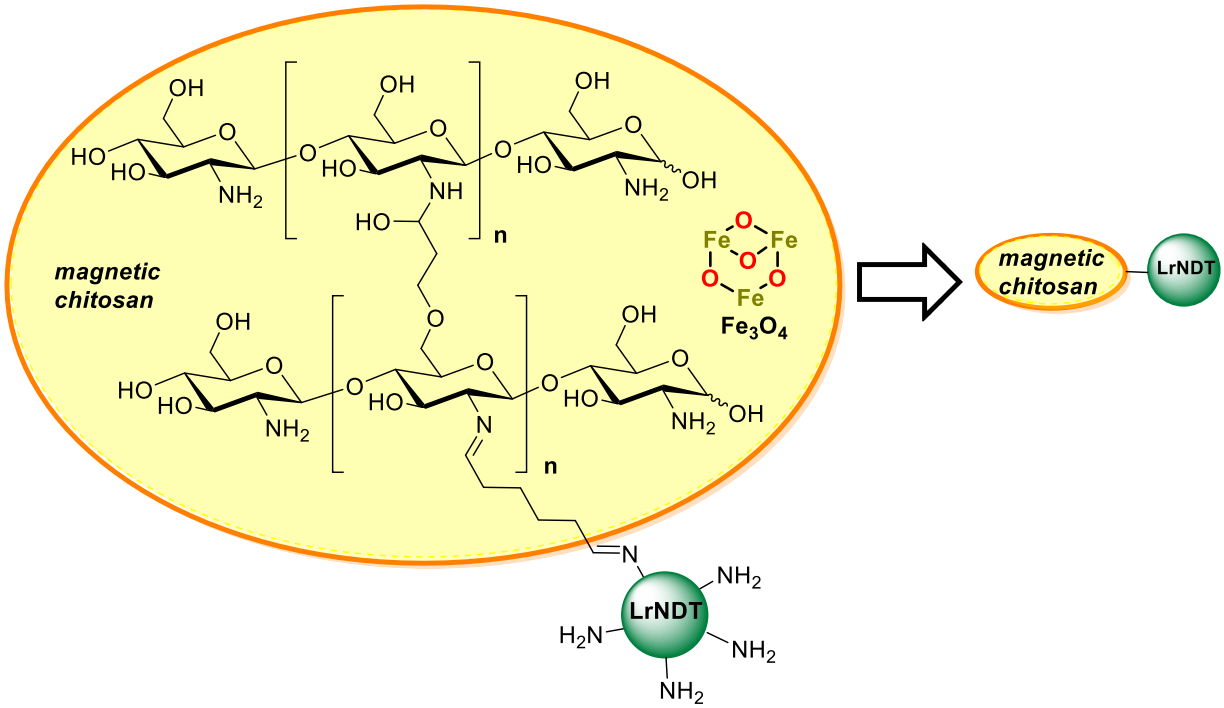

Figure 7. Schematic representation of the immobilization of a recombinant nucleoside 2′-deoxyribosyltransferase from *Lactobacillus reuteri* (LrNDT) on cross-linked magnetic chitosan beads. Adapted from Fernández-Lucas et al. [165].

Hence, by varying the amount of magnetite (Fe_3O4) and epichlorohydrin (EPI), different macroscopic beads were prepared and fully characterized (by scanning electron microscopy, spin electron resonance (ESR), and vibrating sample magnetometry (VSM)) before being used as supports. Once activated with glutaraldehyde, the best support was chosen after assessment of immobilization yield and product yield using as a standard reaction for the synthesis of thymidine (dThd) from 2′-deoxyuridine (dUrd) and thymine (Thy), as depicted in Figure 7. Additionally, optimal conditions for chitooligosaccharides with the highest activity of immobilized LrNDT on magnetic chitosan were carried out using response surface methodology (RSM). Thus, the best-immobilized biocatalyst retained 50% of its maximal activity after 56.3 h at 60 °C and no lost activity was observed after storage at 40 °C for 144 h. Subsequently, this innovative immobilized biocatalyst was employed in the enzymatic synthesis of 2′-deoxyribonucleoside analogues and arabinosyl nucleosides such as vidarabine (ara-A) and cytarabine (ara-C), as depicted in Figure 8, leading to moderate or good yields at 2 h reaction time. Remarkably, the immobilized derivatives could be easily recovered and recycled for 30 consecutive batch reactions without any significant decrease in the catalytic activity in the synthesis of 2,6-diaminopurine-2′-deoxyriboside (2,6-DAPdRib) and 5-trifluorothymidine (5-tFThd).

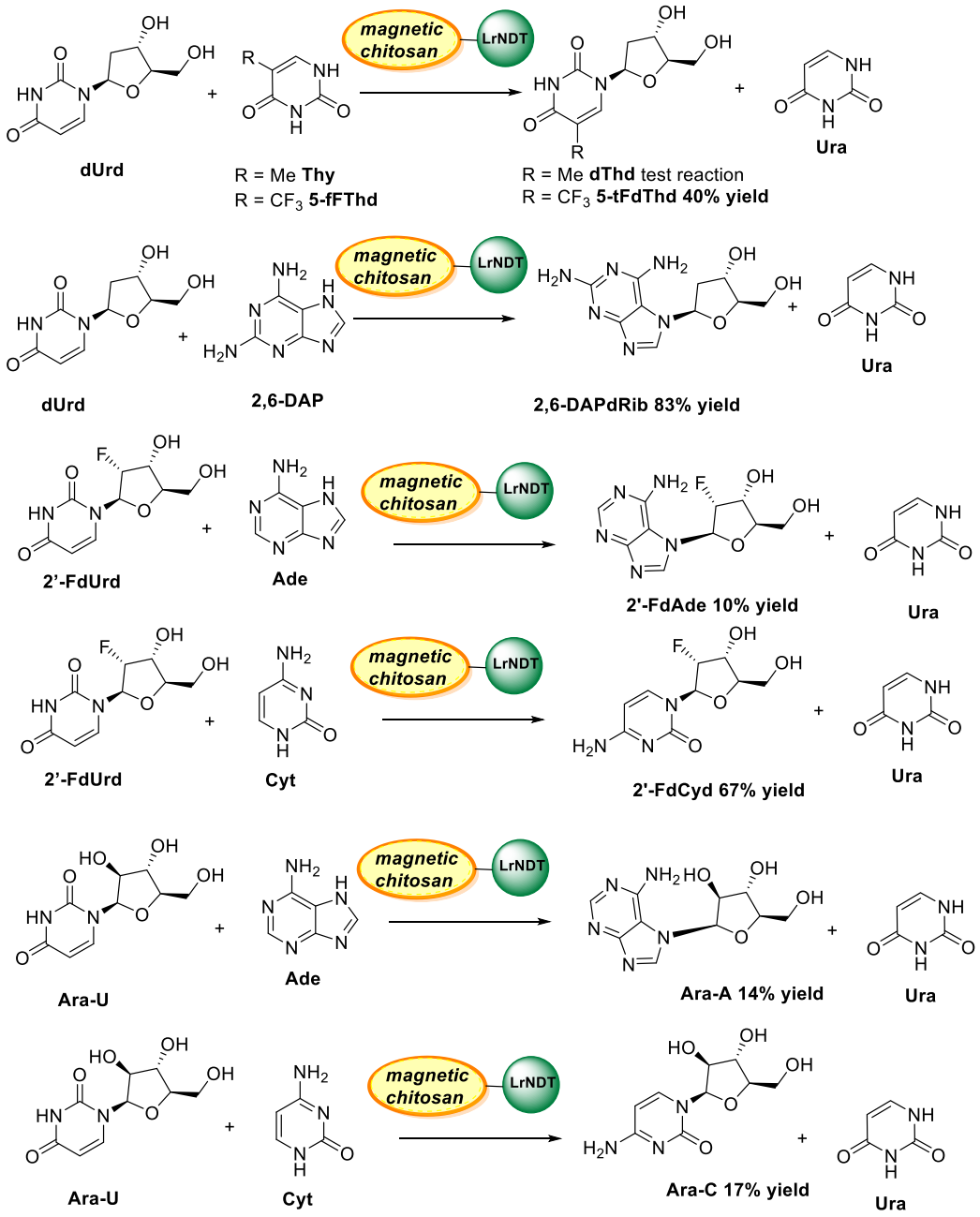

Figure 8. Synthesis of different natural and non-natural nucleosides using a recombinant nucleoside 2′-deoxyribosyltransferase from *Lactobacillus reuteri* (LrNDT) immobilized on cross-linked magnetic chitosan beads [165]. Commission on Biochemical Nomenclature: adenine (Ade), uracil (Ura), cytosine (Cyt), thymine (Thy), 2,6-diaminopurine (2,6-DAP), 5-trifluorothymine (5-tFThy), 2′-deoxyuridine (dUrd), 2′-deoxyadenosine (dAdo), 2′-deoxycytidine (dCyd), thymidine (dThd), 2,6-diaminopurine-2′-deoxyriboside (2,6-DAPdRib), 5-trifluorothymidine (5-tFdThd), 2′-fluoro-20-deoxyuridine (2′-FdUrd), 2′-fluoro-2′-deoxycitydine (2′-FdCyd), ara-uracil (ara-U), ara-adenine (ara-A).

7. Chitosan in Drug Delivery

Since the introduction of the first polymers in drug delivery, chitosan has shown superior biological and physiochemical properties for a wide variety of biomedical and industrial applications. The main feature of this biopolymer is its cationic character due to amino groups. These amino groups are also responsible for properties such as controlled drug release, mucoadhesion, in situ gelation, transfection, permeation enhancement, and efflux pump inhibitory properties [166]. Moreover, interest in this biomaterial due to its

central nervous system (CNS) bio-medical implementation has increased because of its ability to cross the blood brain barrier (BBB) [167].

Therefore, chitosan is widely used in drug delivery due to its technological properties, which allow us to process the polymer in different ways (Table 5).

Table 5. Some examples of chitosan presentations in drug delivery.

Presentation	References
Films	[168–171]
Sponges	[172,173]
Scaffolds	[174,175]
Nanoparticles	[176]
Microspheres	[177–179]
Hydrogels	[180–182]
Aerogels	[183–185]
Fibers	[186,187]
Microneedles	[188,189]
Coated Liposomes	[190,191]
Nanocomposites	[192,193]
Composites	[194]

Initially, a chitosan salt (chitosan hydrochloride) was approved in 2002 by the Pharmacopeia. Chitosan was first introduced as an excipient into the European Pharmacopeia 6.0 and the 29th edition of the United States Pharmacopeia (USP) 34-NF almost ten years later. These monographs contain the assays and establish limits to be observed when the polymer is used as a pharmaceutical excipient [195,196]. The increase in the number of publications regarding the use of this polymer in drug delivery is shown in Figure 9 and reveals a strong increase since 2002 that is still maintained today.

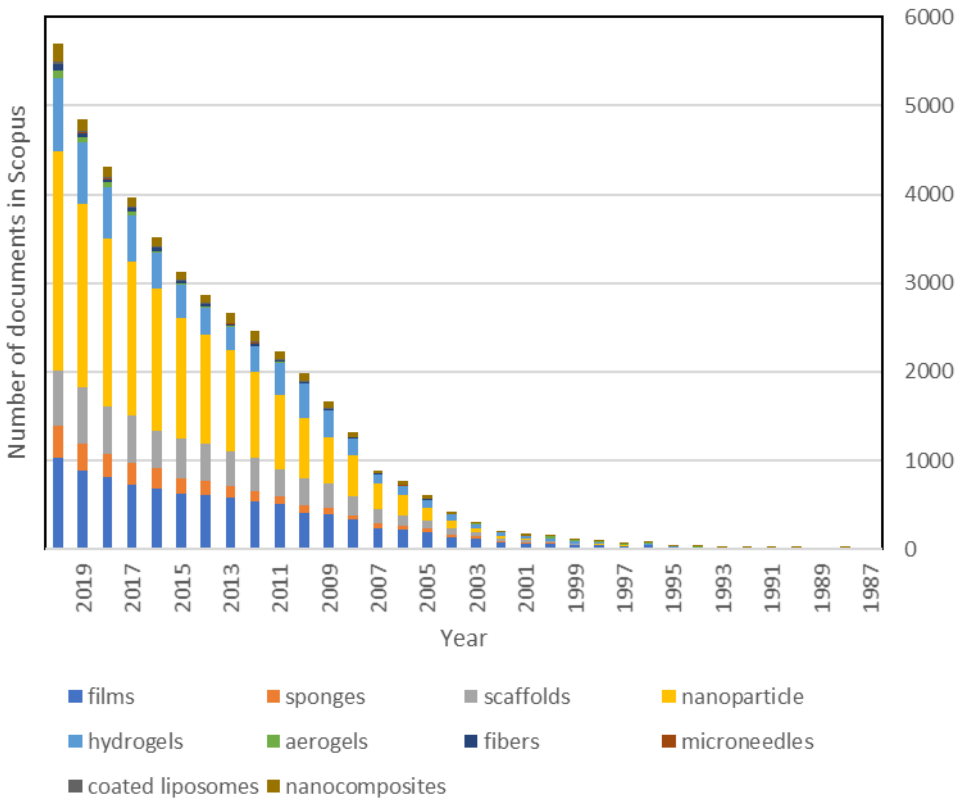

Figure 9. Publications about chitosan drug delivery in Scopus (1987–2020).

Chitosan films are easily produced by solvent-casting methodologies, but more complex systems can be produced by blending the polymer with others such as pectin [197] or by producing layer-by-layer films with negatively charged polymers like polyacid [198], poly (lactic-co-glycolic acid) [199] or polylactic [200], among others. Besides their safety, biocompatibility, and biodegradability, biopolymer-based films have been drawing increasing interest as excellent candidates not only as controlled-drug delivery systems but also as materials to produce contact lenses, wound dressings, and tissue engineering matrices.

Particulate chitosan-based systems (micro and nano systems) are widely used for the encapsulation of a large variety of molecules such as growth factors [178], antimicrobials [201], painkillers [202], anti-tumoral [203] or anti-inflammatory drugs [204].

Recently, chitosan has been used for the fabrication of microneedles (MNs) due to its film-forming ability, biodegradability, and biocompatibility, making it suitable for topical and transdermal drug delivery [188]. In particular, the use of chitosan MNs in vaccination is a hot topic of discussion [205–207]. The use of chitosan MNs in wound healing and point-of-care testing is revolutionary and gives hope of more useful developments in these areas. However, some drawbacks still need further investigation. The development of MNs devices with adequate mechanical strength to penetrate the skin without causing pain and skin damage and the development of efficient methods for their sterilization remain challenging [208].

A comparison of the number of publications containing "Chitosan + drug delivery" in Scopus and patents in Lens portal (free, open patent, and scholarly search) is shown in Figure 10. As observed, the number of patents is almost four times the number of publications, showing the increasing application of this polymer in the drug delivery field. An interesting article by Kurakula and Raghavendra summarizes the chitosan biomedical trends and the related patents [209].

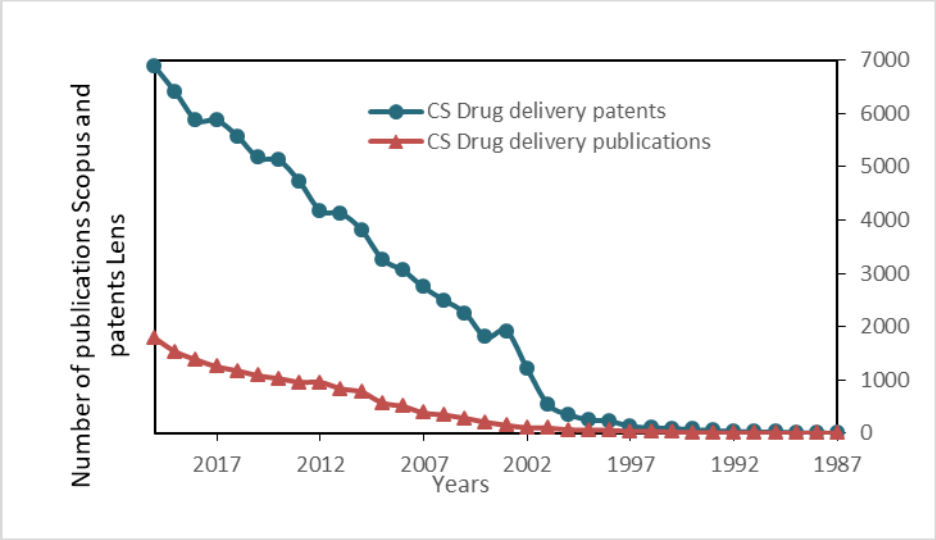

Figure 10. Publications about chitosan drug delivery in Scopus and patents in Lens (1987–2020).

8. Conclusions and Prognosis

Chitosan and its derivatives have been used in a myriad of applications for a long time. The potential interest of these polymers is clear when observing the number of articles and patents that appear every year and the growing market perspective. In some of these applications such as agriculture or the food industry, the use of chitosan in the market is well established. The use of chitosan has extended to a large number of research areas from Materials Science to Arts and the Humanities (Figure 11).

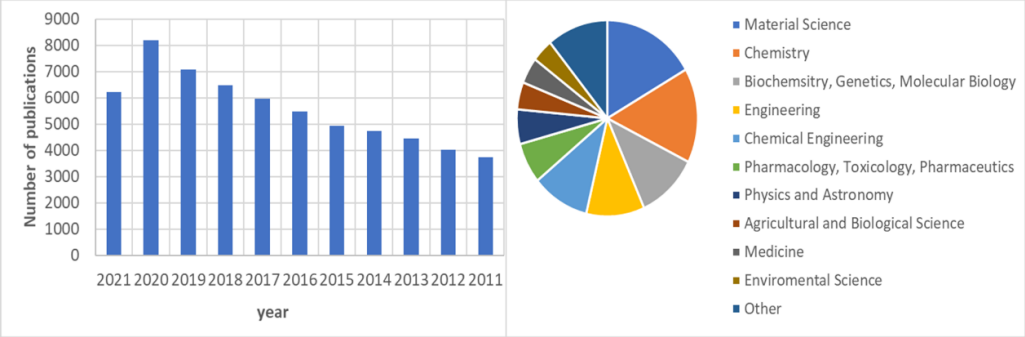

Figure 11. Number of publications and distribution by area in the period 2011–2021. Search of chitosan word in Scopus (abstract, title, keywords).

However, chitosan potentiality is somehow hindered by the inconsistency in the research data and the lack of knowledge in the ultimate mechanism underlying the properties of chitosan. Between 2011–2020, the number of publications on chitosan has displayed a steady growth. In 2021, a drop is observed, which is ascribed in part to the large number of reviews published in 2020, probably due to the COVID-19 pandemic, which has affected normal laboratory work worldwide. Regardless, we consider that this growth will continue in the following years, driven by the strong effort that has been carried out by the Chitin Science Scientific Community in the systematic research on this polymer. In fact, its approval by different agencies has boosted the interest in this polymer both by the industrial and scientific communities.

Chitosan specifications are ultimately related to its final application. Thus, high quality chitosans with low heavy metal and low endotoxin contents are required for biomedical and pharmaceutical uses. Moreover, strict control of production is needed to avoid uncontrollable hydrolysis and chemical modifications during polymer isolation. Therefore, chitosan production is not a trivial issue. To date, chitosan production cannot be considered fully sustainable due to the large amount of acid and basic reagents needed and the high temperatures required. Unfortunately, biotechnological processes using biocatalysts are currently limited to the laboratory scale so that implementation of these greener processes at a large scale is certainly one of the milestones we want to see being achieved in the next decade.

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Ascorbic acid and white wine production: a review of beneficial versus detrimental impacts

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Abstract

We review the use of ascorbic acid in winemaking and the benefits as well as the detrimental outcomes associated with its use. Initial discussion focuses on the antioxidant activity of ascorbic acid. The impact of the wine matrix and wine production practices, especially storage in bottle and oxygen ingress, on its antioxidant efficiency is discussed. The complementary roles of the antioxidant pair, ascorbic acid and sulfur dioxide, are presented. Thereafter, the ability of ascorbic acid to contribute to spoilage processes is covered. This includes both pro-oxidant and non-oxidative mechanisms induced by ascorbic acid that may lead to a reduced shelf life of white wine. Based on this review of scientific literature, the conditions most conducive to the beneficial impacts of ascorbic acid in wine are highlighted. Areas where lack of chemical knowledge still exists are identified for future research.

Keywords: *anaerobic degradation, antioxidant, ascorbic acid, oxidation, pro-oxidant, white wine*

Introduction

The dissolved oxygen present in white wine can be consumed by reaction with wine components and, unless a preservative is present at a sufficient concentration, can cause detrimental changes (Singleton 1987, Danilewicz 2003, Waterhouse and Laurie 2006, Bradshaw et al. 2011). Traditionally, this preservative has been sulfur dioxide (SO_2), and the means by which it protects a wine has recently been well summarised (Danilewicz 2007, Danilewicz et al. 2008, Danilewicz and Wallbridge 2010). Alternatively, ascorbic acid can be used in conjunction with SO_2, which alters the mechanism of oxygen consumption in white wine (Bradshaw et al. 2011). The purpose of this review is to focus specifically upon the beneficial and detrimental aspects of the use of ascorbic acid in white wine, building upon the chemistry of ascorbic acid highlighted in our previous review (Bradshaw et al. 2011). In terms of benefits, the impact of ascorbic acid on the sensory features of wines is discussed, along with the mechanistic aspects of its oxygen scavenging ability and its capacity to reduce oxidised components and allow a higher retention of SO_2 during oxidation. A description of the impact of ethanol on the antioxidant role of ascorbic acid is also reviewed. Discussion of the detrimental aspects of ascorbic acid that can contribute to the spoilage of wine will include pro-oxidant mechanisms, differentiating between direct reactions with oxygen and coupled oxidative processes, as well as non-oxidative mechanisms resulting from the anaerobic degradation of ascorbic acid.

The beneficial antioxidant action of ascorbic acid/sulfur dioxide

Sensory outcomes from bottle aging trials

The combination of ascorbic acid and SO_2 has been shown to have a favourable influence on the sensory features of white wines, including aroma, taste and clarity (Kielhöfer and Würdig 1959, Vecher and Loza 1961a,b, Bauernfeind and Pinkert 1970). In a Riesling and Chardonnay bottle trial (Table 1), Skouroumounis et al. (2005b) showed that addition of 90 mg/L of ascorbic acid at bottling had little impact on wine aroma in the first 6 months. Differences, however, became apparent at 3 years and 5 years of bottle age. The extent of wine oxidation characters, determined via sensory assessment, was either the same or lower for wines containing ascorbic acid compared with those without added ascorbic acid. The largest difference was in the wines bottled under closures that allowed higher ingress of oxygen (i.e. synthetic closures) where lower oxidation attributes were noted in the presence of ascorbic acid. Another study utilising Riesling (Table 1) showed that ascorbic acid addition (250 mg/L) to wines led to increased perception of fruity aromas and a lower intensity of oxidised aromas (oxidised apple, honey and sherry) when assessed 6 months after bottling (Morozova et al. 2015). On the palate, the wines with added ascorbic acid were also perceived as less oxidised, less ripe and fresher. A higher concentration of sulfur dioxide (68 mg/L free SO_2 vs 45 mg/L, Table 1) was found to be less effective than a combination of ascorbic acid and sulfur dioxide in preventing negative sensory attributes in wines exposed to higher oxygen concentration during storage (Table 1). Other long-term bottling studies have been conducted with ascorbic acid added at bottling (Godden et al. 2001, Lopes et al. 2009), but in these studies no ascorbic acid-free control was present to allow an evaluation of the impact of ascorbic acid (Table 1).

The impact of bottle closure in the presence of ascorbic acid has been examined in several studies on Semillon, Sauvignon Blanc, Riesling and Chardonnay (Table 1), utilising a range of ascorbic acid from 40–100 mg/L at bottling (Godden et al. 2001, Skouroumounis et al. 2005a, Lopes et al. 2009). In these studies, the oxygen available to the wine during bottle aging was from three sources; oxygen dissolved in the wine, oxygen trapped in the bottle head space (between the closure and the

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Table 1. Compositional data for white wines utilised for bottle trial studies with ascorbic acid.

Wine	Ascorbic acid (mg/L)	Free and total SO_2 (mg/L)		Dissolved oxygen (mg/L)	Headspace oxygen (mg O_2)	References
Semillon	41–44	30	95	0.6–3.2	Not measured Inert gas used during bottling.	Godden et al. (2001)†
Riesling	0 and 99	29	155	0.8–1.1	Not measured	Skouroumounis et al.
Chardonnay	0 and 92	28	67	1.1–1.5	Inert gas used during bottling.	(2005b)†
Sauvignon Blanc	85	41	132	0.2–2.4	Not measured Inert gas used during bottling.	Lopes et al. (2009)†
Riesling	0 and 250	45 68	149 168	<0.3	0, 2.7, 5.4, 8.2§	Morozova et al. (2015)‡

All values measured just after bottling. †Variable closure types were utilised in these studies including screw cap, cork and synthetic; ‡Each sulfur dioxide concentration was used for wines with and without ascorbic acid, and four different headspace volumes were used for each ascorbic acid and sulfur dioxide treatment. Only screw cap closures were utilised in this study. §Estimated from the volume of air (0, 10, 20, 30) in the headspace cited by Morozova et al. (2015) and assuming 20.95% O_2.

wine surface) and oxygen that permeated through and/or from the closure. In all studies, inert gas was utilised to limit oxygen ingress during bottling, and initial dissolved oxygen concentration was measured at bottling but headspace oxygen was not measured. If removal of the headspace oxygen was not efficient or consistent during bottling, then the oxygen decay of the wine in the first few months after bottling may have been influenced by differences in headspace oxygen. In addition to the widely used wine closures (screw cap, natural cork, synthetic cork, agglomerate cork), wine in two of these studies (Skouroumounis et al. 2005a, Lopes et al. 2009) was also sealed in glass ampoules which were impermeable to oxygen, and hence these wines had access to only the first two sources of oxygen listed above. In the Sauvignon Blanc study (Lopes et al. 2009), after 2 years, wines sealed with a cork or cork agglomerate closure were free from faults, while those sealed with synthetic closures (high oxygen permeability) showed detrimental oxidative characters. The wines sealed under screw cap (tin liner, low oxygen permeability) or ampoule (no oxygen permeability) exhibited detrimental reductive thiol characters. This latter reductive sensory descriptor will be discussed later (see Enhancement of reductive off-odours by ascorbic acid). The earlier studies (Godden et al. 2001, Skouroumounis et al. 2005a) on Semillon, Chardonnay and Riesling gave similar general results to those of Lopes et al. (2009) with regard to spoilage of wines sealed under high oxygen permeable closures (i.e. synthetic) and reductive effects under lowest oxygen permeability closures (screw cap with tin liner).

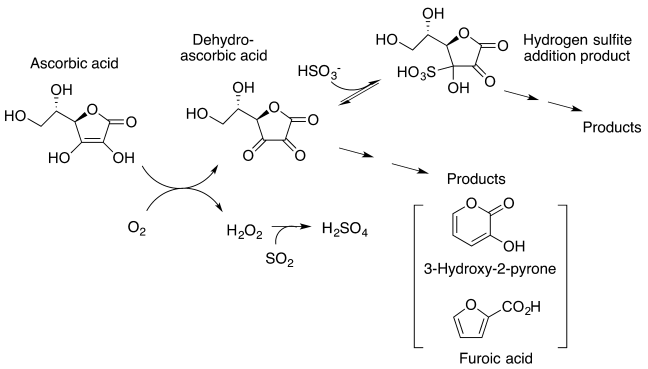

Figure 1. Reaction of ascorbic acid with oxygen in the presence of sulfur dioxide/hydrogen sulfite and the products generated.

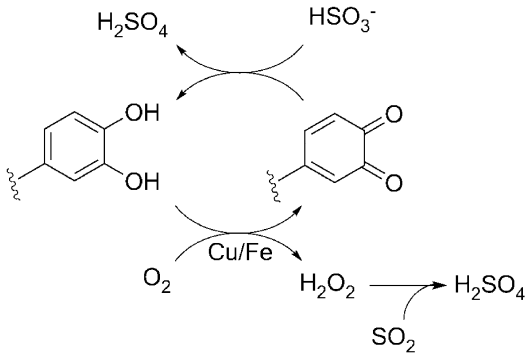

Figure 2. Reaction of phenolic substances with oxygen in the presence of sulfur dioxide/hydrogen sulfite.

Ascorbic acid and oxygen consumption

The mechanism for the antioxidant role of ascorbic acid is well established and reported in detail in our recent review (Bradshaw et al. 2011). In summary (Figure 1), the overall reaction involves the metal ion-mediated reaction between ascorbic acid and molecular oxygen to generate dehydroascorbic acid and hydrogen peroxide (H_2O_2). Hydrogen peroxide then reacts with SO_2 to form sulfuric acid, while dehydroascorbic acid may reversibly bind to SO_2. The latter binding is only weak, however, and instead dehydroascorbic acid itself degrades via intermediates such as diketogulonic acid and xylosone to form a range of degradation products. Although furoic acid and 3-hydroxy-2-pyrone are two of the major products from ascorbic acid degradation in wine-related conditions, many other unidentified products are evident (Barril et al. 2012). The implications of such degradation products will be outlined in the section on Ascorbic acid and colour development in wine conditions, however, in general, their impact is not detrimental provided sufficient SO_2 remains in the wine (threshold levels of SO_2 are discussed in the section on Pro-oxidant activity – oxygen consumption).

In the absence of ascorbic acid, the consumption of oxygen in wine follows a different pathway. In this case, the metal-mediated reaction between molecular oxygen and dihydroxyphenolic compounds results in the production of *o*-quinone compounds and H_2O_2 (Figure 2). Both of these products are then removed by SO_2, and in the case of the *o*-quinone,

this is mostly via reduction either to the parent phenolic substance or otherwise by formation of a sulfonic acid derivative of the parent compound (Danilewicz 2007, Danilewicz et al. 2008, Danilewicz and Wallbridge 2010). The low redox potential for ascorbic acid compared with that of phenolic substances [0.55 vs 0.78–1.07, pH 3.5, Danilewicz (2003)] ensures that the mechanism outlined in Figure 1 will dominate over that of Figure 2 when ascorbic acid is added to white wine.

It must be noted that the summary mechanisms presented in Figures 1 and 2 simplify the reactions, which involve free radical intermediates (i.e. semiquinone, ascorbate and possibly hydroperoxyl radicals) generated during the redox cycling of metal ions (Bradshaw et al. 2011). Importantly, these mechanisms underscore the need for SO_2, as without it the products H_2O_2, *o*-quinone and dehydroascorbic acid can lead to a detrimental impact on wine sensory features (Danilewicz 2003, Waterhouse and Laurie 2006, Bradshaw et al. 2011). The efficiency of oxygen consumption by ascorbic acid is not just confined to wine, and it may be utilised in must or juice to consume oxygen in competition with oxidative enzymes (e.g. polyphenol oxidase, laccase), thereby lowering their activity (Boulton et al. 1999).

Erythorbic acid, the diastereomer of ascorbic acid, may be used in place of ascorbic acid as it exhibits similar antioxidant properties (Fessler 1961, Clark et al. 2010, Grant-Preece et al. 2013). Both ascorbic acid and erythorbic acid can accelerate the consumption of oxygen in wine-related conditions. Grant-Preece et al. (2013) showed the consumption of 15 mg/L oxygen in a model white wine (containing 0.2 mg/L copper and 5.0 mg/L iron, and stored at 45°C) within 2 h when 200 mg/L ascorbic acid or erythorbic acid was present in combination with 120–150 mg/L SO_2. In the absence of either antioxidant, over 13 h was required for the consumption of the same amount of oxygen. Fessler (1961) showed 59% lower dissolved oxygen concentration after bottling a white wine with 120 mg/L of either ascorbic acid or erythorbic acid compared with the control wine (0.77 vs 1.85 mL/L). This was also the case 12 months after bottling although the difference in dissolved oxygen concentration was less pronounced (i.e. 0.46 vs 0.58 mL/L dissolved oxygen, respectively). Although erythorbic acid is cheaper than ascorbic acid, there is marketing evidence that the latter is often preferred by consumers (in, for example, Australia) as it is perceived as a more natural additive.

The impact of SO_2 on the rate of oxygen consumption in wine is also altered depending on the presence of ascorbic acid or erythorbic acid. Danilewicz and Wallbridge (2010) showed that in the absence of ascorbic acid, the consumption of oxygen in wine matrices is accelerated by the presence of SO_2, ascribing this to the ability of SO_2 to remove the *o*-quinone products (Figure 2), thereby accelerating the phenolic oxidation reaction. In contrast, in the presence of erythorbic acid in a model wine system, a higher concentration of SO_2 (120–150 mg/L vs 12 mg/L, 2.9–2.3 mmol/L vs 0.3 mmol/L) led to a slower oxygen consumption (Grant-Preece et al. 2013). Further, the oxidative degradation rate of erythorbic acid and ascorbic acid was lower in the presence of SO_2 (Barril et al. 2012, Grant-Preece et al. 2013). These observations suggest that the weak binding of SO_2 to dehydroascorbic acid (Figure 1) has negligible impact on the ascorbic acid oxidation in Figure 1, as opposed to the significant impact of SO_2 on the *o*-phenol/*o*-quinone reaction in Figure 2. The decrease in the oxidative rate of consumption of ascorbic acid or erythorbic acid in the presence of SO_2 may thus be ascribed to the removal of H_2O_2 by SO_2 (Figure 1), thereby preventing the reaction between H_2O_2 and ascorbic acid (Figure 3).

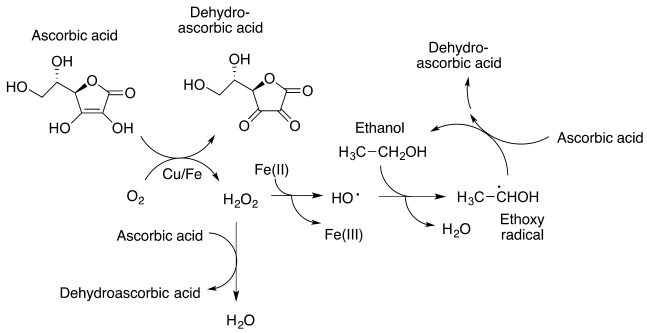

Figure 3. Reaction of ascorbic acid with oxygen in the absence of sulfur dioxide.

Ascorbic acid and the reduction of o-quinone compounds

Traditionally, ascorbic acid has been described as being able to reduce oxidised components in wine back to their reduced state. Most importantly, this includes the reduction of *o*-quinone compounds back to their parent phenolic substance, but the original premise was based upon studies conducted in non-wine media (Rouet-Mayer et al. 1990, Isaacs and van Eldik 1997, Boulton et al. 1999). Since then, Makhotkina and Kilmartin (2009) in a cyclic voltammetric study on wine antioxidants reported that ascorbic acid provided negligible reduction of *o*-quinone compounds in comparison to other agents such as SO_2 and glutathione. More recent kinetic studies by Nikolantonaki and Waterhouse (2012), utilising the synthesised 4-methyl-*o*-quinone, reinforced the earlier proposal that ascorbic acid was indeed as efficient as SO_2 and glutathione in the reduction of the *o*-quinone. To explain the difference of their results to those of Makhotkina and Kilmartin (2009), Nikolantonaki and Waterhouse (2012) suggested that ascorbic acid may be consumed at the cyclic voltammetric working electrode at the same potential at which the *o*-quinone was being generated. Consequently, this would lead to a lower ascorbic acid concentration, compared with that of SO_2 and glutathione (which are not consumed at the electrode), to react with the electrochemically generated *o*-quinone. This issue has not yet been fully resolved.

The ability of ascorbic acid to reduce *o*-quinone compounds would be more critical in must or juice than in wine conditions, as the significant concentration of active enzymes in must and juice allows for rapid generation of *o*-quinone compounds. Rigaud et al. (1991) showed that in oxidising must, a caftaric acid glutathione addition product (otherwise known as grape reaction product) could be formed from the reaction between glutathione with the enzymatically generated caftaric acid quinone. Ascorbic acid has been shown to delay the production of the grape reaction product and this was attributed to the rapid reduction of the caftaric acid quinone compound by ascorbic acid (Singleton et al. 1985, Rigaud et al. 1991). Under wine conditions, the ability of ascorbic acid to reduce *o*-quinones may become more important when the concentration of ascorbic acid is too low to scavenge molecular oxygen effectively, as the latter can then induce the formation of *o*-quinones from phenolic substances. The concentration at which ascorbic acid may not be sufficiently efficient at either scavenging oxygen or reducing *o*-quinones has not been studied but is likely to be dependent on the wine matrix.

Ascorbic acid and SO_2 *consumption*

As mentioned in the section *Ascorbic acid and oxygen consumption*, for ascorbic acid to be effective in wine, it must be used in conjunction with SO_2. This is not only to ensure that SO_2 is

present to allow efficient removal of H_2O_2 (Figure 1) but also to ensure that SO_2 can maintain the microbial stability of wine (Boulton et al. 1999). In white wine, SO_2 will exist in the free form, where it is in a pH-dependent equilibrium between molecular SO_2, hydrogen sulfite and sulfite, and it will also exist in a bound form, whereby it is reversibly bound to wine carbonyl components (often mainly acetaldehyde). Together, the concentration of free and bound SO_2 constitutes the total SO_2 concentration of a wine. Of these forms, it is the free SO_2 form that has antioxidant and antimicrobial action. During gradual depletion of free SO_2 by oxidation, some bound SO_2 will be gradually converted to free SO_2. There would appear, however, to be a threshold level of free SO_2 below which wine oxidation characters may be noted. The threshold level of free SO_2 is wine dependent, but a critical level often proposed by winemakers during bottle aging is 10 mg/L (Godden et al. 2001, O'Brien et al. 2009). In the bottle closure trial by Lopes et al. (2009), with 85 mg/L ascorbic acid at bottling, the wine with the most oxidative characters after 2 years was that sealed under a synthetic stopper which allowed the ascorbic acid concentration to reach near zero (~1–2 mg/L), and the free SO_2 concentration to drop below 10 mg/L. In fact, the actual critical threshold concentration of free SO_2 will depend on the suite of volatile carbonyl compounds present in the wine. Recent work (Grant-Preece et al. 2013, Bueno et al. 2014) has provided equilibrium constant data for the binding and release of several key volatile carbonyl compounds with SO_2, including phenylacetaldehyde and methional.

There have been varying reports on the concentration of SO_2 required when using ascorbic acid (Fessler 1961, Bauernfeind and Pinkert 1970, Zoecklein et al. 1995). Indeed, one impetus for initially utilising ascorbic acid in the wine industry was to allow the use of a lower concentration of allergenic SO_2 (Bauernfeind and Pinkert 1970). In contrast, there is some experimental evidence that suggests a higher concentration of SO_2 is required when ascorbic acid is used (Du Toit et al. 2006).

In a model wine system, Danilewicz et al. (2008) showed a ratio of oxygen to free SO_2 consumption of 1:2 without ascorbic acid present (and given no bound SO_2 was detected, the oxygen to total SO_2 consumption was also 1:2), consistent with Figure 2, while Barril et al. (2012) calculated a ratio of oxygen to ascorbic acid to total SO_2 consumption of 1:1:0.9–2.8. The range in the ratio in Barril et al. (2012) depended on the time after commencement of oxidation at which measurements were made, the ratio of the initial starting concentration of ascorbic acid and SO_2 as well as the presence of copper(II) and iron(III) ions. In the presence of metal ions, the ratio of oxygen to ascorbic acid to total SO_2 consumption became 1:1:0.9–1.3 with an average of 1:1:1.1. The ability of ascorbic acid to lower the consumption of total SO_2 is possibly due to the quasi protective effect resulting from the weak binding of SO_2 by dehydroascorbic acid (Figure 1) and its degradation products, compared with the irreversible oxidation of SO_2 to sulfate by *o*-quinone compounds (Figure 2).

In wine, as opposed to model systems, the reduced consumption of SO_2 in the presence of ascorbic acid is less apparent. In a long-term bottling trial with Riesling and Chardonnay, Skouroumounis et al. (2005b) found that after 4 years, some wines with added ascorbic acid had lower SO_2 depletion (both free and total) while others showed no difference to that of the control wines. The differences did depend on the type of wine and type of bottle closure. The largest difference was observed for Riesling with approximately 33% less total SO_2 lost with added ascorbic acid [27 vs 41 mg/L, roll on tamper evident (ROTE)/screw cap closure]. Based on the ascorbic acid and SO_2 data presented in Godden et al. (2001), the average ratio of ascorbic acid to total SO_2 consumption in Semillon (bottles stored inverted) was 1:2.9 (ranging from 1:2.5–3.4). Estimating the equivalent ratio for the study by Skouroumounis et al. (2005b) is complicated as different methods for measurement of ascorbic acid were used at bottling compared with that at subsequent times. From years 3 to 5 (thereby avoiding any complexity with different measurement techniques), however, the average ascorbic acid to total SO_2 consumption ratio in the Riesling and Chardonnay wines was 1:1.7 but with a range of 1:0.8–2.9, depending on wine type and bottle closure. Similar to the results in model wine systems (Barril et al. 2012), the study of Morozova et al. (2015) in Riesling showed that a decrease in the ratio of free SO_2 to oxygen consumed during wine oxidation in the presence of ascorbic acid was more apparent over storage time, or once a larger amount of oxygen had been consumed. When 5.0 mg/L of oxygen was consumed, the impact of ascorbic acid on decreasing SO_2 consumption was minimal; while on further oxygen consumption the effect became significant. The overall average mole ratio for oxygen to SO_2 loss in the absence and presence of ascorbic acid was 1:1.2 and 1:0.8, respectively.

The possibility of SO_2 consumption in wine by oxygen-independent mechanisms complicates the assessment of the relationship between total SO_2 consumption and oxygen consumption (Boulton et al. 1999, Daniel et al. 2004, Wallington et al. 2013, Arapitsas et al. 2014). These oxygen-independent mechanisms may include the loss of molecular SO_2 as vapour through the closure [although requiring a long storage period and high temperature (Boulton et al. 1999)], the irreversible reaction of SO_2 with α,β-unsaturated aldehydes (Daniel et al. 2004), and in the case of wines with ascorbic acid, via an additional non-oxidative consumption of total SO_2 (see section Degradation of ascorbic acid via oxygen-independent mechanisms). Furthermore, SO_2 has been observed to react slowly, in an irreversible manner, with the middle ring of flavan-3-ols, that is, grape skin and seed-derived phenolic substances (Arapitsas et al. 2014).

Given that, in some cases, ascorbic acid has the ability to maintain a higher SO_2 concentration for a given amount of oxygen consumption, a wine with ascorbic acid may have an increased shelf-life by avoiding excessive oxidation characters and/or microbial spoilage. Further work is required to explain the impact of wine matrix and bottle closure on the variation in the ratio of consumption of ascorbic acid to SO_2.

Ascorbic acid, oxygen consumption and ethanol

As an antioxidant, ascorbic acid undergoes oxidative degradation more rapidly in an aqueous ethanol solution compared with that in a purely aqueous solution (Hsu et al. 2012). The first order rate constants for ascorbic acid loss in 0 and 10% (v/v) aqueous ethanol solutions were 0.075 and 0.089/day at 25°C, with the difference becoming more apparent at higher temperature. The studies of Hsu et al. (2012), however, were conducted in the absence of SO_2 and are most likely complicated by the reaction of ascorbic acid not only with molecular oxygen but also with H_2O_2 formed during the ascorbic acid oxidation (Figure 3). Furthermore, Hsu et al. (2012) did not include metal ions which catalyse the reaction between ascorbic acid and molecular oxygen (Bradshaw et al. 2011), and hence the rates expressed above would be lower than those expected for wine. Nonetheless, the impact of ethanol on the degradation of ascorbic acid has been noted by other researchers in model wine media (Barril 2011). The impact of ethanol on the oxidative degradation rate of ascorbic acid was proposed by Chuang et al.

(2011) to be due to a lowering of water activity, thereby allowing easier dehydration of one particular product of dehydroascorbic acid, specifically xylosone. The rationale is that this would lead to an accelerated decrease in dehydroascorbic acid which would in turn induce the direction of the reaction in Figure 1 towards the products, although the increased instability of xylosone in ethanolic media has not been confirmed. Regardless of the exact mechanism responsible, there is evidence to suggest that ascorbic acid may be more efficient in scavenging molecular oxygen in ethanolic wine media than in juice systems (Barril 2011, Chuang et al. 2011, Hsu et al. 2012).

Ascorbic acid and glutathione

The ability of the ascorbic acid/glutathione pair to act in concert with the ascorbic acid/SO_2 pair with the ultimate aim of lowering the SO_2 concentration in wine has been examined. Glutathione is known to have antioxidant capabilities in physiological systems (Wu et al. 2004), and to efficiently scavenge *o*-quinones (Rigaud et al. 1991) and some aldehyde compounds under wine conditions (Sonni et al. 2011b). Studies in model wine systems containing solely ascorbic acid and glutathione have shown that this pair can prevent oxidative colouration while a threshold concentration of ascorbic acid is present, but once below the threshold concentration, colouration occurs at an accelerated rate (Sonni et al. 2011a). Furthermore, the accumulation of a glutathionyl-vinyl-phenolic product during the protective period has been reported and has been proposed to stem from a glutathionyl radical (Bouzanquet et al. 2012). This would result either from the interaction of glutathione with metal ions (Hamed et al. 1983) or perhaps more likely after glutathione scavenging of 1-hydroxyethyl radicals formed from hydroxyl radical attack on ethanol (Kreitman et al. 2013). In any case, additional work is required to delineate the mechanism. Further, the impact of the ascorbic acid/glutathione pair on wine aroma and how SO_2 may impact the antioxidant contribution of glutathione has not been examined.

Ascorbic acid and pinking

Ascorbic acid has a role to play in protecting white wine from the phenomenon of pinking (Lamuela-Raventós et al. 2001). The pinking phenomenon appears to be random in terms of conditions that cause its initiation and ascorbic acid is claimed to limit the onset of this phenomenon. The chemistry involved would appear to be associated with ascorbic acid inhibiting colour development that results from reactions involving various phenolic substances. While linked to some of the chemistry described here, the pinking concept deserves a review in its own right.

Detrimental pro-oxidant impacts of ascorbic acid

In terms of wine spoilage, a pro-oxidant is considered to be an agent that promotes either the 'browning' colouration of the wine and/or the development of 'oxidation' characters on the aroma or palate of the wine. The brown colour of the wine is typically measured at 420 nm (Simpson 1982), but later studies (Skouroumounis et al. 2005b, Clark et al. 2008) demonstrated in comparing different wines and model wines that absorbance measurements at 500 nm were often better correlated with the visual perception of 'brown' colour. The aroma and palate descriptors for an oxidised wine may include green apple, farmfeed, hay, woody-like, acetaldehyde, honey, rotten potato, cardboard and bitter (Ferreira et al. 2003, Karbowiak et al. 2010). The following sections outline the impact of ascorbic acid on the general colour development of white wine, oxygen consumption, off-aroma production and light-induced effects.

Ascorbic acid and colour development in wine conditions

A summary of the ascorbic acid-independent mechanisms relevant to wine colour are outlined below before discussion of the alternate mechanisms induced by ascorbic acid. During the consumption of oxygen in white wine in the absence of ascorbic acid, H_2O_2 and *o*-quinone compounds (Figure 2) ultimately contribute to the changes in colour, aroma and palate of the wine by several mechanisms that have been widely reviewed (Li et al. 2008, Laurie and Clark 2010, Oliveira et al. 2011). Hydrogen peroxide can induce Fenton chemistry in the presence of metal ions to generate hydroxyl radicals, which in turn oxidise the most concentrated components of wine. After water, which only propagates the hydroxyl radical, these components will typically be ethanol, glycerol and then tartaric acid (in order of decreasing concentration) and their oxidation ultimately leads to acetaldehyde, glyceraldehyde and glyoxylic acid, respectively (Wildenradt and Singleton 1974, Laurie and Waterhouse 2006b, Clark et al. 2007, Clark 2008). The aldehyde moiety of these compounds can bind to SO_2, or otherwise react with grape skin and seed-derived phenolic substances (flavan-3-ols) to form larger polymers by acting as a bridge between flavan-3-ol units (Es-Safi et al. 1999, Laurie and Waterhouse 2006a, Sonni et al. 2011b). Both acetaldehyde and glyoxylic acid have generated coloured solutions or colloidal suspensions upon incubation with flavan-3-ols by this mechanism (Saucier et al. 1997, Es-Safi et al. 1999). In the case of glyoxylic acid, the bridging reaction can result in specific yellow pigments known as xanthylium cations, via the oxidation of a xanthene intermediate (Es-Safi et al. 1999). Hydroxycinnamic acids lower the yield of the yellow xanthylium cations but increase the production of other phenolic pigments that enhance the brown hue, as opposed to the yellow intensity, of the model wine solution (George et al. 2006).

The *o*-quinones formed from oxidation of phenolic substances (Figure 2) are well known to undergo nucleophilic attack by the phloroglucinol-moiety of flavan-3-ol compounds to generate larger polymers that may contribute to brown colouration (Guyot et al. 1996), although there is some suggestion that this is a relatively slow process (Nikolantonaki and Waterhouse 2012). Alternatively, the *o*-quinone can react with various thiol aroma compounds in wine resulting in a decrease in beneficial wine sensory features (Nikolantonaki and Waterhouse 2012).

The majority of studies on the pro-oxidant nature of ascorbic acid has focused on its impact on the colour of model wine systems rather than on flavour or aroma. The ability of ascorbic acid to act as a pro-oxidant in terms of colour enhancement is predominantly a consequence of insufficient SO_2 available to scavenge its initial oxidation products (Figure 1). In this case, ascorbic acid will accelerate the consumption of oxygen according to Figure 3, but the H_2O_2 generated will either induce Fenton chemistry or react with additional ascorbic acid. The competitive reaction kinetics of these two divergent reaction systems is not well understood in wine conditions. Furthermore, if Fenton chemistry is initiated, a high concentration of ascorbic acid may scavenge the radical intermediates, such as the 1-hydroxyethyl radical (Stoyanovsky et al. 1998). While the quenching of the 1-hydroxyethyl radical by various wine components including hydroxycinnamic acids and thiol compounds has been reported in a model wine (Kreitman et al. 2013), its quenching by ascorbic acid has been reported only in non-wine conditions (Stoyanovsky et al. 1998). As a consequence of the

reactions outlined above, the consumption of ascorbic acid in the model wine system without SO_2 for a given amount of oxygen will be higher than that when SO_2 is present (Figure 1 vs Figure 3). This accelerated rate has been demonstrated for ascorbic acid and erythorbic acid in model wine systems (Barril et al. 2012, Grant-Preece et al. 2013). The difference in ascorbic acid consumption with and without SO_2 is more pronounced in the absence of metal ions, most probably because of less iron(II)-induced Fenton chemistry and hence more H_2O_2 available to react with ascorbic acid (Barril et al. 2012). In summary, at high concentration, ascorbic acid alone can prevent oxidative colouration of model wine systems, at the cost of its rapid depletion, although when nearly depleted, it is no longer efficient in scavenging H_2O_2, free radicals and/or maintaining wine components in reduced conditions. The transition from the protective period of ascorbic acid to the onset of oxidative character development has been termed the 'crossover' of ascorbic acid from an antioxidant to a pro-oxidant (Bradshaw et al. 2003).

The crossover from antioxidant to pro-oxidant has been most extensively examined in terms of colour development in model wines containing flavan-3-ols. Models containing ascorbic acid and without SO_2 became more intense in colour than those without ascorbic acid (Bradshaw et al. 2003, Clark et al. 2008, 2010). The enhanced colour is most likely a consequence of increased H_2O_2 and carbonyl compounds generated in the samples with ascorbic acid. The colour enhancement appears dependent, however, on the relative concentration of flavan-3-ol and ascorbic acid as in a model wine system with a relatively low flavan-3-ol concentration (50 mg/L) and high ascorbic acid concentration (500 mg/L), the pro-oxidant colour enhancement by ascorbic acid was not observed (Sonni et al. 2011a). This was attributed to the reaction of all the flavan-3-ol with ascorbic acid degradation products such that when ascorbic acid was fully depleted, no further flavan-3-ol remained to afford conversion of pigment precursors to their coloured products.

As most studies have utilised model systems with saturated oxygen conditions (Peng et al. 1998, Bradshaw et al. 2003, Clark et al. 2008, Barril et al. 2012), oxygen does not become a rate limiting factor, as it would be in bottled wine. As a consequence, for a given reaction period, wine with ascorbic acid will consume a higher amount of oxygen than wines without ascorbic acid, and this in turn will lead to a higher yield of H_2O_2 and carbonyl compounds for the former. In the absence of SO_2 and metal ions, the H_2O_2 can further accelerate the degradation of ascorbic acid into its carbonyl degradation products (Bradshaw et al. 2002). Using square wave voltammetry, Bradshaw (2001) observed a loss of the H_2O_2 peak over time concomitant with the increase in reductive peaks in the potential region for carbonyl compounds. The compounds giving rise to these reductive peaks were not identified. Dehydroascorbic acid, the primary oxidative product of ascorbic acid, is known to degrade to intermediate compounds (e.g. xylosone) that can ultimately contribute, after reaction with a flavan-3-ol, to the production of yellow xanthylium cations (Figure 4) (Barril et al. 2009). Precursors to the xanthylium cation species (see the furanone-catechin-derivative in Figure 4) accumulate, while some ascorbic acid is still in solution, and then on near depletion of ascorbic acid, the precursors are rapidly converted to yellow xanthylium cation pigments (Barril et al. 2009, 2012). In the absence of ascorbic acid, the xanthylium cation pigments are generated only by Fenton chemistry on tartaric acid (Clark et al. 2007), while in the presence of ascorbic acid, the Fenton pathway still occurs with the added dehydroascorbic acid pathway to xanthylium cations being available for colour formation. In essence, the enhanced colouration induced by ascorbic acid after the crossover period is due to its ability to accelerate oxygen consumption with consequent accelerated H_2O_2 production and an increase in the pool of pigment precursors during oxygen consumption.

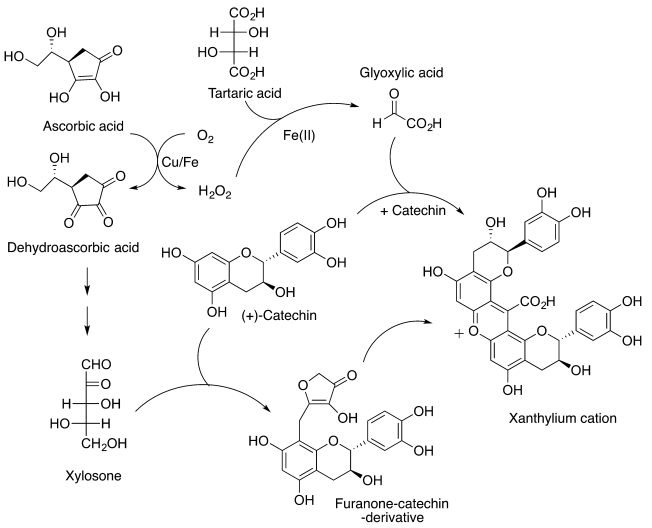

Figure 4. The production of xanthylium cations via a degradation product of ascorbic acid and via the Fenton chemistry degradation of tartaric acid. Adapted with permission from Barril et al. (2009). Copyright 2015 American Chemical Society.

Other pigments besides xanthylium cations are also formed during the ascorbic acid pro-oxidant period. These pigments have not been identified but rather are observed as a general increase in the baseline of chromatographic profiles detected at visible wavelengths (380–520 nm) rather than exhibiting discrete chromatographic peaks as occurs with xanthylium cations (Clark et al. 2008). These observations imply that xanthylium cations are being formed at the same time as a pool of unresolved polymeric pigments that altogether contribute to the colour of the oxidised and yellow/brown model wines.

In bottled wine, ascorbic acid has been shown to contribute to increased yellow, and not brown, colouration of wines even when SO_2 is present (Skouroumounis et al. 2005b). In terms of appeal to consumers, brown and/or orange colouration is negative while yellow colouration is generally taken as an indicator of age (Blackman and Saliba 2009, Blackman et al. 2014). According to data collected using the colour assessment scheme established by the Commission Internationale de l'Eclairage (CIE) and represented by the three dimensional colour space, L* (lightness), a* and b* (red-green and yellow-blue characteristics, respectively), that is CIELab, Chardonnay and Riesling wines with ascorbic acid added at bottling (92 and 99 mg/L, respectively, Table 1) became more yellow after 3 and 5 years of bottle age during which time residual SO_2 and ascorbic acid were still present (Skouroumounis et al. 2005b). At the same time, however, the wines with ascorbic acid tended to have less red colouration than those without ascorbic acid. Sensory colour assessment of the wines after 2 years yielded results consistent with the CIELab data for Chardonnay, that is, more brown colouration in wines without ascorbic acid, but few differences were observed for Riesling (Skouroumounis et al. 2005b). At two and a half years, however, the Riesling wines containing ascorbic acid were assessed as being significantly more yellow. The yellow colouration was reflected by an increase in absorbance at 420 nm while brown colouration, as detected by sensory panellists, was expected to be better correlated to an increase in absorbance at higher wavelengths [500 nm, Skouroumounis et al. (2005b)]. The red and yellow

Table 2. Calculated concentration of ascorbic acid and total sulfur dioxide that would react with different concentration of oxygen in bottled wine. The 'headspace concentration of oxygen' corresponds to the dissolved oxygen concentration increase that would result from all the headspace oxygen dissolving into 750 mL of wine.

Component consumed	1.0 mg/L dissolved O_2	5.0 mg/L dissolved O_2	0.92 mg/L headspace O_2¶	6.3 mg/L headspace O_2††
White wine without ascorbic acid				
Total SO_2†(mg/L)	4.0	20.0	3.7	25.2
Model white wine with ascorbic acid				
Ascorbic acid‡(mg/L)	5.5	27.5	5.1	34.7
Total SO_2§(mg/L)	2.6	13.0	2.4	16.4

†Based on the mole ratio of 1:2 for oxygen consumed to total SO_2 consumed in white wine as per Danilewicz et al. (2008). ‡Based on the mole ratio of 1:1 for the reaction between ascorbic acid and molecular oxygen in model white wines (Bradshaw et al. 2011). §Based on the maximum mole ratio of 1:1.3 for ascorbic acid consumed to total SO_2 consumed in model wine samples with metal ions as per Barril et al. (2012). ¶This headspace corresponds to the maximum oxygen concentration that can exist within the air under a cork with a headspace volume of 0.53 mL (standard headspace under cork). ††This headspace corresponds to the maximum oxygen concentration that can exist within the air under a screw cap closure with a headspace volume of 3.68 mL (standard headspace under screw cap). SO_2, sulfur dioxide.

compounds responsible for the colour in these samples were not identified. Clark et al. (2008) also reported an ascorbic acid-induced increase in yellow colouration (440 nm) and a decrease in red colouration (500 nm) during its antioxidant phase in model wine systems, as supported by UV-visible spectral data (380–780 nm), CIELab data and visual assessment of samples. Recent work [Table 1, Morozova et al. (2015)] also showed an increased 420 nm absorbance for Riesling wines containing ascorbic acid, as well as increased yellow colour as shown by CIELab data. Despite the description by the authors that the 420 nm absorbance indicated 'browning', the CIELab results actually showed similar results to that of Skouroumounis et al. (2005b) and Clark et al. (2008); that is, ascorbic acid induces yellow and decreases red colouration (lower a* CIELab value), and therefore most likely decreases in what is perceived as 'browning'. Morozova et al. (2015) also showed that increasing free SO_2 concentration at bottling (45 vs 67 mg/L SO_2) decreased the 420 nm absorbance induced by ascorbic acid. Although this effect was attributed by Morozova et al. (2015) to SO_2 scavenging radicals, an alternative explanation is improved binding of carbonyl degradation products of ascorbic acid at higher SO_2 concentration.

Pro-oxidant activity – oxygen consumption

Wine industry standards for bottling wine are designed to ensure that only a limited amount of oxygen is available to the wine immediately after bottling with values less than 1.0 mg/L dissolved oxygen being typical (O'Brien et al. 2009). In addition, bottle closures that allow only low oxygen ingress during aging in bottle are often utilised (i.e. screw cap). More recently, consideration has been given to total packaged oxygen (TPO) when evaluating potential for oxidation (O'Brien et al. 2009). The TPO amount includes dissolved oxygen at bottling together with the headspace oxygen (the amount entrained between the bottle closure and the surface of the wine), as well as that enclosed within certain types of seals such as agglomerate cork (Dimkou et al. 2011). In terms of the conversion of ascorbic acid from an antioxidant to a pro-oxidant, determining the amount of oxygen required for the near complete consumption of ascorbic acid and total SO_2 is complicated by the variable ratios observed for the consumption of ascorbic acid to total SO_2 (see section on Ascorbic acid and sulfur dioxide consumption). Using data from Barril et al. (2012), however, an estimate of ascorbic acid and SO_2 consumption at different oxygen concentration was made, recognising that these estimates do not allow for any oxygen-independent mechanism for SO_2 consumption. Table 2 demonstrates the amount of ascorbic acid and total SO_2 that would be theoretically consumed, based on the maximum mole ratio of ascorbic acid : total SO_2 of 1:1.3 consumed in model wine systems containing metal ions (Barril et al. 2012), given different amounts of dissolved oxygen and headspace oxygen in a bottle for a given closure. It is not possible to determine the time required for this ascorbic acid to be consumed as it will depend on the wine matrix, especially the metal ion concentration, as well as bottle storage conditions (i.e. temperature), and it is complicated by the release of reversibly bound SO_2 to free SO_2. There has not been any study on wine that has been specifically designed to examine the impact of the wine matrix on the consumption of ascorbic acid, SO_2 and oxygen. By interpreting the data from a Sauvignon Blanc trial bottled with 85 mg/L ascorbic acid (Lopes et al. 2009, Table 1), it would appear that the dissolved oxygen at bottling (0.2–2.4 mg/L) was completely depleted by the first measurement date at 2 months.

In addition to the amount of oxygen (dissolved plus headspace) resulting from bottling, the other major source of oxygen to wine in bottle is the amount passing through the closure. The data in Table 2 (dissolved and headspace oxygen) and in Table 3 (oxygen transmission rates) identify critical factors that impact on the depletion of ascorbic acid and hence the potential for the crossover from antioxidant to pro-oxidant. It is evident from Tables 2 and 3 that a large amount of oxygen at bottling in combination with a closure with a high oxygen transmission rate would allow the greatest depletion of ascorbic acid. For a wine sealed under a screw cap, the amount of oxygen trapped in the large headspace can be quite significant (Table 2), while for a closure of high oxygen transmission rate (Table 3), the ingress of oxygen over time can be significant.

Whether ascorbic acid or SO_2 will be depleted first depends on the initial starting concentration of the antioxidants (Table 4), and then comparison of the starting mole ratio of the antioxidants to their respective decay mole ratio. The latter can be approximated from studies in model wine systems showing maximum decay mole ratio for ascorbic acid to free SO_2 to total SO_2 of 1:1.7:1.3, assuming the presence of metal ions. Such decay mole ratios, however, are approximations as they tend to gradually decrease with time, oxygen consumption and antioxidant concentration (Barril et al. 2012, Morozova et al. 2015). It is evident from Table 4 and the maximum decay mole ratio of 1:1.7:1.3 (for ascorbic acid : free SO_2 : total SO_2) that at the initial free and total SO_2 concentration of 30 and 100 mg/L, respectively, the ascorbic acid will be depleted before the total SO_2 in all instances, but the free SO_2 would be depleted before or at the same time as ascorbic acid. It must be noted that as the

Table 3. Calculated loss of ascorbic acid and total sulfur dioxide after 1 and 3 years in wine bottled under closures of variable oxygen ingress rate†.

Closure	Screw cap	Cork	Synthetic	Screw cap	Cork	Synthetic
		1 year††			3 years††	
White wine without ascorbic acid						
Total SO_2‡	1.2	2.5	30.0	3.7	7.4	90.0
Model white wine with ascorbic acid						
Ascorbic acid§	1.7	3.4	41.3	5.2	10.2	124.0
Total SO_2¶	0.8	1.7	19.5	2.5	4.8	59.0

†The oxygen transmission rates used were based on the data presented by Lopes et al. (2006): 1.0 μL/L (natural cork), 12.0 μL/L (synthetic stoppers) and 0.5 μL/L for screw caps with tin liner. ‡Based on the ratio of 1:2 for oxygen consumed to total SO_2 consumed in white wine as per Danilewicz et al. (2008). §Based on the ratio of 1:1 for the reaction between ascorbic acid and molecular oxygen (Bradshaw et al. 2011). ¶Based on the maximum ratio of 1:1.3 for ascorbic acid consumed to total SO_2 consumed in model wine samples with metal ions present as per Barril et al. (2012).
††Time in bottle. SO_2, sulfur dioxide.

Table 4. Mole ratio of ascorbic acid to free sulfur dioxide to total sulfur dioxide, at typical wine concentration, as compared with the maximum mole ratio of decay observed in model wine systems: 1:1.7:1.3†.

Ascorbic acid [mg/L, (mmol/L)]	50 (0.28)	100 (0.57)	200 (1.14)
Free SO_2 [mg/L, (mmol/L)]	30 (0.47)	30 (0.47)	30 (0.47)
Total SO_2 [mg/L, (mmol/L)]	100 (1.56)	100 (1.56)	100 (1.56)
Mole ratio	1:1.7:5.5	1:0.8:2.8	1:0.4:1.4

†Barril et al. (2012). SO_2, sulfur dioxide.

concentration of free SO_2 decreases, some reversibly bound SO_2 may be released into the free form; however, this process is matrix dependent and thus difficult to predict. Regardless, the concentration of free SO_2 does not need to drop to 0 mg/L before oxidation characters are noted. The study of Godden et al. (2001) (initial antioxidant concentration shown in Table 1) showed that once the free SO_2 was around 10 mg/L or less, the wine began to show oxidised characters. The same wines, based on the ascorbic acid concentration data presented at 12 months post-bottling, had either no ascorbic acid remaining or a concentration around 10 mg/L.

In essence, it would appear that for a white wine to undergo pro-oxidant spoilage induced by ascorbic acid, higher oxygen concentration, either as a consequence of poor bottling practices and/or poor bottle closures and/or long-term aging (>10 years), is required. Wines with a TPO sufficient to deplete ascorbic acid and generate low free SO_2 concentration will show enhanced colouration faster than that of a similar wine bottled without ascorbic acid. This is due to the ability of ascorbic acid to consume oxygen more rapidly than phenolic substances do (Figures 1–3), as well as the corresponding pool of carbonyl compounds (pigment precursors) that the oxidised ascorbic acid generates. Consequently, a wine bottled with the highest oxygen conditions in Table 2 (high dissolved oxygen and headspace oxygen) may have a shelf life of only a month with ascorbic acid, as compared with 4 months without ascorbic acid, where shelf life is defined as the free SO_2 concentration falling below 10 mg/L. In this sense, ascorbic acid is a pro-oxidant but it is under conditions where even the wine without ascorbic acid will eventually develop oxidised aromas, albeit over a slightly longer period of time.

To date, the pro-oxidant action of ascorbic acid has been described only for white wines in bottle, however, some wine production procedures may utilise ascorbic acid during the storage of white wine in oak vessels. If oxygen access occurs during oak storage, for example, in barrels that are not regularly topped up, then wines with ascorbic acid will continue to consume the oxygen at an accelerated rate leading to proportionally rapid decrease in free and total SO_2. Experiments examining this effect have yet to be performed.

Ascorbic acid and detrimental aroma development

Several studies have shown the ability of ascorbic acid to induce the production of undesirable aroma compounds in wine and related media. These undesirable compounds can be generated either through the direct degradation of dehydroascorbic acid, or via reaction of its degradation products with ethanol or amino acids.

Methional and phenylacetaldehyde are two aldehyde aroma compounds associated with the oxidised aroma of white wine, contributing rotten potato and honey aromas, respectively (Ferreira et al. 2003). These two compounds can be formed from the Strecker degradation of the amino acids methionine and phenylalanine or from the peroxidation of methionol and phenylethanol. Additionally, methional has been implicated as an intermediate in the production of methanethiol from the light-activated reaction of riboflavin with methionine in Champagne and model wine systems (Maujean and Seguin 1983a,b). Grant-Preece et al. (2013) showed that in model systems with saturated oxygen, samples containing ascorbic acid generated a higher concentration of phenylacetaldehyde and methional compared with that of control samples when stored at 25°C. At 45°C, the production of both aldehydes was independent of ascorbic acid and their concentration was higher than that at 25°C. At both 45 and 25°C, the SO_2 initially present in the samples had been depleted by the end of the experiment. Furthermore, in studies conducted to ensure residual SO_2 at the end of the experiment, after the consumption of ~15 mg/L oxygen at 45°C, samples with ascorbic acid generated more methional than the control sample, while no significant difference was observed for phenylacetaldehyde (Grant-Preece et al. 2013). These results suggested that the α-dicarbonyl degradation products of dehydroascorbic acid could participate in the Strecker degradation of methionine for the formation of methional despite the presence of free SO_2. It must be noted, however, that the experimental conditions utilised an oxygen consumption level (~15 mg/L) higher than would be typical of wine, in combination with an elevated storage temperature.

One of the main oxidation degradation products of ascorbic acid is 3-hydroxy-2-pyrone which accumulates during the

oxidation of ascorbic acid (Barril et al. 2012). This compound is volatile and can contribute a caramel-like aroma in aqueous solutions (Kurata and Fujimaki 1976). The contribution of this compound to wine aroma after its production from ascorbic acid has not been reported to date.

Sotolon is linked to the oxidative aromas of white wines and can provide a nutty and spicy aroma (Ferreira et al. 2003). Pons et al. (2010) showed that the ascorbic acid oxidative degradation product 2-ketobutyric acid was a precursor to sotolon, and required an aldol condensation with acetaldehyde to form the furanone. Even wines without ascorbic acid, however, may contain 2-ketobutyric acid as yeast can produce the compound during alcoholic fermentation (Pons et al. 2010). A wine bottled with ascorbic acid and analysed 7 years after bottling under cork ranged from 1.1–1.9 µg/L sotolon, and the higher sotolon concentration was found in the wines with the lowest residual ascorbic acid concentration. The variation in ascorbic acid depletion and sotolon formation was attributed to the variation in oxygen permeability of the corks utilised (Pons et al. 2010). The concentration of sotolon detected in the 7-year-old wines was just below the perception threshold quoted for sotolon in model wine [2 µg/L, Pons et al. (2010)], but well below the value quoted for white wine (7–8 µg/L, Guichard et al. (1993)]. Although SO_2 is an efficient binder of acetaldehyde and thus should limit the production of sotolon, it is only a weak binder of 2-ketobutyric acid. While perhaps speculative for an actual wine (requiring storage at 40°C for 30 days and no SO_2), Pons et al. (2010) observed in model wine that 1 µg/L of sotolon was produced from 10 mg/L 2-ketobutyric acid and 1 mg/L acetaldehyde.

Light-induced ascorbic acid degradation

The impact of light on the degradation of ascorbic acid with the concomitant production of H_2O_2 has been discussed (Sansal and Somer 1997, Tikekar et al. 2011a,b). It was found in studies relevant to microbial sterilisation of juices that aqueous ascorbic acid solutions exposed to UV light without any light filter present, including bottle glass, can induce rapid degradation of ascorbic acid (Tikekar et al. 2011a,b). The UV wavelengths typically utilised (i.e. 257 nm), however, would not be relevant to bottled wine because all glass, regardless of colour, is non-transparent at a wavelength below 300 nm and hence would shield the wine from the short wavelength UV (Grant-Preece et al. 2015). Visible light (350–750 nm) does not impact the stability of ascorbic acid solutions (Sattar et al. 1977), but in combination with riboflavin, ascorbic acid degrades faster under incident visible light (350–750 nm) (Sattar et al. 1977), and results in the production of H_2O_2, even under anaerobic conditions (Sansal and Somer 1997). In white wine, however, the concentration range for riboflavin [average 0.10 µg/L, SD 0.04 µg/L, $n = 83$ (Mattivi et al. 2000)] is much lower than the 12 000 µg/L utilised in the study of Sansal and Somer (1997). Via the riboflavin-mediated mechanism, ascorbic acid could either be beneficial, by preventing the off-flavours caused by light exposure to wine [as facilitated by riboflavin (Grant-Preece et al. 2013)] or detrimental with the production of H_2O_2 and consumption of SO_2. Further work is required on investigating the relevance of the light-induced degradation of ascorbic acid in wine with a wine-like concentration of riboflavin, and to determine if such a process is to the benefit or detriment of the wine. In addition, the potential for photoactivation by iron(III) tartrate (Clark et al. 2011) as another mechanism for light-induced degradation of ascorbic acid requires investigation.

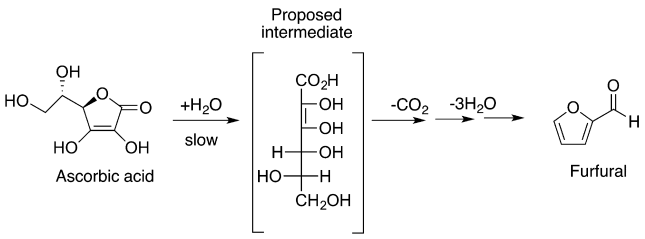

Figure 5. A pathway for the non-oxidative degradation of ascorbic acid.

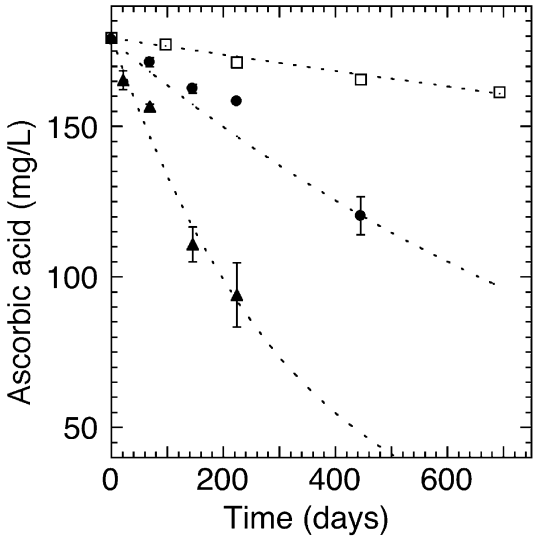

Figure 6. Ascorbic acid concentration in samples stored under low oxygen conditions (<0.1 mg/L dissolved oxygen) at 24°C (□), 36.5°C (●) and 45.0°C (▲). Error bars indicate the standard error ($n = 4$). Reprinted from Wallington et al. (2013) with permission from Elsevier.

Non-oxidative action of ascorbic acid

Degradation of ascorbic acid via oxygen-independent mechanisms

As well as being able to be degraded via an oxidative mechanism, ascorbic acid is also able to degrade via a non-oxidative mechanism (Figure 5). In this case, H_2O_2 is not generated, but instead ascorbic acid undergoes ring opening in a hydration reaction and then degrades into a variety of products. The major product for the degradation of ascorbic acid by this mechanism is furfural, although the yield would appear to depend on the temperature of storage and on the wine composition, including pH and SO_2 (Wallington et al. 2013).

This non-oxidative mechanism occurs regardless of the presence of oxygen, but at normal wine storage temperature (i.e. 10–25°C), the consumption of ascorbic acid by oxygen will be much quicker than its degradation by non-oxidative means. Wallington et al. (2013) provided the rates for the non-oxidative degradation of ascorbic acid at 25, 35 and 45°C, as well as the activation energy of the process. Figure 6 shows that even at a temperature relatively high for wine storage (i.e. 45°C), it takes several months for an appreciable concentration of ascorbic acid to be degraded by a non-oxidative mechanism. While wine is normally maintained in air-conditioned premises, there are occasions during transport and storage (e.g. shipping containers on wharfs) when it may be exposed to extreme temperature, potentially inducing degradation of ascorbic acid by the non-oxidative mechanism (Marquez et al. 2012). Similar to the loss of ascorbic acid, the rate of free and total SO_2 loss was increased at the higher temperature with a significant loss of

free SO_2 being observed in the samples held at 45°C. The kinetic data suggested that at 35 and 45°C, SO_2 was less efficient at binding intermediates of the non-oxidative ascorbic acid mechanism and therefore a higher yield of furfural was observed than at 24°C. As mentioned above (see section on Ascorbic acid and sulfur dioxide consumption), SO_2 can form an addition product with the middle ring of flavan-3-ols, such as (+)-catechin present in the model wine systems of Wallington et al. (2013), in a reaction that is aided by higher storage temperature (Arapitsas et al. 2014). Hence, such a reaction would be in competition with the binding of non-oxidative degradation products of ascorbic acid.

As an aldehyde, furfural can bridge flavan-3-ol compounds resulting in xanthylium cation pigments, similar to those formed from glyoxylic acid (Es-Safi et al. 2002). The study of Wallington et al. (2013), however, showed no accumulation of furfural-derived xanthylium cations or their precursors, despite the flavan-3-ol, (+)-catechin, being present in the model wine systems. Rather, a range of products was observed by MS with *m/z* values suggesting that only one product contained an intact (+)-catechin moiety.

The storage of wine on lees in oak vessels is essentially an anaerobic process and, if ascorbic acid is present, provides an opportunity for its non-oxidative degradation. Based on the reaction rate for the non-oxidative decay of ascorbic acid (Wallington et al. 2013), storage of the wine in barrel on lees would require a temperature higher than normal to induce significant losses via the mechanism outlined in Figure 5. In contrast, there is anecdotal evidence from winemakers that spoilage occurs during this winemaking step. The aldehydes formed from ascorbic acid, coupled with amino acids from the breakdown of yeasts, and residual sugar from the winemaking process could provide conditions for the onset of Maillard-type chemistry (Smuda and Glomb 2013). This has not yet been examined for wine.

Enhancement of reductive off-odours by ascorbic acid

Ascorbic acid has been observed to contribute to the reductive off-odour development during aging of wine in bottle. The reductive off-odour is attributed to the accumulation of several sulfide and thiol compounds including hydrogen sulfide and methanethiol (Skouroumounis et al. 2005b). This effect manifests itself as struck flint, rubber and/or rotten egg in the aroma and often hardness on the palate (Mestres et al. 2000, Skouroumounis et al. 2005a, Goode 2008). A threshold level of oxygen availability to the wine, as regulated by the wine closure and/or oxygen concentration at bottling, was shown to be critical to prevent accumulation of these 'reductive' sulfur compounds (Skouroumounis et al. 2005a, Lopes et al. 2009, Dimkou et al. 2011). More recently, bottle closures have also been shown to adsorb the sulfur compounds with varying efficiency (Silva et al. 2012). That is, increased removal of the reductive thiol compounds occurs in wines bottled with cork and technical closures (i.e. amalgamated cork pieces) compared with those bottled under screw cap with tin liners (Silva et al. 2012). Furthermore, hydrogen sulfide, methanethiol and ethanethiol have been found to give reversible complexes with metal ions (Cu, Zn, Mn) and that their release and accumulation in wine over time may be favoured by low oxygen conditions (Franco-Luesma and Ferreira 2014).

Wines sealed with screw cap (tin liner), however, are much more prone to development of 'reductive' character than wines sealed under closures with higher rate of oxygen transmission. This has been shown in bottling trials on Riesling, Chardonnay, Sauvignon Blanc and Semillon (Godden et al. 2001, Skouroumounis et al. 2005b, Lopes et al. 2009). Most wines in these studies sealed under screw cap and with added ascorbic acid produced reductive flavours at some point. For example, Riesling to which ascorbic acid had been added at bottling and sealed under screw cap (tin liner) gave a higher sensory score for struck flint after 4 years in bottle compared with that without ascorbic acid (Skouroumounis et al. 2005b). No difference was observed in the struck flint rating for Riesling sealed under cork or synthetic closures. In this study, the impact of ascorbic acid on reductive flavours in Chardonnay (even under screw cap with a tin liner) was less apparent than for Riesling (Skouroumounis et al. 2005b). The ability of ascorbic acid to accelerate the removal of TPO from bottled wine (see section Ascorbic acid and oxygen consumption) may produce a low oxygen environment faster than in wines without ascorbic acid, and such conditions may contribute to increased reductive flavours in wine under low oxygen permeable closures. Further work is required to confirm the mechanism by which ascorbic acid contributes to reductive flavour development during bottle aging, particularly the relationship of ascorbic acid to the release of thiols from metal-thiol complexes.

Fessler (1961) also reported that increasing the concentration of ascorbic acid or erythorbic acid at bottling elevated the sensory perception of hydrogen sulfide in a white wine when evaluated after 12 months. Although the wine bottle closure was not stated, it is of interest that the white wine was bottled with a particularly high free SO_2 concentration (i.e. 130–170 mg/L at bottling), implying a link between SO_2 and hydrogen sulfide in low oxygen conditions. A similar link between SO_2 addition, low oxygen concentration and hydrogen sulfide production has been made for a Verdelho wine and Shiraz wine, albeit without added ascorbic acid (Viviers et al. 2014). In these wines, copper(II) in conjunction with SO_2 addition was found to increase substantially the amount of hydrogen sulfide generated.

Recently, an off-flavour compound has been reported after the reaction of ascorbic acid with α,β-unsaturated aldehydes such as (E)-hex-2-enal (Sakamaki et al. 2011). The compound formed is 6-propylbenzofuran-7-ol and is described as having a medicinal flavour with a detection threshold of 19.6 μg/L in water (not known for wine). The product was detected after reaction for 8 weeks at 40°C, but had accelerated formation at higher temperature (50–70°C), with 0.14 mg/L being detected after a week at 50°C. The concentration of the unsaturated aldehyde utilised in this study (50–100 mg/L) was far in excess of the typical concentration (0.02–0.09 μg/L) of such compounds in white wine (Mayr et al. 2015). Earlier work by Sakamaki et al. (2012) showed that various other unsaturated aldehydes [including (E)-non-2-enal and (E)-oct-2-enal], which are also present in wine (Mayr et al. 2015), can generate their respective benzofuran after reaction with ascorbic acid degradation products. Further work is required to determine if ascorbic acid can induce the production of sufficient benzofurans under realistic white wine storage conditions to impact on wine aroma.

Summary

Ascorbic acid is often described as a 'two-headed Janus' and an 'oxymoron of antioxidants' (Porter 1993). The chemistry of ascorbic acid presented in this review reinforces this description. It is clear that ascorbic acid provides considerable protection against white wine oxidation under conditions of low oxygen (i.e. much less than the mole equivalent of ascorbic acid and SO_2

present in the wine). Ascorbic acid has the ability to remove oxygen from wine more quickly than would be the case in its absence. It has been shown in at least one study that the combination of ascorbic acid and SO_2 can preserve fruity aromas and decrease the oxidative aromas of wine, and this antioxidant combination is better than SO_2 alone in this role. Furthermore, although ascorbic acid would appear to induce increased absorbance at 420 nm during its antioxidant phase, it would also appear to reduce absorbance at higher wavelengths, hence inducing a yellowing rather than a browning of the wine. Most importantly, there are certain situations, including wines exposed to a higher oxygen concentration, whereby the presence of ascorbic acid leads to a reduction in the amount of SO_2 consumed for a given amount of oxygen consumed by the wine. Such an outcome has the potential for ascorbic acid to extend the shelf-life of wine, by increasing the time before the free SO_2 falls below a critical threshold level (~10 mg/L), and the concomitant release of oxidative aldehyde aromas. With excessive supply of oxygen, however, rapid consumption of ascorbic acid and SO_2 will lead to a shortened lifetime of the wine compared with that of ascorbic acid-free wine. The latter wine will eventually also spoil, but this will require a longer time period. The critical concentration of ascorbic acid to maintain its antioxidant status has not been fully identified and is an important area of research. It is apparent from the studies summarised in this review that a range of wine components may influence the activity of ascorbic acid. Trials established to determine the critical antioxidant concentration of ascorbic acid would need to be coupled with a complete chemical profile of the wine and subsequent chemometric analysis using, for example, independent components analysis (Ducruet et al. 2014) to identify the specific factors in the wine matrix that influence the activity of ascorbic acid.

Oxygen ingress at bottling or via the closure after bottling is an important factor that contributes to the loss of ascorbic acid. The closures used with wine designed for long-term storage should be selected to minimise oxygen ingress, but at the same time limit the potential for reductive flavour formation that has been observed for ascorbic acid in some cases. It is also important that bottling practices consider the TPO concentration as part of the assessment for ascorbic acid antioxidant behaviour.

Several degradation products of ascorbic acid are ketones and aldehydes that appear to be bound only weakly by SO_2. This means the carbonyl compounds can react with wine phenolic substances to form a pool of pigment precursors capable of rapid conversion to pigments once the concentration of ascorbic acid and SO_2 fall to a critical level. In addition to the carbonyl compounds formed through the oxidative degradation of ascorbic acid, its anaerobic degradation also leads to aldehyde production with consequent loss of SO_2. To assess if the loss of SO_2 under anaerobic conditions is dependent upon ascorbic acid and its products requires a major research effort, as does an examination of the anaerobic degradation of ascorbic acid in wines rather than in model systems.

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Efficacy of fungoid chitosans from *Aspergillus niger* and *Agaricus bisporus* in controlling the oxidative browning of model white wines

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ABSTRACT

The efficacy of two water-insoluble chitosans from *Aspergillus niger* and *Agaricus bisporus*, in controlling the browning of model white wine solutions was assessed and compared with respect to sulfite addition (70 mg/L). A water-soluble oligomeric preparation from *Agaricus bisporus* was also included to test the effect of solubility and reduced molecular weight on the antibrowning capability of the polysaccharide. Chitosans were added at 0.5 g/L and 1 g/L. Color development, iron oxidoreductive equilibrium and generation of phenolic intermediates were monitored. Results demonstrated a significant and comparable anti-browning efficacy of both insoluble formulations (up to 85% reduction in browning development with respect to control samples), which mainly acted by chelating iron (up to around 4.4 mg/g of chitosan) and shifting its oxidoreductive equilibrium toward the reduced form. Oligomeric chitosan was ineffective for this purpose as it completely lacked chelating activity, which it is proposed, depended on its negligible interaction with tartaric acid. Data on browning and oxidation-related phenolic intermediates also revealed that sulfite promotes browning once it is completely oxidized.

Industrial relevance: Following its very recent European authorization as novel food, chitosan from *Agaricus bisporus* has been evaluated for the first time and compared in wine-like conditions with the already known water-insoluble chitosan from *Aspergillus niger*. A further novelty are the data on water-soluble chitosan preparations, not yet permitted in wine but potentially interesting due to the potentially higher specific surface once in solution. The results, apart from providing information on a recently introduced source for enological chitosan, can be useful to producers and winemakers in deciding among fungoid preparations aimed to control the browning of products.

1. Introduction

Chitosan (KT) is a polymer obtained from the partial deacetylation of chitin (poly b(1→4)-2-acetamido-2-deoxy-D-glucose) (Fig. 1), a widely diffused homopolysaccharide extracted from natural sources such as the exoskeleton of arthropods or the cell wall of yeasts and fungi (Dutta, Dutta, & Tripathi, 2004; Rinaudo, 2006; Struszczyk, 2002a). Due to their physical-chemical and biological properties, such as biocompatibility, metal chelation, film-forming capabilities, antioxidant and fungistatic behavior, chitin and chitosan have been the focus of interest in a wide range of industrial and biomedical applications in recent decades (Dutta et al., 2004; Gamage & Shahidi, 2007; Jayakumar, Menon, Manzoor, Nair, & Tamura, 2010; Milhome, Ribeiro, Nascimento, Carvalho, & Queiroz, 2009; Struszczyk, 2002b).

Indeed, the partial deacetylation of chitin imply the generation of free amino groups, randomly distributed along the chitosan backbone (Fig. 1), whose reactivity markedly differentiate the two polymers. In fact, due to the protonation of the -NH2 group, at a pH of <6, KT exhibits a unique polycationic character and increased chelating and antimicrobial activities (Aranaz et al., 2009), making it particularly attractive for the food industry.

The degree of acetylation (DA) and molecular weight (MW) may also affect chitosan behavior since higher bactericidal and antioxidant properties were reported for low MW and low DA chitosan formulations (Sahariah & Másson, 2017; Yang, Shu, Shao, Xu and Gu, 2006).

In addition, solubility itself may depend on the molecular weight of chitosan, with the transition from acid solubility (typical for higher molecular weight) to water solubility (lower molecular weight) reportedly occurring at molecular weights between 4.67 and 3.82 KDa (Tian, Tan, Li, & You, 2015).

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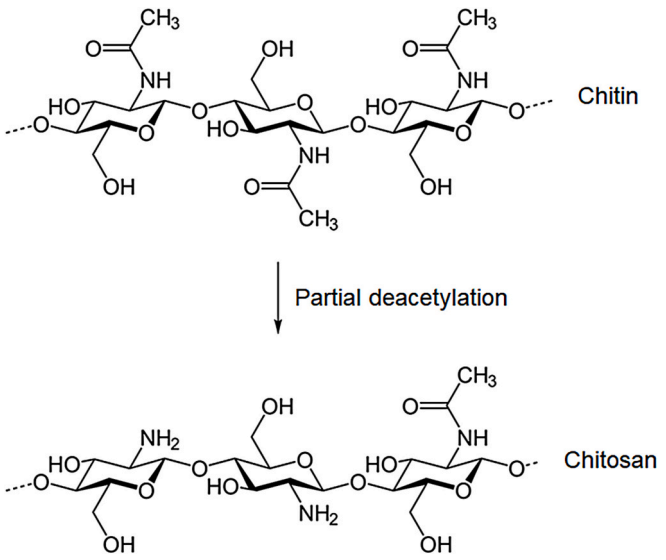

Fig. 1. Chemical structure of chitin and chitosan.

In 2011, water insoluble KT has been admitted in oenology as processing aid with clarifying and metal chelating properties, for the adsorption of contaminants or antimicrobial purposes (EU Commission, 2011).

Due to allergenicity concerns, only chitosan from *Aspergillus niger* had been authorized in wine (European Commission, 2019), excluding shrimps or crustaceans as eligible source for KT extraction. Very recently, fungoid KT from *Agaricus bisporus* has also been admitted in winemaking (European Commission, 2022) following its introduction in the European list of novel foods even if, to our knowledge, its oenological behavior has not been investigated yet.

Oxidation of stored wines is quite an articulate phenomenon, largely driven by the redox cycle of iron (Danilewicz, 2021; Li, Guo, & Wang, 2008), which involves the initial activation of oxygen by Fe^{2+} to generate hydroperoxyl radical and then H_2O_2 (Danilewicz, 2007). If not eliminated by antioxidants in solution (SO_2, ascorbic acid or glutathione, for instance), hydrogen peroxide ignites the Fenton pathway where it is reduced by Fe^{2+} to hydroxyl radical which eventually oxidizes ethanol and tartaric acid (two main constituents of wine) to acetaldehyde and glyoxylic acid respectively (Elias & Waterhouse, 2010). Phenolics with catechol or pyrogallol moieties (e.g. (+)-catechin, ( )-epicatechin, gallic or caffeic acids) fuel this oxidative cascade by reducing Fe^{3+}, generating supplementary hydrogen peroxide and *o*-quinones. These latter are highly reactive toward nucleophiles such as other phenolics, thiols or amines whose interaction ultimately drive to polymerization, browning and flavor changes of wines (Li et al., 2008). In model wine solutions containing (+)-catechin, the described pathway lead to the formation of yellow/brown pigments identified as xanthylium cations, coming from the oxidative condensation of two (+)-catechin molecules bridged by glyoxylic acid (Barril, Clark, & Scollary, 2008; Es-Safi, Le Guernevé, Fulcrand, Cheynier, & Moutounet, 2000).

Tartaric acid itself plays a further role in oxidation as it may lower the reduction potential of Fe^{2+}/Fe^{3+} couple by strongly co-ordinating Fe^{3+}, hence promoting Fe^{2+} oxidation at the expenses of the O_2/H_2O_2 couple, giving rise to the already mentioned O_2 activation (Coleman, Boulton, & Stuchebrukhov, 2020; Danilewicz, 2014).

In this overall context, SO_2 acts as an effective antioxidant because it i) reduces quinones back to the original o-diphenols, ii) quickly scavenges hydrogen peroxide before it oxidizes other wine constituents and iii) binds with carbonylic compounds (e.g. acetaldehyde or glyoxylic acid) responsible for side-reactions which negatively impact the color and the sensory features of oxidized wine (Elias & Waterhouse, 2010; Li et al., 2008).

In the last years, some investigations shed light on the intriguing capability of KT to acts as an antioxidant in wine-relevant conditions thanks to its radical scavenging properties, its metal chelation activity and the absorption of both native and oxidized phenolic species (Castro Marín et al., 2021; Castro Marín, Colangelo, Lambri, Riponi, & Chinnici, 2021; Chinnici, Natali, & Riponi, 2014). This would enlarge the oenological potential of such a polymer, in particular for the production of sulfite-free wines (Castro Marín et al., 2019), a subject of growing interest among producers, researchers and consumers concerned by the possible adverse effects of sulfites on human health (that include dermatitis, asthma or bronchoconstriction, among others) (Vally, Misso, & Madan, 2009).

The aim of this work was hence to compare for the first time the performance of oenological fungoid KT from *Aspergillus niger* and *Agaricus bisporus* in controlling the oxidative decay of model wine solutions in a typical Fenton-like environment where iron, dissolved O_2, and tartaric acid are contextually present. Model wines with and without sulfite acted as positive and negative control respectively. An oligomeric hydrosoluble KT derived from *Agaricus bisporus* was also included to test the effect of this alternative formulation on antibrowning efficacy. Color development, decline of (+)-catechin, generation of phenolic intermediates and iron speciation were used to monitor the progression of oxidative phenomena.

2. Materials and methods

2.1. Reagents

Water insoluble KT from *Aspergillus Niger* (AN), (viscosimetric molar mass = 31.6 KDa) was obtained from Tecnofood srl (Santa Maria della Versa – PV, Italy) while KT from *Agaricus bisporus* was supplied by ChiBio Biotech, (Quingdao, China) as water insoluble (AB) (viscosimetric molar mass = 37.0 KDa) and water soluble (ABsol) oligomeric formulations (viscosimetric molar mass = 1.2 KDa). The deacetylation degree of all the KT formulations was 85–90% according to the suppliers. HPLC grade acetonitrile and acetic acid were obtained from Merck (Darmastdt, Germany). Water was of MilliQ quality. (+)-catechin, (+)-tartaric acid, Fe(II) sulphate heptahydrate, ferrozine (3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p*'-disulfonic acid monosodium salt hydrate), ascorbic acid and EDTA were obtained from Sigma-Aldrich (Milan, Italy).

2.2. Model wine solutions and Fenton environment

4 L of a solution containing 4 g/L (+)-tartaric acid and 12% (*V*/V) ethanol was prepared and adjusted to pH 3.10 with 2 M NaOH. The solution was stirred overnight in an open flask at room temperature to be saturated with O_2, before (+)-catechin addition (90 mg/L). Trials were arranged by transferring 40 mL of this solution in 125 mL inert glass bottles so that air remained in the headspace. An aqueous solution of Fe (II) sulphate heptahydrate was then added to each bottle to give a final concentration of 5 mg/L Fe. When appropriate, 70 mg/L sulphur dioxide (as potassium salt), 0.5 and 1 g/L of each powdered KT formulation were separately added to generate the following solutions: CT (model solution), SO_2 (model solution + sulphur dioxide), AN 0.5 and AN 1 (model solution +0.5 or 1 g/L insoluble KT from *Aspergillus niger*, respectively), AB 0.5 and AB 1 (model solution +0.5 or 1 g/L insoluble KT from *Agaricus bisporus*, respectively), ABsol 0.5 and ABsol 1 (model solution +0.5 or 1 g/L water soluble KT from *Agaricus bisporus*, respectively). KT doses were selected taking into account its mean dosage in winemaking (0.5 g/L) and the maximum admitted dose for chelating and clarification purposes (1 g/L) (EU Commission, 2011). Before being closed with silicone septum and airtight aluminum caps, 100 mM of H_2O_2 was added to the bottles to start the Fenton reaction. Storage was carried out in the dark at room temperature. To facilitate the resuspension of insoluble KT formulations, they were manually shaken for 2 min at the starting of the

trial and every 2 days during the experiment. Triplicate bottles were set up and analyzed in duplicate ($n = 6$), for browning development, total and free SO_2, titratable acidity, the decline of (+)-catechin, the generation of phenolic intermediates, the content of Fe^{2+} and Fe^{3+}, as described below.

2.3. Browning development, SO_2 and acidity parameters

The experiments lasted 30 days during which 3 bottles each of the cited eight thesis were taken at the time points 24, 48, 120 h and 30 days (24 bottles each sampling time). This time series was adopted to focus on the initial phases of the oxidation after hydrogen peroxide addition which represent the key steps for the generation of the yellow pigments with time (Guo, Kontoudakis, Scollary, & Clark, 2017).

Browning development was monitored following the increase in absorbance at 440 nm by using a Jasco 810 spectrophotometer (Tokyo, Japan). Free and total SO_2, pH and titratable acidity (TA) were determined following the official OIV methods (OIV, 2021). All the analysis were done after centrifugation (5 min @ 1200 *g*) and removal of the suspended KT, so as to emulate the winemaking practise and taking into account the removal of compounds by KT itself.

2.4. (+)-catechin and phenolic intermediates generated during oxidation

(+)-catechin, together with compounds generated from its oxidation were quantified according to Chinnici et al. (Chinnici, Sonni, Natali, & Riponi, 2013, 2014) on a Jasco apparatus (Tokyo, Japan) equipped with a quaternary pump Jasco PU-2089, an autosampler Jasco AS-2057, a Jasco UV/VIS MD 910 PDA and a Jasco FD 2020 fluorescence detectors and a Poroshell 120 SB C18, 2.7 mm, 150 × 4.6 mm I.D. C18 column (Agilent, Palo Alto, CA), operating at 30 °C with a flow of 0.8 mL/min. Eluents were 0.2% acetic acid in HPLC grade water (eluent A) and 0.2% acetic acid in HPLC grade acetonitrile (eluent B), with a elution program as follows: from 98% to 95% A in 9 min., from 95% to 90% A in 6 min., from 90% to 82% A in 4 min., from 82% to 80% A in 3 min., from 80% to 70% A in 3 min., from 70% to 50% A in 3 min., from 50% to 0% A in 2 min. A post run of 5 min was applied. Identification was accomplished by comparing UV spectra and retentions times as defined in the previously cited papers (Chinnici et al., 2013, 2014).

Quantification of (+)-catechin was carried out at 280 nm based on a calibration curve made with (+)-catechin solutions of known concentrations. Phenolic intermediates generated by oxidation were monitored according to their maximum absorption wavelengths (440 nm for xanthylim ions and their ethyl esters) or their fluorescence response at λex 280 nm and λem 345 nm (for carboxymethine-linked dimers) and quantified as peak area.

2.5. Fe^{2+}/Fe^{3+} content of model wines

Iron speciation study was based on a previously devised spectrophotometric method (Nguyen & Waterhouse, 2019). For Fe^{2+}determination, to 1.0 mL of sample, 10 μL of ferrozine solution (3.5% *w/v* in water) and, after mixing, a 1.5 mL of 0.005% w/v EDTA (in 10% hydroalcoholic solutio) were added. Reading of the absorbance at 562 nm against a blank (2.5 mL 10% hydroalcoholic solution +10 mL ferrozine) was taken within 1 min from the addition. Total iron analysis followed the same procedure except 1.5 mL ascorbic acid 0.1 mM in 10% hydroalcoholic solution (*w/v*) was pipetted in place of EDTA and readings were done once the absorbances stabilized following the complete reduction of Fe^{3+} to Fe^{2+} and its complexation with ferrozine (about 1 h). Standard solutions of Fe^{2+} in 10% hydroalcoholic solution (0.1–6 mg/L) subjected to total iron procedure were used to quantify total and reduced iron in samples by means of an external calibration curve. Fe^{3+} amounts were calculated by subtracting Fe^{2+} from total Fe. All the readings were duplicated.

2.6. Determination of viscosimetric molar mass of chitosans

An Ubbelhode Capillary Viscosimeter type 531/10 I was used for the determination of average molecular mass. A 1 g/L chitosan was prepared in 0.3 M acetic acid/0.2 M sodium acetate solution (pH 4.6), stirred overnight to facilitate KT dissolution and filtered with 0.45 nm cellulose acetate syringe filters. The flow time of both the solvent (t_s) and the KT solutions (t) was taken at 25 °C each with 5 replicates.

The viscometric molecular weight [M] was calculates according to the Mark-Houwink-Sakurada equation $[\eta] = KM^a$, where [η] = intrinsic vicosity, K = 0.074 mL/g and a = 0.79 (Rinaudo, Milas, & Dung, 1993). Intrinsic viscosity was obtained after a series of single point measurements to determine the near-zero concentration (equal to 1 g/L at our conditions), e.g. the lowest concentration permitting consistent readings of the time needed for solutions to flow through the capillary (110–125 s) (Pamies, Hernández Cifre, Del Carmen López Martínez, & García De La Torre, 2008). Once measured the mean flow time, [η] can be calculated using the Solomon-Ciuta equation (Solomon & Ciută, 1962): $[\eta] = \frac{2*[\eta_{sp} - \ln(\eta_r)]}{c}$ with $\eta_{sp} = (t\text{-}t_s)/t_s$ (specific viscosity), $\eta_r = t/t_s$ (relative viscosity) and c = concentration of chitosan solutions (g/mL). Viscosity-average molecular weight can be expressed as g/mol or KDa (1 KDa = 1000 g/mol).

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and presented as the mean ± standard deviation (SD). Post-hoc comparison (Tukey's test) was used to highlight significant differences with $p < 0.05$ using XLStat ver.2016 statistical package (Addinsoft, Paris, France).

3. Results and discussion

3.1. Effect of chitosan on titratable acidity and pH of model wines

To monitor the extent of KT interaction with tartaric acid, both the titratable acidity and the pH of the solutions were determined during the experiment. As shown by Fig. 2A, the addition of chitosan affected the acidity parameters of model wines depending on the formulation. Regardless of the source, insoluble KT formulations (AB and AN) provoked a prompt, comparable and dose dependent diminution of titratable acidity, up to about 0.5 g/L (for 1 g/L KT addition) and 0.25 g/L (for 0.5 g/L KT addition) reduction, since the first 24 h.

This behavior was not surprising as the removal of carboxylic acids from aqueous solutions after their sorption onto KT has already been demonstrated and proposed to be due to i) the ionic interaction between positively charged amines and carboxylic anions and/or ii) the hydrophobic interaction of KT internal domain with the hydrocarbon chain of acids (Shamov, Bratskaya, & Avramenko, 2002). Gyliene et al., for instance, found that at pH 4, one g of crustacean KT can adsorb up to 1 mM tartaric acid (Gyliene, Nivinskiene, & Vengris, 2008). The same authors did also find lower but appreciable sorption at pH > 6.3 where KT is not protonated, hence accounting for supplementary mechanisms such as hydrophobic or chemical interaction between the -NH_2 groups of KT and the -COOH groups of the acids, with the generation of new amide bonds. In our case, the per gram sorption was somewhat higher than the one reported by Gyliene and colleagues, the differences being probably due to experimental conditions (differences in pH, presence of ethanol, KT source and molecular weight). It is worth to mention that in another study on red wines we found changes in TA of about 0.28 g/L after the addition of 0.5 g/L KT from *Aspergillus niger* (Castro Marín, Colangelo, et al., 2021).

Surprisingly, soluble KT (ABsol) gave almost opposite results as it slightly increased TA of solutions when added at 1 g/L (Fig. 2A). To explain this finding, one should consider that, in this case, KT totally solubilized in the model wines, and after centrifugation, no precipitate

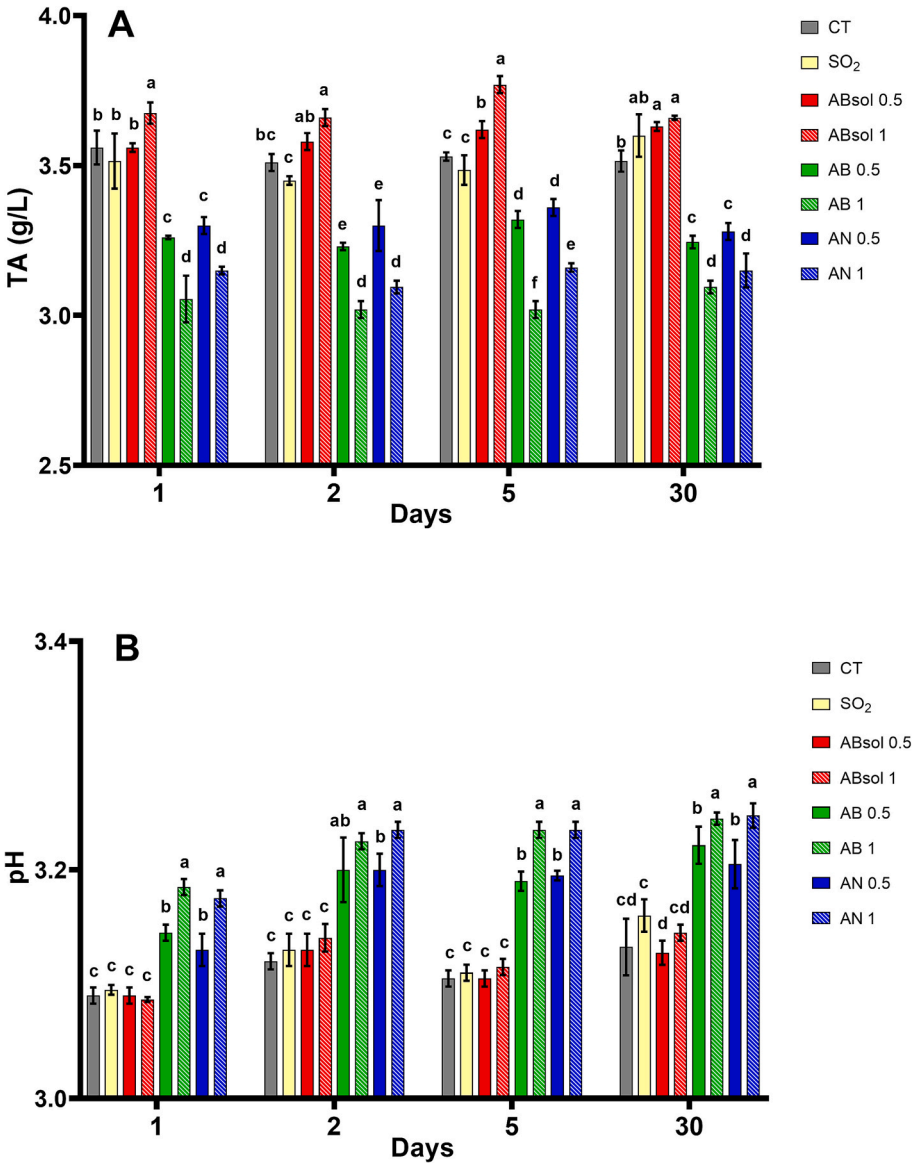

Fig. 2. Evolution of titratable acidity (TA) (panel A) and pH (panel B) of solutions during the experimentation. Data represent mean values ±SD (n = 6). For each sampling time, different letters indicate significant differences at $p < 0.05$.

was formed. Therefore, any potentially weakly bonded carboxylic acid and each -NH_3^+ group of KT would have been titrated during analyses. The greater number of amine groups in ABsol 1 samples with respect to ABsol 0.5 would further justify the tendentially higher acidity of the former. In addition, contrarily to higher MW KT, internal domain of oligomers may not have an adequate hydrophobicity to interact with tartaric acid backbone (Shamov et al., 2002).

The pH of model wines changed according with TA variations (Fig. 2B) as a consequence of the removal of organic acids from the medium. Insoluble formulations raised the pH by 0.11 and 0.15 units after 30 days and KT addition of 0.5 g/L and 1 g/L respectively, while ABsol did not significantly affect this parameter. Apart from the sorption of carboxylic acids, the increase of pH in KT aqueous suspensions could theoretically also be due to the protonation of the amine groups which decreases free [H+] in the medium (Gyliene et al., 2014).

3.2. Iron content of model wines and its speciation

As mentioned, Fe is a key factor in the Fenton chemistry which, in turn, dominates the chemical oxidation of wines. The extent of wine oxidation, in fact, is proportional to the amount of dissolved iron (Danilewicz, 2021; Elias & Waterhouse, 2010) and the claimed capability of KT to chelate this metal (Dutta et al., 2004; Gamage & Shahidi, 2007; Struszczyk, 2002a) might influence the overall kinetics. Accordingly, it has been proposed that the knowledge of Fe^{2+}/Fe^{3+} ratio may permit to estimate the overall redox state of a wine, representing a reliable index of the evolving equilibrium between oxidative and reductive reactions (Danilewicz, 2016).

As shown in Fig. 3, after 5 days of storage all the KT insoluble formulations had chelated Fe to a considerable extent, without a clear dose-dependency and independently from the fungoid source. For those formulations, residual Fe slightly changed at day 30, reaching chelation percentages comprised from 83% to 88% (for AB 0.5 and AB 1, respectively). Similar results were obtained in other studies in model solution and wines at comparable KT concentrations (Bornet & Teissedre, 2007; Chinnici et al., 2014). Once again, hydrosoluble oligomeric KT (ABsol) exerted unexpected results because its presence did not affect the amount of free Fe available to bind with ferrozine (Fig. 3). This result apparently contrasts with previous studies where KT oligomers (5–12 KDa) demonstrated higher chelating capacities with respect to medium

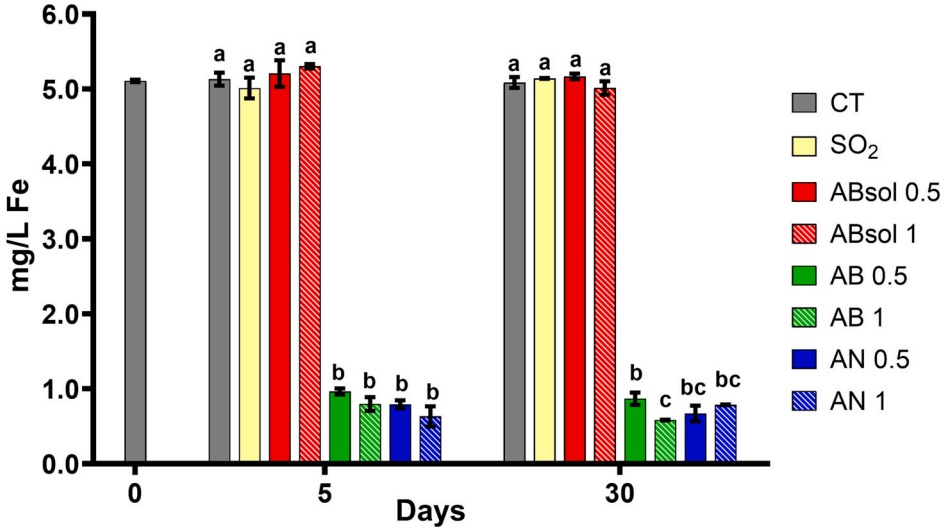

Fig. 3. Total Fe (mg/L) in the samples at days 0, 5 and. 30. Data represent mean values ±SD (n = 6). For each sampling time, different letters indicate significant differences at $p < 0.05$.

or high MW preparations (Chien, Sheu, Huang, & Su, 2007; Jung & Zhao, 2012). However, Rhazi et al. (Rhazi et al., 2002) established for KT a minimum polymerization degree equal to 6 (e.g. about 1.0–1.2 KDa) for noticeable complexation of copper, supposedly due to the need of a favorable spatial organization around the metal ion and to a high amount of molecular chain necessary to initiate the complexation. This would hence give a first plausible justification to our results with the 1.2 KDa hydrosoluble formulation. In addition, it has been speculated that in acidic solutions, chitosan alone may not behave as metal chelator because of the massive protonation of amine groups which impede the coordination with metal cations due to repulsive forces (Rocha, Ferreira, Coimbra, & Nunes, 2020). In these situations, the presence of polycarboxylic acids is thought to be crucial as they form negatively charged complexes with iron (Coleman et al., 2020) which eventually interact with -NH_3^+ groups of KT in a ternary KT-carboxylate-Fe complex, hence promoting metal adsorption (Castro Marín, Stocker, et al., 2021; Rocha et al., 2020). Accordingly, the negligible adsorption of tartaric acid by ABsol formulations (Fig. 2A), could be a further reason for the observed lack of iron chelation by KT oligomers.

Not only did KT formulations affect the total amount of Fe in solution, but they also impacted the oxidation state of Fe during the experiment (Table 1).

As expected, in CT samples the Fe^{2+}/Fe^{3+} ratio was found to be clearly shifted toward the oxidized form of the metal (up to 80–87% of total Fe), indicating strong oxidative environment all along the experiment.

On the contrary, at day 5 the samples added of sulfite only marginally diminished the percentage of Fe^{2+} with respect to time 0 (from 89% to about 85%), as a consequence of the scavenging of H_2O_2 by SO_2 and subsequent blocking of the Fenton cascade. This would decrease the amount of total sulfite (oxidized to sulphate) in the solutions as, in fact, we found for those samples (Table S1). Nevertheless, oxidation probably occurred to some extent, since bound sulfite contextually increased (Table S1) due to the binding with oxidation-derived aldehydes (acetaldehyde and glyoxylic acid) (Danilewicz, 2007; Elias & Waterhouse, 2010). Notably, AB 1 solutions showed 78% of Fe^{2+} after 5 days (Table 1), a value slightly lower than SO_2 but that, however, suggests the decreased rate of iron oxidation in those samples. Also, all the other model wines added of insoluble KT (AB 0.5, AN 1 and AN 0.5) had the prevalence of reduced Fe^{2+} over Fe^{3+}, even if to a lesser extent and commensurate to the dose of added KT. Taking into account that the rate of oxidation is proportional to the amount of total iron in solution (Danilewicz, 2021; Elias & Waterhouse, 2010), these results may well depend on the low amount of free Fe (Fig. 3) available to carry on the oxygen activation and the Fenton reactions e.g. it may depend on the chelating capability of KT. In addition, the scavenging activity against hydroxyl radical and H_2O_2 that insoluble KT may exert in wine relevant environment (low pH, presence of alcohol, phenolics and hydroxycarboxylic acids) (Castro Marín et al., 2019; Castro Marín, Stocker, et al., 2021) could have played a further role.

On the other hand, as it is obvious from Table 1, ABsol did not affect the oxidoreductive equilibrium of model wines, and the Fe^{2+}/Fe^{3+} ratio of those solutions was the same as CT, certainly due to the lack of iron chelation (Fig. 3) and, arguably, to a reduced radical scavenging activity of oligomeric formulation at those conditions. Indeed, when dealing with the scavenging activity of KT oligomers (< 10 KDa) as a function of molecular weight, the limited available literature provides inconsistent results when applied to acidic beverages (Chien et al., 2007; F. Yang

Table 1
Iron speciation in the samples at days 0, 5 and 30 (t = time). For each column, mean values (± SD) are shown ($n = 6$). In the same column, different letters indicate significant differences at $p < 0.05$.

	t0			t5			t30		
	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%	Fe (II) mg/L	Fe (III) mg/L	Fe(II)%
CT	4.59 ± 0.03	0.53 ± 0.01	89.6 ± 1.02	0.69 ± 0.13^{bc}	4.65 ± 0.24^{a}	12.9 ± 1.90^{e}	1.03 ± 0.04^{ab}	4.06 ± 0.13^{a}	20.2 ± 2.36^{d}
SO_2				4.19 ± 0.22^{a}	0.77 ± 0.22^{b}	84.5 ± 2.29^{a}	1.25 ± 0.05^{a}	3.89 ± 0.06^{a}	24.3 ± 1.89^{c}
ABsol 0.5				0.78 ± 0.02^{b}	4.43 ± 0.16^{a}	14.9 ± 0.95^{e}	0.98 ± 0.00^{b}	4.19 ± 0.03^{a}	18.9 ± 1.85^{d}
ABsol 1				0.87 ± 0.09^{b}	4.44 ± 0.11^{a}	16.3 ± 2.02^{e}	1.02 ± 0.02^{b}	3.99 ± 0.10^{a}	20.4 ± 2.54^{d}
AB 0.5				0.56 ± 0.04^{c}	0.40 ± 0.05^{bc}	58.6 ± 2.24^{d}	0.23 ± 0.02^{d}	0.71 ± 0.13^{b}	24.6 ± 3.01^{c}
AB 1				0.62 ± 0.15^{cd}	0.17 ± 0.08^{d}	78.4 ± 2.72^{b}	0.19 ± 0.01^{d}	0.39 ± 0.01^{c}	33.1 ± 2.56^{b}
AN 0.5				0.46 ± 0.03^{d}	0.33 ± 0.01^{c}	58.0 ± 1.10^{d}	0.24 ± 0.10^{d}	0.43 ± 0.08^{c}	35.8 ± 2.31^{b}
AN 1				0.40 ± 0.06^{d}	0.20 ± 0.07^{cd}	66.6 ± 1.85^{c}	0.35 ± 0.05^{c}	0.44 ± 0.01^{c}	44.4 ± 1.98^{a}

et al., 2017). It is worth noting that at day 30, the Fe^{2+} percentages of all the samples dropped considerably, indicating the prevalence of oxidation on the long-term, independently from the addition performed (Table 1). Sulfite itself was unable to prevent Fe from being oxidized because of its eventual complete disappearance as free SO_2 (Table S1).

Further, additional amounts of bound sulfite were consumed during oxidation. Considering that in our model wines SO_2 binders were almost exclusively represented by acetaldehyde and glyoxylic acid, this evidence confirms that even the strongly bound sulfite fractions may dissociate and participate to the overall antioxidant potential of a wine, as recently reported (Sacks, Howe, Standing, & Danilewicz, 2020). Overall, at day 30, in the presence of insoluble KT, the percentage of Fe^{2+} was the highest, especially for KT from *Aspergillus niger* at 1 g/L, indicating the slower rate of oxidation of those media.

3.3. Generation of (+)-catechin intermediates during oxidation and browning of samples

While attempting to study the chemical pathways involved in the browning of white wine, model solutions have often been employed. Indeed, since the late 1990s, the generation of yellow xanthylium pigments from the oxidative decay of (+)-catechin in the presence of glyoxylic acid (coming from tartaric acid oxidation) and iron has been elucidated in those matrixes (Es-Safi et al., 2000; Es-Safi, Le Guernevé, Fulcrand, Cheynier, & Moutounet, 1999). The cascade includes the transient formation of 4 isomeric carboxymethine-linked (+)-catechin dimers, which further undergo to dehydration to give xanthenes isomers whose eventual oxidation generates 6 isomeric yellow pigments and their respective ethyl esters (Clark, Prenzler, & Scollary, 2003; Es-Safi et al., 2000).

In hydroalcoholic model solutions, the ancillary formation of (+)-catechin dimers bridged by acetaldehyde (stemming from ethanol oxidation) was also established, but no evidence emerged so far that those dimers are able to generate yellow pigments equivalent to xanthylium cations (Clark et al., 2003).

The chromatographic analysis carried out on our solutions permitted the identification and semi-quantification of most of those intermediates as illustrated in Fig. S1. In Fig. 4, therefore, the amount of both the (+)-catechin dimers and the xanthylium pigments generated in the samples during the experimentation are shown as sum of peaks area of their respective isomers. As mentioned before, provided the presence of sufficient dissolved O_2, (+)-catechin dimers should evolve to yellow compounds, giving browning extents proportional to their quantity.

Apart from the solutions added with SO_2, (+)-catechin dimers were promptly generated in all the samples since the first 24 h (Fig. 4, panel A) due to the initial addition of hydrogen peroxide to ignite the Fenton

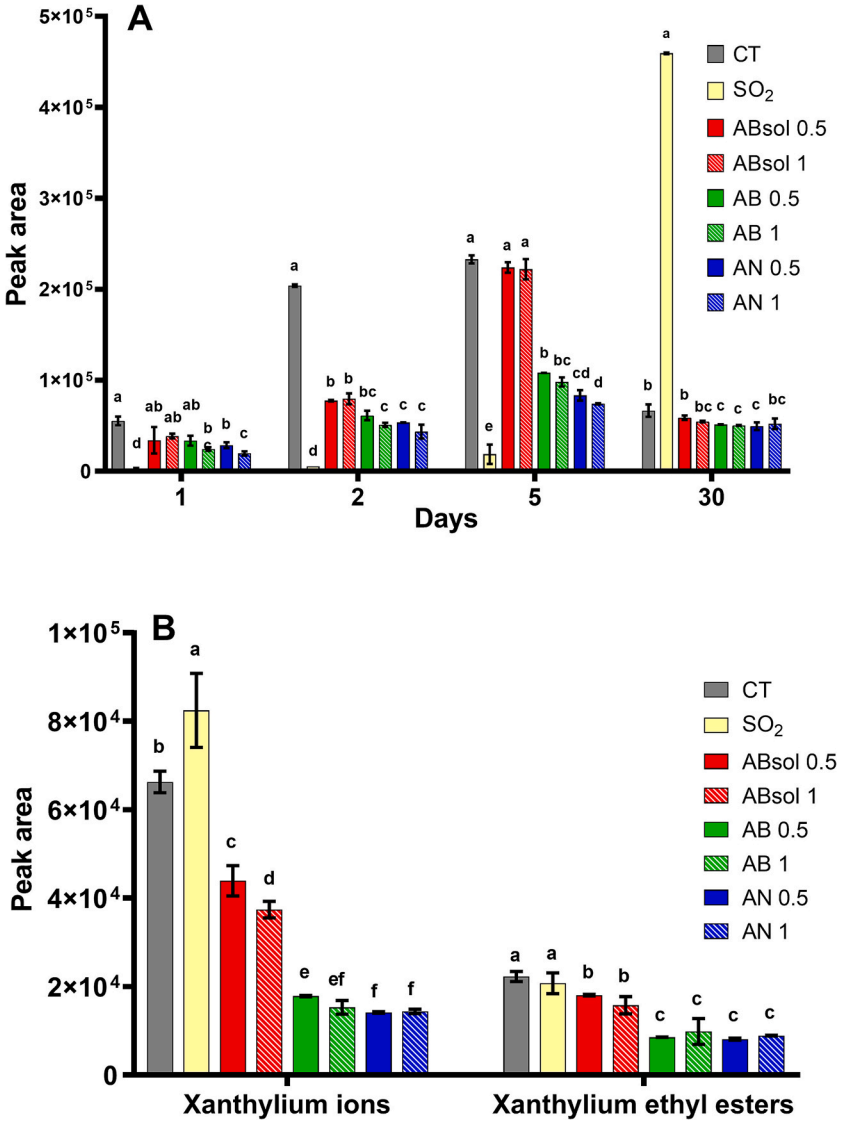

Fig. 4. Phenolic intermediates generated by (+)-catechin oxidation during the storage of model wines. Panel A: evolution of carboxymethine-linked (+)-catechin dimers (as sum of HPLC peak areas of the 4 isomers) during the experimentation. Panel B: Xanthylium pigments and their ethyl esters amounts (as sum of HPLC peak areas of the respective isomers) identified in the solution at day 30. Data represent mean values ±SD (n = 6). For each sampling time (panel A) and for each class of pigments (panel B), different letters indicate significant differences at $p < 0.05$.

reaction. CT and ABsol progressively produced the highest amount of those intermediates up to day 5, while insoluble KT formulations (AB and AN) generated significantly lower quantities of dimers with some differences only between AB 0.5 and AN 1 after 5 days.

On the other hand, it appears that the presence of sulfite strongly interfered with the production of dimers, most likely due to its scavenging of H_2O_2, thereby delaying iron oxidation (Table 1). A further reason would be that any glyoxylic acid possibly generated in those samples would have reacted with free SO_2, in this way becoming unavailable to participate to the formation of dimers.

Xanthylium pigments were found to be produced in little amounts only after 5 days, and solely in CT and ABsol 1 samples (data not shown). This lag period is comparable with the one found by Guo et al. (Guo et al., 2017) in wine-like solutions where the oxidative cascade had been boosted by glyoxylic acid addition and corresponded to the time needed for the generation and subsequent oxidation of (+)-catechin dimers at those conditions. Indeed, after 30 days of storage, xanthylium ions were largely produced in all model wines (Fig. 4, panel B), to which corresponded the generalized and expected depletion of (+)-catechin dimers (Fig. 4, panel A), with the notable exception of SO_2 samples.

In that case, in fact, the formation of dimers appeared to be delayed but, once begun, to proceed toward high accumulation of intermediates up to day 30. It is noteworthy that in both model and real wines, sulfite demonstrated to considerably increase O_2 consumption and redox cycling of (+)-catechin by reducing quinones back to their *O*-diphenol form while oxidizing to sulphate (Danilewicz, 2011). As suggested by table S1, once free sulfite is depleted bound sulfite may dissociate from acetaldehyde and glyoxylic acid which could therefore bridge (+)-catechin molecules to generate dimers. This may probably explain the generation of dimers in SO_2- containing solution only after day 5. Further, the mentioned high rate of oxygen consumption connected to (+)-catechin redox cycles could have played a further role, increase the amount of aldehydes and, hence, of dimers produced. Indeed, supplementary sampling points after day 5 could have provided further insights on the kinetic of dimers and pigments formation but, apart from being this subject beyond the scope of the present work, as discussed below the collected data are adequate enough to explain the color development of the solutions.

The extent of pigments generated at day 30 was the highest for SO_2, CT and ABsol samples (Fig. 4, panel B). Insoluble KT from *Agaricus bisporus* (AB) tended to form marginally higher amounts of pigments with respect to *Aspergillus niger* (AN) especially at 0.5 g/L.

At the end of the experimentation, percentages spanning from 31% to 49% of (+)-catechin were lost, depending on the sample (Table 2) but without an apparent correlation between the amount of intermediates and pigments formed during oxidation (Fig. 4) and the decrease of their initial precursor. However, while for CT and SO_2 the aforementioned loss may be deemed to be only due to oxidative phenomena (e.g. generation of dimers and xanthylium pigments), in the presence of KT (+)-catechin could be additionally removed via adsorption onto the polymer (Castro Marín & Chinnici, 2020; Chinnici et al., 2014). An indirect confirmation of the existence of this mechanism is the lower amount of residual (+)-catechin in all the solutions added with high KT doses (1 g/L) with respect to their respective low dosage (0.5 g/L) (Table 2) despite similar quantities of oxidation intermediates (Fig. 4). It is nonetheless unclear why soluble KT (ABsol 0.5 and ABsol 1) gave the highest loss of (+)-catechin and remains to be verified whether this may depend on its increased interaction with phenolics due to the augmented specific surface once solubilized.

Table 2 also shows the extent of browning of solutions after 5 and 30 days of storage. In that table, the optical densities at T0 are also reported to take into account the impact of KT and SO_2 on the initial color. KT formulations, in fact, appeared to turn the samples slightly yellower since the very beginning, probably due to the presence of residual soluble glucans (Dutta et al., 2004; Struszczyk, 2002a) while sulfite, as expected, bleached the solutions to some extent. The color of all the solutions started to increase at day 5, namely only once xanthylium ions had been generated, and continued up to the end of the storage to various extent depending on the sample. After 30 days, CT, SO_2 and ABsol 1 had the highest color increase, somewhat corresponding to the amount of xanthylium ions present in those samples (Fig. 4, panel B). It seems obvious, hence, that neither the presence of KT oligomer was effective in protecting the solutions from browning nor was sulfite once depleted its free fraction. Both the insoluble formulations, on the contrary, significantly contributed to reduce the oxidative yellowing of model wines, particularly in the case of AN 1. The prolonged presence of the polymer as a suspension permitted, on the long term, to obtain samples with the lowest degree of browning.

Table 2
Residual (+)-catechin content, corresponding percentage loss (in brackets), optical densities of samples (OD 440 nm) at t0, t5 and t30 (days) of storage and browning (Δ D.O. between t0 and t30) of samples. For each column, mean values (± SD) are shown (n = 6). In the same column, different letters indicate significant differences at $p < 0.05$.

	(+)-Catechin at t30		OD 440 nm at t0	OD 440 nm at t5	OD 440 nm at t30	Browning
	(mg/L)		(AU)	(AU)	(AU)	(Δ AU)
CT	63.07 ± 0.85^{a}	(32.1%)	0.027 ± 0.004^{b}	0.044 ± 0.005^{a}	0.203 ± 0.003^{a}	0.176 ± 0.005^{a}
SO_2	63.26 ± 1.39^{a}	(31.9%)	0.015 ± 0.005^{c}	0.017 ± 0.003^{c}	0.187 ± 0.007^{c}	0.172 ± 0.005^{a}
ABsol 0.5	54.15 ± 0.84^{c}	(41.7%)	0.032 ± 0.004^{ab}	0.036 ± 0.003^{b}	0.189 ± 0.003^{b}	0.157 ± 0.004^{b}
ABsol 1	47.34 ± 1.62^{d}	(49.0%)	0.034 ± 0.004^{a}	0.039 ± 0.007^{ab}	0.205 ± 0.005^{a}	0.171 ± 0.004^{a}
AB 0.5	63.27 ± 1.05^{a}	(31.9%)	0.037 ± 0.002^{a}	0.039 ± 0.003^{ab}	0.071 ± 0.001^{d}	0.034 ± 0.002^{c}
AB 1	61.25 ± 0.94^{b}	(34.1%)	0.035 ± 0.004^{a}	0.038 ± 0.003^{ab}	0.068 ± 0.003^{de}	0.033 ± 0.003^{c}
AN 0.5	64.09 ± 0.85^{a}	(31.0%)	0.032 ± 0.001^{ab}	0.036 ± 0.002^{b}	0.064 ± 0.001^{de}	0.032 ± 0.001^{c}
AN 1	61.50 ± 0.97^{b}	(33.8%)	0.036 ± 0.003^{a}	0.037 ± 0.002^{b}	0.062 ± 0.002^{e}	0.026 ± 0.003^{d}

4. Conclusions

Overall, the data discussed in this paper confirmed that insoluble, > 30 KDa MW fungoid KT can reduce the browning of model wines subjected to oxidation, with little differences between doses (0.5 or 1 g/L). The source of KT, whether from Aspergillus niger (AN) or Agaricus bisporus (AB), had only a marginal effect on the efficacy of the polymer, with AN formulations being slightly more effective, particularly at the highest dose. The role of oxidoreductive equilibrium of iron and its chelation by KT on the extent of Fenton reaction was highlighted. Water soluble oligomeric KT from *Agaricus bisporus*, on the other hand, was ineffective for that purpose, and the browning of those solutions was almost the same as the CTRL samples where no antioxidant was added. It is proposed that this behavior may depend on the negligible iron chelation demonstrated by soluble KT which, in turn, could be due to its inability to adsorb tartaric acid. To our knowledge, this is the first report on the use of soluble oligomeric KT in a wine-like medium. Sulfite, on the other hand, may increase browning once fully oxidized. Further research is needed to ascertain whether soluble KT may have increased antimicrobial capabilities with respect to insoluble formulations, as reported in other food matrixes, in this way potentially representing an additional tool for winemakers wanting to reduce the use of sulfite in

wines.

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CRediT authorship contribution statement

Federico Baris: Investigation, Writing – original draft. **Antonio Castro Marín:** Conceptualization, Formal analysis. **Ana Cristina De Aguiar Saldanha Pinheiro:** Investigation, Resources. **Silvia Tappi:** Resources, Visualization. **Fabio Chinnici:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

No.

Data availability

Data will be made available on request.

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Application of low molecular weight chitosan in animal nutrition, husbandry, and health: A review

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ABSTRACT

Chitosan (CHT) is a natural substance widely used in veterinary medicine. However, the application of chitosan in animal production has severe shortcomings. This is because it is insoluble in pH $\geq$ 7 due to its very stable crystalline nature. This has stimulated its derivatization and depolymerization to low molecular weight chitosan. Low molecular weight chitosan (LMWCHT) has unique characteristics such as antibacterial activity and biodegradability, which make it sound for its use in animal nutrition, husbandry, and health. This review has provided sufficient evidence to demonstrate the usefulness of LWMCHT in animal nutrition, husbandry, and health. The review has also shown the benefit of LWMCHT in chemotherapy delivery. Further, the review has demonstrated the potential of LWMCHT as an alternative antibiotic in animal nutrition.

1. Introduction

Livestock is a substantial asset with a global market value of at least $1.4 trillion (Thornton, 2010). As a component of the extensive market chains, livestock industry employs up to 1.3 billion people globally, while 600 million poor smallholder farmers in developing nations directly depend on livestock especially, cattle (Thornton, 2010). In addition to generating milk, meat, and manure, ruminants perform several other tasks, which include draught force. Animal protein makes up a significant portion of the typical person's diet. Thirty-three percent (33%) of the daily protein requirement worldwide is met by dairy, meat, and eggs from animals (Thornton, 2010). The demand for livestock products is increasing daily due to population expansion and urbanisation (Thornton, 2010). For rural households in developing nations, livestock species perform essential economic, social, and cultural services to boost farm revenue and households' well-being. Livestock is beneficial for a variety of reasons, including food production, family nutrition, family income, asset preservation, and soil productivity. Others include transportation, agricultural traction, agricultural diversification, sustainable agricultural production, family and community employment, ritual purposes, and social status (Bettencourt et al., 2015; Moyo et al., 2010). In many Sub-Saharan African (SSA) countries, livestock production accounts for about 35% of the agricultural GDP and employs approximately 70% of the population, especially in rural areas (Moyo, 2016). Smallholder farmers produce 90% of SSA's agricultural products Giller et al., 2021). According to Giller et al. (2021), livestock in this region meets up to 18% of the population's dietary protein needs with high-quality animal protein, which helps to balance the people's high-carbohydrate diets.

Feeding is critical in producing livestock. However, the sector has to deal with inadequate feed resources especially, during the dry season, which results in weight loss of animals and lower market value. Further, due to the high price and limited supply of conventional feedstuffs, like soybean meal, which is a significant source of protein for the preparation of animal concentrates, feeding livestock with agricultural and industrial feed has dramatically improved in recent years (Hassan et al., 2013). The ever-increasing prices of cereals and protein concentrates have increased the cost of producing livestock. These problems necessitated the intensive study of less expensive non-traditional feed ingredients (Khattab et al., 2011). Also, the rising global cost of energy, purchased feeds, equipment, and pharmaceutical drugs have led to a sharp drop in poultry production and the exit of many poultry farms has placed the industry's survival in jeopardy (Attia et al., 2022).

Feed additives in animal nutrition increase immunity, and metabolic function and promote livestock growth in general (Kobayashi et al., 2002). Antibiotics have been used for decades to treat diseases and boost agriculture development (Kobayashi et al., 2002). The prolonged application of conventional antibiotics could result in microbial

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resistance and the residual effect on animal products. As a result, several non-conventional antibiotics have been developed. Chitosan is an alternative antibiotic and feed additive currently being used to formulate an animal's diet (Li et al., 2018, 2021).

Chitosan is created by alkali-deacetylating chitin. D-glucosamine and N-acetyl-D-glucosamine are two monosaccharides randomly arranged to form the linear polysaccharide called chitosan.

Chitosan has uses in various industries, including the agricultural sector and more cutting-edge biotechnology and nanotechnology disciplines. Oral, nasal, and other routes deliver different medications into the body (Je et al., 2004). Various chitosan-based films are used in food coating as an antimicrobial agent and a flocculating and adsorbing agent (Priya et al., 2014; Vargas & González-Martínez, 2010). Jayakumar et al. (2010) asserted that, chitosan could aim in delivering different genes utilised in siRNA technology, cancer, and gene therapy.

Further, chitosan has been used in 3D networks and microelectrochemical systems are created using tungsten carbide chitin whiskers, graphitic carbon nanocapsules, and other materials (Wang et al., 2009), which are used in heart regeneration therapy, corneal and bone regeneration, including brain and skin regeneration (Dastidar & Ghosh, 2018). According to Yamazaki et al. (2009), combining chitosan and sulphuric acid can potentially discharge high voltage for electrolyte application. Chitosan can remove effluents which include suspended particles from various processing plants, such as whey, dairy, poultry, and seafood (Alishahi & Aïder, 2012).

Chitosan is also used to produce filter papers, biodegradable packaging, and water-resistant papers which are used as a fixative for colour photography and making colour films. Chitosan is used as a wood adhesive, fungicide, quality enhancer, and preservative (Cheba, 2011). Chitosan, as an ingredient is employed in various cosmetic products due to its fungicidal properties, UV absorption capacity, and biocompatibility (Dutta et al, ., 2009). In plants, for example, chitosan's antibacterial properties have made it beneficial for suppressing some plant diseases (Xing et al., 2015). Additionally, it increases the germination potential, root length and activity and auxin concentration. It also increases seedling height and urea release in the soil. Further, chitosan by products provide animals with additional protein (Kashyap et al., 2015; Xing et al., 2015). Chitosan is effective to remove organic and inorganic contaminants from the environment (Boamah et al., 2015; Mohanasrinivasan et al., 2014; Rajeswari et al., 2016). Also, chitosan is used to treat obesity by taking advantage of the reducing effects of cholesterol and LDL (Huang et al., 2015). Chitosan and its derivatives are permeation enhancers that can be employed in drug-delivery systems because they can increase the permeability of intestinal, nasal, and buccal epithelial cells (Mourya et al., 2010). Chitosan is a material that could create solid-state batteries. This is because it provides ionic conductivity in an acetic acid solution. The use of chitosan beads makes it possible to immobilise cells like *E. coli* (Fierro et al., 2008; Strand et al., 2003). The two main criteria used to assess the physical, biological, and chemical characteristics of chitosan (CHT) are its molecular weight (Mw) and degree of deacetylation (DD) (Domard, 2011). The most common commercial chitosan available is those with DD and kDa of 70-90% and 50 and 2000, respectively, derived from crustacean shells (Seyfarth et al., 2008).

There are three groups of CHT based on their molecular weight: LMWCHT(<150 kDa), medium-molecular-weight-chitosan(MMWCHT) (150-700 kDa), and high- molecular-weight-chitosan(HMWCHT) (>700 kDa) (Mishra, 2015).

LMWCHT is obtained through physical, chemical, or enzymatic procedures (Holappa et al., 2006; Swiatkiewicz et al., 2015). The polydispersity index (PDI), degree of deacetylation (DD), and Mw of LMWCHT are influenced by biological activity (Zargar et al., 2015). LMWCHT has been used in the field of agriculture as seed-coating (Badawy & Rabea, 2011; Boamah et al., 2023; Hadwiger, 2013; Sharp, 2013), particularly in plants. However, reviews on chitosan application in animal nutrition, husbandry, and health are limited, especially for LMWCHT. Therefore, the aim of this review paper is to use LMWCHT in animal nutrition and the health of farm animals. Fig. 1 shows the structure of $LMWCHT_n$ (n = 8, 11)

2. Overview of LMWCHT in farm animals

Due to the increase in demand for organic meat and egg products, organic and free-range animals are more widely available to animal food consumers (Siegrist & Hartmann, 2019). With the contemporary agriculture production intensified, many customers believe that organic products are healthier for them (Śmiglak-Krajewska & Wojciechowska-Solis, 2021). The issue of antibiotic resistance and the growing consumer demand for organic animal products has made researchers to investigate new strategies to improve farm-animal performance while protecting environmental and human health. As a result, several feed additives are being produced in animal production to improve productivity. Studies have reported that LMWCHT is an alternative to synthetic antibiotics (Hashem et al., 2021; Swiakiecz et al., 2015). According to Duffy et al. (2018), the antimicrobial properties of LMWCHT are very effective against viruses and bacteria. Chitosan has been used in beef and dairy cattle feed to improve rumen fermentation and digestibility (Gandra et al., 2016; Goiri et al., 2009). CHT has been employed as a rumen modulator in beef and dairy cattle as well as an addition in the preparation of silage (Araujo et al., 2015; de Paiva et al., 2016; Gandra et al., 2016). Furthermore, chitosan is commonly used in feed preservation due to its antimicrobial activity (Kong et al., 2010).

According to de Paiva et al. (2016), adding CHT to dairy cow feed is an alternate strategy for lowering the energy needs of animals that produce much milk. Beef calves fed being chitosan-ensiled grass have improved dry matter digestibility, neutral detergent fibre (NDF), acid detergent fibre, and live weight (Henry et al., 2015).

3. Chitosan in nanosize for farm animals

The application of nanotechnologies such as nano antibiotics, nano photogenic, nano hormones, and nano minerals has recently gained attention in livestock production (Hashew & Gonzalez-Bulnes, 2021). According to Rikta (2019), chitosan's effectiveness as an antiviral agent has been applied recently in nanotechnology. The distinctive nanoparticles of chitosan have promoted its use in animal nutrition and health management (Anwar et al., 2019). Chitosan has contributed significantly to developing nanoparticle vaccines for treating arbovirus infections because of its adjuvant qualities (Amenta et al., 2015). CHT nanoparticles boost the potency of antivirals due to their exceptional physicochemical properties.

According to Enoka et al. (2020)), chitosan nanoparticles have an antibacterial activity comparable to fosfomycin, which makes them a potent alternative to antibiotics for treating *Staphylococcus aureus* and *Escherichia coli (E. coli)*. Chitosan nanoparticles are, therefore, a viable substitute for antibiotics. When 3.0 and 4.5 g/ml of nano chitosan were added to a broiler meal, Abdeltwab et al. (2019) discovered that fungal

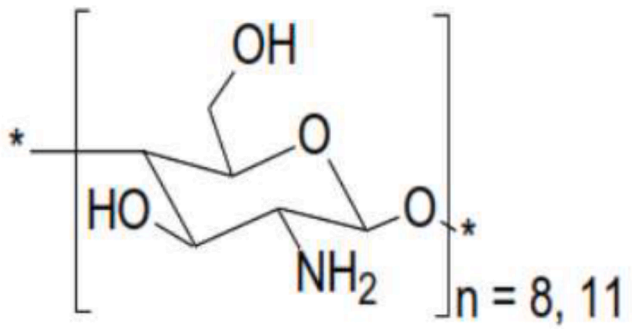

Fig 1. Structure of $LMWCHT_n$ (n = 8, 11)

activity was delayed. Additionally, Hassanein et al. (2021) found that feeding rabbits with nano chitosan decreased the number of hazardous bacteria (*E. coli* and *Salmonella spp*.) while increasing the number of Lactobacillus, a helpful bacterium. One recognized indicator of a healthy gut is the lactobacillus index. When goats were fed an ensiled grass diet (GnRH), chitosan nanoparticles were conjugated with tripolyphosphate, and the Gonadotropin-releasing hormone (GnRH) dosage was reduced three to four times. However, neither fertility nor prolificacy was noticeably affected (Hashem & Sallam, 2020).

In order to trigger ovulation in rabbits, Hassanein et al. (2021) investigated different delivery strategies and GnRH-loaded chitosan nanoparticle dosages (GnRH-ChNPs). This revealed its utility in reducing the GnRH dose, controlling animals, and enhancing the success of in vitro fertilization. GnRH-ChNPs added to semen had negative consequences for fertility despite effectively inducing ovulation. Further, when chitosan dextran sulphate nanoparticles were included, they had no impact on the vitality, motility, or membrane operation of rabbit semen.

In another study, Tufan et al. (2015) fed quails with a diet supplemented with 150 mg of chitosan and observed a significant reduction in the *E. coli* load compared to the control diet. Similarly, Elnesr et al. (2022) also observed a substantial decrease in the *E. coli* population and an increase in *Bacillus* population when broilers were fed a 1 and 2-g/kg chitosan-based diet. Tufan et al. (2015) study also, reported a significant decrease in intestinal pathogenic bacteria when Japanese quails were fed a 75 or 150 mg/kg chitosan diet. Further, when 100 mg of chitosan was added to the broiler meal, Xu et al. (2013) saw a reduction in the amount of *E. coli* present. Several authors have reported similar results when chitosan was added to chicken diets (Spring et al., 2000; Tufan et al., 2015). They all reported a decrease in the *E. coli* population and an increase in the *Lactobacillus* population. In addition, the Japanese quails' antioxidative state was improved by nano chitosan supplementation, and catalase (CAT), a typical enzymatic antioxidant in poultry, showed higher antioxidant activity in Hassan et al. (2021) study.

4. Preparation, features, and antibacterial capabilities of LMWCHT

4.1. Preparation of LMWCHT

LMWCHT is prepared using chemical, physical or enzymatic methods (Aranaz et al., 2021; de Farias et al., 2019; Doan et al., 2021). Each method offers benefits and drawbacks depending on the reagents employed and the treatment conditions (Doan et al., 2021). In the chemical depolymerization operations, oxidants (Hydrogen peroxide, Potassium dithionite) or acids (Hydrochloric acid, Nitrous acid, Hydrofluoric acid, Phosphoric acid) are employed (Aljbour et al., 2019; Tsao et al., 2011). The acid approach has benefits, which include the ability to generate LMWCHT on a large scale at low cost of production (Minh et al., 2020). Also, vast amounts of tiny pieces are produced, and has the ability to speed up chitosan hydrolysis. However, using concentrated acids at large concentrations harms the ecosystem (Gonçalves et al., 2021). Aside that, the hazardous effects of severe acid depolymerization of chitosan prevent its use in medicine (Schmitz et al., 2019). Strong acids are not the only option for depolymerization. Mild acids are also used for the depolymerization of chitosan, and weak acids are considered safer than strong acids when producing LMWCHT for pharmaceutical use. According to Savitri et al. (2014), chitosan can be broken down with insufficient weak acid. As a result, depolymerization can be accomplished with formic acid, a weak acid. The molecular weight of chitosan is markedly reduced by formic acid depolymerization in the presence of H_2O_2 (Purwanto et al., 2019).

Together with acid approaches, physical techniques like gamma irradiation (Co-60), microwaves, and electrochemical degradation (ultrasonication) decrease the Mw of CHT (Alves et al., 2018; Liu et al., 2006). The sonication technique breaks down the chemical bonds of chitosan through energy application (Kritchenkov et al., 2020; Liu et al., 2006; Singh et al., 2019). The characterization results demonstrate that ultrasonic depolymerization does not alter the structure of chitosan (Popa-Nita et al., 2009). However, few products are produced with the required special equipment.

LMWCHT is also prepared via biological or enzymatic depolymerisation techniques using generic or specialised enzymes (chitinase, chitosanases, and lysozyme). In using enzymes to produce LMWCHT, it increases the quantity and the products are safe. However, the method is expensive for commercial production (Naveed et al., 2019; Zhang & Zhang, 2013). The preparation of LMWCHT using nonspecific enzymes is less expensive and aids commercial production (Kumar & Tharanathan, 2004). Three enzymes, which are cellulase, chitosanase, and chitinase are commonly used to break down chitosan bonds (Naveed et al., 2019). Chitinases have the unique capacity to hydrolyze A-A bonds, unlike chitosanase, which can cleave A-D and D-D links. The A-A bonds in chitosan are discovered to be cut arbitrarily by cellulase (Xia et al., 2008). Chitinase, lysozyme, and cellulose are used successfully to prepare several LMWCHT products (Lin et al., 2009). Similarly, when cellulase, lipase, and bromelain are combined during catalysis instead of employing available enzymes, the rate of hydrolysis increased (Tishchenko et al., 2011).

4.2. Features of LMWCHT

The solubility, chain length, Mw distribution, and DD affect CHT's properties in solution (Birth & Dautzenberg, 2002). The polymer and bulk phases are the most significant characteristics of chitosan in solution. Several authors have reported a connection between chitosan and solvent phenomena (Ogawa et al., 2004; Roy et al., 2017). In Roy et al. (2017) study, a looser chain changed into a coiled sphere, when the CHT solution's concentration was raised. Osorio-Madrazo et al. (2010) discovered that its properties remained unchanged when chitosan was hydrolyzed in 2 mol of L^{1} HCl. Through a variety of processes, including oxidative degradation, acidic cleavage, enzymatic degradation, and ultrasonic degradation, HMWCHT can be converted into LMWCHT (Bartkowiak, 2002; Orive et al., 2005). Due to its protonated pH in solution or lower producing charged polycations, LMWCHT is employed as a drug delivery system carrier (Hayashi & Ito, 2002; Kato et al., 2003).

4.3. Antimicrobial capabilities of LMWCHT

The primary features of CHT are the cationic and unique behaviour in solution (Muzzarelli & Muzzarelli, 2005; Rinaudo, 2006). Chitosan is a biopolymer with many NH_2 and OH groups and a stiff D-glucosamine structure (Muzzarelli et al., 1986). The NH_2 groups in CHT are often protonated at a pH of 6.3, which gives the polymer a polycationic behaviour (Muzzarelli & Muzzarelli, 2005). Moreover, chitosan is more soluble in acid at a lower acetylation level (50%) (Rauh & Dornish, 2006). According to Morin-Crini et al. (2019), ultra-pure, pure and technical are the main ways of determining chitosan purity. Biologically, chitosan is non-toxic; however, it cannot be digested by humans (Rabea et al., 2003). Chitosan has bioactivities such as drug-releasing activities in vaccine delivery due to its antibacterial, antifungal, antioxidant, and anti-inflammatory properties (Vasconcelos & Pomin, 2018). Chitosan's biological characteristics and mode of action are listed in Table 1.

5. Effect of LMWCHT on farm animals

The nutritional effect of LMWCHT's supplementation in agricultural animals has been reported by Walsh et al. (2012). The dosage and molecular weight determine the effectiveness of chitosan's supplementation in animal production (Walsh et al., 2012). Using LMWCHT as a supplement in animal feed reduces gastrointestinal ulcers and chronic constipation (Suthongsa et al., 2017). Further, it improves microbiota,

Table 1
Biological characteristics of CHT and action strategy (Kamal et al., 2022).

Discipline	Action strategy	Reference
Medicine	Multiple medications are administered through various internal passageways, including the mouth and nose.	(Nagpal et al., 2010)
Nanotechnology	Chitosan has an exceptional capacity for positively charging nanoparticles, enhancing their ability to interact with cell membranes and allowing nanocapsules to enter cells.	(Guo et al., 2013; Nagamoto et al., 2004)
Antimicrobial	The negative charged microbial cell membrane interacts with the positive charged chitosan molecules, which force them to pull apart. Chitosan possesse santibacterial characteristics as a result.	(Dong et al., 2019; Klaykruayat et al., 2010)
Anti-inflammatory	Given the potential harmful effects of an inflammatory response that is heightened and prolonged in several disorders, chitosan has strong anti-inflammatory properties that have been repeatedly characterised.	(Ma et al., 2011; Villiers et al., 2009)
Antioxidant	Chitosan has antioxidant characteristics such as DPPH radical and superoxide protection	(Tomida et al., 2009; Xu et al., 2018)

villus structure, and apparent nutrient digestibility in ruminants and monogastric animals' (Magalhaes et al., 2019). Several authors have observed significant improvement in animal performance when chitosan was used in dietary supplementation of weaned piglets (Duan et al., 2020; Hu et al., 2018), rabbits (Kamal et al., 2022) and lambs (Magalhaes et al., 2019). In addition, Li et al. (2019) in their study found that broiler immunity and growth performance improved when their diet was supplemented with LMWCHT.

According to Araujo et al. (2015), there is enough data to prove that chitosan and its derivatives positively impact the flora gut. Henry et al. (2015) also found that dry matter digestibility was affected when chitosan was included in the beef heifer diet. In a study in UK, acetate and propionate production in the rumen was found to alter with time, according to Belanche et al. (2016). It has been demonstrated that chitosan increases the quantity and quality of milk produced by dairy cows and sows (Ho et al., 2020). Furthermore, the sexual behaviour, sperm quality, and reproductive efficiency of New Zealand rabbit bucks have improved in Kamal et al. (2023) study on multifunctional role of chitosan in farm animals. In Table 2, studies on the effects of LMWCHT on farm animals are compiled and summarized.

6. Mode of action of LMWCHT on farm animals

The physicochemical characteristics of chitosan regulate its mode of action. The mode of action which is often associated with its functional groups and Mw of CHT determine its antimicrobial effectiveness (Klaykruayat et al., 2010). LMWCHT kills bacteria by inhibiting RNA transcription and halting cell growth when it touches the bacterial cell membrane (Klaykruayat et al., 2010). The molecule in chitosan interacts with microbe proteins to compromise permeability, results in cell death (Lim & Hudson, 2004). The chitosan's biodegradation kinetics affected the polymer's chain length and how the acetyl groups were distributed within the polymer (Zhang & Neau, 2001). Additionally, CHT increases CD4+ cells and humoral immunity, enhancing farm animals' immune response (Sarwar et al., 2021; Zaharoff et al., 2007).

LMWCHT is a potential antibiotic replacement (Park & Kim, 2010). Antibiotics prepared from chitosan releases cation chelation and cytoplasmic membrane (Park & Kim, 2010). Chitosan enables the entry of nanocapsules into cells Because of its interactions with cell membranes (Guo et al., 2013).

Table 2
Animals' reactions to LMWCHT supplementation.

Animal species	Results	Reference
Sheep, goat, cattle	• Chitosan lowers the rumen fluid's acetate-to-propionate ratio. • The formation of volatile fatty acids decreased biohydrogenation in the rumen.	(Goiri et al., 2010; Araujo et al., 2015) (Del Valle et al., 2017)
Poultry	• The cumulative feed efficiency of chickens improved after receiving 3,600 Da COS as a meal. • The average daily gain and feed conversion ratio in broilers were enhanced. • liver lipase activities increased in the groups who received chitosan supplements.	(Huang et al., 2005)
Swine	• Chitosan enhanced the weaner health or performance by improving gastrointestinal absorption and nutrient bioavailability. • Chitosan reduced intestinal inflammation, increased intestinal barrier performance, and improved growth performance in weaned pigs. • Piglet diets containing chitosan enhanced growth rate and boosted immune response.	(Suthongsa et al., 2017) (Hu et al., 2018).(Duan et al., 2020)
Poultry	Chitosan increased the ratio of feed to body weight rise and body weight. Increased blood serum protein, dietary lactobacillus, and nutritional digestibility. With no impact on triglyceride, total cholesterol, high-density lipoprotein levels, or growth performance, there was a decreased blood low-density lipoprotein concentration in birds fed a chitosan-supplemented diet. The addition of chitosan encouraged sexual activity.	(Liu et al., 2017) (Keser et al., 2012) (Kamal et al., 2022)

7. The use of LMWCHT in farm animals

Chitosan stimulates digestive enzymes, making it a good feed additive (Hou & Gao, 2001). According to Suthongsa et al. (2017), chitosan enhances growth and nutrient absorption when fed to pigs as a dietary supplement. Further, growing piglets (Liu et al., 2008), lambs (Magalhaes et al., 2019; Pereira et al., 2020), chicks, and quail have all demonstrated enhanced growth performance when exposed to chitosan (El-Ashram et al., 2020; Khambualai et al., 2009; Kong et al., 2010; Shi-bin & Hong, 2012).

LMWCHT reduces intestinal disaccharide activity and fat absorption, preventing weight gain in rats (Chiu et al., 2017). Some research revealed no appreciable differences between piglets given chitosan-supplemented and non-chitosan-supplemented diets while others discovered that low molecular weight chitosan favours weaning pig growth performance (Hu et al., 2018; Xu et al., 2013). Walsh et al. (2012) reported that 10 to 50 kDa CHT feed supplements enhanced intestinal structure most, and 5 to 10 kDa CHT had an antibacterial impact in weaned pigs.

7.1. The use of LMWCHT in animal health

Chitosan has been used in veterinary medicine for several purposes, including bone regeneration, analgesic effects, and wound healing (Senel & McClure, 2004). Senel and McClure (2004) have studied the potential of chitosan as a method of administering medications and vaccines to veterinary animals and as a food additive. They found that chitosan is being used for therapy in veterinary medicine due to its

various biological properties. With regards to animal health, chitosan is advantageous for transporting immunostimulatory and chemotherapeutic drugs to the mucosal epithelium and is more beneficial for veterinary services (Underwood & van Eps, 2012). Chitosan is cited by Underwood and van Eps (2012) as a potential nanoparticle including nanomedicine as a cutting-edge nano-system for medication delivery. Also, commercial nutritional supplements made from chitosan called Nutri+Gen and EpakitinTM are used to treat canine and feline chronic kidney disease. The therapeutic use of chitosan to combat dangerous germs and fungi has been demonstrated by Yan et al. (2021) in their study on antimicrobial properties of chitosan and chitosan derivatives in the treatment of enteric infections. Atay (2020) showed that low molecular weight chitosan and its derivatives are more effective than high molecular weight chitosan against bacteria, yeast, and fungus. LMWCHT, made from Pacific white shrimp shells, according to Sugiyanti et al. (2018), has antioxidant and antibacterial properties against both gram (+) and gram (-) bacteria. Underwood and van Eps (2012) examined the reality and the practicality of nanomedicine and veterinary medine. They reported that more antioxidant and antibacterial activity are present in LMWCHT. Minh et al. (2020) obtained similar results, demonstrating the antioxidant and antibacterial properties of the chitosan hydrochloride salt in combination with LMWCHT. According to reports, LMWCHT can break through bacterial membranes, which accounts for its more vigorous antibacterial and antioxidative activity (Yin et al., 2009).

Moreover, Jaime et al. (2012) concluded in their study in chemogenomics that LMWCHT could increase cell membrane permeability, and destroys fungal cells. Studies have shown that chitosan binds to the cell walls of microorganisms, causing extracellular acidification that increases the uptake of calcium ions into the cell and ultimately results in cell death (Goy et al., 2009). Chitosan has the potential to be widely used in the field of animal husbandry as a feed additive, a disease-resistant agent, or an adjuvant in vaccinations. According to Walsh et al. (2012), chitosan therapy at a dose of 250 mg/kg significantly reduced the number of numerous bacteria, such as *Lactobacillus* and *Escherichia coli*, in piglets' colons in Ireland.

Other reported benefits of LMWCHT include an increased villus height and an improved villus height to crypt depth ratio in the duodenum and jejunum as indicators that chitosan supports intestinal structural integrity (Walsh et al., 2012).

Liu et al. (2008) examined the effect of ultrasonic treatment on the biochemphysical properties of chitosan and posited that adding CHT to pigs' diets improved the structure of their digestive tracts, decreased the amount of *Escherichia coli* in their faeces, and decreased their likelihood of getting diarrhoea. Several observations made shown that chitosan boosted the immune-stimulating effects of the *Vibrio anguillarum* vaccination in fish aquaculture (Kumar et al., 2008). Also, chitosan evoked the humoral immune response, which was better suited to prevent pathogens from infecting the host (Boroumand et al., 2021).

The immunogenicity of two inactivated porcine circovirus type 2 vaccines (PCV2-COS-1 and PCV2-COS-2) was boosted when chitosan was covalently bonded to them to generate vaccine conjugates (Zhang et al., 2017). The degree of chitosan deacetylation was further discovered to correlate positively with the adjuvant effect (Zhang et al., 2017). Furthermore, chitosan's affinity for a specific carrier protein (ovalbumin, or OVA) may help the PCV2-COS vaccine's immunological effect (Zhang et al., 2018).

7.2. The use of LMWCHT in animal husbandry

LMWCHT has been employed as an alternative feed additive to promote the development of the livestock sub-sector (Yang et al., 2012). Due to its numerous advantageous biological qualities, which include its ability to support growth performance (Chen et al., 2009; Yang et al., 2012), fight free radicals (Anandan et al., 2012), and modify resistance (Kobayashi et al., 2002), low molecular weight chitosan has reportedly been utilized extensively as a viable antibiotic replacement in feed for piglets and broiler chickens. Chitosan has been shown to increase milk production and quality in various farm animals, including dairy cows and sows (de Paiva et al., 2016; Ho et al., 2020).

The ability of animals to reproduce, including pregnancy and its outcomes, milk production in dairy cows, egg production, and swine development performance, are enhanced by giving them chitosan (Del Valle et al., 2017; Hamady & Farroh, 2020; Wan et al., 2018). In addition, CHT can enhance placental activities, a dam's capacity for immunity and antioxidants, a dam's or offspring's ability to use nutrients, and a dam's capacity for antioxidants, all of which can create the best conditions for fetus growth and survival (Del Valle et al., 2017). CHT also increased feed consumption, body weight gain, the ratio of feed to energy, the weights of immunological organs, and lymphocyte proliferation in broiler chickens (Shi-bin & Hong, 2012).

7.3. The application of LMWCHT in animal nutrition

In the rumen of sheep, steers, and dairy cows, chitosan was observed to decrease the acetate-to-propionate ratio (Del Valle et al., 2017). Gorelik et al. (2021) study in Russia reported an improved blood physiological condition and metabolism in black and white dairy cows whose diet was supplemented with 2% chitosan succinate. Additionally, Li et al. (2022) reported an enhanced dry matter intake, milk production, and antioxidant capacity in dairy cows fed chitosan at 0, 500, 1000, 1500, and 2000 mg/kg inclusion levels in their study in China.

In a study by Wencelova et al. (2014) that examined the effects of chitosan, plant oils, and different diets on rumen metabolism and protozoan population in sheep found that the generation of volatile fatty acids in a goat's rumen, the overall amount and motility of ruminal protozoa, were not significantly affected by chitosan. According to the authors, the differences in diets, SF (Sunflower oil) or RP (Rape seed oil) composition, and different microbial activity in the batch cultures could be the reasons for the insignificant results. However, chitosan was observed to alter ruminal fermentation by changing the volatile fatty acids (VFAs) profile and raising the propionate level in Goiri et al. (2009) study involving dose–response effects of chitosans on in vitro rumen digestion and fermentation of mixtures differing in forage-to-concentrate ratios in Spain. Chitosan was added to the diet of Chinese Marino sheep by Zhang et al. (2022), who noted better growth rates, wool output, and blood parameters.

Further, chitosan and its derivatives are excellent in neutralising free radicals, such as hydroxyl radicals and superoxide anions (Sun et al., 2007). Earlier studies found that chitosan can prevent oxidative damage to the body by controlling the activity of linked antioxidant defence enzyme systems, reducing lipid peroxidation and active oxygen in animals (Ivanova & Yaneva, 2020). Studies have shown that the addition of CHT to laying hen meals at varied concentrations of 0, 200, 400, 800, and 1600 ppm caused a linear increase in the plasma antioxidant capacity (Farivar et al., 2018; Kosaka et al., 1996).

7.4. Application of LMWCHT as an animal growth enhancer

Chitosan has been successfully used as a feed supplement for cattle, pigs, and poultry, which has helped to regulate the initiation of inflammation, oxidative stress, and in vivo immune response. Chitosan supplementation in broiler chicken diets has improved lipid metabolism in broiler chicken diets as reported in Shenghe et al. (2017) study in China. Also, chitosan reduces belly fat and enhances breast meat quality. In addition to lowering the proportion of chicken caspase-3-positive cells, chitosan has stimulated the growth of immunological organs and prevented lymphocyte death (Shenghe et al., 2017; Zhou et al., 2009). This has impacted on poultry performance as a result of CHT's treatment (Shenghe et al., 2017). In Shi et al. (2005) study in China, favourable effects were observed on feed conversion ratio and live body weight gain in chickens fed with a diet containing 0.05 to 0.1% chitosan. These

include growth performance, energy availability and protein retention. Shi et al. (2005) again reported that chitosan can improve laying hen production. As observed, fowls fed with a meal containing 0.06% chitosan showed better body weight gain and average feed consumption (Khambualai et al., 2009). According to Świątkiewicz et al. (2013) study, a meal treated with chitosan boost daily egg mass and hen-day egg output while lowering yolk cholesterol levels. Meng et al. (2010) also found that adding LMWCHT to the feed of layer chickens boosted average egg weight and quality in China. Similarly, when chitosan dosages of 250 g/kg and 500 g/kg were administered to fowls, it increased the daily egg production and feed conversion ratio (Li et al., 2022). However, Swiqtkiewicz et al. (2018) found that adding 100 mg/kg chitosan to the meals of layer chickens had no discernible impact on the digestion of nutrients. Similar findings were made by Chang et al. (2022), who fed broiler 50 g/kg of chitosan and found no discernible difference in weight gain, feed intake, or feed conversion ratio. Besides, chitosan's use in feed and water enhances their immune system's response, antioxidative activity, and capacity to survive bacterial infection in *Aralichthys olivaceus* and *Litopenaeus vannamei* (Li et al., 2015). In a study, supplementing sows' food with chitosan throughout late pregnancy and lactation significantly enhanced the suckling piglets' daily growth and weaning weight (Xie et al., 2015). Chitosan made crude protein and fat more digestible in piglets' ilea, resulting in better nutritional digestion and absorption (Thongsong et al., 2018). Moreover, the molecular weight and level of deacetylation of chitosan affected sow and piglet growth differently (Xie et al., 2015).

The addition of LMWCHT to the diet of weaning pigs was found to exhibit potent anti-inflammatory characteristics that prevented intestinal discomfort in Yang et al. (2018)) study. Thongsong et al. (2018)) also found that adding dry chitosan to weaned pigs' diets increased their feed intake, body weight gain, and daily gain. In addition, Wencelova et al. (2014) asserted that chitosan reduces nutrient digestibility in dairy cows due to its antibacterial properties against rumen microbes. Kirwan et al. (2021) also noted that supplementing beef heifers with chitosan raised the pH and ammonia levels in the rumen but not the levels of butyric acid. That is the CH_3COOH to CH_3CH_2COOH ratio decreased when chitosan was added to the low-protein total mixed ratio (Kirwan et al., 2021).

Del Valle et al. (2017) studied chitosan's effects on rumen fermentation in living things, particularly dairy cows in Brazil. They found that when chitosan was included in the diets of dairy cows, feed intake with regards to dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fibre (NDF), and unsaturated fatty acid concentrations increased. However, nutrient digestibility (DM, OM, NDF, and crude fat) decreased. Sannes et al. (2002) study found that chitosan raised ruminal pH and propionate molar proportion compared to calves fed without chitosan. They also discovered that chitosan levels were lower in the Butyri vibrio population when compared to the other treatments, as well as decarboxylation of the branch-chain amino acids.

In another study, ammonia concentration increased when chitosan was added to a high-protein diet, and this was also noticed in steers that had chitosan supplements (Araújo et al., 2015). The observed ammonia concentration increase was not associated with an increase in protein cleavage but rather with an addition of ammonia from the amine group of the chitosan and a decrease in the amount of ammonia taken up by rumen bacteria (Belanche et al., 2016). According to Kang-Meznarich and Broderick (1980), low-protein diets did not affect the concentration of ruminal ammonia because they produced less ammonia than was necessary for the creation of microbiota and the digestion of fibre. de Paiva et al. (2016), observed an enhanced feed intake and nutrient digestibility. These were caused by chitosan supplementation, that raised propionic acid levels. Also, chitosan degrades in the rumen, certain bacteria may utilise the remaining carbon skeleton to produce the altered rumen fermentation products (Chen et al., 2002). Chitosan supplementation is believed to have a detrimental effect on nutrient digestibility, a potential lead in an overproduction of volatile fatty acids. On the other hand, Haraki et al. (2018) study discovered that the ratio of CH_3COOH to CH_3CH_2COOH was significantly higher, which indicates a more substantial contribution from the NDF, the animals' feeding behaviour as well as the levels of volatile fatty acids. The CP presence, however, changed the rumen's volatile fatty acid composition. Thus, the increased CP levels in the food led to lower CH_3COOH and higher CH_3CH_2COOH concentrations in the rumen.

8. Conclusion and future outlook

LMWCHT is a byproduct of hydrolysis, and has better solubility than the polymer it started with. LMWCHT is a potential biomaterial in animal nutrition, husbandry, and health because of the of the abrupt global increase in the price of purchased feeds and veterinary medications coupled with dramatic decline in livestock productivity. It's anti-inflammatory, anti-bacterial and reduced oxidative stress properties among others makes it application relevant in livestock husbandry. Low-molecular-weight chitosan is made via physical, chemical, and enzymatic processes and each method has benefits and drawbacks. The biological activity of LMWCHT is significantly influenced by its molecular size. There is sufficient research to demonstrate its usefulness in animal nutrition and health, such as feed additives, wound healing, bone regeneration, analgesic, and antimicrobial effect. Low molecular weight chitosan is a potential alternative antibiotic in livestock production to address the rising global cost of veterinary drugs. However, there a need for more literature on its utilization, especially in livestock production. Hence, more in-depth studies should be carried out.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Data availability

No data was used for the research described in the article.

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Metschnikowia pulcherrima as biocontrol agent and wine aroma enhancer in combination with a native *Saccharomyces cerevisiae*

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Sulfur dioxide

ABSTRACT

One of the main objectives for a sustainable winemaking process is the reduction of the use of sulfur dioxide. In this regard, non-*Saccharomyces* wine yeasts are proposed as biocontrol agent in different steps of wine production chain. Here, a selected strain of *Metschnikowia pulcherrima* (DiSVA 269) and a native *Saccharomyces cerevisiae* low sulfite producer strain (DiSVA 708) were investigated. After preliminary laboratory trials, winemaking process at industrial level showed an effective biocontrol action (reduction of c.a. 1 Log order of wild yeasts) of *M. pulcherrima* inoculated at prefermentative stage in cold clarification (48 h at 10 °C) and during the subsequent fermentation process. The combination of *M. pulcherrima/S. cerevisiae* led a distinctive aromatic profile of wines both in laboratory and winery trials with a significant enhancement of ethyl butyrate, ethyl hexanoate, isoamyl acetate and β-phenyl ethanol. Moreover the use of the two selected strains was the best combination to enhance volatile thiols (3-mercaptohexan-1-ol and 3-mercaptoexil acetate) that well correlate with the sensory analysis (tropical fruits). The overall results indicate that the combined use of *M. pulcherrima* DiSVA 269 and native *S. cerevisiae* DiSVA 708 led a biocontrol action and an improvement of aromatic and sensorial profile of wine with low SO_2 content.

1. Introduction

The use of non-*Saccharomyces* selected strains in sequential fermentation with *Saccharomyces cerevisiae* starter strains is a current well-established winemaking strategy to produce wines with distinctive sensorial properties. Among them, *Metschnikowia* is one of the most investigated genera due to its multiple contribution in winemaking. *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, and *Metschnikowia viticola* are the most species naturally found in wine environments with well-established antimicrobial activities (Belda et al., 2016b; Brysch--Herzberg et al., 2015; Morata et al., 2019; Vicente et al., 2020). *M. pulcherrima* is a well characterized species for several positive features in winemaking: Indeed, it can modulate the synthesis of secondary metabolites to improve the sensorial profile of wine and to act as biocontrol agent. (Varela et al., 2016; Zhang et al., 2018). Recently, a selected strain of *M. pulcherrima* in mixed fermentation with two different *S. cerevisiae* strains determined an impact on the analytical and sensorial profile due to an increase in the levels of the thiol 4-MSP (4-methyl-4-sulfanylpentan-2-one) above the sensory threshold, together with a decrease in higher alcohol production (Ruiz et al., 2018). Another important feature of this non-*Saccharomyces* yeast is the wide possess among the strains of the enzymatic activities such as pectinase, protease, glucanase, lichenase, β-glucosidase, cellulase, xylanase, amylase, sulphite reductase, lipase and β-lyase activity (Barbosa et al., 2018; Vicente et al., 2020). Regarding to the proteolytic activity of *M. pulcherrima* is important feature in mixed fermentation to release amino acids as nutrient for *S. cerevisiae* and act as control of protein haze formation in wines as a biological fining agent. (Marangon et al., 2012). Pectinase activity is strain-dependent on *M. pulcherrima* (Hong et al., 2019; Marangon et al., 2012) while the glucosidase activity promotes the release of varietal aromas from the grape (Belda et al., 2016a). *M. pulcherrima* sequential fermentations seem to increase the final amino acid concentration in wine (Benito et al., 2015).

According to several works, when *M. pulcherrima* is used the reduction of volatile acidity seems to be a trend, with variations estimated between 10% and 75% (Hranilovic et al., 2020; Roca-Mesa et al., 2020).

M. pulcherrima can be also used as biocontrol agent, due to the production of pulcherrimin, a red pigment with antifungal activity (Csutak

Abbreviations: 4-MSP, (4-methyl-4-sulfanylpentan-2-one); DiSVA, Dipartimento Scienze della Vita e dell'Ambiente-Dep. Life Environmental Sciences; OIV, Organizzazione Internazionale della Vigna e del Vino.

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et al., 2013; Kántor & Kacániová, 2015; Oro et al., 2018; Saravanakumar et al., 2008). Based on these attitudes, *M. pulcherrima* species could be used as a strategy to contain spoilage microorganisms and reduce the use of sulfur dioxide particularly in organic wine production.

In this context, the investigations are directed to the reduction of compounds that bind SO_2, the management of pH and towards the search of new compounds of natural origin with antimicrobial activity.

A microbiological approach based on the selection of bio-protective strains can be a useful tool to reduce sulfite concentration in wine-making (Di Gianvito et al., 2022; Escribano-Viana et al., 2022; Windholtz et al., 2021). In this way, the use *S. cerevisiae* strains characterized by the absence or the reduced production of sulfur compounds and which nevertheless highlight the aromatic imprint of the wine is one of the main goals of researchers to satisfy winemakers and consumer requests (Agarbati et al., 2020; Linderholm et al., 2010).

In this work, the combined use of different yeast strains, each with a specific functional role during fermentation and both enhancers of the final wine aroma, could contribute to improve the overall wine quality. Selected strains of *M. pulcherrima* and native *Saccharomyces cerevisiae* have been set up with a dual role: i) play a biocontrol activity with consequent reduction in added sulphites; ii) enhance the final wine aroma profile. The suitability of the studied yeast strains to naturally control the fermentation process will be addressed.

2. Materials and methods

2.1. Yeast strains

M. pulcherrima strain DiSVA 269, already characterized for its biocontrol ability, was used (Oro et al., 2014b). The *S. cerevisiae* native strain DiSVA 708, previously selected and featuring (Agarbati et al., 2020), was used as fermenting yeast. A commercial *S. cerevisiae* starter strain, Lalvin ICV OKAY® (Lallemand Inc., Toulouse, France) were used as control commercial strain. YPD agar medium (yeast extract 1%, peptone 2%, dextrose 2% and agar 1.8%) at 4 °C was used for short-term storage while for long-term storage was used YPD broth supplemented with 80% (w/v) glycerol at 80 °C.

2.2. Preliminary laboratory scale fermentation trials

The Verdicchio grape juice (vintage 2017) was used for laboratory scale fermentations. The main composition of grape juice was as follows: pH 3.22; initial sugar content 212 g/L; total acidity 4.58 g/L; malic acid 2.7 g/L; nitrogen content YAN (60 mg/L) and total SO_2 27 mg/L. Fermentations were conducted in 250 mL Erlenmeyer flasks locked with a Müller valve containing 200 mL of Verdicchio grape juice at the temperature of 22 °C ± 0.5 under static condition in triplicate. Pre-cultures of strains were carried out using modified YPD (0.5% w/v yeast extract, 2% w/v glucose, and 0.1% w/v peptone) in an orbital shaker (150 rpm) at 25 °C for 24h. The inoculum grape juice was carried out at an initial concentration of approximately 1×10^6 cells/mL. Sequential fermentations were carried out inoculating *M. pulcherrima*, followed after 48 h, by *S. cerevisiae* DiSVA 708 and OKAY®, respectively. Pure cultures of *S. cerevisiae* were used as control trial. The weight loss of the apparatus due to the CO_2 evolution was monitored to evaluate the fermentation kinetics until constant weight (for 2 consecutive days).

2.3. Fermentation trials in winery at industrial level

After the preliminary laboratory trials, *M. pulcherrima* DiSVA 269 strain was inoculated at pre-fermentative stage (during clarification procedures) followed by the inoculation of native *S. cerevisiae* DiSVA 708 or commercial strain OKAY®, or to carry out the fermentation process at industrial level.

2.3.1. Preparation of starter inoculum

To prepare the inoculum all the yeast strains were pre-cultured using a modified YPD medium (0.5% yeast extract, 0.1% peptone and 2% glucose) for 48 h at 25 °C under agitation (150 rpm). 30-L bioreactor (Biostat® C; B. Braun Biotech Int., Goettingen, Germany) containing 25 L of modified YPD was then inoculated (5% vol/vol). Fermentation condition were: 400 rpm/min; air flow of 1 vvm (L/L/min). Yeast biomass production was carried out using a feed batch procedure and, at the end of the process, the cells were collected by centrifugation, and washed three times with sterile distilled water. The inoculum of grape juice was conducted in the form of cream (80% humidity) at a concentration of approximately 1×10^6 cell/mL. This cell concentration was used for *M. pulcherrima* before cold clarification and for both *S. cerevisiae* starter strains for fermentation of the respective vats. The growth kinetics of the yeast strains were monitored during the fermentation at established time.

2.3.2. Winemaking process procedures

Fermentation trials were performed using Verdicchio grape juice coming from vintage 2020. Freshly harvested grapes) were treated following the standard winemaking procedure: soft pneumatic pressing, cold clarification without SO_2 addition at 10 °C for 48 h. The analytical characters of the grape musts were initial sugars 216 g/L, pH 3.34, total acidity 4.37 g/L, malic acid 1.7 g/L, and nitrogen content 90 mg/L. Yeast assimilable nitrogen were adjusted to 250 mg N/L with diammonium phosphate and yeast derivative (Genesis Lift® Oenofrance, Bordeaux, France).

Using two consequential Verdicchio grape juice lots of 600 hL each coming from two consecutive working days (1° and 2° lots), four vats of 300 hL were filled: two of these were inoculated with 1×10^6 cells/mL of *M. pulcherrima* DiSVA 269 strain to assess the potential biocontrol action during cold clarification during 48 h, while the other two vats were not inoculated. After 48 h the four vats were inoculated with *S. cerevisiae* DiSVA 708 and OKAY® strains, respectively with the following scheme:

M. pulcherrima DISVA 269/*S. cerevisiae* OKAY® (1° lot).
S. cerevisiae OKAY® (1° lot).
M. pulcherrima DISVA 269/*S. cerevisiae* DiSVA 708 (2° lot).
S. cerevisiae DiSVA 708 (2° lot).

The fermentations were carried out at 19 ± 1 °C and were monitored by sugar consumption using Baumé (°Bé) densimeter.

2.3.3. Monitoring of yeast population

The evolution of the wild and inoculated yeast strains was followed during the fermentation by viable cell count using lysine agar medium (Oxoid, Hampshire, UK) as selective medium for non-*S. cerevisiae* strains and WL nutrient agar medium (Oxoid, Hampshire, UK) for the differential recognition of form and color diversity of colony. The plates, after incubation at 25 °C for four days, were evaluated for the detection of inoculated and wild yeasts. The combination of the results of lysine agar enumeration and macro- and micro-morphological estimation in WL nutrient agar medium permitted the distinction between inoculated and wild yeasts. The presumptive identities of the yeasts were confirmed by sequencing using ITS 1 and 4 as target region. The ITS1-5.8S rRNA-ITS2 region was amplified by PCR (Polymerase Chain Reaction) using primer pair ITS1 (50-TCCGTAGGTGAACCTCGCG-30) and ITS4 (50-TCCTCCGCTTTATTGATATGC-30), as described by White et al. (1990). The BLAST program and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) were used to compare the sequences provided with those already in the data library. With the aim to discriminate the inoculated *S. cerevisiae* strains (native or commercial starter) by wild strains, intraspecies characterization of isolates were carried out using primer pairs δ 12/21 as described by Legras & Karst, 2003. The length of the PCR products was estimated by comparing them with 100-bp marker DNA standards (GeneRuler 100-bp DNA Ladder; AB Fermentas).

2.4. Analytical procedures

Total acidity (OIV-MA-AS313-01), volatile acidity (OIV-MA-AS313-02), pH (OIV-MA-AS313-15), and ethanol content (OIV-MA-AS313-24) were evaluated according to the use the standard methods of OIV (https://www.int/standards/compendium-of-international-methods-of-wine-and-must-analysis, *OIV*). Enzymatic kits (Megazyme International Ireland) were utilized to determine glucose and fructose (K-FRUGL) and malic acid (K-DMAL) following the manufacturer procedures. The ammonium content was determined using a specific enzymatic kit (kit no. 112732; Roche Diagnostics, Germany) while the free α-amino acids were evaluated following Dukes and Butzke protocol (1998). Ethyl acetate, acetaldehyde, and higher alcohols were quantified by direct injection using a gas-chromatograph with flame ionization detector (GC-2014; Shimadzu, Kjoto, Japan). The final wines, prepared following the instruction of Canonico et al. (2018), were analyzed to quantify the main volatile compounds as described by Canonico et al. (2019) using the solid-phase microextraction (HS-SPME) method with the fiber Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) (Sigma-Aldrich, St. Louis, MO, USA).

Volatile thiols 3-mercaptohexan-1-ol (3-MH), 3-mercaptohexylacetate (3-MHA), were determined by derivatization and SPE online extraction and High-Performance Liquid Chromatography Tandem Mass Spectrometry (HPLC-MS/MS) following the methodologies of Capone et al. (2015). Internal standard calibration was used to quantify the thiols concentration in the wine samples.

2.5. Sensory analysis

At the end of the fermentation carried out in winery, the wines were transferred into full 750 mL bottles with 30 mg/L of SO_2 closed with the crown cap and maintained at 4 °C until sensory analysis. After 6 months of refinement, the wines were subjected to sensory analysis based on principal sensory category. A group of 15 trained testers, 10 males and 5 females aged 25–45 years and composed by oenologists, sommeliers and wine producers, conducted the sensory analysis. The score scale was from 1 to 10, where 10 was the score that quantitatively represented the best judgment (maximum satisfaction), while 1 was the score to be attribute in case of very poor satisfaction. The sensory analysis was conducted from 10:00 to 12:00 a.m. in the following ways: 30 mL of each wine were served at 22 ± 1 °C (room temperature) in glasses labeled with code and covered to prevent volatile loss. The order of presentation was randomized among judges.

2.6. Statistical analysis

The data of analytical character of wines were elaborated using the analysis of variance (ANOVA). The means were analyzed using the statistical software package JMP® 11. The significant differences were detected using Duncan tests and the experimental data were significant with a p-values <0.05.

3. Results

3.1. Fermentation trials at laboratory scale: biomass evolution and main volatile compounds

The growth kinetics of pure (control) and sequential fermentations carried out at lab scale are reported in Fig. 1. *S. cerevisiae* pure fermentations carried out using native DiSVA 708 strain and starter strain OKAY® as control, respectively are reported in Fig. 1a and b. As expected, native and commercial strains showed a similar behavior between them, as well as the wild yeasts population exhibited an initial comparable trend in both fermentations achieving over 10^7 CFU/mL at 5th day of fermentation. After that, *S. cerevisiae* DiSVA 708 (Fig. 1a) led a slower decrease of wild yeasts from 5th day until the end of fermentation in comparison with *S. cerevisiae* OKAY® (Fig. 1b) that disappear after 8 days.

During sequential fermentations with *M. pulcherrima* DiSVA 269, wild yeasts did not exceed 10^6 CFU/mL disappearing in both cases (Fig. 1c and d) at 8th day of fermentation. Both S. *cerevisiae* strains inoculated after 48 h, maintained the similar trend observed during pure fermentations, while *M. pulcherrima* DiSVA 269 population disappeared after the 8th day either in the presence of *S. cerevisiae* DiSVA 708 and OKAY®.

These preliminary results indicated that *M. pulcherrima* DiSVA 269 with both native *S. cerevisiae* DiSVA 708 and OKAY ®strains determined an effective control on the development of wild yeasts.

However, the analysis of the main volatile compounds of resulting wines (Table 1) showed that *M. pulcherrima* DiSVA 269 in sequential

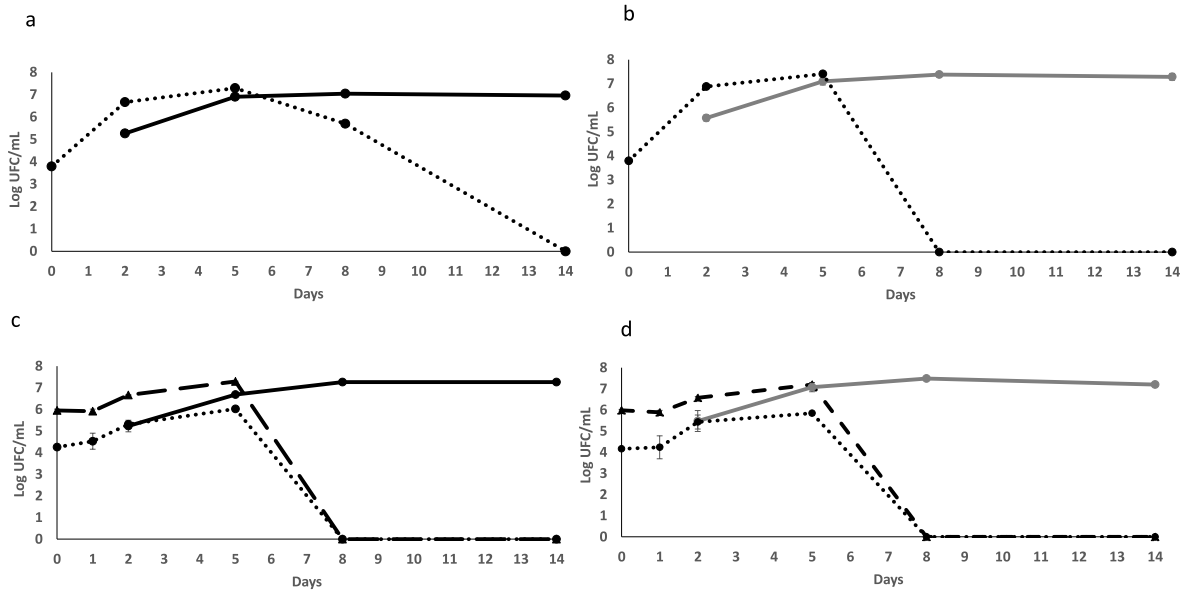

Fig. 1. Growth kinetics of pure and sequential fermentations carried out at lab scale. a) *S. cerevisiae* DiSVA 708 pure fermentation and b) *S. cerevisiae* OKAY® pure fermentation; c) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708; d) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* OKAY®. *S. cerevisiae* D (—●—); OKAY® (—●—); *M. pulckerrima* (—▲—) and Wild yeasts (•••●•••).

Table 1
Some main volatile compounds (mg/L) of the fermentation trials carried out at laboratory scale. Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c,d}) within each line are significantly different according to Duncan tests ($p < 0.05$).

	S. cerevisiae DiSVA708	*M. pulcherrima*/*S. cerevisiae* DiSVA708	*S. cerevisiae* OKAY	*M. pulcherrima*/*S. cerevisiae* OKAY
Ethyl butyrate	0.121 ± 0.016^{b}	0.410 ± 0.033^{a}	0.429 ± 0.016^{a}	0.453 ± 0.07^{a}
Isoamyl acetate	0.867 ± 0.172^{d}	1.08 ± 0.23^{c}	1.630 ± 0.031^{b}	2.493 ± 0.13^{a}
Ethyl exanoate	0.107 ± 0.020^{b}	0.147 ± 0.006^{a}	0.063 ± 0.012^{c}	0.048 ± 0.00^{c}
Hexanol	0.012 ± 0.001^{c}	0.013 ± 0.006^{c}	0.054 ± 0.009^{a}	0.038 ± 0.00^{b}
Linalol	0.079 ± 0.044^{b}	0.117 ± 0.055^{a}	0.043 ± 0.007^{b}	0.062 ± 0.01^{b}
β-Phenyl Ethanol	33.4 ± 0.05^{b}	57.8 ± 0.072^{a}	42.2 ± 0.019^{b}	32.5 ± 0.010^{b}

fermentations with both *S. cerevisiae* strains differently influenced the main volatile compounds of the final product. Indeed, sequential fermentation *M. pulcherrima*/OKAY® led only significant increase of isoamyl acetate, while sequential fermentation *M. pulcherrima/S. cerevisiae* DiSVA 708 determined a significant enhancement of several volatile compounds as ethyl butyrate, isoamyl acetate, ethyl hexanoate, β-phenyl ethanol and linalool, indicating a possible positive interaction in the formation of these compounds.

3.2. Fermentation trials in winery at industrial level

3.2.1. M. pulcherrima DiSVA 269 as biocontrol agent during clarification procedures

Based on the results obtained at laboratory scale, the selected strain *M. pulcherrima* was used at pre-fermentative stage in cold clarification and then inoculated with *S. cerevisiae* as reported above.

Results reported in Fig. 2 showed that the presence of *M. pulcherrima* determined a significant reduction (approximately 1 log, 90% of reduction) of wild yeast population, mainly represented by *H. uvarum* (data not shown), in both inoculated vats, while in the vats without the inoculum of *M. pulcherrima,* no wild yeast population reduction was shown.

3.2.2. Biomass evolution and sugar consumption of fermentation processes

Growth kinetics of fermentations inoculated and uninoculated with *M. pulcherrima* strain are reported in Fig. 3. The results showed that *S. cerevisiae* DiSVA 708 exhibited a similar trend in comparison to OKAY®. Indeed, the two *S. cerevisiae* strains (Fig. 3 a, b) exhibited the maximum cell concentration at 7th day of fermentation (c.a 10^8 cell/ml) to remain constant until the end of fermentation. The results showed that *S. cerevisiae* starter strain OKAY® exhibited a more effective control on the wild yeasts in comparison to DiSVA 708. However, in both fermentation trials the wild yeasts disappear a 7th day. *M. pulcherrima* sequential fermentation with OKAY® (Fig. 3d) showed a decrease of wild yeasts to disappear at 3rd day of fermentation. Moreover, the biomass evolution of OKAY® did not affect by *M. pulcherrima*. The inoculum of *M. pulcherrima* DiSVA 269 improved the control on wild yeasts in both inoculated fermentations even though in *M. pulcherrima/S. cerevisiae* DiSVA 708 fermentation showed a lower control on wild yeasts if compared to *M. pulcherrima*/OKAY® fermentation.

Regarding the sugar consumption (Fig. 4), all fermentations exhibited a similar trend in fermentation kinetics with the only exception of *S. cerevisiae* DiSVA 708 pure culture that exhibited a slower sugar consumption that other trials. All fermentations showed a complete sugar consumption at the end of fermentation. Moreover, the results highlighted a positive interaction on fermentation kinetics of *M. pulcherrima* when used in sequential fermentation with *S. cerevisiae* DiSVA 708.

3.2.3. Frequency and dominance of S. cerevisiae starter strains

The results using of interdelta sequences indicated that, *S. cerevisiae* DiSVA 708 showed a lower ability to dominate the fermentation process carried out at industrial level (Table 2). Indeed, *S. cerevisiae* DiSVA 708 was 60% in both pure and sequential fermentation while the commercial starter strain OKAY® showed a percentage of was 80 and 90% in pure

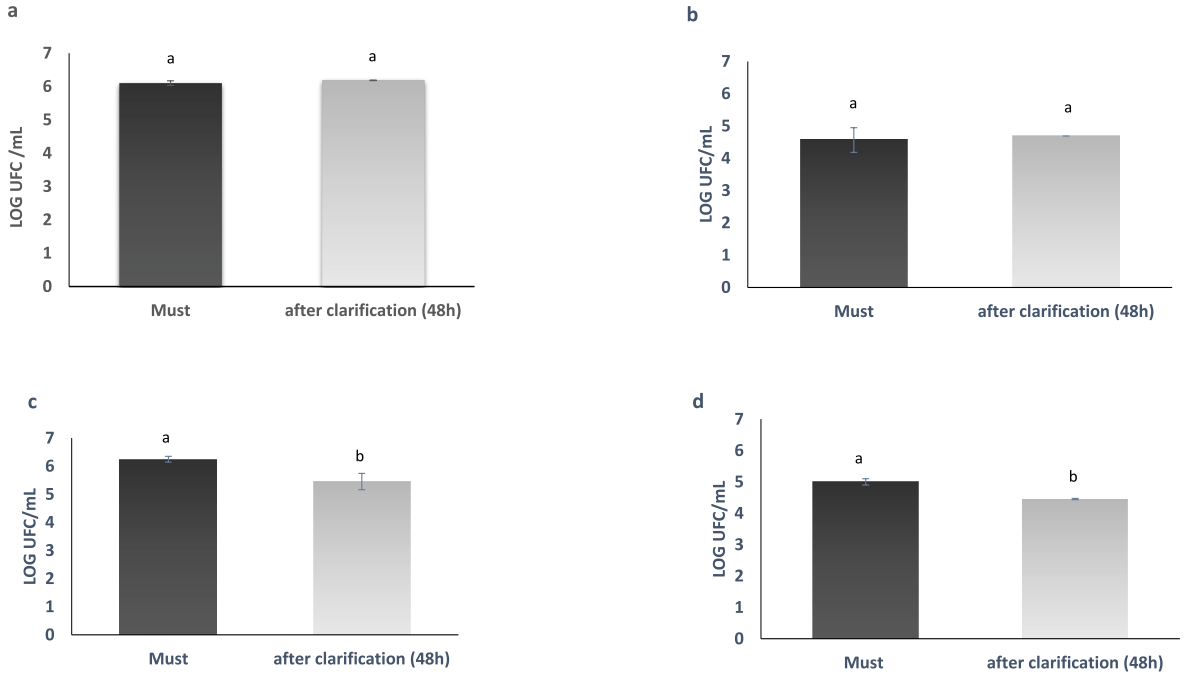

Fig. 2. Effect of *M. pulcherrima* DiSVA 269 on wild yeasts after clarification (48h). 1° Lot: a) with *M. pulcherrima* inoculum and b) without inoculum respectively; 2 Lot c) with *M. pulcherrima* inoculum and d) without inoculum respectively.

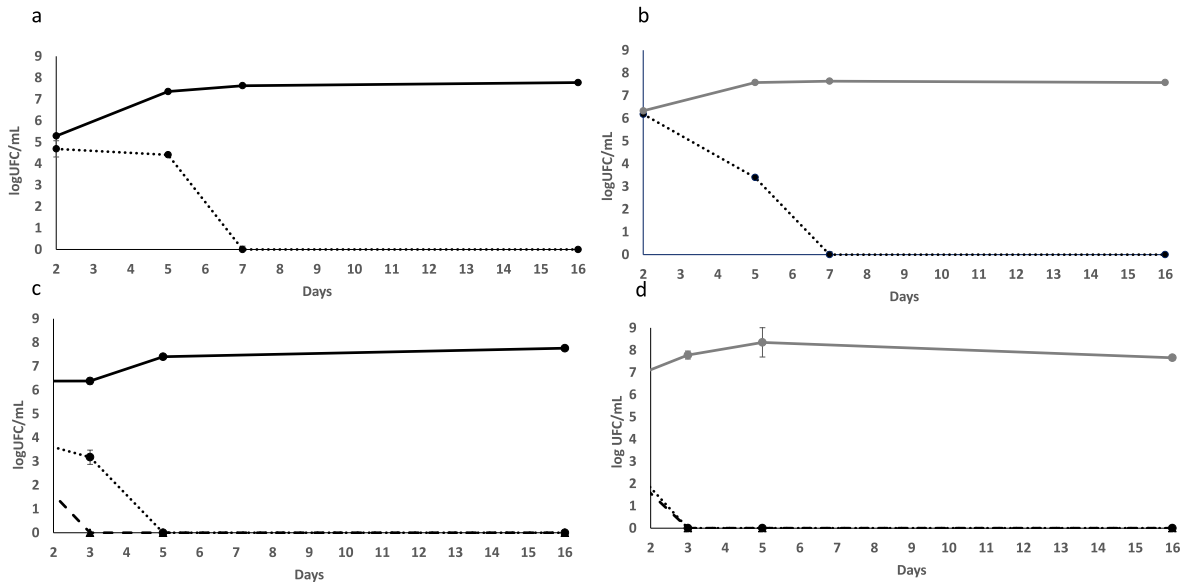

Fig. 3. Growth kinetics of pure and sequential fermentations carried out at industrial level. a) *S. cerevisiae* DiSVA 708 pure fermentation and b) *S. cerevisiae* OKAY® pure fermentation; c) *M. pucherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708; d) *M. pucherrima* sequential fermentation with *S. cerevisiae* OKAY®. *S. cerevisiae* DiSVA 708 (—●—); OKAY® (—●—); *M. pulcherrima* DiSVA 269 (—▲ -) and Wild yeasts (••●••).

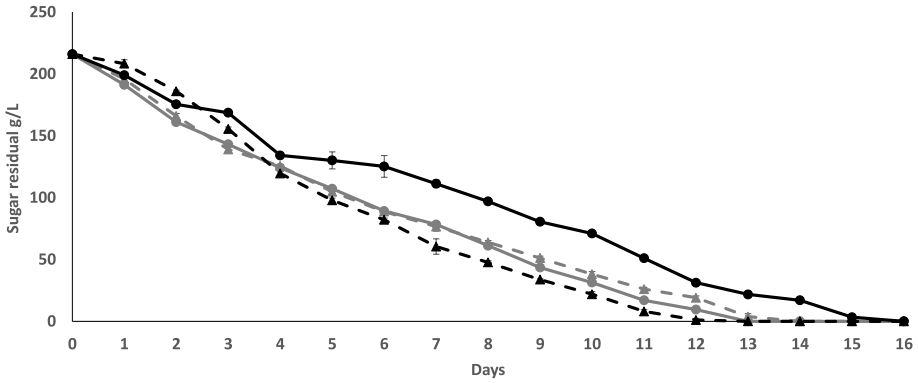

Fig. 4. Kinetics of sugar consumption of the pure and sequential fermentation carried out at industrial level. *S. cerevisiae* Disva 708 (—●—); OKAY® (—●—); *M. pulcherrima* DiSVA 269/*S. cerevisiae* Disva 708 (—▲ -) and *M. pulcherrima*/OKAY® (- -▲- -).

Table 2
Percentage values of isolates of *S. cerevisiae* detected close to the end of fermentation process in the fermentation assayed.

Fermentation trials	% of isolates
M. pulcherrima DISVA 269/*S. cerevisiae OKAY®*	90
S. cerevisiae OKAY®	80
M. pulcherrima DISVA 269/*S. cerevisiae* DiSVA 708	60
S. cerevisiae DiSVA 708	60

and sequential fermentation, respectively.

3.2.4. Main oenological characters of wine

The results of the main analytical characters of wines are shown in Table 3. The presence of *M. pulcherrima* during cold clarification showed a significant reduction of total and volatile acidity indicating its influence in both *S. cerevisiae* starter strains. The production of malic acid is comparable in both pure *S. cerevisiae* fermentations, while the *M. pulcherrima*/OKAY® showed a significant lower malic acid content.

3.2.5. Volatile compounds of wine

In Table 4 are shown the results concerning the principal volatile

Table 3
Chemical characterization of resulting wine. The analytical characters of the grape musts were initial sugars 216 g/L, pH 3.34, total acidity 4.37 g/L, malic acid 1.7 g/L, and nitrogen content 90 mg/L.
Data are means ± standard deviations. Values displaying different superscript letters (^{a,b,c}) within each column are significantly different according to Duncan tests ($p < 0.05$).

	Ethanol (%v/v)	Total Acidity (Tartatic Acid g/L)	Volatile Acidity (Acetic Acid g/L)	Malic Acid (g/L)
M. pulcherrima/ OKAY	13.90 ± 0.02^{a}	5.07 ± 0.04^{c}	0.24 ± 0.01^{b}	1.25 ± 0.07^{b}
OKAY	14.06 ± 0.09^{a}	5.41 ± 0.03^{b}	0.25 ± 0.01^{ab}	1.55 ± 0.07^{a}
M. pulcherrima/*S. cerevisiae* DiSVA 708	13.92 ± 0.04^{a}	5.12 ± 0.014^{c}	0.21 ± 0.02^{b}	1.5 ± 0.14^{a}
S. cerevisiae DiSVA	13.93 ± 0.06^{a}	6.28 ± 0.01^{a}	0.29 ± 0.014^{a}	1.55 ± 0.07^{a}

Table 4
The main by-products and volatile compounds in final wines in presence and absence of *M. pulcherrima.*
MP: *M. pulcherrima*; OAV: Odor Activity value
Data are the means ± standard deviation. Data with different superscript letters (^{a,b,c,d}) within each row are significantly different (Duncan tests; $p < 0.05$).

	MP OKAY	OAV	OKAY	OAV	MP DiSVA 708	OAV	DiSVA 708	OAV
Esters (mg/L)								
Ethyl butyrate	0.126 ± 0.00^{b}	0.31	0.064 ± 0.035^{b}	0.16	0.303 ± 0.075^{a}	0.757	0.148 ± 0.01^{b}	0.37
Ethyl acetate	27.01 ± 0.39^{b}	2.25	42.96 ± 0.36^{a}	3.58	25.67 ± 0.83^{b}	2.13	12.28 ± 0.97^{c}	1.02
Phenyl ethyl acetate	0.038 ± 0.00^{c}	0.52	ND		0.10 ± 0.01^{a}	1.36	0.049 ± 0.02^{b}	0.067
Ethyl exanoate	0.194 ± 0.011^{a}	2.42	0.041 ± 0.003^{b}	0.51	0.130 ± 0.020^{a}	1.625	0.037 ± 0.001^{b}	0.462
Ethyl octanoate	0.005 ± 0.00^{a}	0.008	0.006 ± 0.000^{a}	0.01	0.005 ± 0.001^{a}	0.0086	0.002 ± 0.000^{a}	0.003
Isoamyl acetate	0.914 ± 0.28^{ab}	5.71	0.517 ± 0.171^{ab}	3.23	1.029 ± 0.314^{a}	6.43	0.307 ± 0.001^{b}	1.91
Alcohols (mg/L)								
n- propanol	75.83 ± 0.33^{b}	0.247	104.79 ± 5.04^{a}	0.342	31.83 ± 0.15^{c}	0.104	13.98 ± 0.18^{d}	0.045
Isobutanol	11.54 ± 0.76^{b}	0.288	13.83 ± 0.57^{a}	0.345	13.12 ± 0.52^{ab}	0.328	13.79 ± 0.83^{a}	0.344
Amyl alcohol	6.41 ± 0.90^{b}	0.1	9.76 ± 0.09^{a}	0.15	9.87 ± 3.41^{a}	0.15	19.50 ± 1.35^{b}	0.304
Isoamyl alcohol	89.49 ± 1.08^{b}	1.41	110.18 ± 0.01^{a}	1.83	13.99 ± 7.09^{c}	0.23	94.73 ± 3.08^{b}	1.57
β-Phenyl Ethanol	13.12 ± 0.33^{ab}	0.92	16.05 ± 0.20^{a}	1.14	19.04 ± 0.27^{a}	1.36	8.08 ± 0.23^{b}	0.57
Carbonyl Compounds (mg/L)								
Acetaldehyde	1.40 ± 0.24^{c}	2.8	3.80 ± 0.93^{c}	7.6	7.97 ± 1.16^{b}	15.94	13.98 ± 1.36^{a}	27.96
Monoterpenes (mg/L)								
Linalool	0.18 ± 0.100^{ab}	3.2	0.371 ± 0.147^{a}	14.84	0.221 ± 0.054^{ab}	8.84	0.028 ± 0.008^{b}	1.12
Geraniol	0.025 ± 0.01^{a}	0.83	0.036 ± 0.015^{a}	1.2	0.038 ± 0.012^{a}	1.26	0.014 ± 0.008^{a}	0.466
Nerol	0.074 ± 0.05^{a}	4.93	0.202 ± 0.140^{a}	13.46	0.136 ± 0.022^{a}	9.06	0.028 ± 0.008^{a}	1.86
Thiols (ng/L)								
3-mercaptohexan-1-ol	367.1 ± 0.0^{b}	6.11	35.7 ± 0.0^{d}	0.59	1215.1 ± 0.0^{a}	20.25	190.6 ± 0.00^{c}	3.17
3-mercaptoexil acetate	388.9 ± 0.0^{a}	92.56	52.8 ± 0.0^{c}	12.57	181.8 ± 0.0^{b}	43.28	17.4 ± 0.00^{d}	4.14

compounds. Regarding to the esters content, the presence of *M. pulcherrima* (inoculation at cold clarification stage) led an increase in ethyl butyrate, ethyl hexanoate, phenyl ethyl acetate and isoamyl acetate content that resulted significant with the starter *S. cerevisiae* DiSVA 708. *M. pulcherrima/S. cerevisiae* OKAY® showed the only appearance of phenyl ethyl acetate. Regarding to the higher alcohols both *S. cerevisiae* fermentations without the inoculation of *M pulcherrima* were characterized by a high final content of amylic alcohols that were strongly reduced in inoculated fermentations with *M. pulcherrima. S. cerevisiae* OKAY® was characterized by a high production of n-propanol (in both fermentations) while *M. pulcherrima* determined a significant increase in β-phenyl ethanol but only with DiSVA708. Regarding to the monoterpenes a relevant high content was detected for linalool in OKAY® fermentation trials and in presence of *M. pulcherrima*/DiSVA708 in comparison with DiSVA708 pure culture. while no significant differences were shown for the other terpenes production. The acetaldehyde was significant higher in wine fermented by DiSVA708. The presence of *M. pulcherrima* led a significant increase of 3-mercaptohexan-1-ol and 3-mercaptoexil acetate particularly in *M. pulcherrima*/DiSVA708 trial.

The enhancement of volatile compounds found with *M. pulcherrima* in the laboratory trials were substantially confirmed by the results obtained in the winery, particularly with the DiSVA708 starter strain. These results, confirming the positive role on fruity characters and wine complexity of *M. pulcherrima*, also indicated differences in the interactions with *S. cerevisiae* starter strain.

3.2.6. Sensory analysis

To establish a further role of *M. pulcherrima* in aroma complexity. the wines produced with and without it in cold clarification, were undergo to sensory analysis. Results reported in Fig. 5 highlighted a general positive appreciation by the tasters of the wines, each distinguished by specific aromatic notes and without defects. Wines obtained with pure *S. cerevisiae* DiSVA708 were perceived more balanced and structured and significantly characterized by citrusy, and softness note, with a low perception of bitter notes. Instead, *M. pulcherrima/S. cerevisiae* DiSVA 708 led a wine with tropical fruit notes. This result matches the results for the main volatile compounds evaluated. Indeed, the fermentation carried out with *M. pulcherrima/S. cerevisiae* DiSVA708 showed a

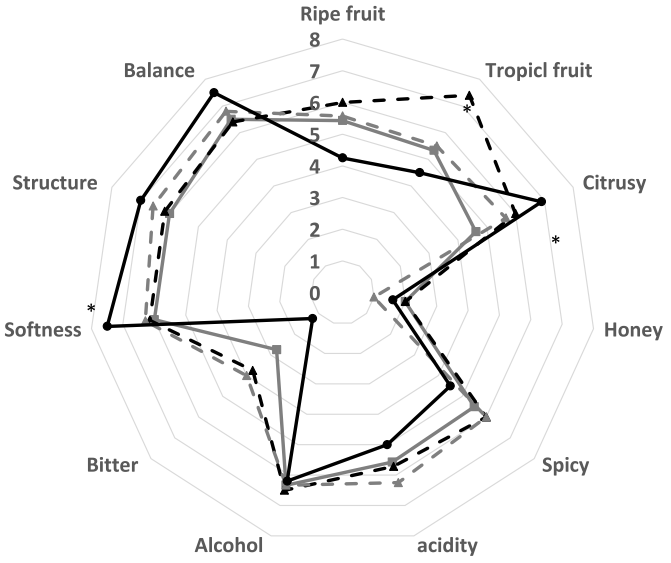

Fig. 5. Sensory analysis of Verdicchio wine fermented by *S. cerevisiae* DiSVA 708 (—●—)and OKAY® (—■—) pure fermentation; *M. pulcherrima* DiSVA 269 sequential fermentation with *S. cerevisiae* DiSVA 708 (—▲—)and OKAY®.

significant increase in 3-mercaptohexan-1-ol and 3-mercaptoexil acetate responsible of passion fruit and grapefruits notes.

No significant differences were shown regarding to the other aromatic descriptors.

4. Discussion

Nowadays great attention is focused on the concept of bioprotection, consisting of the inoculation viable antagonist microorganisms (bacteria, yeasts, or a mixture of them) or the addition of their antimicrobial products in purified form, during, at the end or after the production chain of food and beverages (Comitini et al., 2017; Di Gianvito et al., 2022; Oro et al., 2014a; Simonin et al., 2020). Biological

control implies the reduction or even the elimination of chemical compounds such as sulfur dioxide (SO_2) the most common chemical additive used by winemakers (zhang et al., 2013). leading to the production of high-quality wines with higher add value. In this regard, bio-protection is a tool in fast development, and several formulations based on viable microorganisms or on their antimicrobial compounds have recently started to be proposed in agriculture and food industry (Di Gianvito et al., 2022). To protect grapes, strawberry, and tomato against *Penicillium, Botrytis* and *Monilinia,* a commercial preparation with *Aureobasidium pullulans* has been set up, while a product based on *M. fructicola i*s employed to protect strawberry, blueberry, grape, stone fruit, and pome against *Botrytis, Penicillium, Rhizopus, Aspergillus* and *Monilinia* spp. (Mukherjee et al., 2020; Zhang et al., 2020).

In the winemaking sector several technological alternatives were set up able to control microorganisms such as ultrasound, ultraviolet radiation, pulsed electric field, electrolyzed water, high hydrostatic pressure pre-treatments, or the addition of lysozyme, sorbic acid, dimethyl dicarbonate and chitosan (Guerrero & Cantos-Villar, 2015), a valid and complete substitute for SO_2 has not been found, particularly during prefermentative stage (Giacosa et al., 2019).

The biocontrol action by using non-*Saccharomyces* yeasts have been proposed as a possible alternative to sulphite addition (Comitini et al., 2011). In this regard, be going to appear recent studies carried out by using selected strains of *M. pulcherrima* or a mix of *M. pulcherrima* and *T. delbrueckii* in the red winemaking process at the prefermentative stage (Chacon-Rodriguez et al., 2020; Simonin et al., 2020; Windholtz et al., 2021).

Here, after the preliminary sequential fermentations carried out at laboratory level the biocontrol action found in *M. pulcherrima* DiSVA 269 were confirmed under winery condition, where the biocontrol was exerted at prefermentative stage of a white winemaking process of Verdicchio grape juice determining an improvement of the control on wild yeasts during the subsequent fermentation process.

Other important feature of this oenological practice is the effects on analytical and volatile compounds. The combined use of *M. pulcherrima* DiSVA 269 and native *S. cerevisiae* DiSVA 708 showed a relevant impact on the aromatic profile of wines in both laboratory and winery trials. Indeed, the combined use of the yeasts led a significant enhancement of ethyl hexanoate, isoamyl acetate, phenyl ethyl acetate and β-phenyl ethanol with an OAV higher than 1 (calculated with OTH reported in Table1s supplementary materials). Higher alcohols also contribute to define the overall sensory characteristics of wines. Although the higher alcohols contribute positively to the overall wine flavors, low levels (below 300 mg/L) increased the perception of the varietal aroma of grapes (Escribano et al., 2018). In our study the presence of *M. pulcherrima,* reducing the higher alcohols, may contribute to emphasize the specificity of Verdicchio grapes.

Another relevant result of the investigation regarding to the yeast-yeast interactions in metabolome profiling of wines. *M. pulcherrima* DiSVA 269/*S. cerevisiae* DiSVA 708 turned out the best combination led and enhancement of volatile thiols (3-mercaptohexan-1-ol and 3-mercaptoexil acetate) that good correlate with the sensory analysis of tropical fruit note (Ruiz et al., 2018; Vicente et al., 2020; Zott et al., 2011). *M. pulcherrima* was indicated in several works to improve the concentration of volatile compounds (Zott et al., 2011; Ruiz et al., 2018, Vicente et al., 2020). Indeed, this species positively contribute to volatile thiol release in wines, especially during the pre-fermentation stage in winemaking, (Zott et al., 2011). Intriguingly, it is needed to underline the different results of *M. pulcherrima* strain with the two different *S. cerevisiae* starters strains indicating that different interaction between the inoculated yeasts can take place. In this regard, native *S. cerevisiae* DiSVA 708 was specifically improved and selected for specific characters such as low sulfites production and valuable analytical a sensory profile (Agarbati et al., 2020). The combined use of *M. pulcherrima* DiSVA 269 an *S. cerevisiae* DiSVA 708 specifically selected for the sulfites reduction showed a valuable result.

In conclusion, the results indicate that, under winery condition, the combined use of *M. pulcherrima* DiSVA 269 at the prefermentative stage during cold clarification exerted an effective biocontrol toward wild yeast population. The combination with the native *S. cerevisiae* DiSVA 708 enhanced some aromatic and sensorial characters producing a wine with distinctive features and low SO_2 content.

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Laura Canonico: Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – original draft, Writing – review & editing, Supervision. **Alice Agarbati:** Data curation, Validation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Edoardo Galli:** Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization. **Francesca Comitini:** Conceptualization, Investigation, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Maurizio Ciani:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114758.

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fermentation

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Review

Chitosan Production by Fungi: Current State of Knowledge, Future Opportunities and Constraints

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Abstract: Conventionally, the commercial supply of chitin and chitosan relies on shellfish wastes as the extraction sources. However, the fungal sources constitute a valuable option, especially for biomedical and pharmaceutical applications, due to the batch-to-batch unsteady properties of chitin and chitosan from conventional ones. Fungal production of these glycans is not affected by seasonality enables accurate process control and, consequently, more uniform properties of the obtained product. Moreover, liquid and solid production media often are derived from wastes, thus enabling the application of circular economy criteria and improving the process economics. The present review deals with fungal chitosan production processes focusing on waste-oriented and integrated production processes. In doing so, contrary to other reviews that used a genus-specific approach for organizing the available information, the present one bases the discussion on the bioprocess typology. Finally, the main process parameters affecting chitosan production and their interactions are critically discussed.

Keywords: chitosan; fermentation; waste upgrading; integrated bioprocesses; fungi

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1. Introduction

Chitin, a structural glycan composed of randomly distributed *N*-acetyl-D-glucosamine (GlcNAc) residues (Figure 1a) [1], is the second most abundant biopolymer on earth (more than 100 billion tons) [2]. Chitosan, a linear heteroglycan mainly made of β-(1-4)-linked D-glucosamine (GlcN) units (Figure 1b), is often derived from chitin deacetylation, the extent of which, however, is never quantitative. Consequently, chitosan is a copolymer made of GlcNAc and GlcN residues, where the latter account for at least 60% of total residues [1,3,4]. However, the degree of deacetylation (DD) of commercially available chitosans generally amounts to or exceeds 80% [3].

Over a 2020–2027 period, the Global Industry Analysts Inc. [5] estimated growth of the market of chitin and chitosan from 106.9 × 10^3 to around 282 × 10^3 tons, with a compounded annual growth rate equal to 14.8%; this growth estimate was attributed to the ever-increasing applications of these polymers in various end-use sectors.

Chitin occurs in nature in different crystalline forms denominated α-, β-, and γ-chitin, exhibiting distinct physicochemical properties (Figure 1c). The differences among these polymorphs are due to the mode with which crystalline regions' chains are reciprocally arranged. In the α and β forms, all the chains are arranged in an antiparallel and parallel mode, respectively, while in the γ form, there is an alternation of sets of two parallel strands with single antiparallel ones [6]. Among these allomorphs, α-chitin is the most widespread being found in arthropods and fungi; β-chitin generally occurs in cephalopods, while γ-chitin is rather rare.

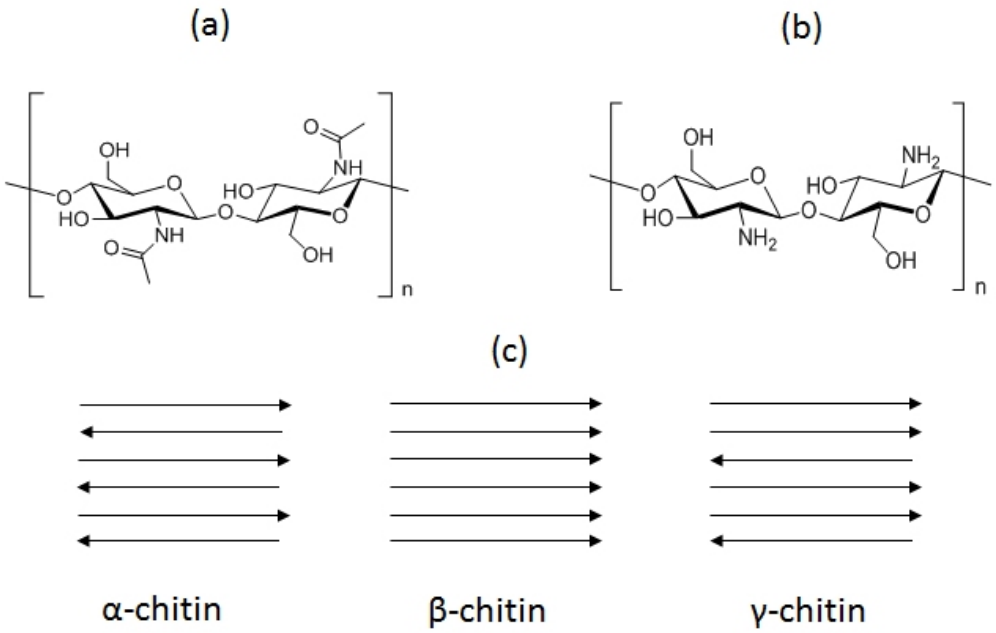

Figure 1. Basic structures of chitin (**a**) and chitosan (**b**) and chitin allomorphs (**c**). The tips of the arrows indicate the positions of the reducing ends of the chains.

At present, the industrial production of chitin and chitosan relies on crustacean wastes. Chitin contents in crab and shrimp processing wastes range from 13% to 15% and 14% to 27% dry weight, respectively [7]. The heterogeneous composition of crustacean shell wastes requires stepwise chemical methods to extract chitin and chitosan from these sources [8].

The extraction scheme generally involves a demineralization step with strong acids, followed by alkaline deproteinization and, frequently, a decolorization step. The first step, generally relying on HCl solutions (concentration ranges 0.6–11.0 M), is generally conducted at room temperatures, while the second uses NaOH solutions (concentration ranges 0.12–5.0 M) and temperature up to 160 °C; the final step, frequently added to remove pigments, such as β-carotene and astaxanthin, generally relies on acetone as the extraction solvent [9]. The chitin thus obtained undergoes alkaline deacetylation under very harsh chemical conditions (NaOH solutions from 30% to 60%) under variable temperature and contact times to yield chitosan [10]. The variability of the raw materials and the harsh conditions that characterize some extraction steps can lead to unsteady physicochemical properties of chitosan from batch to batch [9].

The occurrence of chitin and chitosan in fungi has opened the door to a promising alternative route for their productions [11]. Since fungal chitin has a lower ash content than crustacean shell wastes, the demineralization step is not required during its processing [12,13]. Moreover, chitin and chitosan of fungal origin provide a non-seasonal and reliable source of these polymers and consistent properties of the product. The extraction of a value-added product, such as chitosan, may afford a profitable solution to mushroom growers and biotechnological industries considering the vast quantities of fungal-based wastes accumulated and the ensuing expense in waste management.

2. Physicochemical and Functional Properties of Chitin and Chitosan

The chemical structures of chitin and chitosan resemble that of cellulose, a glycan composed of hundreds of D-glucose residues connected by β-(1-4) linkages [1]. In chitin and chitosan, however, an acetamide or amino group replaces the hydroxyl group at the C-2 position of glucose residues. Thus, the nitrogen content of chitin and chitosan ranges from 5% to 8%, and the presence of amino groups gives these glycans distinctive biological functions and susceptibility to chemical modification reactions [14]; chitosan, in particular, owing to the presence of free amino groups is susceptible to *N*-acylation and

Schiff's reactions paving the way to a variety of chemical modifications. Moreover, the joint presence of the amino and hydroxyl groups on each deacetylated unit renders chitosan more water-soluble and chemically reactive than chitin. Due to their pk_a values (≈6.3), the free amino groups in GlcN residues are protonated at slightly acid pHs, making chitosan the only naturally occurring cationic glycan [15]. A further consequence is the polymer's solubility in slightly acid aqueous solution as opposed to chitin [16].

The majority of the biological properties of these glycans are due to their physico-chemical features, such as solubility, deacetylation degree, molecular weight, and inherent moisture content [17]. For instance, the inhibition of fungal and bacterial growth exerted by chitosan relies principally on the extent of positively charged groups and molecular mass. Two main mechanisms have been suggested to explain the antimicrobial activity of chitosan. The first outlines the importance of its molecular weight and postulates that the smaller chito-oligosaccharides can easily penetrate the cellular membrane, thus preventing cell growth via inhibition of DNA transcription [18,19]. The second suggests that the positively charged groups of chitosan interact with anionic components of the microbial cell membrane resulting in cell death [20,21]. Moreover, chitosan can operate as a chelator of essential elements [22]. Chitosans with a degree of deacetylation (DD) larger than 97.5% have a higher positive charge density and an ensuing stronger antibacterial activity than those with moderate DD (83.7%), as shown by Kong et al. [23]. Some properties of chitin and chitosan, such as non-toxicity, biodegradability, biocompatibility, and non-allergenicity, associated with bioactivity and suitable adsorption properties, render them appropriate alternative options to artificial polymers [24,25]. Another reason underlying their success is that they can be manufactured to yield several forms, including beads, flakes, membranes, gels, and fibers [15]. As a consequence, they have been exploited as carriers for enzyme immobilization [26,27], coagulating agents in effluents treatment, as food preservatives [28], hypocholesterolemic, and wound healing agents, and as components of several drug delivery systems [29,30].

3. General Aspects of Chitin and Chitosan Production from Fungal Sources

Since the beginning of this century, many countries have focused attention on using fungal sources for the commercial production of chitin and chitosan due to the remarkable disadvantages that burden the conventional process. Table 1 comparatively summarizes the advantages and disadvantages of chitin and chitosan production from fungal and crustacean sources [12,13].

Among the advantages of the fungal approach, there is the possibility of obtaining chitosans with different properties by varying species and culture conditions [31,32]. For instance, the chitosan derived from shellfish wastes has a high molecular mass (around 1.5×10^6 Da), while the MW of chitosan from fungal sources widely ranges from 6.4×10^3 to 1.4×10^6 Da [33,34]. High MW chitosans are sparingly soluble in neutral pH aqueous solutions and yield high viscous solutions that limit their exploitation in the food, health, and agricultural sectors [35]. Fungal-derived chitosan with medium-low MW can be used as hypocholesterolemic agents in healthcare products and as a thread or membrane in a variety of biomedical applications [36,37]. Moreover, chitosan extraction from fungal sources is more environmentally benign than that from shellfish wastes since the latter source requires highly concentrated acid and alkaline solutions for demineralization and chemical deacetylation that have to be disposed of. Another advantage associated with fungal chitosan encompasses the absence of allergenic proteins, such as tropomyosin [9]. Chitin and chitosan extraction from fungi can lower disposal costs of fungal-based waste materials in association with the production of value-added products, which may offer a lucrative opportunity to the biotechnological industries [38–40].

3.1. Chitin and Chitosan Biosynthesis and Their Biological Functions in Fungi

Arthropods and fungi share a common biosynthetic pathway that uses glucose and its storage carbohydrates, such as trehalose and glycogen, as the starting materials. The

pathway of chitin biosynthesis is organized into three groups of reactions, the first leading to the formation of GlcNAc, the second yielding its activated counterpart uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) through a modification of the Leloir pathway, and the third resulting in polymer formation using UDP-GlcNAc as the GlcNAc donor to the growing chitin chain (Figure 2) [41].

Table 1. Benefits and drawbacks of fungal chitosan compared to those from conventional sources with reference to a series of evaluation criteria.

Evaluation Criterion	Benefits	Drawbacks
Biomass supply	Not affected by seasonal and geographical factors. Possible biomass supply from pharmaceutical and biotechnological industries.	Lower biomass amounts than those made available from the shellfish industry.
Extraction process	Process scheme simpler (no demineralization and decolorization steps) and lower amounts of chemicals employed as compared to those used for crustacean sources.	Less established as compared to that from shellfish waste.
Environmental impact and disposal costs of process wastes	More environmentally friendly and lower disposal costs of effluents as compared to the shellfish waste process.	Potential risks of dispersal of pathogenic fungi when dealing with species not satisfying the generally regarded as safe requirements.
Inorganic and organic contaminants in the product	Absence of heavy metals and allergenic proteins as opposed to chitosan preparations from shellfish waste.	Some chitosan preparations might contain residual phosphates.
Production costs	They can be modulated by the choice of low-cost substrates and low equipment-intensive fermentation techniques, such as solid-state fermentation.	Not yet competitive in terms of production costs compared to the conventional process.
Physicochemical properties of the products	Molecular weights and degree of deacetylation of fungal chitosans frequently lower and higher, respectively, than those from conventional sources with ensuing positive impacts on their antimicrobial and antioxidant activities.	The lower MW of fungal chitosans than those from conventional sources make them less suitable as anti-lipidemic and hypocholesterolemic agents.

Free glucose or that derived from trehalase-catalyzed hydrolysis of trehalose is converted to glucose-6-phosphate (G-6-P) by hexokinase. If the starting material is glycogen, its depolymerization, catalyzed by glycogen phosphorylase, releases glucose-1-phosphate, which is also converted to glucose-6-phosphate by phosphoglucomutase-catalyzed isomerization. Irrespective of its origin, G-6-P is then isomerized to fructose-6-phosphate (F-6-P) by phosphoesoisomerase. F-6-P is then converted to *N*-acetyl-D-glucosamine-6-phosphate (GlcNAc-6-P) through two consecutive transfer reactions of an amino and acetyl group where glutamine and acetyl CoA, respectively, act as the donors. Isomerization step of GlcNAc-6-P catalyzed by phospho-*N*-acetyl glucosamine mutase yields 1-phospho-*N*-acetyl-D-glucosamine (GlcNAc-1-P). The chitin precursor, UDP-GlcNAc, is formed upon reaction of GlNAc-1-P with uridine triphosphate (UTP) and serves as a N-acetylglucosamine (GlNAc) donor for the sequential addition of GlcNAc units to the non-reducing terminus of the growing chain catalyzed by chitin synthase [42]. The linear chains spontaneously assemble to form microfibrils with varying diameters and lengths. In a further step, chitin deacetylase (CDA, E.C. 3.5.1.41) brings about the deacetylation of GlcNAc residues of chitin, thus leading to chitosan [43]. The formation of GlcNAc and UDP-GlcNAc takes place in the cytosol, while chitin synthesis occurs in specialized domains of the cell membrane. Some enzymes involved in chitin syntheses, such as glutamine-fructose-6-phosphate ami-

dotransferase (EC 2.6.1.16), UDP-N-acetylglucosamine pyrophosphorylase (EC 2.7.7.23), and chitin synthase (EC 2.4.1.16), are subjected to tight regulation and limit the rate of chitin production [41,44].

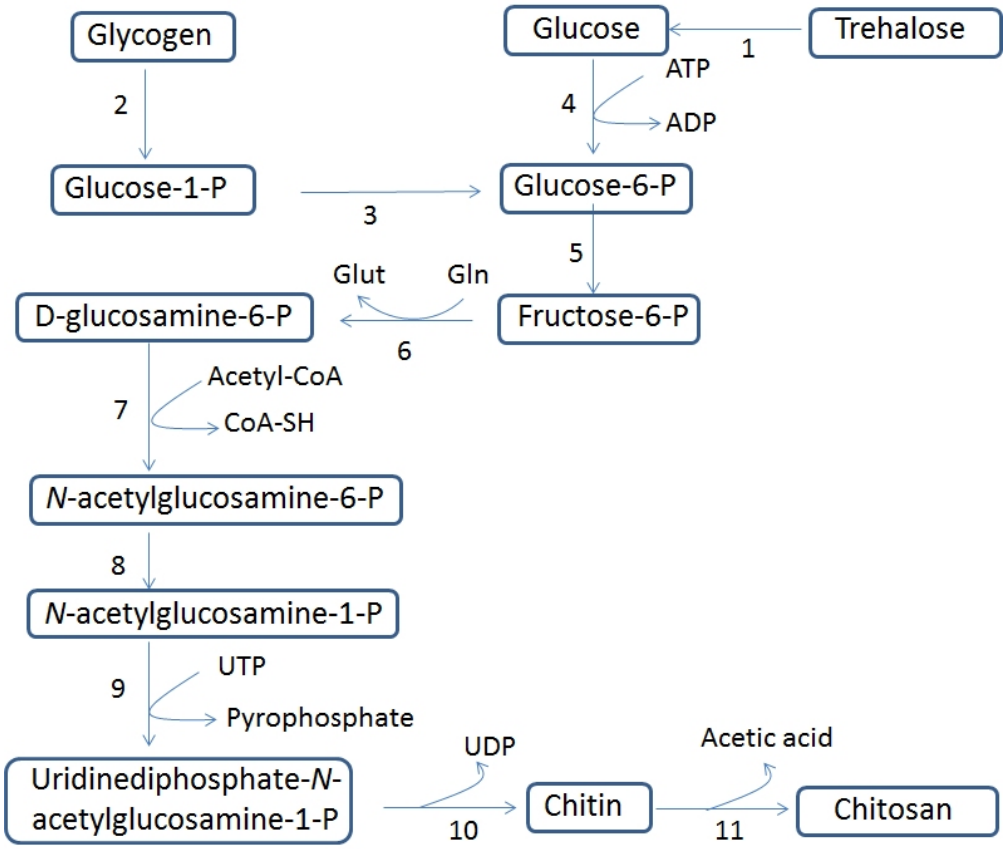

Figure 2. Biosynthetic pathway of chitin and chitosan. The following numbering has been assigned to the enzymes that catalyze each reaction: 1, trehalase; 2, glycogen phosphorylase; 3, phosphoglucomutase; 4, hexokinase; 5, glucose-6-phosphate isomerase; 6, glutamine-fructose-6-phosphate amidotransferase; 7, glucosamine-6-phosphate *N*-acetyltransferase; 8, *N*-acetylglucosamine-phosphate mutase; 9, UDP-*N*-acetylglucosamine pyrophosphorylase; 10, chitin synthase and 11, chitin deacetylase. The abbreviations used are as follows: acetyl CoA, acetyl coenzyme A; ADP, adenosine diphosphate; CoASH, coenzyme A; Gln, L-glutamine; Glu, L-glutamate; UDP, uridine diphosphate; UTP, uridine triphosphate.

Table 2 summarizes the taxonomic classification of chitosan-producing species and range of chitosan contents (referred to dry biomass) and degree of deacetylation.

Table 2. Taxonomic classification of chitosan-producing species and range of chitosan contents referred to their biomass dry weight and degree of deacetylation (DD).

Genus and Species	Class	Order	Family	Chitosan Content (g/kg)	DD (%)	References
Absidia						
A. blakesleeana				10–170	85	
A. coerulea	Zygomycetes	Mucorales	Cunninghamellaceae	30–300	93–95	[31,45–51]
A. glauca				52–59	75–80	
A. orchidis				18–69	68–85	
Cunninghamella						
C. bertholletiae				55–128	87–90	
C. echinulata	Zygomycetes	Mucorales	Cunninghamellaceae	50–130	85	[27,51–59]
C. elegans				35–78	72–90	
C. ramose				50–123	n.r.	
Gongronella						
G. butleri	Zygomycetes	Mucorales	Cunninghamellaceae	58–216	89–92	[52,60–65]
Benjaminiella						
B. poitrasii	Zygomycetes	Mucorales	Mucoraceae	51–78	94–95	[66]
Mucor						
M. racemosus				12–117	70–84	
M. rouxii	Zygomycetes	Mucorales	Mucoraceae	33–204	80–90	[27,38,40,67–73]
M. rouxianus				181	80–90	
M. indicus				94–235	72–89	
Rhizomucor						
R. pusillus	Zygomycetes	Mucorales	Mucoraceae	80	n.r.	[71,74]
R. miehei				14–137	81	
Rhizopus						
R. oryzae				44–138	85–89	
R. oligosporus	Zygomycetes	Mucorales	Mucoraceae	32	n.r.	[51,52,55,68,75–81]
R. arrhiizus				21–58	82	
Syncephalastrum						
S. racemosum	Zygomycetes	Mucorales	Syncephalastraceae	74–152	72–77	[27,82]
Aspergillus						
A. niger	Eurotiomycetes	Eurotiales	Trichocomaceae	70–209	81–90	[28,38,68,83–86]
A. terreus				69–141	85–88	
Penicillium						
P. chrysogenum	Eurotiomycetes	Eurotiales	Trichocomaceae	29–57	84–86	[87–90]
P. waksmanii				297	65	

In fungi, both vegetative and sporulating cells are capable of chitin synthesis, and its secretion occurs in a polarized mode. As a matter of fact, chitin accumulation occurs at growth sites such as hyphal tips and cross-walls in filamentous fungi and emerging buds in yeasts. Chitin and chitosan accumulation mostly occurs in the cell wall's layers adjacent to the plasmalemma, where these glycans play a fundamental role in maintaining the cell wall's shape and integrity; moreover, they provide protection against foreign materials (e.g., cell inhibitors) and environmental stressors to which fungi might be exposed [91–94]. Owing to its positive charge, chitosan is capable of retaining anionic storage materials, such as polyphosphates, which are highly abundant in the Zygomycetes' cell wall [72]. Chitosan also exerts a role in some pathogenic fungi such as *Colletotrichum graminicola* [95] and *Magnaporthe oryzae* [96]. During infection by these species, the chitin deacetylase-catalyzed conversion of chitin into chitosan seems to preserve the appressorium from the hydrolysis by plant chitinases [95].

3.2. Fungal Producers of Chitin and Chitosan

In cell walls of fungal species belonging to the classes Deuteromycetes, Ascomycetes, and Basidiomycetes, chitin is regarded as the second most abundant component [97].

As opposed to chitin, chitosan is a less widespread cell wall constituent. However, Zygomycetes have significantly higher amounts of chitosan in their cell walls than other fungal classes. Within Zygomycetes, the most productive genera belong to the order of Mucorales and to two families, namely Cunninghamellaceae and Mucoraceae. The former family includes a variety of highly producing genera, such as *Absidia* [31,48,50], *Cunninghamella* [53,54,56,58] and *Gongronella* [60–62,64] while the latter includes the genera *Mucor* [27,40,67–73], *Rhizomucor* [71,74], and *Rhizopus* [68,76,77,80,81,98].

Although chitosan production from fungal sources can be regarded as a greener alternative to the shellfish-based process, it is not devoid of risks, depending on the selected fungal species. To exemplify, *Colletotrichum lindemuthianum* has been used as a valuable source of chitin deacetylase [99], although this fungus is the causative agent of 'anthracnose', a plant disease that can affect a variety of crops grown in both temperate and tropical climates. Within the class of Zygomycetes, some species belonging to *Absidia* and *Rhizopus* genera can be pathogens either to animals or humans. Some species of *Absidia* are causative agents of mucormycosis in humans with low immune systems [100] and zygomycosis, causing spontaneous abortion in cows. In addition, *Rhizopus oryzae* can act as an opportunistic human pathogen causing pulmonary mucormycosis [101]. As a consequence, the handling of these potentially pathogenic strains requires the adoption of specific measures to prevent their accidental dispersal. The hope is that the research and development of the chitosan fungal production process can be oriented in the future only and exclusively on strains that meet the generally regarded as safe (GRAS) requirement.

3.3. Production Processes of Fungal Chitin and Chitosan

The chitin and chitosan contents are species-specific and are largely affected by the growth medium and fermentation system. Although a variety of chitosan production processes have been conducted in solid-state fermentation (SSF) (Table 3), the majority of studies have relied primarily on liquid submerged fermentation (LsF) (Tables 4 and 5).

The marked preponderance of studies conducted in liquid fermentation is likely due to several advantages of this technique compared to SSF. These advantages include facile control of process parameters, especially at the reactor level, higher amenability to scale transfer, and straightforward biomass recovery from the growth medium [13]. In SSF, the fungal colonization occurs either on inert and homogeneous solid substrates or on natural ones moistened in such a way as to ensure the absence of free water [102]. On the one hand, using homogeneous solid substrates is advantageous as it allows better control of medium composition and improved oxygen and nutrients transfer; moreover, it facilitates the recovery of fungal biomass. However, no reports dealing with the chitin/chitosan production on inert substrates are available to the best of our knowledge. On the other hand, the natural use of solid substrates, due to their intrinsic heterogeneity, implies mass-transfer limitations, which do not enable accurate process control and enhance the difficulty of recovering fungal biomass [34].

Contrary to other reviews that have used a genus-specific approach for organizing literature data [3,103], a discussion based on the fermentation technique appeared to be more valuable and informative here (the present review). Considering the relative scarcity of studies on the solid-state production of chitosan and, in general, their poorly equipment-oriented character, only a single section of this review has been dedicated to this topic. Conversely, this review offers an articulated discussion of LSF production studies. These were divided based on the nature of the production medium into two sections, the first of which focused on studies conducted on synthetic media and the second on waste- or effluent-derived media. Finally, the present review devotes a section to process parameters that directly influence fungal chitosan production and properties. The reader is referred

to the conclusions section for any comparative considerations between the production approaches in liquid and solid-state culture.

3.3.1. Solid-State Production of Fungal Chitosan

As mentioned in Section 3.3, solid-state chitosan production studies have relied solely on naturally occurring substrates. This approach has offered the opportunity to explore economically sustainable production solutions and, at the same time, to exploit processing residues that would otherwise have little economic value. A variety of plant residues have been used for this purpose, including wheat and rice straw [104,105], soybean residues [76,106,107], hardwood sawdust [108], sweet potato pieces [60], potato chip processing waste [79], and cottonseed hulls [34].

Table 3, summarizing SSF chitosan production studies, shows that this technique enabled the achievement of high product concentration, referred to dry weight of solid substrate, which widely ranged from 1.6 to 17 g kg^{-1} depending on strain and substrate combinations.

The average volumetric productivities (r_P) were also interesting in some cases, such as in cultures of *A. niger* and *M. rouxii* grown on soy-derived solid matrices (59 and 119 mg kg^{-1} h^{-1}, respectively) [107,109]; these productivity values were attributable to a high product concentration rather than to rapid growth since the product peak was obtained in 12-day-old cultures in both cases.

Table 3. Chitosan production yields (CPY) referred to unit mass of the solid substrate, average volumetric productivity (r_P), degree of deacetylation, and viscosity of chitosan obtained in solid-state cultures of several fungal strains.

Fungal Strain	Solid Substrate	Cultivation Mode	CPY (g/kg)	r_P (mg/kg*h)	DD (%)	Viscosity (cP)	References
Absidia coerulea CTCC AF 93105	Non-supplemented cotton seed hulls	Conical flask	1.62	9.64	85	n.r.	[34]
Absidia coerulea CTCC AF 93105	Potato pieces added with sucrose and urea	Conical flask	6.12	36.4	85	n.r.	[34]
Aspergillus niger n.s.	Rice straw	Plastic bag with sterile filters	5.26	24.4	84.2	59	[105]
Aspergillus niger TISTR3245	Mung bean residues	Conical flask	1.39	19.3	n.r.	n.r.	[76]
Aspergillus niger BBRC 20004	Soybean residues	Conical flask	17.03	59.1	n.r.	n.r.	[109]
Gongronella butleri USDB0201	Sweet potato pieces supplemented with urea	Tray reactor	3.7	22.0	92–96	n.r.	[61]
Gongronella butleri USDB0201	Sweet potato pieces supplemented with urea	Tray reactor	4.31	25.7	n.r.	n.r.	[62]
Lentinus edodes SC-495	Wheat straw	Plastic bag with sterile filters	6.18	21.5	87.5	n.r.	[104]
Mucor rouxii ATCC 24905	Soybean meal	Autoclavable plastic bag with sterile filters	32.4	119.4	55–60	n.r.	[107]
Penicillium citrinum n.s.	Rice straw	Plastic bag with sterile filters	5.12	17.8	78.5	4.6	[105]
Penicillium expansum	Corn straw	Conical flask	4.31	n.r.	80.2	4.8	[110]
Rhizopus oryzae n.s.	Rice straw	Plastic bag with sterile filters	5.63	19.6	90.2	6.8	[105]
Rhizopus oryzae TISTR3189	Potato peel	Conical flask	6.6	55.0	87.5–90	3.1–6.1	[79]
Rhizopus oryzae (local isolate)	Corn straw	Conical flask	8.57	29.8	91.5	7.2	[110]
Rhizopus oryzae TISTR3189	Soybean residue	Conical flask	4.3	29.9	n.r.	n.r.	[76]

Different evaluation, on the other hand, can be performed for potato- or sweet potato-derived substrates, which provided productivity values higher than those obtained on cereal residues owing to the comparatively lower time requirements to attain the product peak (Table 3). For example, the time required to achieve the product peak in *R. oryzae* TISTR3189 and *A. coerulea* CTCC AF 93105 cultures grown on solid potato-based matrices was 5 and 7 days [34,79]. Conversely, *L. edodes* SC-495, *R. oryzae*, and *P. citrinum* ATCC 24095 cultures grown on wheat straw, corn straw, and rice straw, respectively, reached the product peak 12 days after the inoculation [104,105,110].

Noteworthy, *Absidia coerulea* AF93105 solid-state cultures on potato waste provided a direct method of producing low molecular weight chitosan, which, due to its compatibility with agricultural and biomedical applications, is generally obtained by thermochemical or enzymatic depolymerization starting from high molecular weight chitosans [34]; in particular, the polymer obtained with a yield of 6.1 g kg^{-1} showed an average molecular weight of 6.4 kDa associated with a very low degree of polydispersity.

Twelve-day-old *R. oryzae* solid-state cultures on nutrient-supplemented rice straw yielded 5.63 g chitosan kg^{-1} substrate; the chitosan thus obtained exerted a higher antibacterial activity toward a variety of pathogenic bacteria, such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella* sp., and *Escherichia coli* as compared to chitosan from crab shells [105].

However, all these studies were performed either on stationary flasks or using autoclavable plastic bags. Although a variety of solid-state reactors are available, unique exceptions are the studies of one research group [60–62,111] that used a perforated tray reactor to perform chitosan production (3.7–4.3 g kg^{-1}) with excellent DD (92–96%) from *Gongronella butleri* USDB0201 cultures grown on potato peel wastes. An additional exception is the study of Dhillon et al. [3] who used a rotary tumbling drum reactor to investigate the coproduction of citric acid and chitosan.

3.3.2. Fungal Chitosan Production in Liquid Submerged Bioprocesses

Several screening studies aimed at identifying valuable chitosan-producing strains relied on synthetic media, such as the yeast extract-malt extract medium (YM) [45], potato dextrose broth (PDB) [68], yeast extract-peptone-dextrose (YPD) medium [27,46], glucose-peptone [88] and glucose-peptone-yeast extract (GPY) [75,112]. Moreover, a variety of strain-oriented studies, summarized in Table 4, used these production media in original or slightly modified formulation. Particularly in the last decade, the high costs of these media have shifted the attention of researchers toward cheaper solutions. Moreover, the temporal distribution of studies conducted on synthetic media shows that a non-negligible part of them is far from recent. This review, however, considered it appropriate to reserve them a section. The reasons underlying this choice are many and include a high relevance from the production point of view, the provision of information relating to the choice of strains, physiology of production, and reactor configuration.

Chitosan Production on Chemically Defined Liquid Media

Table 4 shows comparatively volumetric biomass and chitosan productions (X and CVP, respectively) and r_P values of chitosan in liquid cultures of several fungal strains grown on synthetic media either in the shaken flask or in the reactor.

Table 4. Volumetric productions of biomass (X) and chitosan (CVP) and chitosan average volumetric productivity (r_P) in liquid cultures of several fungal strains grown on synthetic media either in shaken flask or in reactor.

Fungal Strain	Culture Condition and Growth Medium	X (g L^{-1})	CVP (g L^{-1})	r_P (mg L^{-1} h^{-1})	References
Absidia butleri NCIM977	Shaken cultures on GYT medium	6.78	0.57	7.9	[51]
Absidia coerulea ATCC14076	Aerated shaken cultures on YM broth	6.2	1.86	26.0	[47]
Absidia coerulea ATCC14076	Batch cultures in 2.5 L STR at 250 rpm and 2 vvm with adaptive pH control at 4.5 on GY medium supplemented with $(NH_4)_2SO_4$	20	2.33	63.8	[49]

Table 4. *Cont.*

Fungal Strain	Culture Condition and Growth Medium	X ($g\ L^{-1}$)	CVP ($g\ L^{-1}$)	r_P ($mg\ L^{-1}\ h^{-1}$)	References
Absidia coerulea ATCC14076	Batch cultures in 20 L STR at 200 rpm and 1 vvm on PGY medium	13.9	0.55	11.5	[113]
Absidia coerulea ATCC14076	Continuous cultures in 2.5 L STR with pH control at 4.5 on GY medium supplemented with $(NH_4)_2SO_4$ at a dilution rate of 0.05 h^{-1}	7.0	1.04	50.0	[49]
Absidia coerulea ATCC14076	Continuous cultures in BioFlo C30 chemostat on GYP medium at a dilution rate of 0.025 h^{-1}	2.3	1.37	41.0	[46]
Absidia coerulea CTCC AF 93105	Shaken cultures on a glucose-based medium added with 0.5 g L^{-1} as $(NH_4)_2SO_4$	11.4	2.86	19.9	[50]
Absidia coerulea CCRC 30897	Batch cultures in airlift with double-net draft tube on GYP medium	30.8	3.16	65.8	[114]
Absidia coerulea CCRC 30897	Batch cultures in bubble column reactor on GYP medium	11.3	1.36	28.3	[114]
Absidia glauca (+)	Shaken cultures on GYP medium	8.8	0.65	13.5	[112]
Absidia orchidis NCAIM F 00642	Batch cultures in 5 L STR on GYP medium supplemented with ferrous ions	45.3	1.79	37.3	[31]
Absidia orchidis NCAIM F 00642	Batch cultures in 5 L STR on GYP medium supplemented with Mn^{2+} ions	15.2	1.05	21.9	[31]
Absidia repens CBS 102-32	Batch cultures in 10 L STR at 350 rpm on a medium made of glucose, yeast extract, and $(NH_4)_2SO_4$	12.9	2.8	58.3	[115]
Aspergillus niger MTCC 872	Shaken cultures on a medium made of potato dextrose broth (24 g L^{-1}), glucose (80 g L^{-1}), L-Asparagine (6 g L^{-1})	15.9	3.35	46.5	[84]
Aspergillus niger BBRC 20004	Shaken cultures on Sabouro dextrose broth added with 2% glucose	5.17	0.84	17.5	[83]
Aspergillus nidulans NS	Shaken cultures on peptone-glucose-yeast extract (PGY) salt broth	5.15	0.20	4.19	[112]
Benjaminiella poitrasii CSIR isolate	Batch cultures in 2 L STR on medium containing (g L^{-1}): yeast extract, 6.0; peptone, 10.0; soluble starch,10.0	10.0	0.51	10.6	[66]
Cunninghamella bertholletiae IFM 46.114	Shaken cultures on yeast extract-peptone-dextrose medium	7.1	0.39	5.5	[56]
Cunninghamella echinulata	Shaken cultures on glucose-peptone-yeast extract medium added with $(NH_4)_2SO_4$	5.6	0.40	3.3	[52]

Table 4. *Cont.*

Fungal Strain	Culture Condition and Growth Medium	X ($g\ L^{-1}$)	CVP ($g\ L^{-1}$)	r_P ($mg\ L^{-1}\ h^{-1}$)	References
Cunninghamella elegans IFM 46109	Shaken culture on a medium mainly made of glucose, asparagine, and MgSO4 (60, 3.0, and 0.25 g L^{-1}, respectively)	11.0	0.86	8.9	[53]
Cunninghamella elegans UCP 542	Shaken cultures on Sabouraud-sucrose medium	12.0	0.42	8.7	[57]
Gongronella butleri USDB 0201	Shaken cultures on glucose-peptone-yeast extract medium added with $(NH_4)_2SO_4$	8.2	0.47	3.9	[52]
Mucor racemosus (soil isolate)	Shaken cultures on Sabouraud dextrose broth	3.8	0.45	2.6	[71]
Mucor rouxii ATCC 24905	Shaken cultures on glucose-peptone-yeast extract medium	3.8	0.28	5.8	[67]
Mucor rouxii ATCC 24905	Shaken cultures on peptone-yeast extract-glucose (PYG) salt broth	5.6	0.21	4.4	[112]
Mucor rouxii DSM 1191	Batch cultures in 30 L STR on glucose-peptone-yeast extract medium	8.6	0.30	21.2	[116]
Rhizomucor miehei ATCC 26282	Shaken cultures on Sabouraud dextrose broth	4.1	0.56	3.4	[71]
Rhizopus oryzae USDB 0602	Shaken cultures on glucose-peptone-yeast extract medium added with $(NH_4)_2SO_4$	5.7	0.28	2.3	[52]
Syncephalastrum racemosum UCP148	Shaken cultures on yeast extract-peptone-dextrose medium	8.0	1.26	26.1	[27]

By sorting the data shown in Table 4 based on the volumetric production, it is evident, with a few exceptions, that the best performing strains belong to the *Absidia* genus. This outcome does not change using the r_P of chitosan as the sorting criterion, and it is no coincidence that 8 of the 10 highest values are related to studies conducted in reactors. Furthermore, it is in agreement with the investigation of Shimahara et al. [117], who had already concluded several years earlier at the end of a screening conducted on 125 strains of Zygomycetes, that those belonging to the genus *Absidia* were by far the most productive.

Wu et al. [114] achieved the best r_P (65.8 $mg\ L^{-1}\ h^{-1}$) ever reported with *A. coerulea* CCRC 30897 batch cultures grown in an airlift reactor modified with a double-net draft tube. This modified reactor enabled an excellent oxygen transfer to the liquid medium resulting in the achievement of a CVP value more than two times higher than that observed in a conventional bubble column reactor (3.16 vs. 1.36 g L^{-1}). The same study also compared the performance of the modified airlift with that of a mechanically agitated reactor and found that CVP values were more than two-fold and 55% higher than those achieved in an STR with an impeller speed of 600 and 300 rpm, respectively.

Kim et al. [49] also obtained very relevant results with another *A. coerulea* strain, viz. 14076, grown in a 2.5 L STR operated either in batch or continuous mode and using a glucose-YE medium supplemented with $(NH_4)_2SO_4$. In this study, the strategy of pH control at 4.5 led to a higher maximal growth rate and smaller pellets than cultures where pH was left to fluctuate freely, leading to 1.8- and 3.5-fold improvement in CVP and r_P, respectively. The same study claimed that when the STR was operated in continuous mode at a dilution rate of 0.05 h^{-1}, the r_P of the process was 52 $mg\ L^{-1}\ h^{-1}$, a value higher than

that obtained in a chemostat with the same strain by Rane and Hoover [46]. Noteworthy, batch cultures of the same strain grown in a 20 L STR in a similar medium [113] exhibited lower performance than those of Kim et al. [49], presumably owing to either the larger process scale or omitted control of medium's pH.

Another species belonging to the genus *Absidia* that proved to be a suitable producer of chitosan was *A. repens* CBS 102.32; this strain, grown in a 10 L STR on a medium consisting of glucose and YE and added with $(NH_4)_2SO_4$, provided CVP and r_P values equal to 2.8 g L^{-1} and 58.3 mg L^{-1} h^{-1}, respectively [115]. Although the CVP value was moderate (0.3 g L^{-1}), the study by Gözke et al. [116], conducted in a 30 L STR reactor with *M. rouxii* DSM 1191 cultures on GYP medium, is noteworthy due to the scale of the reactor and the short process duration reaching maximum productivity after 14–16 h from the inoculation. The use of inorganic supplements, such as manganese and ferrous ions able to affect the chitin synthase and chitin deacetylase activities was tested by Jaworska and Konieczna [31]. This study was conducted in a 4.5 L STR with *A. orchidis* NCAIM F 00642 grown on iron-supplemented yeast extract-peptone glycerol medium led to CVP and r_P values equal to 1.79 g L^{-1} and 58.3 mg L^{-1} h^{-1}, which were several folds higher than those of non-supplemented cultures.

Chitosan Production on Waste- and Effluent-Based Liquid Media

As discussed in the previous section, the liquid media for fungal chitosan production often include organic components, such as YE, D-glucose, and peptone, which are costly growth substrates. For this reason, several studies investigated the exploitation of cheap carbon and nitrogen sources derived from wastes [64,81,86,90] to mitigate the production costs and compete commercially with crustacean's shell-based processes [43].

With regard to their origin, these wastes derived from crop residues [118], corn wet-milling operations [119] or were byproducts of the dairy industry [77,78], sugar manufacturing [56,70,81,120], fruit juice industry [64,65] or distilleries [121,122]. In several cases, the liquid medium was derived from a solid substrate either by acid hydrolysis, such as for corn straw [118], or via its aqueous extraction, such as in the case of apple pomace [64,86] and date syrups [28]. Other studies, instead, relied on liquid byproducts, such as sugarcane or sugar beet molasses [56,81,82] and deproteinized whey [77]. Suitable liquid production media have also been derived from a variety of process effluents, such as cassava wastewater [54], paper mill effluent [90], thin stillage [122], and xylose-rich wastewater from a bioethanol plant [74] (Table 5).

In several cases, the approach adopted involved only a partial replacement of the expensive organic components of the medium, intended to act as sources of carbon or nitrogen, with others derived from residues of agro-industrial origin. For example, Jiang et al. [50] obtained excellent CVP (4.11 g L^{-1}) and r_P (28.54 mg L^{-1} h^{-1}) values in *A. coerulea* CTCC AF 93105 cultures on a glucose-based medium by replacing commercial sources of inorganic nitrogen with soybean pomace.

Table 5. Volumetric productions of biomass (X) and chitosan (CVP) and chitosan average volumetric productivity (r_P) in liquid cultures of several fungal strains grown on liquid byproducts or process effluents either in shaken flask or in reactor.

Fungal Strain	Culture Condition and Growth Medium	X (g L^{-1})	CVP (g L^{-1})	r_P (mg L^{-1} h^{-1})	References
Absidia coerulea CTCC AF 93105	Shaken cultures on a glucose-based medium (20 g L^{-1}) added with 0.5 g L^{-1} nitrogen as soybean pomace	15.4	4.11	28.5	[50]
Aspergillus awamori MTCC6995	Shaken cultures on thin stillage from rice-based distillery	5.2	0.39	4.0	[122]

Table 5. *Cont.*

Fungal Strain	Culture Condition and Growth Medium	X (g L^{-1})	CVP (g L^{-1})	r_P (mg L^{-1} h^{-1})	References
Aspergillus braziliensis ATCC16404	Shaken cultures on syrup from date waste	13.3	2.78	19.3	[28]
Aspergillus niger NRRL567	Batch cultures in 7.5 L STR (impeller speed at 200 rpm; adaptive flow rate to ensure 20% dissolved oxygen saturation) on apple pomace sludge	12.6	0.64	4.9	[3]
Cunninghamella bertholletiae IFM 46.114	Shaken cultures on sugarcane juice (10.5 g L^{-1} sucrose) added with YE (3 g L^{-1})	4.2	0.53	11.1	[56]
Cunninghamella elegans UCP 0542	Shaken cultures on a medium made of cassava wastewater (CWW, 10%) and corn steep liquor (CSL, 4%)	5.7	0.33	4.6	[54]
Gongronella butleri CCT4274	Shaken cultures on aqueous extract of apple pomace supplemented with NaNO3 (2.5 g L^{-1})	5.5	1.19	16.4	[64]
Gongronella butleri IFO8081	Shaken cultures on sweet potato shochu distillery wastewater	6.2	0.73	6.1	[121]
Gongronella butleri CCT 4274	Batch cultures in 6.5 L airlift reactor with external loop circulation (aeration rate, 0.6 vvm) on apple pomace extract added with 5 g L^{-1} $(NH_4)_2SO_4$	6.7	0.93	62.2	[65]
Lichtheimia hyalospora UCP1266	Shaken cultures on a medium made of CWW (4%) and CSL (6%)	11.9	0.75	6.3	[119]
Mucor rouxii MTCC 386	Shaken cultures on molasses salt medium added with indole-3-acetic acid (1.0 mg L^{-1})	9.1	0.95	29.7	[70]
Mucor subtilissimus UCP 1262	Shaken cultures on a medium made of CWW (4%) and CSL (6%)	4.8	0.16	1.3	[119]
Penicillium citrinum (local isolate)	Batch cultures in 3 L stirred tank reactor (200 rpm and 2.0 vvm) on paper mill effluent added with 50 mg L^{-1} acetic acid	n.s.	0.14	2.9	[90]
Rhizopus arrhizus UCP 0402	Shaken cultures on a medium made of CSL (4%) and honey (13%)	11.7	0.34	3.6	[80]
Rhizopus oryzae MTCC262	Shaken cultures on deproteinized whey added with gibberellic acid (0.1 mg L^{-1})	8.3	1.13	15.7	[77]
Rhizopus oryzae 00.4367	Batch cultures in 7 L STR (340 rpm, 2.1 vvm) on untreated sugarbeet molasses (45.4 g L^{-1} total sugars)	10.7	1.06	14.7	[120]

Table 5. *Cont.*

Fungal Strain	Culture Condition and Growth Medium	X ($g\ L^{-1}$)	CVP ($g\ L^{-1}$)	r_P ($mg\ L^{-1}\ h^{-1}$)	References
Rhizopus oryzae AS 3.819	Batch cultures in 3 L stirred tank reactor (200 rpm, 1 vvm) on corn stover hydrolysate supplemented with urea (4 $g\ L^{-1}$)	11.0	0.99	13.8	[123]
Rhizopus oryzae PAS 17	Shaken cultures on medium made of molasses (7%, *v/v*) and supplemented with $MgSO_4$	10.7	1.50	7.8	[81]
Rhizopus oryzae ME-F12	Shaken cultures on corn straw hydrolysate	5.2	0.58	n.s.	[118]
Rhizopus oryzae MTCC262	Shaken culture on deproteinized whey supplemented with $(NH_4)_2HPO_4$ (8 $g\ L^{-1}$) and YE (2 $g\ L^{-1}$)	6.2	0.62	n.s.	[78]
Syncephalastrum racemosum UCP148	Shaken cultures on sugarcane juice (10.5 $g\ L^{-1}$ sucrose) added with YE (3 $g\ L^{-1}$)	8.1	0.60	5.0	[82]
Syncephalastrum racemosum UCP148	Batch cultures in 5 L STR on sugarcane juice (10.5 $g\ L^{-1}$ sucrose) added with YE (3 $g\ L^{-1}$)	8.0	0.96	32.0	[82]

S. racemosum UCP148 cultures grown in a 5 L STR containing sugarcane juice supplemented with 0.3% YE yielded CVP and r_P values as high as 0.93 $g\ L^{-1}$ and 32 $mg\ L^{-1}\ h^{-1}$, respectively [82].

In other studies, instead, the approach was to integrally eliminate the expensive organic components of the medium with the liquid residue used as it was or, possibly, supplemented with inorganic nitrogen sources. For instance, *G. butleri* CCT 4274 cultures grown in a 6.5 L airlift reactor on an aqueous extract of apple pomace (AEAP) supplemented with an inexpensive source of nitrogen, such as ammonium sulfate (5 $g\ L^{-1}$) provided a CVP value of 0.93 $g\ L^{-1}$ but, above all, one of the highest r_P values of chitosan ever reported for reactor cultures (62 $mg\ L^{-1}\ h^{-1}$) [65]. The use of the airlift enabling better gas exchanges associated with the use of a more readily available nitrogen source provided much better results than those reported by Streit et al. [64] with the same strain grown on sodium nitrate-supplemented AEAP. Göksungur [120] used untreated and non-supplemented sugar beet molasses as the liquid medium for chitosan production by *R. oryzae* 00.4367 in a 7 L STR; the use of a response surface methodology approach allowed the investigators to optimize statistically both agitation and aeration regimes and sugar concentration. In particular, under the optimization of these variables (impeller speed, 340 rpm; aeration rate, 2.1 vvm; sugar concentration, 45.4 $g\ L^{-1}$), the CVP and r_P amounted to 1.06 $g\ L^{-1}$ and 14.5 $mg\ L^{-1}\ h^{-1}$, respectively.

Whey, a byproduct of the dairy industry, the world production of which amounts to 121 million tons [124], was also used as the basis for the development of a chitosan production medium. In particular, *R. oryzae* MTCC262 cultures, grown on deproteinized whey (DW) and added with 0.3% YE and 0.1 $mg\ L^{-1}$ of gibberellic acid (GA3), provided CVP and r_P values equal to 1.13 $g\ L^{-1}$ and 15.72 $mg\ L^{-1}\ h^{-1}$ [77]. In a subsequent study, the same research group reported for the same strain on a DW-based medium, but differently formulated (Table 5), CVP and r_P values equal to 0.62 $g\ L^{-1}$ and 8.6 $mg\ L^{-1}\ h^{-1}$ [78].

With regard to the use of process effluents for the development of chitosan production media, several studies focused their attention on that produced by the cassava (*Manihot esculenta*) processing industry, termed cassava wastewater (CWW). Recent estimates es-

tablished that the processing of 1 ton of tubers generates around 60 m^3 of effluent [125]. Several studies shared the combination of CWW with corn steep liquor, a byproduct of corn wet-milling, frequently used as a low-cost nitrogen source [54,63,119]. Among these studies, the best process performance was obtained with *Lichtheimia hyalospora* UCP1266 yielding CVP and r_P values amounting to 0.75 g L^{-1} and 4.56 mg L^{-1} h^{-1} [119].

Grain-based distilleries generate thin stillage (TS) as the high-strength process effluent, the volume of which is ten-fold higher than that of the ethanol produced. Ray and Ghangrekar [122] used *Aspergillus awamori* MTCC6995 to reduce the organic load of a rice-based TS and to extract chitosan from the residual fungal biomass; at its endpoint (96 h), the process yielded a 60% reduction in the effluent's COD and CVP and r_P of chitosan amounted to 0.39 g L^{-1} and 4.02 mg L^{-1} h^{-1}, respectively. Yokoi et al. [121] used sweet potato shochu wastewater as the chitosan production medium for *G. butleri* IFO 8081, which attained a CVP of 0.73 g L^{-1} after 120 h from the inoculation.

3.4. Relevant Factors in Chitin and Chitosan Production from Fungi

Production levels of these glycans can be achieved via an increase in the biomass yield or an increase in their contents in the cell wall [31], and several process parameters were found to be relevant to this goal. These parameters affect not only the production of these glycans but also their physicochemical properties [31,69,77].

3.4.1. Fungal Morphology

Fungal morphology, especially during cultivation in reactors, frequently evolves in a way resulting in a decline in the growth rate [126]. The growth mode involving the formation of dispersed mycelium leads to a highly viscous medium with ensuing agitation and aeration problems [127]. Several strains, instead, have a marked propensity to form pellets, and, in that instance, an increased pellet diameter might result in dropped growth owing to diminished mass transfer to the pellet's innermost part [126]. Several studies highlighted the importance of the pellet size, showing that diameters in the 4.0–5.0 mm were most conducive to the maximization of D-glucosamine yield in *R. oligosporus* [128] or chitosan production by *Lichtheimia hyalospora* UCP 1266 [119]. Similar results were obtained with *Absidia repens* CBS 102.32 [115], grown at different stirring regimes (i.e., 350, 200, 200 for the early 24 h followed by 350 rpm) in a 10 L STR; growth at 350 rpm, leading to the formation of pellets with an average diameter of 0.5 mm was the condition enabling best chitosan production (2.8 g L^{-1}) with an r_p of 58 mg L^{-1} h^{-1}.

Macro-morphology can also largely affect chitosan production of several dimorphic species, such as *M. indicus* [129] and *M. subtilissimus* [119]. The growth mode in dimorphic fungi is affected by several factors, including initial spore concentration, sugar content in the growth medium, and oxygen availability; moreover, the shift to a yeast-like morphology can be promoted by the addition of compounds acting as cytochrome oxidase inhibitors. Noteworthy, de Souza et al. [119] reported that the increase in concentrations of cassava wastewater, containing cyanides, promoted a yeast-like growth mode and negatively affected the chitosan production in *M. subtilissimus* UCP 1262 cultures. The same study found that the best chitosan production conditions were those leading to the mycelial form in agreement with other studies conducted with *M. indicus* CCUG 22424 [129] and *M. rouxii* [130].

3.4.2. Harvesting Time

Although chitin and chitosan productions are obviously growth-associated processes, their maximum yields do not necessarily occur at the biomass peak [4]. With this regard, in fact, the amount of extractable chitosan was highest at the late exponential growth phase in *Asidia coerulea* [50], *Rhizopus oryzae* 00.4367 [120], *R. oryzae* USDB 0602 [52], and *Cunninghamella bertholletiae* [56] to decline significantly thereafter. Tan et al. [52] suggested that free chitosan molecules were largely abundant during the exponential phase, owing to active growth; during the stationary growth phase, instead, a higher proportion of

chitosan bound to other cell's wall constituents, thus rendering extraction less effective. This effect was also evident in *R. oryzae* solid-state cultures on rice straw, where the ratio between chitosan and alkali-insoluble material (AIM) dropped from 61%, in concomitance with biomass peak, to 42% when culture had entered the stationary phase [105]. Albeit not yet proven in vivo, an alternative explanation might be a time-dependent increase in chitin crystallinity due to the failure of the majority of CDAs to perform the in vitro deacetylation of crystalline chitin [131]. A notable exception to this time-dependent trend in chitosan production was the study of Davoust and Persson [115], who observed chitosan accumulation in the late stationary phase of *Absidia repens* cultures; they hypothesized that since the synthesis of chitin and chitosan occurs at the hyphal apex during the elongation process, the observed increase was due to continued apical growth at the expense of products derived from partial cell lysis.

3.4.3. Medium's Ph

By using a modified GPY medium adjusted to several pH values (range: 3.0–6.5), Rane and Hoover [46] found that the chitosan-producing ability and DD of chitosan were largely unaffected in *M. rouxii* DSM1191; the same study, however, found a significant strain-dependent effect of the pH on the same descriptors in *A. coerulea* ATCC 14076 and *A. coerulea* NRRL 1315. Kim et al. [49] observed that pH affected fungal morphology in *A. coerulea* ATCC 14076 cultures, and adaptive control at pH 4.5 enabled the formation of smaller pellets than those without pH control in batch cultures conducted in an STR. This pH control strategy enabled higher CVP and r_P as compared to cultures without pH control (2.3 vs. 1.3 g L^{-1} and 63.9 vs. 36.1 mg L^{-1} h^{-1}, respectively). Solid-state *G. butleri* cultures conducted on sweet potato pieces at various initial pH (i.e., 3.77, 4.92, 5.46, and 5.52) provided better chitosan yields at pH 5.46 and 5.52, and the chitosans obtained had higher average molecular weights than those at more acidic pHs [62].

3.4.4. Nitrogen Source and Concentration

Both the nitrogen source and its amounts are among the most relevant process parameters since chitosan is a nitrogen-containing glycan. In general, fungi are able to directly exploit ammonium ions, while other inorganic nitrogen sources have to be reduced to the redox level of ammonium [132]. Nwe and Stevens [62] showed that urea was a valuable N source for chitosan production by *Gongronella butleri* solid-state cultures. By increasing the urea levels from 5 to 14 g per kg of solid substrate, in addition to obtaining a significant increase in the yield of chitosan (from 0.082 to 0.114 g g^{-1} mycelium), there was a disproportionate increase in weight average molecular weight (Mw) as compared to number average molecular weight (M_n), thus increasing polydispersity (Mw/M_n) [62]. A dose-dependent effect of the N source on the molecular weight of *Mucor rouxii* chitosan was also observed by Arcidiacono and Kaplan [33] by doubling either peptone or yeast extract concentration in a YPG medium. Several studies claimed that the impact of yeast extract, a costly organic nitrogen source derived from cells autolysis, was beneficial to chitosan production [33,56,82]. To improve the economic feasibility of the chitosan production process, Abasian et al. [73] replaced YE with an autolysis-derived *M. rouxii* extract in a glucose-based medium; this approach improved the yields of the AIM and led to increased GlcN and decreased GlcNAc concentrations in AIM in *M. rouxii* CCUG 22424 cultures.

3.4.5. Plant Growth Hormones

Some studies showed that the addition of phytohormones to *R. oryzae* and *Mucor rouxii* liquid cultures grown on deproteinized whey [77] and molasses salt medium [70], respectively, stimulated both fungal biomass and chitosan productions although in a dose-dependent manner. Among the tested phytohormones (i.e., indole-3-acetic acid, IAA; indolebutyric acid, IBA; gibberellic acid, GA3 and kinetin, KIN), GA3 was the most effective. In fact, the addition of GA3 at 0.1 and 3.0 mg L^{-1} concentration to *R. oryzae* [77] and *M. rouxii* [70] cultures resulted in a 50% and 69% increase in chitosan, respectively;

however, in both studies, higher GA3 doses had an inhibitory effect on chitosan production. Chatterjee et al. [77] also showed a 27% increase in the specific activity of chitin deacetylase in GA3-added cultures as compared to control ones, thus leading to an increased chitosan/chitin ratio (0.72 vs. 049). Another impact of GA3 addition was an increase in MW of chitosan derived from hormone-added cultures as compared to control ones [70,77].

3.4.6. Organic Stimulators

Another range of potential stimulators of chitosan biosynthesis emerged indirectly from a study reporting on the superiority of a corn straw hydrolysate for chitosan production by *R. oryzae* ME-F12 over glucose-, and xylose-based media [118]. This acid hydrolysate, in addition to containing xylose, as the main component and other pentoses, also contained furfural, acetic acid, and formic acid. The last three compounds added to a xylose medium exerted a dose-dependent stimulatory effect on both chitosan production and chitosan yield [118]; the authors speculated that the enhanced chitosan synthesis was a compensatory response of the fungus to increase the thickness and density of the cell wall to better cope with the presence of potentially inhibitory compounds.

3.4.7. Inorganic Supplements

Some studies have taken into consideration the effect deriving from the addition of some inorganic supplements, such as Mg^{2+}, Co^{2+}, Mn^{2+} and Fe^{2+} ions, capable of influencing the in vitro activity of chitin deacetylase and chitin synthetase [131,133]. For instance, the wide recognition of the stimulatory effect of Mg^{2+} ions on both CS and CDA have led to the inclusion of $MgSO_4$ in several chitosan production media at a concentration ranging from 0.5 to 5.0 g L^{-1} [31,46,49].

For some microelements such as cobalt, the effect was strongly concentration dependent or even led to growth failure due to the intrinsic toxicity of this metal. In fact, Rane and Hoover [46] reported that while the addition of 5 mg L^{-1} to a modified GPY medium led to a 20% increase in chitosan production in *A. coerulea* ATCC 14076 cultures, a four-fold increase in its concentration led to inhibitory effects. In another study attempting the exploit the stimulatory effect of several ions, the addition of Co^{2+} (2.3 and 4.5 g L^{-1}) to a YPG medium led to severe growth inhibition in *A. orchidis* NCAIM F 00642 cultures [31]. Conversely, the same study found that the addition of Mn^{2+} ions led to an improved CVP as compared to control cultures (1.03 vs. 0.71 g L^{-1}), increased viscosimetric molecular weight (1156 vs. 751 kDa), and decreased DD (69.3% vs. 84.4%).

Phosphates are the primary constituents in the cell walls of Zygomycetes, and several studies indicate that their contents can vary from 8.3% to 23% [130,134]. Phosphates interact with chitosan and other structural polysaccharides to yield complexes that are not broken down by conventional acid treatment. As a consequence, a significant amount of chitosan can remain associated with both acid- and alkali-insoluble materials during extraction. Several studies showed that low concentrations of phosphates in the growth medium led to increased GlcN yields [39,135]. In Zygomycetes, the accumulation of anionic storage materials, such as polyphosphates, is thought to be performed by chitosan due to its polycationic nature [72]. Consequently, under P-limiting conditions, chitosan synthesis is probably boosted to enhance the uptake of phosphates from the growth medium. Moreover, in media containing inorganic nitrogen sources, such as ammonium sulfate (AS), the limitation of inorganic phosphorus (Pi) stimulates the overproduction of chitin/chitosan in some Zygomycetes [136]. Acidic stress caused by the low concentrations of Pi in the growth medium was suggested to be the primary reason explaining this phenomenon in AS-Pi media at low Pi concentrations [137]. These findings are in agreement with a chitosan optimization study conducted with *Mucor indicus* CCUG22424 cultures, where a phosphate-free medium led to the highest chitosan production [39].

4. Integrated Bioprocesses

In recent decades, significant advances have been made toward promoting a sustainable bio-based economy. A positive outcome of these endeavors primarily depends on the enactment of the concept of biorefinery, namely a facility able to integrate processes and equipment to yield a wide array of marketable products and energy [138]. Pursuing the concept of coproduction can increase the economic viability of the microbial processes currently in place [3,136]. Moreover, there is an undeniable urgency to promote integrative technology to exploit the vast amounts of mycelial waste from industrially relevant processes. In this framework, the mycelial wastes from various bioprocesses can be exploited as feasible chitosan sources in compliance with the biorefinery concept. These fungal strains mostly belong to the *Aspergillus* and *Mucor* genera, the species of which are used in several relevant processes. Unfortunately, microbial productions are sometimes governed by very different factors, and, therefore, the concept of coproduction can be difficult to pursue. This may mean sacrificing one of the two coproducts or, otherwise, using tools capable of safeguarding the production levels of both. Among these tools, we can mention the use of high-throughput culture techniques [136] and that of statistical optimization methods enabling the identification of variable combinations maximizing both coproducts, such as in response surface methodology [13,40].

In a very recent study, the combination of high-throughput culture technique with fast Fourier transform infrared spectroscopy allowed a fast determination of the impact of two relevant variables (i.e., two nitrogen typologies and inorganic phosphate) on the coproduction of single-cell oils, polyphosphates, and chitin/chitosan in nine different Zygomycetes strains [136].

One relevant bioprocess is the annual production of citric acid by *Aspergillus niger* strains, estimated to be around 1.7 million tons and generating 0.34 million tons of fungal waste [3]. The average chitin contents in *A. niger* strains are around 15–22% of the dry mycelium, and the polymer can be easily extracted and converted into chitosan [139]. Dhillon et al. [3] integrated citric acid (CA) production by *A. niger* NRRL567 cultures on apple processing wastes and subsequent extraction of chitosan from the waste stream; under LsF conditions, 132 h-old cultures grown in a 7.5 L STR on apple pomace sludge, provided volumetric CA and chitosan productions of 18.4 and 0.64 g L^{-1}, respectively. Solid-state cultures on apple pomace performed well, leading to CA and chitosan production of 182 and 64 g kg^{-1} dry substrate, respectively, after 120 h from the inoculation [140]; the viscosity (1.02–1.18 mPa s^{-1}) and DD (78%–86%) of chitosan preparations from SSF and LSF cultures resembled those of chitosan from crab shells. A previous study conducted on the *A. niger* spent biomass from a production plant of citric acid showed that the use of an enzymatic extraction method yielded chitosan with higher MW and D-glucosamine content and a similar DD as compared to the conventional alkali-acid reflux method [139].

Liao et al. [98] investigated the coproduction of fumaric acid and chitin by *Rhizopus oryzae* ATCC 20344 using a N-nitrogen-rich liquid fraction of dairy manure and a DM-derived hydrolysate from sequential alkaline peroxide and enzymatic treatment; under optimal conditions, volumetric fumaric acid and chitin productions were 31 and 2.4 g L^{-1}, respectively.

The lipid-accumulation ability of several members of the Mucorales order makes them valuable candidates in second-generation biodiesel production [141]. This feature, combined with the greater proportion with which chitosan and chitin enter the constitution of the cell wall, opens up the possibility of profitably using their residual biomass. Zininga et al. [40] seized this opportunity by associating the production of biodiesel by *M. circinelloides* ZKT with the chitosan recovery from the spent biomass; the lipid and chitosan contents of the biomass grown on a glucose-YE-peptone medium were 21.4% and 11.2%, respectively.

Vinche et al. [142] developed an anaerobic chitosan-ethanol coproduction process by *Rhizopus oryzae* cultures grown on wheat hydrolysate containing variable glucose amounts (15–190 g L^{-1}). The initial sugar concentration markedly affected chitosan and ethanol

production. The yield of the latter ranged from 0.28 to 0.46 (g g^{-1} sugar consumed), and the best value of this parameter was observed at 45 g L^{-1} glucose. The AIM was a major fraction (17–25%) of the *R. oryzae* dry mycelium, and GlcN and GlcNAc constituted 61–65% of this fraction. In particular, the GlcN content reached its maximum (0.46 g g^{-1} AIM) at a glucose concentration of 45 g L^{-1}.

Penicillium chrysogenum is extensively exploited for the industrial production of antibiotics, thus generating large amounts of mycelial wastes mostly disposed of by landfilling or incineration. On average, the production of 1 ton of penicillin generates 8–10 tons of waste biomass, the annual amount of which is estimated to amount to 1.2 million tons [143].

P. chrysogenum mycelial waste supplied by a pharmaceutical company [144] underwent an integrative extraction method aimed at recovering (1-3)-α-D-glucan, ergosterol, and chitosan; since the presence of alkali was required for the chitin deacetylation and for saponification during ergosterol isolation, the extraction scheme integrated these two steps for simplification.

5. Conclusions

There are several companies on the market that commercialize chitosan-based products of fungal origin, such as Kitozyme (http://www.kitozyme.com; accessed on 30 December 2021), MycoDev Group Inc. (https://mycodevgroup.com; accessed on 30 December 2021) and Chibio (https://www.chibiotech.com; accessed on 30 December 2021). This suggests an autonomous production capacity or, alternatively, the exploitation of fungal biomass derived from mycobiotechnological processes. Although the scientific literature currently available witnesses several efforts toward process upscaling to a diverse range of LSF reactors, studies reporting process scales on the order of cubic meters are absent. The solid-state production of chitosan has been regarded as a promising route since it enables high product concentration and is less equipment oriented than LsF. However, the use of SSF is negatively affected by mass-transfer limitation phenomena, heat dissipation problems above all, which become critical in large-scale processes. Although SSF seems to provide better performance than LSF in some cases [104,109], a non-negligible factor is the low bulk density of several solid substrates, which negatively affects the loadable mass inside the reactor. Therefore, comparisons between SSF and LSF, based on mass balances, should be made considering the masses of solid and volumes of liquid media, respectively, that can be used for the same working capacity of the reactor.

Despite these limitations, there are several issues that witness in favor of fungal chitosan. The advantages of fungal approaches to chitosan production are well documented, with relatively uniform physicochemical properties of the product made possible by accurate bioprocess control. As discussed earlier, a variety of studies shows that MW, polydispersity, and DD can be manipulated by deliberate variations in process conditions. Obtaining reproducible values of these parameters is fundamental to guaranteeing the acceptance of chitosan in critical sectors such as the medical and pharmaceutical sectors. The possibility of controlling MW and polydispersity of the polymer allows satisfying specific application requirements. This target is made difficult with the conventional extraction process relying on shellfish wastes due to the variability and non-uniformity of the raw material and the relative complexity of the extraction scheme. Switching from conventional to mushroom-based processes requires economic and environmental factors to be balanced and carefully evaluated. A likely scenario involves an increased environmental unacceptability of the conventional process associated with raised pollution abatement costs. An ever-increasing adoption of integrated bioprocesses that, in addition to the primary product, yields chitosan as a co-product from waste mycelia, might boost and increase the diffusion of this technology.

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Abbreviations

AEAP, aqueous extract of apple pomace; AIM, alkali-insoluble material; CDA, chitin deacetylase; CS, chitin synthase; CSL, corn steep liquor; CVP, chitosan volumetric production; CWW, cassava wastewater; DD, degree of deacetylation; DW, deproteinized whey; F-6-P, fructose-6-phosphate; GA3, gibberellic acid; GlcN, D-glucosamine; GPY, glucose-peptone yeast extract; GYT, glucose-yeast extract-tryptone; GlcNAc, *N*-acetylglucosamine; GlcNAc-6-P, *N*-acetyl-D-glucosamine-6-phosphate; GlcNAc-1-P, *N*-acetyl-D-glucosamine-1-phosphate; LsF, liquid submerged fermentation; Mn, number average molecular weight; MW, molecular weight; PDB, potato dextrose broth; P_i, inorganic phosphorus; r_P, average hourly volumetric productivity; SSF, solid-state fermentation; UDP-GlcNAc, uridine 5′-diphospho-N-acetylglucosamine; YE, yeast extract; STR, stirred tank reactor; X, biomass volumetric production; YPD, yeast extract-peptone-dextrose.

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Defining winery processing conditions for the decontamination of must and wine spoilage microbiota by Pulsed Electric Fields (PEF)

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ABSTRACT

This study investigated the PEF-resistance of *Saccharomyces bayanus*, *Brettanomyces bruxellensis, Lactobacillus plantarum*, and *Oenococus oeni* in must or wine under continuous PEF processing. Results showed the capacity of PEF to achieve 3.0-$\log_{10}$-cycles (CFU/mL) of inactivation of all the microorganisms under moderate conditions (< 155 kJ/kg). Developed tertiary models accurately predicted the effect of PEF parameters on microbial inactivation, and Monte Carlo simulation considered the variability of factors and the maximum assumable microbial load in the final treated product. Results showed that PEF-treatments at 15 kV/cm and 129 or 153 kJ/kg would ensure the adequate decontamination (< 10 CFU/mL) of spoilage microorganism in must or wine, respectively.

Industrial relevance: PEF technology has been shown to achieve adequate levels of microbial inactivation (3-$\log_{10}$) in must and wine under industrial applicable processing parameters, making it a suitable alternative to SO_2 or sterilizing filtration for microbial control in winemaking. Reductions of 3-$\log_{10}$ CFU/mL of must and wine microbiota were found by continuous flow PEF-processing at 15 to 25 kV/cm and 175 to 148 kJ/kg, parameters applicable at industrial scale at 1 ton/h.

1. Introduction

Winemaking is a traditional process with thousands of years of history in which the apparently simple concept of transforming grape must into wine has undergone significant developments. Today, the procedure of obtaining a stable, defect-free wine can be facilitated by using coadjutants, enzymes, additives, or disruptive methodologies with technological benefits (Cosme, Filipe-Ribeiro, & Nunes, 2021; Pérez-Coello & Díaz-Maroto, 2009). Microorganisms play an essential role in the steps of the fermentation process, and the use of commercial starter cultures has improved yields and outcomes. However, the primary control strategy used against microbial spoilage in wineries still consists in applying one of the oldest preservatives: sulfur dioxide (SO_2). This can be explained by the fact that SO_2 not only has antiseptic but also antioxidant activity, which makes it a virtually irreplaceable preservative (Giacosa et al., 2019). In the last decades, however, concern has grown regarding the extensive use of SO_2 in wineries due to its toxic and allergenic nature, which can affect consumer health. It is nevertheless challenging to find a good alternative to replace SO_2 as an antimicrobial, considering its broad spectrum of activity. The main microbial groups of concern in wine spoilage are yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) by virtue of their high level of tolerance to ethanol and low pH. The most common spoilage yeasts are *Saccharomyces*, due to their capacity to re-ferment wines, the highly undesirable *Brettanomyces bruxellensis*, which produces a typical "horse sweat" taint, and other non-saccharomyces genera (*Zygosaccharomyces*, *Kloeckera/Hanseniaspora*, *Pichia*, and *Candida)*, which can generate turbidity, cause film layers, and/or produce undesired metabolites. The growth of LAB species, including *Lactobacillus*, *Pediococcus*, and certain *Oenococcus* species, is associated with undesirable aroma and flavor compounds that generate bitterness; moreover, all AAB species are regarded as spoilage bacteria capable of producing a vinegary aroma (Bartowsky, 2009). Consequently, in order to substitute SO_2 in wineries, any potential alternative method needs to be effective in controlling all the microbial groups enumerated above. Although several new chemicals (dimethyl dicarbonate, lysozyme, and sorbic acid) as well as a series of physical methods (high hydrostatic pressure, ultrasound, ultra-high pressure homogenization) have been proposed, their effectivity and real applicability on an industrial scale has been called into question (Lisanti, Blaiotta, Nioi, & Moio, 2019). Conversely, Pulsed Electric Fields (PEF)

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have the capacity to inactivate vegetative cells in continuous flow, and the procedure is easy to install in a wine production line. Moreover, flexible pulsed power devices that cover industrial processing volumes have recently been developed. For all these reasons, PEF has become a promising technology for microbial control in the wine industry.

In PEF processing, the delivery of microsecond pulses at high voltages generates an electric field strength (kV/cm) in the chamber, which is made up of two electrodes. When microbial cells are subjected to an external electric field of sufficient intensity, their cytoplasmic membrane becomes more permeable, causing a homeostasis imbalance that can lead to cell death. Furthermore, the lethality of PEF, combined with even moderate temperatures (< 60 °C), maintains the nutritional, organoleptic, and fresh-like characteristics of heat-sensitive food products (Timmermans, Nierop Groot, & Matser, 2022). Moreover, the application of PEF at these elevated temperatures has been demonstrated to enhance the electroporation rates and thus rising the lethality of the treatments by a synergetic effect (Saldaña et al., 2010; Timmermans et al., 2019). The application of PEF technology has been especially studied in the area of liquid foods: results are particularly encouraging when it is applied as a cold pasteurization method for acidic products such as fruit juices (Bevilacqua et al., 2018; Katiyo, Yang, & Zhao, 2017; Marsellés-Fontanet, Puig, Olmos, Mínguez-Sanz, & Martín-Belloso, 2009; Min, Jin, Min, Yeom, & Zhang, 2003; Raso & Álvarez, 2016). The use of PEF has also resulted in effective microbial inactivation of the typical spoilage microorganisms in wine under laboratory growth conditions (González-Arenzana et al., 2015; Puértolas, López, Condón, Raso, & Álvarez, 2009). However, most of those studies were conducted with non-scalable PEF parameters including batch conditions and intense electric field strengths (> 30 kV/cm), which would be difficult to apply with current commercial PEF devices at the processing capacity required in the industry (González-Arenzana et al., 2018; van Wyk, Farid, & Silva, 2018; van Wyk, Silva, & Farid, 2019). On the other hand, most studies have not considered the potential effect on microbial inactivation of temperature increase during PEF application in continuous flow configuration. Consequently, there is still a lack of fundamental, systematic research in this area; further investigations are needed in order to obtain a sufficient body of data to define PEF protocols that meet the microbial standards of wineries. Such an approach would require the development of predictive models and the identification of the limiting microorganisms, thereby allowing for the implementation of risk-based management strategies. Predictive microbiology has proven to be an essential tool capable of linking scientific development with practical application in the food industry (Membré & Lambert, 2008). Therefore, to determine whether PEF could serve as an effective preservation technology for microbial control in wineries, microbial resistance characterization needs to be assessed under industrially feasible PEF conditions. In this study, it was evaluated the inactivation of several spoilage microorganisms of concern (*Saccharomyces*, *B. bruxellensis*, *L. plantarum*, and *O. oeni*) in terms of the main PEF processing parameters in conjunction with controlled wine-intrinsic parameters (pH, ethanol content, sulfite concentration) that could be used in future predictive models. Although (Puértolas et al., 2009) already applied a similar approach, our study's significant contribution lies in the procurement of inactivation data under industrially scalable PEF parameters and continuous flow conditions. Furthermore, the developed models were validated on wines provided by a winery at two different steps of the winemaking process.

2. Materials and methods

2.1. Microorganisms and cultivation conditions

Table 1 details the conditions of culture media and treatment media used in this study for each microorganism. The strains used in this study, *Saccharomyces cerevisiae* var. *bayanus* (CECT 1969), *Brettanomyces bruxellensis* (CECT 11045), and *Lactobacillus plantarum* (CECT 220), were supplied by the Spanish Type Culture Collection (CECT); the commercial culture of *Oenococcus oeni* was purchased from Enoferm BETA (Lallemand, St. Simon, France). Strains were revitalized from freeze-dried samples and maintained on slants of the corresponding media (Table 1). A broth subculture was prepared from each slant by inoculating a single colony in a test tube of 10 mL of broth, followed by incubation as indicated. From subcultures, flasks with 50 mL of the corresponding culture media were inoculated to a final concentration of 10^4 for yeast and 10^5 CFU/mL for bacteria. To achieve alcoholic fermentation, flasks containing 3 L of a commercial red grape must (GREIP, PepsiCo, Vitoria, Spain) were inoculated with *S. bayanus*. Before inoculation the must was added with 70 g/L of sucrose (Oxoid) to achieve a final concentration of around 220 g/L, corresponding to a potential ethanol content of 12.9% (*v*/v). Microbial cells reached the stationary growth phase after the incubation time indicated in Table 1. Prior to PEF treatments, microbial cultures were centrifuged at 10,000*g* for 4 min and resuspended in a commercial red grape must (GREIP, PepsiCo, Vitoria, Spain) or in the wine after removing by centrifugation (10.000 $g \times 10$ min) the cells of *S. bayanus* involved in the fermentation. In the case of *S. bayanus*, the wine was directly treated with the suspended yeast cells at the end of the fermentation period. The use of a wine obtained under laboratory conditions ensured a lower degree of variability in terms of wine properties and allowed us to avert the potential influence of sulfites, which are abundantly present in commercial wines. The pH and electrical conductivity of the must were 3.2 and 1.4 mS/cm, and 3.1 and 1.3 mS/cm for the wine.

2.2. PEF treatments

Monopolar square waveform pulses were applied with a commercial PEF generator (Vitave, Prague, Czech Republic) capable of applying a maximum voltage of 20 kV and current up to 500 A. PEF treatments were applied to must and wine in a continuous flow system fixed at 5 L/h by means of a peristaltic pump (BVP, Ismatec, Wertheim, Germany) sent

Table 1
Culture media, temperatures, growth times, and treatment media of the strains used, at each stage of the investigation.

Microorganism	Slant	Preculture	Culture	Treatment medium	Initial microbial concentration $log_{10}N_0$ ±SD (CFU/mL)
S. bayanus (CECT 1969)	PDA	SD broth (25 °C, 24 h)	SD broth (25 °C, 48 h) Must + Sucrose (20 °C, 9 days)	Must	6.37 ± 0.10
				Wine	7.65 ± 0.04
B. bruxellensis (CECT 11045)	Brett. Agar	SD broth (25 °C, 24 h)	SD broth (25 °C, 5 days)	Must	6.26 ± 0.16
				Wine	6.45 ± 0.16
L. plantarum (CECT 220)	MRSA	MRS broth (37 °C, 24 h)	MRS broth (37 °C, 24 h)	Must	7.24 ± 0.14
				Wine	7.24 ± 0.28
O. oeni (Enoferm BETA)	MRSA	MRS broth (30 °C, 24 h)	MRS broth (30 °C, 24 h, anaerobic conditions)	Wine	7.85 ± 0.02

SB broth: Sabouraud Dextrose broth. PDA: Potato Dextrose Agar. MRS broth: Mann Rogosa Sharpe broth. MRS agar: Mann Rogosa Sharpe agar. (Oxoid, Basingtok, UK) Brett. Agar: Brettanomyces Agar (Scharlab, Sentmenat, Barcelona).

through a parallel titanium chamber of 0.4 cm gap (3 cm length, 0.5 cm width), generating a residence time of 0.43 s. Pulse waveform and treatment chamber configuration is shown in Fig. 1. Samples were pre-tempered at 20 °C by a coil heat exchanger before entering the PEF chamber. Upon leaving the chamber, samples were cooled down by a further cooling exchanger that reduced the product temperature below 15 °C in less than 5 s after the PEF treatment. Actual voltage during treatments was measured by a high-voltage probe (Tektronik, P6015A, Wilsonville, Oregon, USA) connected to an oscilloscope (Tektronik, TDS 220). Inlet and outlet temperatures were measured by a type-K thermocouple (Ahlborn, Holzkirchen, Germany) located in the circuit before (inlet temperature) and after (outlet temperature) the treatment chamber. Total specific energy (W) of treatments was estimated by calculating the temperature increase during pulses delivering presumable adiabatic conditions (Heinz, Alvarez, Angersbach, & Knorr, 2001), using the following equation:

$$W = (T_{outlet} \quad T_{inlet})^{*}Cp \tag{1}$$

where T_{outlet} is the temperature of the sample immediately after the PEF treatment, T_{inlet} is the temperature of the sample just before entering the treatment chamber (20 °C), and Cp is the specific heat capacity of must or wine according to the average values from Genc, Genc, and Goksungur (2017). Outlet temperatures were constant during experimental times of PEF processing and the thermal conduction of the electrodes was negligible.

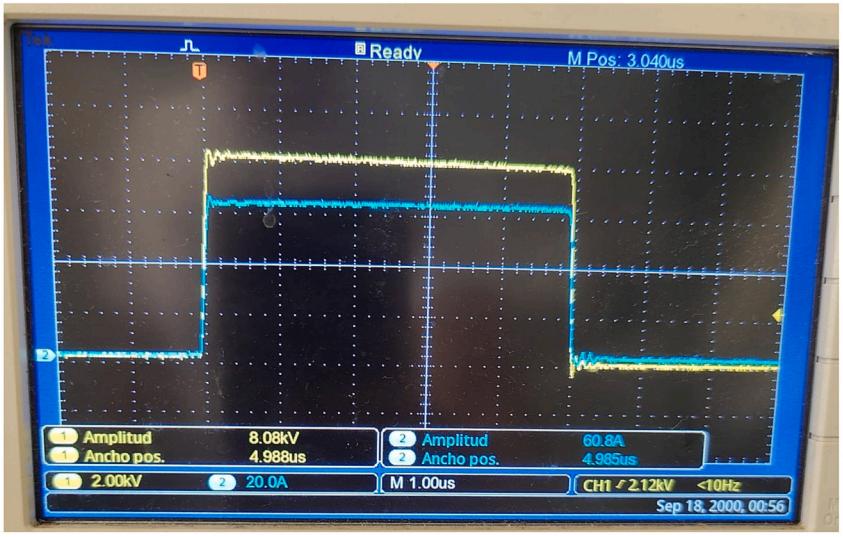
To obtain a substantial amount of microbial inactivation data under industrially scalable conditions, different PEF parameters of electric field strength (10, 15, 20, and 25 kV/cm) and total specific energies (30 to 175 kJ/kg) were tested at a constant pulse width of 5 μs by modifying the pulse rate from 20 to 170 Hz. After treatments, *S. bayanus* and *B. bruxellensis* were recovered, respectively, by plating onto PDA and incubating 48 h at 25 °C, and onto Brettanomyces agar incubating for five days at 25 °C. *O. oeni* and L. *plantarum* were recovered onto MRS agar and incubated for 24 h at 30 °C and 37 °C, respectively, under anaerobic conditions (< 1% O_2) (MACS VA500 Microaerophilic Workstation, DW Scientific, UK). After the incubation times, the number of colonies counted corresponded with the number of viable microorganisms expressed as colony-forming units per milliliter (CFU/mL). The survival fraction was calculated by dividing the number of microorganisms that survived the treatment (N_t) by the initial number of viable cells (N_0) in the treatment media before PEF. Survival curves ($\log_{10}$ of the survival fraction vs. total specific energy) for each electric field strength and investigated medium were obtained.

2.3. Curve fitting

In order to describe the survival curves obtained for the different microorganisms and treatment media, a mathematical equation (primary model) based on Weibull distribution was used (primary model):

$$Log_{10}\frac{N_t}{N_0} = \left(\frac{W}{\delta}\right)^{\rho} \tag{2}$$

where N_t is the number of microorganisms that survived the treatment, N_0 is the initial number of the microbial population, W is the specific energy, δ is the scale parameter, and ρ the shape parameters. The δ parameter represents the specific energy (kJ/kg) required to inactivate



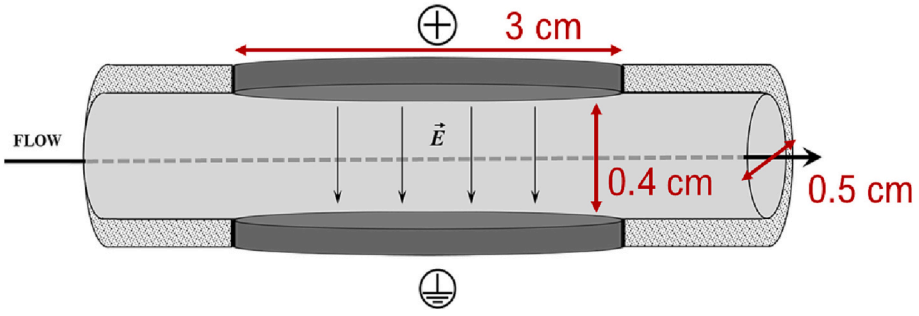


Fig. 1. Picture of the waveform of pulses in the oscilloscope (top) and scheme of the parallel electrode configuration of the treatment chamber and its dimensions (bottom).

the first $\log_{10}$ cycle of the microbial population. The ρ parameter accounts for upward concavity of a survival curve ($\rho < 1$), a linear survival curve ($\rho = 1$), and downward concavity ($\rho > 1$) (van Boekel, 2002).

The determination of the δ and ρ values was performed by fitting survival curves with the freeware model-fitting tool GInaFiT 1.7 (KU Leuven, Belgium) and the Solver function of the Excel 16.0 package (Microsoft, Seattle, WA, USA). The mathematical relationship (secondary model) between parameters δ and ρ with the electric field strength for each studied treatment medium and microorganism was defined by using GraphPad Prism (Graph- Pad Software, San Diego, California, USA) to obtain secondary models. Global equations were developed by substituting the secondary equations of δ and ρ in Eq. (2).

To establish the models' accuracy, R^2 (determination coefficient) and RMSE (root mean square error) were calculated based on Baranyi, Pin, and Ross (1999).

2.4. Experimental validation

For the validation of the models obtained under laboratory conditions, two red wines provided by a winery (San Juan Bautista cooperative, Fuendejalón, Spain) after alcoholic fermentation (AF) and malolactic fermentation (MLF) were used. *S. cerevisiae* var. *bayanus* (CHP, Levuline, OENOFRANCE, Magenta, France) and *O. oeni* (Viniferm OE AG-20, Agrovin, Ciudad Real, Spain) performed each fermentation process, respectively, in the winery facilities. At the end of each fermentation, a matrix of 15 to 20 different PEF treatments was applied to wines in the same ranges previously investigated (see Section 2.2). The pH, % of ethanol content, free SO_2, and electrical conductivity were, respectively, 3.8, 15.7%, 19 ppm, and 1.9 mS/cm for the wine that contained *S. bayanus* and 3.5, 14.3%, 2.0 mS/cm, and 10 ppm in the case of the wine containing *O. oeni* cells. Inactivation rates were assayed by plating on the corresponding media and incubating as indicated in Table 1.

2.5. Monte Carlo simulation

Response surface methodology (RSM) was employed using Design-Expert 13 software to establish a matrix of electric field strengths (ranging from 10 to 25 kV/cm) and specific energies (ranging from 15 to 155 kJ/kg) to develop the subsequent Monte Carlo simulation (MC). Rather than assigning discrete levels to the factors, a continuous range of experimental conditions was specified.

By means of the tertiary models developed for the microorganisms *S. bayanus* and L. *plantarum* in both must and wine, and along with the parameter deviation of each model, Monte Carlo simulation (MC) was conducted to assess the risk of obtaining more than 10 CFU/mL of *S. bayanus* and *L. plantarum* in must or wine after PEF processing, depending on process parameters. The initial microbial concentrations (N_0) taken into account for the MC simulation were based on experimental data obtained from previous studies performed in a winery. The function used to assess the risk was:

$$Risk(\%) = \frac{\#Runs\ with\ N_t < 10}{\#Runs} \times 100 \quad (3)$$

where N_t is the number of microorganisms that survived the treatment. Subsequently, a MC simulation was performed with the Monte Carlo Simulation App from OriginLab in the Origin Pro 2021 software (OriginLab, Northampton, Massachusetts, USA) with 9000 runs per point as previously established with Design-Expert. The percentage of Monte Carlo runs that achieved a remaining amount of microorganisms corresponding to a maximum of 10 CFU/mL were used as responses for the RSM. Finally, response surfaces were compared for both must and wine to establish treatment conditions that would achieve a risk lower than 10%. The response surface was then optimized to pinpoint the conditions that meet a criterion of a maximum 10% risk of the aforementioned microbial survival rate after PEF processing.

3. Results

Fig. 2 shows the inactivation of *S. bayanus*, *B. bruxellensis*, and L. *plantarum* obtained after PEF treatments in must (Fig. 2A) and of the same microorganisms and *O. oeni* in wine (Fig. 2B). Graphs shows the experimental data at different electric field strengths and the curves obtained after fitting data to Weibull distribution. Since the total specific energy was calculated based on the temperature increase due to the PEF treatment (Eq. (1)), the outlet temperature achieved immediately after the PEF treatments is also represented (secondary OX axis). For all the microorganisms and independently of the treatment medium, the inactivation rate increased with the total specific energy for all the electric fields. However, the inactivation kinetics were different depending on the treatment media and the type of microorganism. Yeast strains treated in must presented linear kinetics up to around 75 kJ/kg, after which the inactivation velocity slowed down, generating a concave upward shape. Meanwhile, the inactivation kinetics of L. *plantarum* in must and of all microorganisms treated in wine exhibited a downward concave shape. The shoulders observed in those survival curves were more or less pronounced depending on the microorganism. For example, for L. *plantarum* in wine, the shoulder extended up to 125 kJ/kg, whereas in the case of *B. bruxellensis*, it was around ten times lower. For all studied microorganisms and all treatment media, PEF treatments were able to attain between 2.0 and 3.0 $\log_{10}$ cycles of inactivation with total specific energies up to 136 kJ/kg and maximum temperatures of 55 °C. Nevertheless, PEF treatments of lower intensities (up to 97 kJ/kg; 45 °C) were capable of achieving the inactivation of yeasts and *O. oeni* in the same ranges (2.0 to 3.0 $\log_{10}$).

To determine the influence of PEF parameters and treatment media on the inactivation kinetics of the different microbial strains, survival curves were mathematically described by fitting the obtained survival curves to equations. Since survival curves did not follow a first-order inactivation kinetics, a mathematical model based on the Weibull distribution (Eq. (2)) was used which enabled to fit concave upward and downward curves (Mafart, Couvert, Gaillard, & Leguerinel, 2002). Table 2 shows the values of the model parameters δ (scale) and ρ (shape) determined for each microorganism, treatment medium, and electric field strength. As can be observed, the scale parameter (δ value) decreased when the electric field increased, and it was generally higher for treatments in wine than in must, as well as for both media containing L. *plantarum*. This relationship was not as evident in the case of the shape parameter (ρ value). It was <1 when survival curves presented an upward concave behavior (yeasts in must), but generally, it was >1, meaning downward concave shapes. The accuracy values of R^2 and RMSE ranged from 0.85 to 0.99 and from 0.07 to 0.56, respectively, indicating adequate goodness of fit.

Based on the obtained value of the primary model parameters, as shown in Table 2, the relationship between the calculated δ and ρ parameters and the applied electric field strength was carried out. The equations (secondary models) that describe the relationship between the model parameters and the treatment conditions (electric field strength and specific energy) for each microorganism and treatment medium are shown in Table 3. Fitting was performed according to the equations that better described each correlation among data. The associated standard deviations of each parameter, along with the RMSE and R^2 values, are also shown. Although in thermal treatments the logarithm value of δ has usually been found to be linearly dependent on the treatment temperature (van Boekel, 2002), our results show that this is not always the case in the relationship between the logarithm value of δ and, in this case, the electric field. In some of the data, the δ values followed a good linear relationship with the electric field (as was the case for *S. bayanus* and *B. bruxellensis* in wine) or were even independent of the field strength (as was the case for *O. oeni*). Similar relationships between shape parameter and electric field strength have been described in the

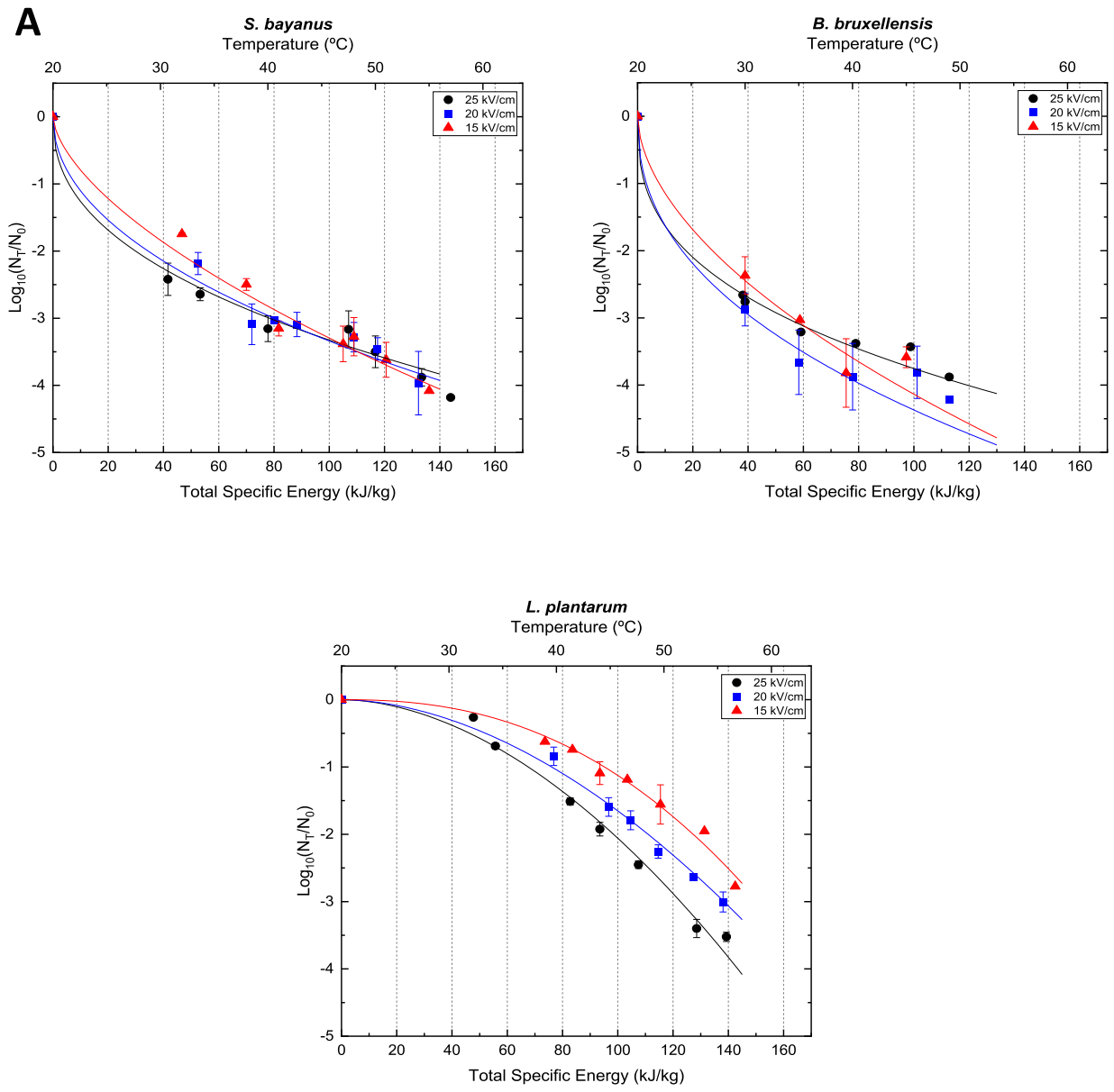


Fig. 2. Survival curves of *S. bayanus*, *B. bruxellensis, L. plantarum* and *O. oeni* at different electric field strengths: 10 kV/cm (◆),15 kV/cm (▲), 20 kV/cm (■), and 25 kV/cm (●) treated in must (A) and wine (B). Dots represent the experimental data and continue lines represents the obtained fitted curves to Weibull distribution.

literature, but mainly when static treatments were applied (Saldaña, Puértolas, Condón, Álvarez, & Raso, 2010).

Based on these equations (secondary models) (Buzrul, 2022) and including them in the Weibull model (primary model), equations (tertiary models) that permitted to estimate the rate of microbial inactivation for each microorganism and treatment medium under all types and combinations of PEF treatment conditions were obtained (tertiary models). Values predicted by the obtained equations versus experimental values corresponding to the inactivation of the different microorganisms in must and wine are shown in Fig. 3. RMSE and R^2 values reflect the good correlations between experimental values and those estimated by the global equations obtained independently of the microorganism or the treatment medium. In general, R^2 values were higher than 0.90, and RMSE lower than 0.16. Based on these results, the obtained equations allow for an accurate description of the lethality of PEF treatments against the investigated microorganisms suspended in must or wine in the range of electric field strengths and specific energies under investigation.

Based on those validated equations (tertiary models), the specific energy and electric field strength required to reach a certain level of inactivation (1.0 or 3.0 $\log_{10}$ cycles) for each microorganism studied in must and wine are shown in Fig. 4. As can be observed, the specific energy or the outlet temperature required to obtain a given inactivation in must or in wine was lower at higher electric field strengths, with the exception of *O. oeni* in wine, for which the achieved inactivation only depended on the total specific energy and was independent of the electric field strength. Generally, however, for all the investigated microorganisms, the decrease in total specific energy required to achieve a given level of inactivation occurring at higher electric field strength was very low. For example: in order to obtain 3.0 $\log_{10}$ cycles of inactivation of *S. bayanus* in wine, an increment in electric field strength from 10 to 25 kV/cm only reduced the total specific energy from 128 to 104 kJ/kg (e.g., a 19% reduction in energy requirement) or from 190 to 150 kJ/kg (21% reduction) for L. *plantarum*. On the other hand, Fig. 4 shows that L.

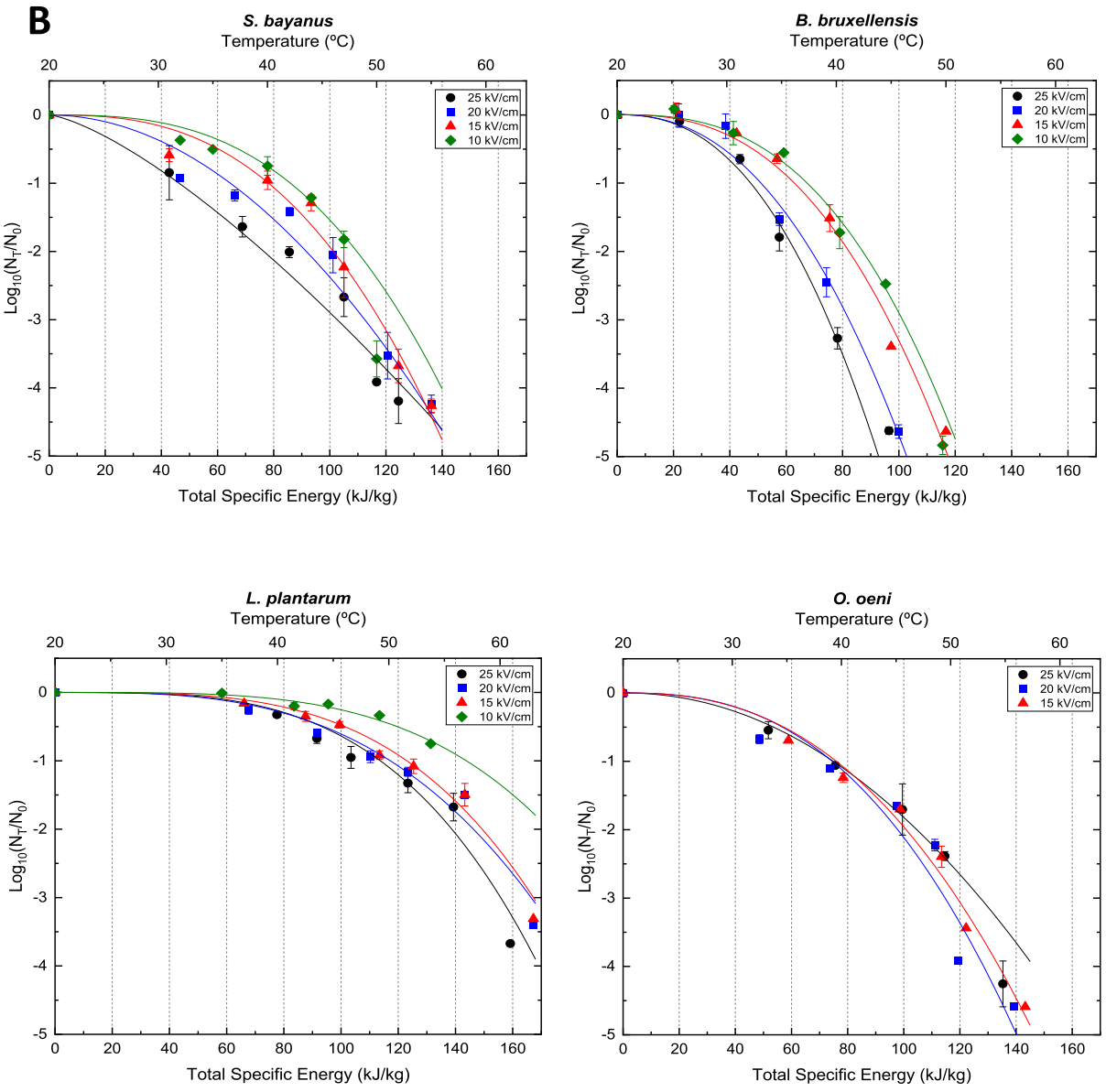


Fig. 2. (*continued*).

plantarum was the most resistant microorganism in both must and wine, followed by *O. oeni*, *S. bayanus*, and *B. bruxellensis*. Furthermore, microbial resistance to PEF was higher in wine than in must in all cases. Thus, for example, treatments of 15 kV/cm, 90 kJ/kg, and a final temperature of 42 °C would be sufficient to inactivate 3.0 $\log_{10}$ cycles of yeasts in the must; in wine, however, 125 kJ/kg (52 °C) at 15 kV/cm or 101 kJ/kg (46 °C) at 25 kV/cm would be necessary to achieve the same lethal outcome. Similarly, to achieve 3.0 $\log_{10}$-reduction of L. *plantarum*, the required total specific energy increased from 125 kJ/kg (52 °C) in must to 148 kJ/kg (58 °C) in wine at 25 kV/cm.

In order to validate the tertiary models with independent data, two red wines provided by a winery immediately after alcoholic and malolactic fermentations were subjected to a series of different PEF treatments. The inactivation results obtained from that independent data were compared with the predicted values estimated by the developed equations. Fig. 5 compares the experimental data with the data predicted by the equation developed for each microorganism. The equation obtained for the most resistant microorganism (*L. plantarum*) (Fig. 5A) underpredicts the observed rate of inactivation (RMSE = 2.76). Similarly, the equation that predicts the inactivation of *O. oeni* in wine also underpredicted the inactivation of *O. oeni* in the wine supplied by the winery immediately after malolactic fermentation (Fig. 5C). On the other hand, a perfect correspondence was observed between estimated inactivation data of *S. cerevisiae* in the wine supplied by the winery just after alcoholic fermentation and data corresponding to the inactivation predicted by the model developed for *S. bayanus* (RMSE = 0.137; R^2 = 0.914) (Fig. 5B). In any case, none of the equations overpredicted the inactivation ultimately obtained in the wines containing the microorganisms supplied by the winery after alcoholic or malolactic fermentation.

To investigate the effects of energy and electric field strength as continuous entry variables (factors) on the risk of microorganism prevalence, we carried out a response surface experimental design (see above, Section 2.5). An energy range of 15 to 155 kJ/kg was considered, while the electric field strength varied between 10 and 25 kV/cm. The experimental design and models used for the Monte Carlo simulation are summarized in Table 4. MC input parameters for the initial microbial concentration (N_0) were selected based on experimental data obtained in must and wine from wineries. Fig. 6 represents the conditions (continuous lines) of electric field strength and specific energy necessary

Table 2
δ and ρ values from the fitting of the mathematical model based on the Weibull distribution to the experimental data at different electric field strengths for each studied microorganism and treatment medium.

Microorganism	Treatment medium	Field strength (kV/cm)	δ value			ρ value			R^2	RMSE
S. bayanus	Must	15	14.53	±	4.15	0.62	±	0.08	0.96	0.29
		20	8.16	±	3.26	0.48	±	0.07	0.95	0.31
		25	5.76	±	2.51	0.42	±	0.06	0.97	0.27
	Wine	10	85.97	±	3.55	2.85	±	0.61	0.97	0.14
		15	78.19	±	5.26	2.68	±	0.35	0.97	0.29
		20	64.57	±	6.40	1.98	±	0.31	0.93	0.38
		25	46.29	±	5.60	1.38	±	0.17	0.95	0.30
B. bruxellensis	Must	15	7.82	±	12.72	0.56	±	0.23	0.85	0.71
		20	3.20	±	2.80	0.43	±	0.10	0.92	0.52
		25	2.57	±	7.29	0.36	±	0.13	0.95	0.37
	Wine	10	67.41	±	3.87	2.70	±	0.36	0.97	0.19
		15	62.87	±	3.33	2.57	±	0.20	0.99	0.21
		20	51.03	±	3.81	2.30	±	0.22	0.98	0.25
		25	47.34	±	3.32	2.39	±	0.28	0.98	0.20
L. plantarum	Must	15	95.18	±	7.20	2.39	±	0.41	0.95	0.22
		20	76.08	±	5.72	1.84	±	0.19	0.97	0.19
		25	67.60	±	8.37	1.84	±	0.19	0.95	0.30
	Wine	10	143.82	±	5.12	3.77	±	1.02	0.93	0.07
		15	123.04	±	3.63	3.58	±	0.44	0.95	0.18
		20	117.26	±	4.14	3.14	±	0.39	0.95	0.19
		25	113.77	±	4.33	3.49	±	0.58	0.94	0.24
O. oeni	Wine	15	75.98	±	4.03	2.45	±	0.27	0.99	0.15
		20	74.53	±	7.40	2.55	±	0.88	0.94	0.34
		25	74.88	±	8.43	2.07	±	0.47	0.95	0.37

Table 3
Equations used to describe the relationship between the δ and ρ values and the electric field strength in must and wine for each investigated microorganism along with their associated deviation and accuracy parameters.

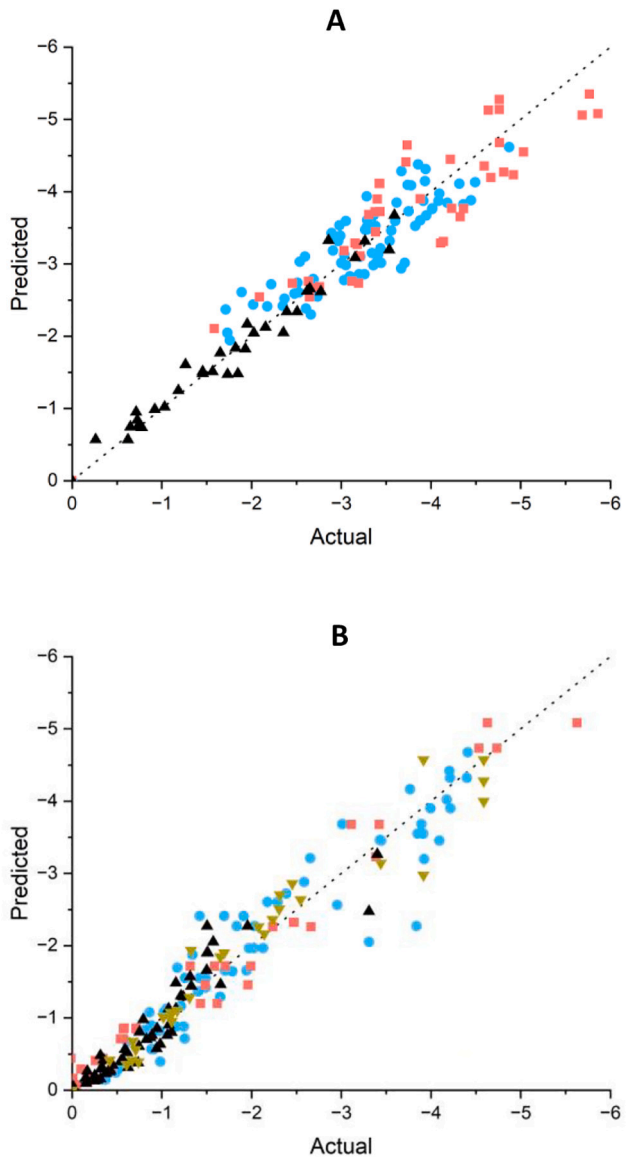
Microorganism		Secondary models	Associated std. deviation of each parameter			
			Slope	Y intercept	R^2	RMSE
S. bayanus	Must	$\log_{10}\delta = 0.04020\,\bar{E} + 1.749$	0.0138	0.2406	0.7687	1.8800
		$\log_{10}\rho = 0.01761\,\bar{E} + 0.051$	0.0048	0.0922	0.8465	0.0257
		$\delta = 2.653\,\bar{E} + 115.2$	0.2866	5.2660	0.9925	1.6550
	Wine	$\rho = 0.1022\bar{E} + 4.011$	0.0196	0.3597	0.8682	0.6259
B. bruxellensis		$\log_{10}\delta = 0.04795\,\bar{E} + 1.568$	0.0338	0.4319	0.9758	0.6901
	Must	$\rho = 0.01954\,\bar{E} + 0.8397$	0.0072	0.1331	0.9863	0.0145
		$\delta = 1.441\,\bar{E} + 82.38$	0.0016	0.0266	0.8087	4.5010
	Wine	$\rho = 2.4903$	0.0033	0.0542	–	0.3180
L. plantarum		$\log_{10}\delta = 0.01539\,\bar{E} + 2.204$	0.0032	0.0613	0.9720	2.3620
	Must	$\log_{10}\rho = 0.01237\,\bar{E} + 0.550$	0.0052	0.1023	0.7732	0.1608
		$\log_{10}\delta = 0.00688\,\bar{E} + 2.214$	0.0011	0.1089	0.9146	4.1650
	Wine	$\rho = 3.495$	–	0.1756	–	0.6084
O. oeni		$\delta = 75.131$	–	1.9980	–	5.9950
	Wine	$\rho = 2.355$	–	0.1876	–	0.5629

to guarantee that in 90% of samples the final microbial counts (Sb for *S. bayanus* and Lp for L. *plantarum*) lie below 10 CFU/mL. The fields marked in yellow represent the combinations of electric fields and specific energy that would ensure that less than 10% of must or wine samples would contain more than 10 CFU/mL of L. *plantarum* (the most resistant microorganism studied). According to Fig. 6, PEF treatments of total specific energies in the range of 113 or 141 kJ/kg at 25 kV/cm and 130 or 153 kJ/kg at 15 kV/cm would be necessary in must or wine, respectively, to achieve the defined requirements, assuming a 10% risk. In general, the required energy in both media could be reduced by increasing the electric field strength, but the impact would be moderate. In wine, for example, the energy could be reduced from 155 to 141 kJ/kg by increasing the electric field from 13 to 25 kV/cm, implying a reduction of less than 1.2 kJ/kg per unit increment of electric field.

According to our MC simulation (Table 4), treatments of 15 kV/cm and 153 kJ/kg in wine or 15 kV/cm and 129 kJ/kg in must would be sufficient to attain that risk (e.g., a < 10% probability of finding >10 CFU/mL in PEF-treated must or wine), compared to the 3-$\log_{10}$-reductions treatments previously defined based on a deterministic approach in wine (25 kV/cm;150 kJ/kg) and must (25 kV/cm;124 kJ/kg or 15 kV/cm;150 kJ/kg). On the other hand, according to the MC simulation, PEF treatments at 15 kV/cm of 68 or 145 kJ/kg of total specific energy would be required in must or wine, respectively, for effective decontamination of *S. bayanus* to be achieved.

4. Discussion

In the present study, we conducted a systematic investigation under continuous process conditions of the PEF resistance of different wine-associated microorganisms, including microorganisms involved in wine fermentation (*S. bayanus* and *O. oeni*) and spoilage microorganisms (*B. bruxellensis* and L. *plantarum*). In the course of the winemaking process, microorganisms involved in alcoholic and malolactic fermentation need to be controlled once fermentation has finished, in order to prevent further re-fermentation that can spoil the wine. In initial experiments, microbial suspensions of *S. bayanus*, *B. bruxellensis*, *L. plantarum*, and *O. oeni* were obtained under laboratory-controlled conditions and treated in commercial must that did not contain any preservative that might exert an influence on lethal outcome, as well as in wine obtained under laboratory conditions by must fermentation



	Must		Wine	
	RMSE	R^2	RMSE	R^2
S. bayanus	0.102	0.947	0.154	0.922
B. bruxellensis	0.155	0.926	0.110	0.977
L. plantarum	0.025	0.975	0.052	0.904
O. oeni	-	-	0.089	0.955

Fig. 3. Correlation between experimental and predicted data obtained with the tertiary models in must (A) or wine (B) for *S. bayanus* (●), *B. bruxellensis* (■), *L. plantarum* (▲) or *O. oeni* (▼).

without added SO_2, which would otherwise also have affected inactivation.

With the aim of obtaining a comprehensive overview of the PEF resistance of microorganisms involved in must and wine spoilage, we investigated the influence of electric field and the specific energy (Fig. 2A and B). Electric field strength and total specific energy (instead of treatment time) have been propounded as key factors for the definition of PEF treatments, as a manner of facilitating comparison among results obtained under varying experimental conditions and different types of PEF generators (Heinz et al., 2001; Raso et al., 2016). This approach is especially convenient when data are obtained under continuous processing conditions, where the total specific energy determines the temperature increment of the samples due to the Joule effect.

The survival curves obtained after PEF treatments showed two different inactivation kinetics. While the survival curves for yeast strains in must (*S. bayanus* and *B. bruxellensis*) had a concave upward shape, all the other survival curves presented a concave downward shape. The differences in inactivation kinetics of *S. bayanus* could be related to the composition of the media in which the yeast was grown. The inactivation of *S. bayanus* in wine was conducted on the same cells that had participated in the must fermentation. The presence of ethanol in growth media has been shown to modify the composition of the cytoplasmic membrane of yeasts by increasing the unsaturated fatty acid content (Huffer, Clark, Ning, Blanch, & Clark, 2011). These changes in the cytoplasmic membrane of *S. bayanus* during must fermentation might affect that microorganism's resistance to PEF and its inactivation kinetics observed in wine. In the case of *B. bruxellensis*, the change in inactivation kinetics in wine as compared with must could be related to changes in the size of cells grown in laboratory media when they are inoculated in wine, as an adaptive response to wine's low pH and to the presence of ethanol. Cell size is a parameter that plays an important role in terms of the amount of external electric field required to achieve electroporation (Kotnik, Pucihar, & Miklavčič, 2010).

Conversely, although the shape of the survival curves of L. *plantarum* did not drastically change when the treatment was applied in must, a noticeable increase could be observed in the δ parameter (first $\log_{10}$ decimal reduction) when PEF was applied in wine (Table 2). This increment in the δ parameter resulted in an increase of the total specific energy required to obtain a given level of inactivation in wine as compared with must. These findings run counter to results obtained by Heinz and Knorr (2000), who reported that the presence of ethanol (5%) increased the lethal effect of PEF on *Bacillus subtilis* when the pH of the treatment medium was reduced; however, at pH 7, the presence of ethanol led to a higher resistance to PEF. The increment in resistance of L. *plantarum* when transferred to a medium with low pH and a high concentration of ethanol – such as wine – could be caused by a rapid adaptive response of the cells to the new environment, as in the case of yeasts. Furthermore, acid-ethanol shock (pH 3.5 / 10% *v*/v) has been shown to produce a significant rigidification in the cytoplasmic membrane of *O. oeni* (lactic acid bacteria) within minutes (Chu-Ky, Tourdot-Marechal, Marechal, & Guzzo, 2005). A similar phenomenon presumably also occurred the in L. *plantarum* cells in our study. However, when attempting to develop a proper characterization of resistance, it is not possible to obtain high concentrations of L. *plantarum* cells adapted to wine conditions, as opposed to nutrient-fed cells grown in laboratory media. Our results therefore stem from conditions in which these microorganisms are more resilient. Nevertheless, it is highly important to evaluate the resistance of wine-related spoilage microorganisms to PEF if we want to estimate this technology's potential. The initial approach implemented in this study therefore provides a foundation that needs to be validated in real wines.

Most survival curves obtained in this investigation displayed a downward concave shape ($\rho > 1$), which indicates that microorganisms gradually became more susceptible to damage inflicted by PEF with higher specific energies (van Boekel, 2002). Survival curves of similar shape have been reported for the inactivation of several different microorganisms in continuous flow (Mendes-Oliveira, Jin, & Campanella, 2020; San Martín et al., 2007). Such behavior might be the outcome of the temperature increment that occurs in the treatment medium when total specific energy is increased. The lethal effect of the rise in temperature in the treatment chamber can be considered practically negligible in view of the sample's short residence time therein (<1 s) and its

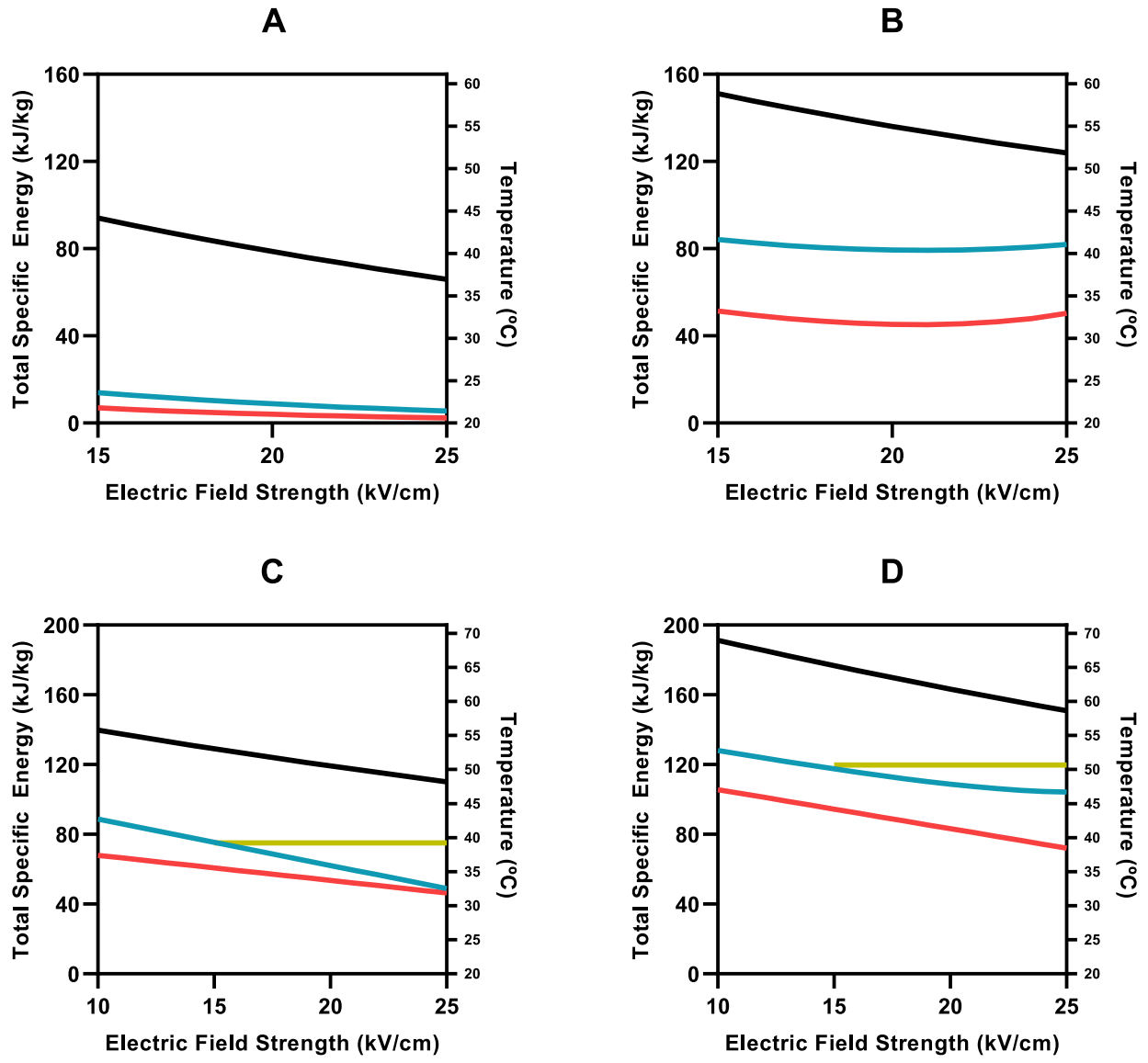


Fig. 4. Treatment conditions of electric field strength and total specific energy to achieved 1.0 $\log_{10}$ (A, C) or 3.0 $\log_{10}$ (B, D) cycles of inactivation in must (A, B) or in wine (C, D) of the different microorganisms. *S. bayanus* (■), *B. bruxellensis*(■), *L. plantarum* (—), *O. oeni* (■).

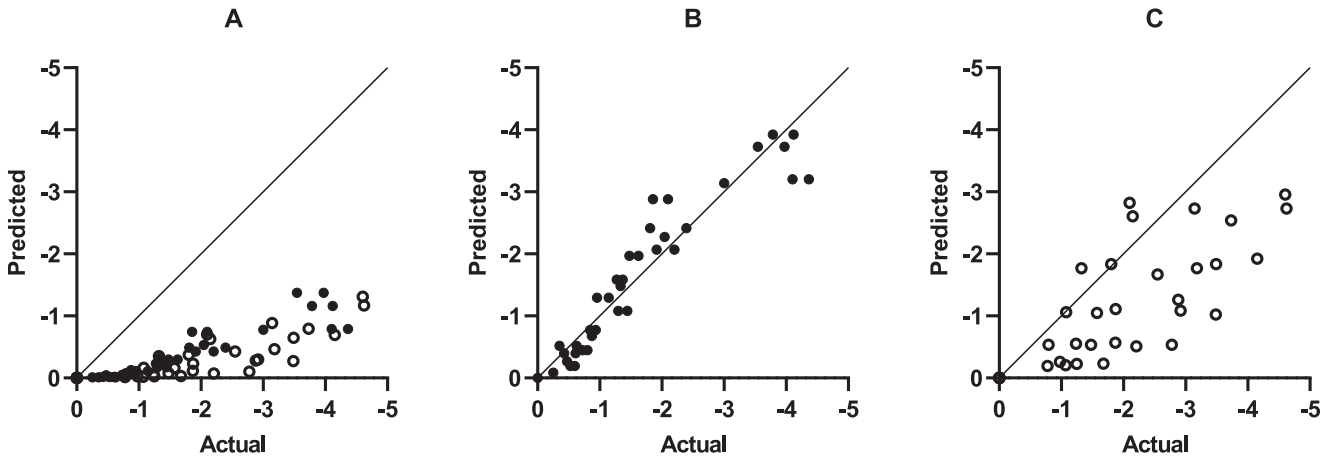


Fig. 5. Validation of tertiary models that describes inactivation in wine of L. *plantarum* (A), *S. bayanus* (B) and *O. oeni* (C) with independent inactivation data obtained after PEF treated wines from a winery after alcoholic (●) or malolactic fermentation (○) governed respectively by *S. cerevisiae* or *O. oeni*.

Table 4
Range, values, and characteristics of the input factors included in the Monte Carlo simulation and the simulation models obtained.

Experimental design				
Study type:	Randomized response surface – optimal – 37 experimental points			
Factor	Unit	Type	Minimum	Maximum
Energy	kJ/kg	Numeric/Continuous	15	155
Electric Field	kV/cm	Numeric/Continuous	10	25

Microorganism		Simulation models	$log_{10}N_0 \pm$SD (CFU/mL)
S. bayanus	Must	$N_t = 10^{log_{10}N_0}\left(\frac{w}{10^{\delta_s\bar{E}+\delta_y}}\right)^{10^{\rho_s\bar{E}+\rho_y}}$	3.4 ± 0.35
	Wine	$N_t = 10^{log_{10}N_0}\left(\frac{w}{\delta_s\bar{E}+\delta_y}\right)^{\rho_s\bar{E}+\rho_y}$	3.0 ± 0.50
L. plantarum	Must	$N_t = 10^{log_{10}N_0}\left(\frac{w}{10^{\delta_s\bar{E}+\delta_y}}\right)^{10^{\rho_s\bar{E}+\rho_y}}$	2.1 ± 0.20
	Wine	$N_t = 10^{log_{10}N_0}\left(\frac{w}{10^{\delta_s\bar{E}+\delta_y}}\right)^{\rho_y}$	2.0 ± 0.20

rapid cooling in the heat exchanger located after the treatment chamber. Consequently, the observed rapid increment in lethality of the PEF treatments by increasing total specific energy could be attributed to the high efficacy of PEF in the electroporation of cells at moderate temperatures. A significant rise in lethal outcome by applying PEF treatments at mild temperatures (30–50 °C) has been reported in different microorganisms (Saldaña, Monfort, Condón, Raso, & Álvarez, 2012; Timmermans et al., 2019). Changes in the phospholipids of the cytoplasmic membrane from gel to liquid-crystalline phase at higher temperature would make the membrane more vulnerable to electroporation (Liu, Zeng, Ngadi, & Han, 2017; Stanley & Parkin, 1991; Wang, Ou, Zeng, & Guo, 2019). Differences among microorganisms in terms of cytoplasmic membrane composition and intrinsic PEF-sensitivity would explain the observed differences among inactivation kinetics. Typically, fundamental studies on microbial inactivation by PEF conducted in batch tend to yield concave upward survival curves (Delso et al., 2022). Such kinetics would imply a limitation for the commercial exploitation of PEF as a food preservation technology, since treatments of a very high total specific energy would be required to obtain substantial microbial inactivation (Monfort, Gayán, Raso, Condón, & Álvarez, 2010; Puértolas et al., 2009; Qin et al., 2015; Walter, Knight, Ng, & Buckow, 2016). From a practical point of view, the use of PEF for wine decontamination in a winery requires continuous processing, in which an increment in temperature would contribute to obtain the required inactivation while applying moderate PEF treatment intensities.

As depicted in Fig. 3, the equations (tertiary models) based on the Weibull model developed demonstrated an overall high goodness of fit for the prediction of experimental data. These equations permitted to compare the investigated microorganisms' resistance to PEF under different levels of inactivation (Fig. 4). In must as well as in wine, the most sensitive strain was *B. bruxellensis*, while the most resistant one was L. *plantarum*. Similar findings were reported by Puértolas et al. (2009), who found that *Lactobacillus* strains were the most resistant ones in must and wine. It is widely reported that yeasts are more sensitive to PEF than bacteria due to their larger size, which makes them more susceptible to electroporation (Delso et al., 2022; Hülsheger, Potel, & Niemann, 1981; Sale & Hamilton, 1967; Wouters, Alvarez, & Raso, 2001). Further intrinsic microbial characteristics such as structure and composition of the cellular envelopes might exert an influence on microbial resistance to PEF, as previously indicated (Liu et al., 2017; Wang et al., 2019). In addition, as can be observed in Fig. 4, the influence of the electric field at equivalent specific energies was not very marked, while no impact on the inactivation of *O. oeni* was observed. In the best-case scenario, each unit increment in the electric field only allowed for a reduction of 2.5 kJ/kg of the total specific energy required to reach the target inactivation level. In contrast, when a similar evaluation was performed in static configuration (no temperature increase), up to 30 kJ/kg were reduced per unit of electric field increment (Puértolas et al., 2009). Furthermore, in that study, specific energies from 300 to 450 kJ/kg were reported to have reached similar lethal goals in must and wine at electric fields of 19–25 kV/cm, hence 2.5-fold the energies reported in our study. These observations reinforce the assumption that in continuous PEF processing, total specific energy is a crucial factor in describing PEF protocols

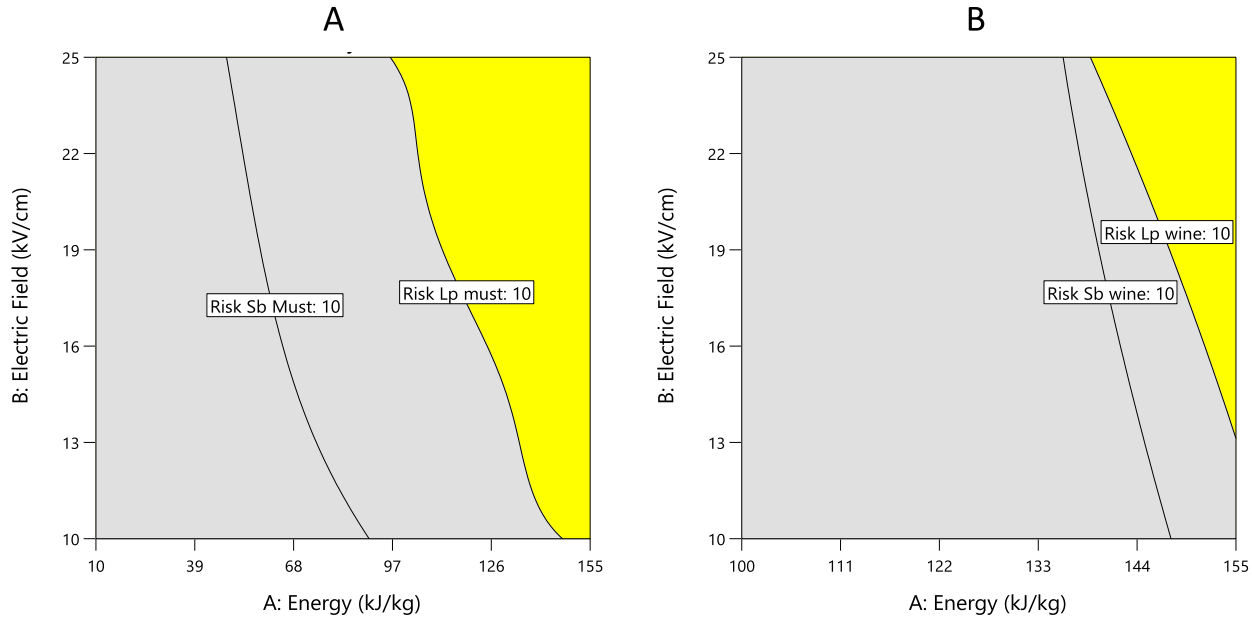


Fig. 6. Electric field strength and total specific energy treatment conditions to fulfill the requirement of MC simulation, ensuring a concentration lower than 10 CFU/mL assuming 10% of risk for *S. bayanus* (Sb) and L. *plantarum* (Lb) in must (A) and wine (B).

and seems to exert a greater impact than the electric field strength. The fact that even mild temperatures can provoke changes in the lipid conformation of the cytoplasmic membrane (thereby reducing the electroporation threshold and hence facilitating electroporation) would explain why electric field strength had less impact on lethality under continuous conditions compared to static ones. Consequently, continuous processing configuration would not only allow for a significant reduction of the total specific energy, but also of the electric field strength. The possibility of reducing electric field strength while maintaining high inactivation rates and moderate specific energies is of considerable importance for the application of PEF on an industrial scale. The lower the electric field required, the larger the PEF treatment chamber volume (distance between electrodes) and hence the lower risk of arching, the lower power requirements of PEF devices, and the greater the flow rates, thereby managing to meet the productive requirements and capacities of wineries.

Predictive microbiology is essential when evaluating the implementation of new decontamination technologies in the food industry. By assembling inactivation data and developing mathematical equations capable of describing a series of different scenarios, more far-reaching and efficient operational decisions can be made. Although the developed mathematical equations (tertiary models) had a good predictive capacity (Fig. 3), the conditions under which data are obtained to develop models do not necessarily represent real food conditions. We were nevertheless able to use the independent data obtained by inactivating microorganisms that had grown in wines from a winery in order to validate those tertiary models. *L. plantarum* proved to be the most PEF-resistant microorganism under the experimental conditions we investigated; it is thus the target microorganism when defining PEF processing conditions for microbial decontamination of must and wine. In view of the target microorganism's observed resistance to PEF, our model predictions underpredicted observations obtained independently of the group of microorganisms (yeasts or lactic acid bacteria) (Fig. 5A). Consequently, this validation demonstrated that by implementing the most preventive scenario, the required selected treatment conditions more than guarantee effective microbial decontamination of wine.

On the other hand, when lethal treatments are applied in the food industry, there is a huge variability in terms of efficacy and in terms of microbial load; additionally, at each stage of processing, there is a probability that the microbial load count can increase or decrease. Therefore, instead of defining PEF treatment conditions for a concrete inactivation value as indicated in Fig. 4, it may be of more interest to provide a probability or risk value of the presence of a certain level of microorganisms in the final product. Consequently, the microbial concentration of those microorganisms, combined with the risk boundaries, can be estimated by means of a probabilistic approach better suited to define PEF protocols, depending on the winemaking step and/or scenario. To achieve this, MC simulation was run in order to stablish with the purpose of establishing the PEF conditions required to accomplish a maximum assumable concentration load defined for *S. bayanus* and L. *plantarum* in must or wine, assuming a normal distribution of the initial microbial load (Table 4). Despite highly restrictive requirements (the most resistant microorganisms, a high initial microbial load, $<$10 CFU/mL of final concentration, and $\leq$ 10% of assumable risk), several combinations of moderate electric field strengths and energies could be selected that would ensure 90% probability of zero spoilage. In any case, the intensity of PEF treatments can vary, depending on the amount of risk that a winery is willing to assume. In comparison with the deterministic approach (Fig. 4), this probabilistic approach allowed to further optimize the PEF parameters by reducing the intensity requirements of PEF parameters in both must and wine. Consequently, total specific energy can be reduced by 10% and by 20% in must and wine, respectively, thereby allowing for the implementation of PEF treatments below 60 °C of maximum temperature, while maintaining low electric field strength (15 kV/cm) and fulfilling the restrictive MC input conditions. Furthermore, the initial count of L. *plantarum* used in the MC simulation was based on the experimental data obtained from the number of total lactic acid bacteria found in real wines from a winery: in other words, we deliberately overestimated the input value of the initial load of L. *plantarum*. As this limiting microorganism was considerably more resistant than the remaining ones under study, the defined protocols obtained by MC represented a "worst case scenario". Despite this, those protocols would be more than reasonably applicable on an industrial scale. Moreover, as mentioned above, we investigated L. *plantarum* cells under stress conditions that might trigger cross-resistance responses to PEF. This thus also suggests that even PEF processing conditions of lower intensity would be able to ensure the decontamination of L. *plantarum*, and thus of all the microorganisms under study. Nevertheless, a complete evaluation of the concentration probabilities of L. *plantarum* in all steps of the winemaking process would allow researchers to improve the risk assessment of PEF processing on an industrial scale.

5. Conclusions

As opposed to most previous modeling studies on PEF inactivation, which were performed in static processing, our research was carried out in continuous flow, thus allowing us to determine this technology's lethal effect in a more practical/realistic setting. Moderate PEF parameters of electric field strength (15 kV/cm) and total specific energy (129 or 153 kJ/kg) were shown to achieve adequate lethality levels for the decontamination of spoilage-related microorganisms in must or wine. These results underscore the promising potential of PEF technology in this area, particularly in view of these parameters' applicability using the current industrial-scale commercial PEF generator, while ensuring the fulfillment of winery production rates. Furthermore, the incorporation of probabilistic risk evaluation into PEF protocols is better adjusted to the standards and requirements of wineries, and will allow for more flexible processing strategies adapted to specific scenarios.

CRediT authorship contribution statement

Carlota Delso: Methodology, Investigation, Writing – original draft, Visualization. **Sebastián Ospina:** Data curation, Formal analysis, Software. **Alejandro Berzosa:** Validation, Visualization. **Javier Raso:** Supervision, Project administration, Conceptualization. **Ignacio Álvarez-Lanzarote:** Funding acquisition, Writing – review & editing, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Article

Copper Content and Export in European Vineyard Soils Influenced by Climate and Soil Properties

Boris Droz,* Sylvain Payraudeau, José Antonio Rodríguez Martín, Gergely Tóth, Panos Panagos, Luca Montanarella, Pasquale Borrelli, and Gwenaël Imfeld*

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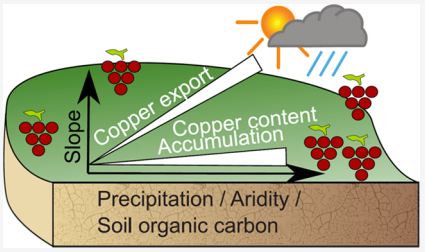
ABSTRACT: Copper-based fungicides (Cu_f) are used in European (EU) vineyards to prevent fungal diseases. Soil physicochemical properties locally govern the variation of the total copper content (Cu_t) in EU vineyards. However, variables controlling Cu_t distribution at a larger scale are poorly known. Here, machine learning techniques were used to identify governing variables and to predict the Cu_t distribution in EU vineyards. Precipitation, aridity and soil organic carbon are key variables explaining together 45% of Cu_t distribution across EU vineyards. This underlines the effect of both climate and soil properties on Cu_t distribution. The average net export of Cu at the EU scale is 0.29 kg Cu ha^{-1}, which is 2 orders of magnitude less than the net accumulation of Cu (24.8 kg Cu ha^{-1}). Four scenarios of Cu_f application were compared. The current EU regulation with a maximum of 4 kg Cu ha^{-1} $year^{-1}$ may increase by 2% of the EU vineyard area, exceeding the predicted no-effect concentration (PNEC) in soil in the next 100 years. Overall, our results highlight the vineyard areas requiring specific remediation measures and strategies of Cu_f use to manage a trade-off between pest control and soil and water contamination.



KEYWORDS: *copper-based fungicides, total copper content, predicted no-effect concentration*

INTRODUCTION

Copper-based fungicides (Cu_f) are intensively used in European vineyards since the end of the 19th century to prevent fungal diseases such as "downy mildew."[1] Cu_f application doses and timing vary across regions depending on practices, regulation, hydroclimatic conditions, and vine variety. The European Union (EU 2018/1981) regulation recently decreased the maximal dose allowed from 6 to 4 kg Cu ha^{-1} $year^{-1}$ over seven years.[2] However, doses as high as 50 kg Cu ha^{-1} $year^{-1}$ were applied frequently for decades in the mid-20th century.[3] Today, the total copper content in topsoil (Cu_t) exceeds 100 mg Cu kg^{-1} in 15% of the European vineyard area. This corresponds to the average proposed threshold values for which soil remediation is needed.[4−6] European vineyards represent more than 50% of the total vineyard surface worldwide.[7] Although vine growing accounts for only 3.3% of the agricultural area in Europe, it uses 86% of total fungicides consumed in Europe.[8] Hence, vineyard soil is often more contaminated by Cu_f than soils of any other agricultural soils. Cu_f accumulates in soil and can impact soil organisms and plants,[9] thereby reducing soil fertility[10] and productivity.[11] In contrast, leaching[12] and surface runoff[13,14] export Cu_f from vineyard soil, which can contaminate aquatic ecosystems[15] and drinking water resources.[16]

Cu_f mobility in topsoil controls Cu_t accumulation and off-site export. Cu mobility in soil is mainly controlled by (i) sorption on organic matter,[17] and to a lesser extent on clay and Fe-, Mn-(hydr)oxide, and carbonate;[18] (ii) pH-redox dependency since alkaline soil increases the proportion of available binding sites on organic matter.[17,19] Cu sorbed on fine particle matter may result in Cu export in runoff.[13,14] Hydroclimatic and field conditions indirectly control Cu export by (i) affecting soil properties,[20] (ii) mediating dissolved and particulate Cu_f transport, and (iii) modifying preferential transport.[21] Accordingly, presumed or established processes governing Cu_t mobility in vineyard soil may co-occur and/or may be interrelated (Table 1). Variables governing Cu_t and dynamics in vineyard soil are, however, examined in the laboratory and field studies separately. As a result, the understanding of variables governing Cu_t in soil remains study- or site-specific. The identification of variables governing Cu_t in vineyard soil and leading to Cu accumulation and/or off-site export over time is necessary to evaluate the current and future distribution of Cu_t at the European scale.

In this context, a major issue is to predict the spatial variation of Cu_t in the European vineyards to quantify Cu accumulation and export and to identify priority areas requiring soil remediation. The purpose of this study was (i) to identify key variables potentially governing Cu_t in vineyard

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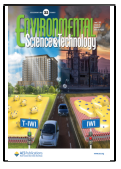

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Table 1. Hypothetic Predictive Variables Governing the Total Copper Content in Vineyard Topsoil (Cu_t), Leading to Cu_t Accumulation and/or Off-Site Export

variables[a]	processes
soil properties	
soil physicochemical properties affect Cu_t mobility	Cu_t correlates positively to soil organic matter since Cu preferentially sorbs to organic matter in soil,[13,17] which affects Cu_t mobility. Higher pH increases sorption sites (e.g., negative charge) for Cu_t on clay, organic matter, and sand-containing carbonate,[19] and reduces Cu_t leaching.[12]
soil moisture influences soil biogeochemistry	Soil moisture directly affects microbial respiration and activities in soil, resulting in biogeochemical gradients.[27,28] Cu occurs as Cu(II) in oxic soils. In contrast, Cu(II) may potentially precipitate to less mobile Cu(I) forms in anoxic soils.[17,29]
topography/climate	
topography and climate conditions drive soil erosion	Greater rainfall depth and a higher slope length enhance soil erosion by runoff.[21] Cu_t is mostly bound to suspended particles matter (SPM) in topsoil (~85%[13,14]) where SPM-bound Cu can account for 84.4% of the total Cu_t exported by runoff.[14]
hydroclimatic conditions influence Cu application doses	Intense rainfalls during warm conditions favor the development of downy mildew. This increases the frequency and amount of Cu_t use in some areas to control it.[30,31]
hydroclimatic conditions influence	Arid environments (with higher AI values), characterized by higher soil pH and oxidizing soils,[20] favor Cu_t accumulation (see soil physicochemical properties above). In contrast, arid soils tend to lose more soil organic matter and associated Cu_t since Cu is preferentially associated with soil organic matter.[13,17]
(i) soil chemistry	
(ii) vegetation growth	Optimal conditions for vegetation growth depend on temperature, soil moisture, and solar radiation, and vary across plant growth stages.[32] Vegetation growth controls Cu_t mobility indirectly by limiting erosion via surface runoff and thus can be related to Cu_t.
(iii) plant uptake	Cu uptake from soil by plants remains limited: 0.1−7.2% by wild[33] and 0.1−3.8% by the vineyard[34] plants of Cu_t. Hence, plants only marginally decrease Cu_t in soil.
plant cover	
plant cover affects surface runoff and organic matter content in topsoil	Plant cover, i.e., vine and grass in vineyards, can stabilize the vineyard soil and thus limit erosion via surface runoff.[35] In addition, plant decomposition can slightly increase organic matter content in topsoil,[36] thereby reducing Cu mobility.

[a]Hypothetic predictive variables governing Cu_t in vineyard soil. Variables associated with each process are provided in the Supporting Information, Table S2. Processes were identified based on experimental studies in vineyard or related soil.

topsoil (0−30 cm), (ii) to predict average Cu_t at the European vineyard scale using machine learning techniques, and (iii) to estimate Cu_t accumulation, export, and associated toxicity for terrestrial trophic levels, for historical, current, and future scenarios of Cu_f application (i.e, 0, 2, 4, or 8 kg Cu ha^{-1} $year^{-1}$).

MATERIALS AND METHODS

Topsoil Total Copper Data Sets. Cu_t data sets ($Cu_{t,dat}$; i.e., soils within 0−30 cm) were obtained from several soil surveys across European vineyards (−9.4° to 42° E; 35−53° N; Figure S1) delineated by the Corine Land Cover (CLC 2012; v18.5.1; http://land.copernicus.eu). In total, 1202 $Cu_{t,dat}$ measurements (average: 27.8 mg Cu kg^{-1}; min−max: 0.54−774 mg Cu kg^{-1}) were sampled between 1992 and 2016 (median = 2008, standard deviation (SD) = 5) and used in our study. The summary of the studied $Cu_{t,dat}$ is provided in Table S1. Although the Cu quantification methods differ among data sets, intercomparability between data sets has been demonstrated previously for the methods used in our study.[22] The impact of topsoil sampling depth (first 20 or 30 cm) and sampling time span on the average prediction of Cu_t (Cu_{pred}) at the European scale remains low (Supporting Information, "Influence of Heterogeneous Sampling Depths and Time on the Cu Prediction" section). The heterogeneity of topsoil sampling depth and time span did not significantly (within 2σ) impact the average predictions of Cu_t at the European scale.

Predictive Variables. Soil properties, topology, climate, and land cover were selected and evaluated as hypothetic predictive variables of Cu_t (Tables 1 and S2). Geogenic, i.e., from bedrocks,[23] and atmospheric nonagricultural anthropogenic inputs of Cu, i.e., mining, smelter, sewage sludge, and vehicle brake pads are sources of Cu contributing from 5 to 100 g Cu ha^{-1} $year^{-1}$, whereas about 8 kg Cu ha^{-1} $year^{-1}$ of Cu_f was typically applied on vineyards.[24] Topsoils surrounding Cu mining or smelter, which may reach up to 6 g Cu kg^{-1},[25,26] were not identified close to vineyard areas. Consequently, geogenic and industrial sources of Cu were not considered in this study.

Predictive variables were computed or directly obtained on a grid cell of 250 × 250 m^2 for the study area. Details concerning building spatial variables are provided in the Supporting Information, Tables S2 and S3. $Cu_{t,dat}$ coordinates were used to extract values of each predictive variable in the corresponding grid cells. Extracted data were then used to select variables and calibrate the model (details are given in the Supporting Information). Extracted data represented adequately the range of predictive variables within the prediction domain (details are given in the Supporting Information; the paired *t*-test; $p\text{-value}_{average} > 0.01$) and did not display any spatial correlation (Moran's test; $I_{average} = -0.211 \pm 0.09$; *p*-value > 0.01). The selection of variables was carried out from the 24 original predictive variables (Table S2) to limit collinearity between predictive variables and optimize model predictions as described elsewhere:[37] (i) Pearson correlation was used to discard variables providing similar information and (ii) principal component analysis (PCA) was used to discard noise variables containing the least information.[38]

Prediction of the Total Copper Content in European Vineyard Topsoil. The variable selection procedure highlights that the following variables were predictive of the average Cu_t in European vineyards: clay and silt fractions (%), soil organic carbon content (C_{org}; %), soil pH (unitless), aridity index (AI; unitless), annual average temperature (T_{av}; °C), annual rainfall (rainfall; mm), soil moisture content (moisture; %), the enhanced vegetation index (EVI; unitless), and slope (degree; Table S3). This selection is consistent with predictive variables reported previously (Table 1). Three independent machine learning techniques, i.e., neural networks (R package nnet v 7.3-12), random forest (R package randomForest v.4.6-12), and bagging tree (R package ipred v.0.9-5), were used in parallel and were averaged into an ensemble model (ENS;

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details are given in the Supporting Information) for the final prediction, as described elsewhere.[39] The ENS resulted in fewer false predictions and higher predictive accuracy than a single model. Consistency of Cu_{pred} was evaluated for each grid cell on an SD of 1000 predictions for each machine learning technique. Model performance was evaluated using a 10-fold cross-validation (CV). Model spatial transferability was evaluated using a spatially constrained 10-fold CV (details are given in the Supporting Information). For each model, the averaged relative importance (ARI) of each variable in the model was calculated with an input permutation technique and one-factor-at-a-time (OFAT) sensitivity analysis (details are given in the Supporting Information). The complete modeling framework to select variables, calibrate the model, and predict Cu_t is available for download at https://github.com/Boris-Droz/ML_soil_predi_conc.

Copper Accumulation and Export. Cu_t net mass accumulation since the beginning of the Cu_f application was estimated assuming that Cu_t is distributed similarly across a homogenous topsoil layer, following eq 1

$$\text{net acc.} = (Cu_{Pred} - Cu_{bgd}) \times BD \times h_{topsoil} \quad (1)$$

where net acc. is for the Cu_t net mass accumulation (kg Cu ha^{-1}), Cu_{bgd} is the background of geogenic and atmospheric nonagricultural anthropogenic Cu_t (mg Cu kg^{-1}; Figure S2), and BD is the soil bulk density (https://soilgrids.org/, series M_sl1, v18.4.2017; in kg m^{-3})[40] and the average topsoil thickness where Cu mainly accumulates ($h_{topsoil}$ = 30 cm; Table S1). The net mass export of Cu_t (net exp.; kg of Cu ha^{-1}) was assumed to be the sum of Cu_t exported from topsoil via runoff and leaching and was calculated yearly following eq 2 [13,14]

$$\text{net exp}. = (q_{RUSLE} + f_{sol} \times BD \times h_{topsoil}) \times Cu_{Pred} \quad (2)$$

where q_{RUSLE} is the soil loss rate (kg ha^{-1}) as defined elsewhere[21] and f_{sol} is the soluble fraction of Cu_{Pred} estimated from soil properties (both details are given in the Supporting Information).

Scenarios of Copper-Based Fungicide Application Doses. Four scenarios of Cu_f application doses were compared: (i) a historical scenario with 8 kg Cu ha^{-1} $year^{-1}$ inferred from the sold Cu_f amount before the 2002 European regulation,[2] and/or speculated illegal usage in some areas,[41] current regulation scenarios, with either (ii) 2 or (iii) 4 kg Cu ha^{-1} $year^{-1}$ as average and maximum doses, respectively,[2,42] and (iv) a scenario of Cu_f substitution, i.e., no Cu_f application or 0 kg Cu ha^{-1} $year^{-1}$.

For the scenario with 8 kg Cu ha^{-1} $year^{-1}$ and current regulation scenarios, a stepwise mass balance calculation based on a year t was established (eq 3 and details are given in the Supporting Information), assuming no temporal variation of the topsoil volume and a homogeneous topsoil layer (0−30 cm)

$$Cu_{pred,t+1} = Cu_{pred,t} + (APP_{Cu,f} - Cu_{pred,t} \times \text{net exp.} - Cu_{input} \times f_{sol})/(BD \times h_{topsoil}) \quad (3)$$

where Cu_{pred} is the average predictions of Cu_t (mg Cu kg^{-1}), $APP_{Cu,f}$ is the yearly application dose of Cu_f (kg Cu ha^{-1} $year^{-1}$), and f_{sol} is the soluble fraction, as calculated in eqs S3−S6. The net exp. was previously calculated in eq 2. $C_{upred,t+1}$ accounted for the yearly Cu application dose to which the soluble fraction of Cu_f (f_{sol}) and the net exp. were deducted. For each pixel, the scenario was applied until Cu_{pred} at year t + 1 reaches the predicted no-effect concentration for the given pixel (PNEC; Supporting Information, building spatial variables). In contrast, the time required to export the accumulated Cu_f via runoff and associated suspended solids and reach back Cu_{bgd}, i.e., assuming the existence of alternatives to Cu_f, was estimated by dividing eq 1 by eq 2.

The uncertainty associated with each scenario was estimated by numerical propagation of uncertainties across the entire modeling framework. Briefly, each variable was associated with lower and upper uncertainty limits accounting for the analytical or monitoring uncertainty or calculation steps, depending on the variable (Table S4). All combinations of lower and upper uncertainty limits for the selected variables were then included to propagate uncertainties and estimate the lower and upper uncertainty limits associated with each scenario for each pixel independently.

RESULTS AND DISCUSSION

Prediction of the Total Copper Content in Topsoil. Ten variables governing Cu_t in vineyard topsoil were considered (see the Materials and Methods section and the Supporting Information, Figure S4b). Using these climate and soil variables, the ENS accurately predicted Cu_t (slope of 1.13, intercept of 0.01; Figure S4a), and predictions were fairly precise (averaged R^2 = 0.61). Comparison between ENS and CV (averaged R^2-CV = 0.58 ± 0.06) indicates no overfitting of the ENS. For the three machine learning techniques, Cu_{pred} fit $Cu_{t,dat}$ (SD $Cu_{pred}/Cu_{t,dat} \times 100$ = 13 ± 2%), indicating a good overall agreement of predictions between the three techniques. Average uncertainty of the Cu_{pred} falls within ± 11 mg Cu kg^{-1} for 75% of the data (RMSE of 0.63 ± 0.06). The scattering observed in the predictions (Figure S4) mainly concerns data above 130 mg Cu kg^{-1}, corresponding to 6.3% of the data set only. ENS predictions failed to reproduce observed Cu_t in the corresponding vineyard areas, which potentially received larger historical Cu_f applications than other areas under similar climatic and soil conditions. Additional model performances and limits are provided in the Supporting Information.

Climatic Variables Rather than Soil Variables Determine the Total Copper Content in Topsoil. Climatic variables (i.e., rainfall and aridity index) mainly determined Cu_{pred} at the European scale, possibly by controlling physicochemical processes, including Cu leaching and the redox state of soil. Hydroclimatic conditions likely determined regional Cu application practices as downy mildew's pressure increases in more humid and warmer areas in early summer.[30,31] In contrast, soil physicochemical properties (i.e., pH) predominantly governed Cu_t on the local scale and under homogeneous climatic conditions. The averaged relative importance (ARI) from the ENS and the sensitivity analyses confirmed the relationship between climatic variables and Cu_{pred}. Cu_{pred} correlated positively with both the annual rainfall (rain; R^2 = 0.71) and the aridity index (AI; potential evapotranspiration divided by rainfall; R^2 = 0.79), while the combined effect of rainfall and AI accounted for 32.6% of the Cu_{pred} ARI (rainfall = 17.4 ± 0.9%, AI = 15.2 ± 1.3%; Figure S4b).

Although rainfall was negatively correlated with AI, both variables were positively correlated to Cu_{pred}, suggesting that two different mechanisms control Cu_t in vineyard soil. First, rainfall could temporally increase the soil water content and reduce oxygen diffusion in water, leading to reducing soil

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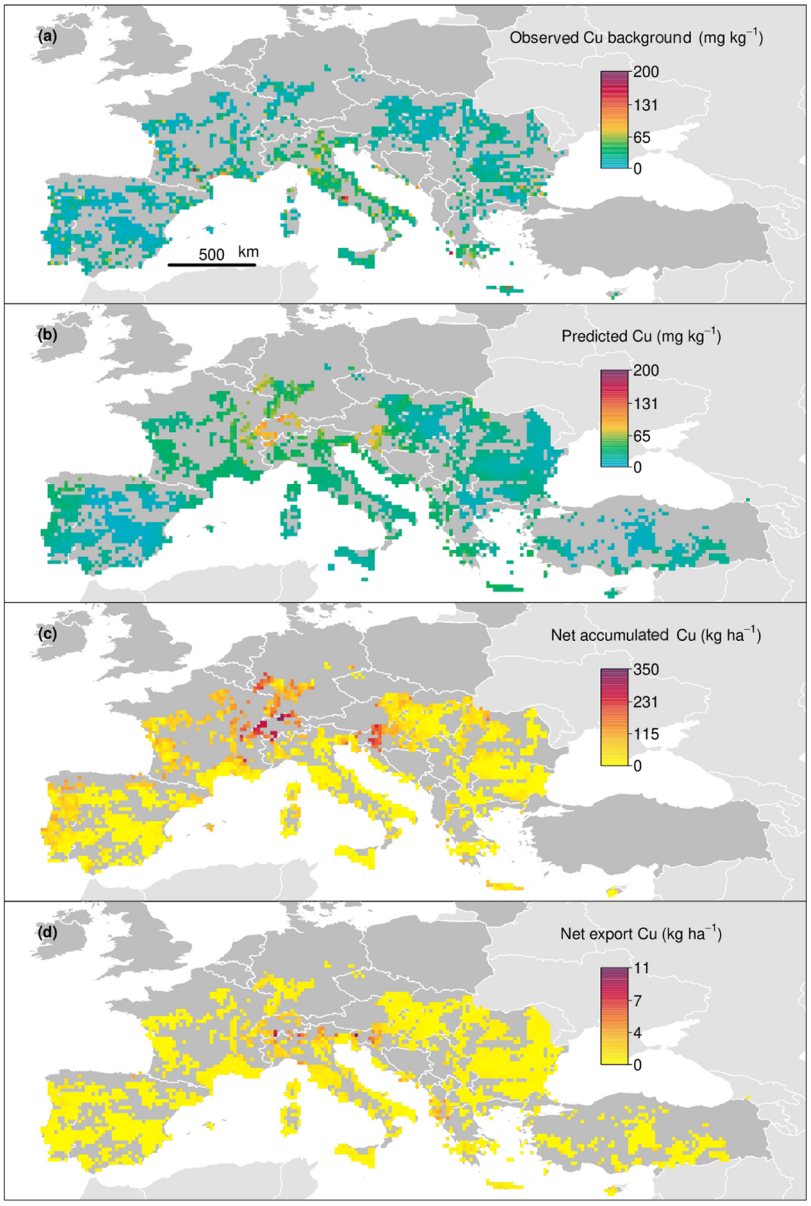

Figure 1. Distribution of the total copper content in vineyard topsoil (Cu_t), accumulation, and net export of Cu in European vineyards. (a) Observed background Cu_t. (b) Predicted Cu_t (Cu_{pred}). (c) Estimated net accumulation of Cu_t. (d) Estimated net export of Cu_t. Dark gray: The area of the European Corine Land Cover (CLC). Pixels depict average predictive values at a spatial resolution of 20 km^2. Net accumulation was not computed for Turkey due to the absence of background data. Overall, Cu_{pred} was higher for vineyards in the alpine regions ((b) average = 57 mg Cu kg^{-1}) than in other European biogeographic regions (Table S5).

conditions.[43] Under anoxic conditions, biological activity may decrease Cu mobility by precipitating soluble Cu(II) to Cu(I),[44,45] which is strongly complexed and stabilized with reduced organic sulfur.[46] Additionally, depending on the solutes present in soil, e.g., carbonate and sulfide, biotic and abiotic processes may form minerals and further reduce the Cu_t mobility.[33,47,48]

In contrast, higher AI leads to drier soil,[49] which facilitates the formation of soil macropores favoring oxygen transfer.[50] As a result, Cu(I) may partly reoxidize into mobile Cu(II) in oxic soil. However, an opposite trend is observed as AI may indirectly increase the Cu-binding affinity of soil organic matter (ARI of 13.1 ± 0.1%). Indeed, the oxidation of organic matter under oxic conditions increases the contribution of carboxylic and hydroxylic groups as preferential binding partners for Cu in organic matter.[51] In addition, pH (ARI of 7.5 ± 1.3%) directly controls the deprotonation of sorption sites on soil constituents. Hence, higher pH increases available sorption sites for Cu binding.[19]

Distribution of the Total Copper Content in Topsoil Across European Vineyards. Cu_{pred} in European vineyards was successfully made as the data sets covered the range of predictive variables within the domain of prediction. The ENS also displays good model transferability in space evaluated on four equivalent geographical subdomains (average R^2 = 0.84 ± 0.07, details are given in the Supporting Information). This suggests a homogeneous influence of predictive variables within the European vineyards. Overall, Cu_{pred} for vineyards is 25.4 mg Cu kg^{-1} which is 30.5% higher than Cu_{bgd} including

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geogenic and atmospheric nonagricultural anthropogenic Cu (Figure 1a,b).

The threshold of contamination (>100 mg Cu kg^{-1})[5] was reached for 2% of Cu_{pred} within the prediction domain. Contaminated areas were mainly located in central Western Europe, i.e., northern Italy, eastern France, Switzerland, and Slovenia. In those regions, C_{org} is medium (up to 14%), while Cu_{pred} >100 mg Cu kg^{-1} accounted for 70% of vineyard soil.[52] Data points above the contamination threshold in those regions are characterized by high AI, soil moisture, and annual rainfall, although only C_{org} was significantly higher (>9.0%) than in other regions of southern Europe (*t*-test; *p*-value < 0.01). This suggests that higher annual rainfall correlates with higher temperature in central Western Europe. This may favor downy mildew emergence and concomitant use of higher doses and more frequent applications of Cu_f. In central Western Europe, soil with higher C_{org} may influence Cu_{pred} predominantly and locally, although C_{org} accounted in total for 13.1% ARI of the Cu_{pred}.

Accumulation and Export of Copper in Topsoil. The estimated average net accumulation and net export of Cu in topsoil in European vineyards are 24.8 (SD = 42.7) and 0.29 kg Cu ha^{-1} (SD = 0.64), respectively (Figure 1c,d). This corresponds to 112 400 tons of Cu accumulated over time, and a yearly Cu export from vineyards of 1466 tons across Europe. The distribution of Cu net export fitted with that of Cu_{pred} (R^2 = 0.65). On average, the predicted loss rate of vineyard soil (9.47 tons ha^{-1} $year^{-1}$) is 3.5 times greater than any other European arable land due to low vegetation and sloping area in vineyards. Consequently, net Cu export is particularly large in the Southern Alps due to intense soil erosion. Large soil loss is associated with a higher slope (>25°) and greater rainfall amounts (>1600 mm $year^{-1}$) in the Southern Alps than in other vineyard areas.[21]

Potential Future Copper Accumulation in European Vineyards. Overall, the proportion of European vineyard area exceeding PNEC values (i.e., ranged from 30 to 290 mg Cu kg^{-1} according to soil properties calculate elsewhere,[53,54] details are given in the Supporting Information) may largely vary in the future depending on the application dose of Cu_f (Figure 2). Cu accumulation due to Cu_f application doses of 2, 4, and 8 kg Cu ha^{-1} $year^{-1}$ can be balanced with Cu export after 1200, 750, and 150 years, with 75, 89, and 97% of European vineyard areas exceeding PNEC values, respectively. Assuming constant soil properties, we speculate that the current European regulation with a maximum application of 4 kg Cu ha^{-1} $year^{-1}$ will increase by only 2% of the proportion of vineyard areas exceeding PNEC in the next 100 years.

In contrast, 94% of vineyard areas may exceed PNEC in the historical application scenario of 8 kg Cu ha^{-1} $year^{-1}$. With an application of 2 kg Cu ha^{-1} $year^{-1}$, vineyard areas exceeding PNEC values may increase by less than 0.5% for the next 100 years. In contrast, a substitution of Cu_f (0 kg Cu ha^{-1} $year^{-1}$) may decrease the proportion of vineyard areas exceeding the background Cu_{bgd} from 48 to 38% after 100 years. Such long-term indicative estimation for different Cu application doses provides a critical temporal trend to support the reduction of Cu application in a vineyard.

Our back-envelope is a first attempt to evaluate Cu accumulation and export at the European scale, and the proposed mass balance approach may lead to inaccurate estimations of Cu_t export. For instance, freshly applied Cu_f is a Cu pool that can be directly exchanged with the labile cation

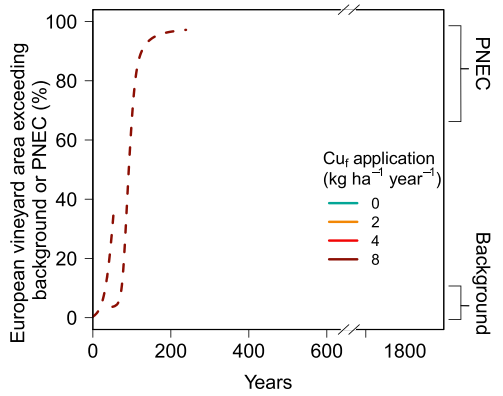

Figure 2. Proportion of European vineyard area exceeding predicted no-effect concentration (PNEC) values of total Cu in topsoil (scenarios with Cu_f application doses of 2, 4, and 8 kg ha^{-1} $year^{-1}$) or equal to or lower than the background (bgd) Cu content in topsoil (scenario with no Cu_f application). Predictions account for the balance between yearly Cu_f application at a given application dose and Cu export over time. Dashed lines indicate the upper and lower uncertainties of predictions.

present in vineyard soil following Cu_f application. Depending on the time following Cu_f application, Cu_f may be partly mobilized during rainfall-runoff events[14] and thus bias estimations of Cu_t in soil. Since rainfall erosivity is predicted to increase by 18% in Europe over time, Cu_f export may also increase.[55] In addition, estimations using the semiempirical model account for Cu aging in contaminated soil and rely on realistic desorption experiments, which may underestimate Cu_f export.[12] However, Cu_f accumulation over time may be also underestimated. Climate change models predict an average of 10% increase of soil organic matter content by 2100 in regions of European vineyards,[56] which may, in turn, reduce Cu_t mobility and increase the amount of Cu accumulated in the vineyard soil.

Implications for Wine Growing Practices. Cu is an element that cannot be degraded in soil, and soil remediation processes, such as phytoextraction[57] or enhanced mobilization by plants,[58] have limited efficiencies. The most efficient strategy to limit the increase of Cu_t is thus to reduce application doses of Cu_f. Reducing strategies consist of applying Cu_f during long humid periods only[42] and in preventively reducing the use of Cu_f. Forecast models of grapevine downy mildew[59] relying on climatic variables can help to improve the efficiency of Cu_f reduction strategies. Alternatives to Cu_f include replacing current grape varieties with new grape varieties with higher resistance to fungus pests,[60] using microorganisms for biocontrol,[61] introducing plant defense stimulators,[62] and/or applying plant extracts to substitute Cu_f.[63] Alternative scenarios without Cu_f are still debated but are currently used in some biodynamic approaches accounting for only ca. 1% of vineyards worldwide (http://www.demeter.net). Additional benefits of biodynamic wine growing include the higher soil biodiversity and microbiological activity[10] and potentially greater resilience to climatic and pathogenic threats in vineyards[64] as opposed to most conventional wine growing.[65]

Cu export is mainly driven by soil erosion with concomitant loss of solid-bound Cu, which should be prevented and lessened in regions prone to soil loss to reduce contamination of connected aquatic ecosystems. In fact, 10% of the vineyards

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are located close to a river (<500 m)[66] and the associated catchment may act as a point source of contamination. The transport of solid-bound Cu into aquatic ecosystems may also affect the downstream areas due to the progressive release of toxic Cu(II), resulting from a change in pH and/or redox conditions. Antierosion soil management strategies should thus be prioritized and designed to retain Cu on the vineyard plots. In particular, traditional soil management strategies, including grass or mulch cover, reduced tillage, contour planting, and terraced vineyards, are most effective in limiting soil erosion and associated Cu runoff.[35] Complementarily, stormwater wetlands may be deployed at the outlet of vineyard catchments. Stormwater wetlands have the potential to retain >68% of the dissolved Cu and >92% of the solid-bound Cu, the principal contributions to Cu export in runoff.[14]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02093.

Text, tables, and figures provide detailed methods (the total copper content in the topsoil data set, spatial variable building, data preprocessing, variable selection, machine learning building, performance and evaluation, and scenario of copper-based fungicide application) and supporting results and discussion (model performance, regional prediction, and the limit of the approach) (PDF)

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Author Contributions

B.D., S.P., and G.I. designed research; B.D. performed research; S.P., J.A.R.M., G.T., P.P., L.M., and P.B. contributed to the acquisition of data/soil samples; B.D. and S.P. analyzed data; and B.D., S.P., and G.I. wrote the manuscript with critical review from all authors.

Notes

The authors declare no competing financial interest.

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Sulfites: Allergen of the Year 2024

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Abstract

Sodium disulfite, also known as sodium metabisulfite or sodium pyrosulfite, is an inorganic compound, which may cause allergic contact dermatitis. Sulfites act as antioxidants and preservatives; common sources include food/beverages, pharmaceuticals, and personal care products. Importantly, sulfites are not included in most screening patch test series and thus may be missed as a relevant contact allergen. The American Contact Dermatitis Society chose sulfites as the Allergen of the Year for 2024 to raise awareness about this significant allergen.

Related information

Cited in Books
PubChem Compound (MeSH Keyword)

LinkOut - more resources

Full Text Sources
Atypon

EPA Shares Data on Chitosan Salts for Public Comment

Released on May 6, 2022

Today, in support of transparency and sound science, the U.S. Environmental Protection Agency (EPA) is making available for comment two aquatic toxicity reports related to the ongoing rulemaking in response to a petition to add chitosan to the minimum risk pesticide <https://epa.gov/minimum-risk-pesticides> exemption list. The purpose of the exemption list is to eliminate the need for the Agency to expend significant resources to regulate products deemed to be of minimum risk to human health and the environment. Products that contain only those active and inert ingredients allowed by the exemption and meet certain Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) requirements are exempt from the normal FIFRA registration requirements. Approximately a decade has passed since a substance was added to the list of ingredients eligible for the minimum risk pesticide exemption.

Chitosan is a naturally occurring substance found in the cell walls of all crustaceans, most fungi, and the exoskeletons of most insects. It is currently registered with EPA under FIFRA as a fungicide, antimicrobial agent, and plant growth regulator that boosts the ability of plants to defend against fungal infections. Chitosan is currently widely available to the public for non-pesticidal uses, and has established applications in various industries including textiles, cosmetics, beverage processing, and water treatment.

On October 10, 2018, EPA received a petition from Tidal Vision Products, LLC requesting that chitosan be added to the list of active ingredients allowed in exempted minimum risk pesticide products. In November 2020, EPA requested comments from the public on a proposed rule to add chitosan to the list of active ingredients eligible for the

exemption. The public comments received on the proposal expressed concerns regarding derivatives of chitosan that are likely to be produced when chitosan is mixed with certain acids and on the potential hazard for aquatic organisms exposed to chitosan salts.

In November 2021, EPA requested additional information from the petitioner on chitosan salts and their potential effect on the environment and, in response, received two aquatic toxicity reports. Because these reports were not available when the rule was proposed, EPA is now seeking public input on how the reports may be used to inform the Agency's assessment of the aquatic toxicity of chitosan and its salts. Stakeholders are invited to submit any questions, comments, and concerns related to these reports so EPA may consider them before deciding whether to add chitosan to the minimum risk exemption.

The public comment period will be open for 30 days in docket EPA-HQ-OPP-2019-0701 <https://www.regulations.gov/document/epa-hq-opp-2019-0701-0022> at www.regulations.gov <https://www.regulations.gov>, starting May 6, 2022.

Last updated on April 1, 2024

European Food Safety Authority

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SCIENTIFIC OPINION

Scientific Opinion on the substantiation of health claims related to chitosan and reduction in body weight (ID 679, 1499), maintenance of normal blood LDL-cholesterol concentrations (ID 4663), reduction of intestinal transit time (ID 4664) and reduction of inflammation (ID 1985) pursuant to Article 13(1) of Regulation (EC) No 1924/2006[1]

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

SUMMARY

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to provide a scientific opinion on a list of health claims pursuant to Article 13 of Regulation (EC) No 1924/2006. This opinion addresses the scientific substantiation of health claims in relation to chitosan and reduction in body weight, maintenance of normal blood LDL-cholesterol concentrations, reduction of intestinal transit time and reduction of inflammation. The scientific substantiation is based on the information provided by the Member States in the consolidated list of Article 13 health claims and references that EFSA has received from Member States or directly from stakeholders.

The food constituent that is the subject of the health claim is chitosan. The Panel considers that chitosan is sufficiently characterised.

Reduction in body weight

The claimed effect is "weight management". The target population is assumed to be overweight individuals in the general population who wish to reduce their body weight. In the context of the proposed wordings and references provided, the Panel assumes that the claimed effect relates to a

[1] On request from the European Commission, Question No EFSA-Q-2008-1466, EFSA-Q-2008-2236, EFSA-Q-2008-2718, EFSA-Q-2010-00616, EFSA-Q-2010-00617, adopted on 08 April 2011.

[2] Panel members: Carlo Agostoni, Jean-Louis Bresson, Susan Fairweather-Tait, Albert Flynn, Ines Golly, Hannu Korhonen, Pagona Lagiou, Martinus Løvik, Rosangela Marchelli, Ambroise Martin, Bevan Moseley, Monika Neuhäuser-Berthold, Hildegard Przyrembel, Seppo Salminen, Yolanda Sanz, Sean (J.J.) Strain, Stephan Strobel, Inge Tetens, Daniel Tomé, Hendrik van Loveren and Hans Verhagen. The members of the Claims Sub-Working Group on Weight Management/Satiety/Glucose and Insulin Control/Physical Performance: Kees de Graaf, Joanne Harrold, Mette Hansen, Mette Kristensen, Anders Sjödin and Inge Tetens. Correspondence: nda@efsa.europa.eu

[3] Acknowledgement: The Panel wishes to thank for the preparatory work on this scientific opinion: The members of the Working Group on Claims: Carlo Agostoni, Jean-Louis Bresson, Susan Fairweather-Tait, Albert Flynn, Ines Golly, Marina Heinonen, Hannu Korhonen, Martinus Løvik, Ambroise Martin, Hildegard Przyrembel, Seppo Salminen, Yolanda Sanz, Sean (J.J.) Strain, Inge Tetens, Hendrik van Loveren and Hans Verhagen. The members of the Claims Sub-Working Group on Weight Management/Satiety/Glucose and Insulin Control/Physical Performance: Kees de Graaf, Joanne Harrold, Mette Hansen, Mette Kristensen, Anders Sjödin and Inge Tetens.

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reduction in body weight. The Panel considers that a reduction in body weight is a beneficial physiological effect.

In weighing the evidence, the Panel took into account that a meta-analysis of randomised controlled trials, which included all the individual human intervention studies submitted for the scientific substantiation of the claim and which investigated the effects of chitosan consumption on body weight, did not show a significant effect of chitosan when only studies that met the allocation concealment quality criteria were considered for analysis.

On the basis of the data presented, the Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and reduction in body weight.

Maintenance of normal blood LDL-cholesterol concentrations

The claimed effect is "stimulates the regulation of cholesterol levels due to O-carboxymethyl chitosan". The target population is assumed to be the general population. In the context of the proposed wordings, the Panel assumes that the claimed effect relates to the maintenance of normal blood LDL-cholesterol concentrations. The Panel considers that maintenance of normal blood LDL-cholesterol concentrations is a beneficial physiological effect.

In weighing the evidence, the Panel took into account that a meta-analysis of randomised controlled trials which investigated the effects of chitosan consumption on blood lipids showed a small but statistically significant reduction in total and LDL-cholesterol concentrations.

On the basis of the data presented, the Panel concludes that a cause and effect relationship has been established between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations.

The Panel considers that in order to obtain the claimed effect, 3 g of chitosan should be consumed daily. The target population is adults.

Reduction of intestinal transit time

The claimed effect is "stimulates the intestinal transit by volume effect". The target population is assumed to be the general population. In the context of the proposed wordings, the Panel assumes that the claimed effect refers to a reduction in intestinal transit time. The Panel considers that reduction of intestinal transit time may be a beneficial physiological effect, provided that it does not result in diarrhoea.

No references were provided from which conclusions could be drawn for the scientific substantiation of the claim.

On the basis of the data presented, the Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and reduction of intestinal transit time.

Reduction of inflammation

The claimed effect is "réduit l'inflammation". The target population is assumed to be the general population.

In the context of the proposed wordings, the Panel assumes that the claimed effect refers to the reduction of inflammation in the context of maintaining joint flexibility.

The Panel considers that the evidence provided does not establish that a reduction of inflammation in relation to the maintenance of joint flexibility in the general population is a beneficial physiological effect.

On the basis of the data presented, the Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and a beneficial physiological effect for the general population related to the reduction of inflammation.

KEY WORDS

Chitosan, fibre, body weight, LDL-cholesterol, intestinal transit time, inflammation, health claims.

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TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

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EFSA DISCLAIMER

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INFORMATION AS PROVIDED IN THE CONSOLIDATED LIST

The consolidated list of health claims pursuant to Article 13 of Regulation (EC) No 1924/2006[4] submitted by Member States contains main entry claims with corresponding conditions of use and literature for similar health claims. EFSA has screened all health claims contained in the original consolidated list of Article 13 health claims which was received by EFSA in 2008 using six criteria established by the NDA Panel to identify claims for which EFSA considered sufficient information had been provided for evaluation and those for which more information or clarification was needed before evaluation could be carried out[5]. The clarifications which were received by EFSA through the screening process have been included in the consolidated list. This additional information will serve as clarification to the originally provided information. The information provided in the consolidated list for the health claims which are the subject of this opinion is tabulated in Appendix C.

ASSESSMENT

1. Characterisation of the food/constituent

The food constituent that is the subject of the health claim is chitosan.

Chitosan is a linear cationic polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine produced commercially by the deacetylation of chitin, which is a component of the exoskeleton of crustaceans and the cell walls of fungi. The degree of deacetylation can be measured by established methods, and ranges from 60-100 % in commercial preparations. The molecular weight of chitosan in commercial preparations ranges from 3,800 to 20,000 Da. Chitosan is insoluble in water.

The Panel considers that the food constituent, chitosan, which is the subject of the health claims, is sufficiently characterised.

2. Relevance of the claimed effect to human health

2.1. Reduction in body weight (ID 679, 1499)

The claimed effect is "weight management". The Panel assumes that the target population is overweight individuals in the general population who wish to reduce their body weight.

In the context of the proposed wordings and references provided, the Panel assumes that the claimed effect relates to a reduction in body weight.

Weight loss can be interpreted as the achievement of a normal body weight in previously overweight subjects. In this context, weight loss in overweight subjects without the achievement of a normal body weight is considered to be a beneficial physiological effect.

The Panel considers that a reduction in body weight is a beneficial physiological effect.

[4] Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods. OJ L 404, 30.12.2006, p. 9–25.

[5] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2011. General guidance for stakeholders on the evaluation of Article 13.1, 13.5 and 14 health claims. EFSA Journal, 9(4):2135, 24 pp.

2.2. Maintenance of normal blood LDL-cholesterol concentrations (ID 4663)

The claimed effect is "stimulates the regulation of cholesterol levels due to O-carboxymethyl chitosan". The Panel assumes that the target population is the general population.

In the context of the proposed wordings, the Panel assumes that the claimed effect relates to the maintenance of normal blood LDL-cholesterol concentrations.

Low-density lipoproteins (LDL) carry cholesterol from the liver to peripheral tissues, including the arteries. Elevated LDL-cholesterol, by convention >160 mg/dL (>4.14 mmol/L), may compromise the normal structure and function of the arteries.

The Panel considers that maintenance of normal blood LDL-cholesterol concentrations is a beneficial physiological effect.

2.3. Reduction of intestinal transit time (ID 4664)

The claimed effect is "stimulates the intestinal transit by volume effect". The Panel assumes that the target population is the general population.

In the context of the proposed wordings, the Panel assumes that the claimed effect refers to a reduction of intestinal transit time.

The Panel considers that reduction of intestinal transit time may be a beneficial physiological effect, provided that it does not result in diarrhoea.

2.4. Reduction of inflammation (ID 1985)

The claimed effect is "réduit l'inflammation". The Panel assumes that the target population is the general population.

The Panel notes that the claimed effect refers to reduction of inflammation in the context of maintaining joint flexibility. Inflammation is a non-specific physiological response and changes in markers of inflammation such as various interleukins do not indicate a beneficial physiological effect *per se*. In the context of the proposed wordings, the Panel assumes that the claimed effect refers to a reduction of inflammation in the context of maintaining joint flexibility. The Panel considers that the evidence provided does not establish that a reduction of inflammation in relation to the maintenance of joint flexibility is a beneficial physiological effect for the general population.

The Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and a beneficial physiological effect related to the reduction of inflammation.

3. Scientific substantiation of the claimed effect

3.1. Reduction in body weight (ID 679 and 1499)

The references provided for the scientific substantiation of the claim included narrative reviews and book chapters which did not provide original data for a scientific evaluation, one human intervention study which investigated the effects of a combination of chitosan and glucomannan on body weight, and one intervention study using chitosan which did not report on body weight. Two references on internal reports were not available to the Panel even after every reasonable effort had been made to

retrieve them. The Panel considers that no conclusions can be drawn from these references for the scientific substantiation of the claim.

In addition, one meta-analysis of randomised controlled trials (RCTs, Ernst and Pittler, 2000), one Cochrane systematic review and meta-analysis of RCTs (Ni Mhurchu et al., 2005) and seven human intervention studies on the effect of chitosan on body weight were provided. The Panel notes that all of the intervention studies provided, and those included in the systematic review and meta-analysis, were considered in a more recent update of the Cochrane systematic review (Jull et al., 2008), which was considered by the Panel for the scientific evaluation of the claim. This systematic review was restricted to RCTs which assessed the effects of chitosan on body weight, compared to placebo or standard care in adult overweight or obese males and females, and which had a minimum duration of four weeks. A total of 15 studies out of 42 identified met the inclusion criteria, and these studies comprised a total of 1,216 participants (640 allocated to chitosan and 640 to placebo) with a mean age of 44 years (range 18 to 70 years). Mean trial duration was 8.3 weeks (range 4-24 weeks) and mean study size was 81 subjects (range 24 to 250). All trials compared chitosan at doses ranking from 0.24 g/day to 15 g/day (mean 3.7 g/day) to placebo, but five studies did not report any dose (Colombo and Sciutto, 1996; Giustina and Ventura, 1995; Sciutto and Colombo, 1995; Veneroni et al., 1996; Woodgate and Conquer, 2003). Sufficient data on body weight were available only for 13 trials, which were considered for data analysis (Colombo and Sciutto, 1996; Giustina and Ventura, 1995; Ho et al., 2001; Kaats et al., 2006; Macchi, 1996; Ni Mhurchu et al., 2004; Pittler et al., 1999; Schiller et al., 2001; Sciutto and Colombo, 1995; Veneroni et al., 1996; Williams, 1998; Woodgate and Conquer, 2003; Zahorska-Markiewicz et al., 2002). Seven studies (Colombo and Sciutto, 1996; Girola et al., 1996; Giustina and Ventura, 1995; Kaats et al., 2006; Sciutto and Colombo, 1995; Veneroni et al., 1996; Woodgate and Conquer, 2003) used treatment preparations which contained other active ingredients in addition to chitosan, while the remainder used chitosan alone. The treatment preparations contained, in addition to chitosan, guar gum, ascorbic acid and other micronutrients (Colombo and Sciutto, 1996; Giustina and Ventura, 1995; Sciutto and Colombo, 1995; Veneroni et al., 1996), glucomannan, fenugreek, *Gymnema sylvestre* and vitamin C (Woodgate and Conquer, 2003), *Garcinia cambogia* extract and chrome (Girola et al., 1996), and beta-glucan, snow white oat fibre, betamine HCL and aloe saponins (Kaats et al., 2006). The Panel considers that no conclusions can be drawn from these studies, and thus from the meta-analysis in which they were included, for the scientific substantiation of the claim.

When the analysis was limited to trials which used chitosan alone as intervention (Ho et al., 2001; Macchi, 1996; Ni Mhurchu et al., 2004; Pittler et al., 1999; Schiller et al., 2001; Wuolijoki et al., 1999; Zahorska-Markiewicz et al., 2002), a small but statistically significant weight loss of -0.9 kg (95 % CI -1.4 to -0.4, p=0.0009) was observed with chitosan compared to placebo. However, when the analysis was limited to trials that met the allocation concealment quality criteria (Ni Mhurchu et al., 2004; Pittler et al., 1999; Schiller et al., 2001), no significant differences between the effect of chitosan and placebo on body weight changes were observed (-0.6 kg, 95 % CI -1.3 to 0.1, p=0.09). Similar results were obtained when the analysis was limited to studies of six months duration (Ni Mhurchu et al., 2004; Zahorska-Markiewicz et al., 2002). The Panel notes that this meta-analysis does not show an effect of chitosan consumption on body weight loss.

The mechanism by which chitosan is presumed to exert the claimed effect is by binding to negatively charged lipids and hence reducing their gastro-intestinal uptake, and these effects were observed in some animal studies (Deuchi et al., 1995; Sugano et al., 1980; Zacour et al., 1992). However, the effects of chitosan on 24 h faecal fat excretion in healthy human volunteers at doses of about 3 g daily were not statistically significant, and thus were unlikely to have an impact on body weight (Guerciolini et al., 2001).

In weighing the evidence, the Panel took into account that a meta-analysis of RCTs, which included all the individual human intervention studies submitted for the scientific substantiation of the claim

and which investigated the effects of chitosan consumption on body weight, did not show a significant effect of chitosan when only studies that met the allocation concealment quality criteria were considered for analysis.

The Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and reduction in body weight.

3.2. Maintenance of normal blood LDL-cholesterol concentrations (ID 4663)

Five animal studies and one human intervention study on the effects of chitosan on blood lipids were provided for the scientific substantiation of the claim.

The Cochrane systematic review (Jull et al., 2008) cited in section 3.1. also reported on the effects of chitosan on blood lipids and included the only human intervention study submitted for the scientific substantiation of the claim (Macchi, 1996).

Statistical analyses combining the nine trials that provided data on total cholesterol concentrations (Colombo and Sciutto, 1996; Ho et al., 2001; Kaats et al., 2006; Macchi, 1996; Ni Mhurchu et al., 2004; Pittler et al., 1999; Veneroni et al., 1996; Wuolijoki et al., 1999; Zahorska-Markiewicz et al., 2002) were reported in the meta-analysis. However, the Panel notes that some of these studies used treatment preparations which contained other active ingredients in addition to chitosan, and considers that no conclusions can be drawn from these analyses for the scientific substantiation of the claim. When the trials were limited to those that used chitosan alone as intervention (Ho et al., 2001; Macchi, 1996; Ni Mhurchu et al., 2004; Pittler et al., 1999; Zahorska-Markiewicz et al., 2002), a small but statistically significant reduction in total cholesterol concentrations of -0.15 mmol/L (95 % CI -0.23 to -0.07, p=0.0002) was observed. Similar results were obtained when the analyses were limited to trials that met the allocation concealment quality criteria (Ni Mhurchu et al., 2004; Pittler et al., 1999) (-0.15 mmol/L; 95 % CI -0.23 to -0.07, p=0.0004). The I^2-statistic indicated substantial heterogeneity (I^2=59.5 %).

Statistical analyses combining the seven trials that included data on LDL-cholesterol concentrations (Colombo and Sciutto, 1996; Ho et al., 2001; Kaats et al., 2006; Ni Mhurchu et al., 2004; Veneroni et al., 1996; Wuolijoki et al., 1999; Zahorska-Markiewicz et al., 2002) were provided in the meta-analysis. However, the Panel notes that four of these trials used treatment preparations which contained other active ingredients in addition to chitosan (Colombo and Sciutto, 1996; Kaats et al., 2006; Veneroni et al., 1996; Wuolijoki et al., 1999), and that no separate analysis of the trials using chitosan alone was provided. The Panel notes, however, that whereas the studies by Ho et al. (2001) and Zahorska-Markiewicz et al. (2002), including 68 and 32 subjects respectively, did not show a significant effect on LDL-cholesterol concentrations, the largest study, by Ni Mhurchu et al. (2004), which included 250 subjects (125 per group), observed a small but statistically significant reduction in LDL-cholesterol concentrations in favour of chitosan (-0.12 mmol/L, 95 % CI -0.19 to -0.05). Similar results were obtained when the analysis was limited to the two studies of 6 months duration (-0.14 mmol/L, 95 % CI -0.19 to -0.06) (Ni Mhurchu et al., 2004; Zahorska-Markiewicz et al., 2002).

Statistical analyses combining the seven trials that provided data on HDL-cholesterol concentrations (Colombo and Sciutto, 1996; Ho et al., 2001; Kaats et al., 2006; Macchi, 1996; Ni Mhurchu et al., 2004; Veneroni et al., 1996; Zahorska-Markiewicz et al., 2002) were also provided. The Panel notes that three of these trials used treatment preparations which contained other active ingredients in addition to chitosan (Colombo and Sciutto, 1996; Kaats et al., 2006; Veneroni et al., 1996), and that no separate analysis of the trials using chitosan alone was provided in the meta-analysis. The Panel also notes that only the smallest study using chitosan alone showed a statistically significant increase in HDL-cholesterol concentrations compared to placebo (0.15 mmol/L, 95 % CI 0.03 to 0.27; 10 subjects per group) (Macchi, 1996), whereas no significant differences between chitosan and

placebo were observed in any of the other three studies, including the largest study by NiMhurchu et al. (2004), which had the longest duration (6 months).

The Panel notes that while chitosan consumption at doses of about 3 g/day showed, in the meta-analysis by Jull et al. (2008), a small but statistically significant effect on the reduction of both total (combining five studies) and LDL-cholesterol (combining two studies) concentrations, no effect was observed on HDL-cholesterol concentrations.

The mechanism by which chitosan is presumed to exert the claimed effect is by binding to negatively charged lipids and hence reducing their gastro-intestinal uptake, and these effects were observed in some animal studies (Deuchi et al., 1995; Sugano et al., 1980; Zacour et al., 1992). The effects of chitosan on 24 h faecal fat excretion in healthy human volunteers at doses of about 3 g daily were not statistically significant (Guerciolini et al., 2001), and it is unclear whether this could play a role on the claimed effect.

In weighing the evidence, the Panel took into account that a meta-analysis of RCTs, which investigated the effects of chitosan consumption on blood lipids, showed a small but statistically significant reduction in total and LDL-cholesterol concentrations.

The Panel concludes that a cause and effect relationship has been established between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations.

3.3. Reduction of transit time (ID 4664)

Only one reference was provided in relation to the claim.

In a double-blind, placebo-controlled study, Kaats et al. (2006) evaluated the safety and efficacy of chitosan on body composition in a group of 134 overweight/obese adults. The Panel notes that the study did not address outcome measures related to the claimed effect, and considers that no conclusions can be drawn from this study for the scientific substantiation of the claim.

The Panel concludes that a cause and effect relationship has not been established between the consumption of chitosan and reduction of intestinal transit time.

4. Panel's comments on the proposed wording

4.1. Maintenance of normal blood cholesterol concentrations (ID 4663)

The Panel considers that the following wording reflects the scientific evidence: "Chitosan may contribute to maintaining normal blood cholesterol levels".

5. Conditions and possible restrictions of use

5.1. Maintenance of normal blood cholesterol concentrations (ID 4663)

The Panel considers that in order to obtain the claimed effect, 3 g of chitosan should be consumed daily. The target population is adults.

CONCLUSIONS

On the basis of the data presented, the Panel concludes that:

- The food constituent, chitosan, which is the subject of the claim, is sufficiently characterised.

Reduction in body weight (ID 679, 1499)

- The claimed effect is "weight management". The target population is assumed to be overweight individuals in the general population who wish to reduce their body weight. A reduction in body weight is a beneficial physiological effect.

- A cause and effect relationship has not been established between the consumption of chitosan and reduction in body weight.

Maintenance of normal blood LDL-cholesterol concentrations (ID 4663)

- The claimed effect is "stimulates the regulation of cholesterol levels due to O-carboxymethyl chitosan". The target population is assumed to be the general population. Maintenance of normal blood LDL-cholesterol concentrations is a beneficial physiological effect.

- A cause and effect relationship has been established between the consumption of chitosan and maintenance of normal blood LDL-cholesterol concentrations.

- The following wording reflects the scientific evidence: "Chitosan may contribute to maintaining normal blood cholesterol levels".

- In order to obtain the claimed effect, 3 g of chitosan should be consumed daily. The target population is adults.

Reduction of intestinal transit time (ID 4664)

- The claimed effect is "stimulates the intestinal transit by volume effect". The target population is assumed to be the general population. Reduction of intestinal transit time may be a beneficial physiological effect, provided it does not result in diarrhoea.

- A cause and effect relationship has not been established between the consumption of chitosan and reduction of intestinal transit time.

Reduction of inflammation (ID 1985)

- The claimed effect is "réduit l'inflammation". The target population is assumed to be the general population. In the context of the proposed wordings, it is assumed that the claimed effect refers to a reduction of inflammation in the context of maintaining joint flexibility. The evidence provided does not establish that a reduction of inflammation in relation to the maintenance of joint flexibility is a beneficial physiological effect for the general population.

- A cause and effect relationship has not been established between the consumption of chitosan and a beneficial physiological effect related to the reduction of inflammation.

DOCUMENTATION PROVIDED TO EFSA

Health claims pursuant to Article 13 of Regulation (EC) No 1924/2006 (No: EFSA-Q-2008-1466, EFSA-Q-2008-2236, EFSA-Q-2008-2718, EFSA-Q-2010-00616, EFSA-Q-2010-00617). The

scientific substantiation is based on the information provided by the Member States in the consolidated list of Article 13 health claims and references that EFSA has received from Member States or directly from stakeholders.

The full list of supporting references as provided to EFSA is available on: http://www.efsa.europa.eu/panels/nda/claims/article13.htm.

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APPENDICES

APPENDIX A

BACKGROUND AND TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The Regulation 1924/2006 on nutrition and health claims made on foods[6] (hereinafter "the Regulation") entered into force on 19th January 2007.

Article 13 of the Regulation foresees that the Commission shall adopt a Community list of permitted health claims other than those referring to the reduction of disease risk and to children's development and health. This Community list shall be adopted through the Regulatory Committee procedure and following consultation of the European Food Safety Authority (EFSA).

Health claims are defined as "any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health".

In accordance with Article 13 (1) health claims other than those referring to the reduction of disease risk and to children's development and health are health claims describing or referring to:

a) the role of a nutrient or other substance in growth, development and the functions of the body; or

b) psychological and behavioural functions; or

c) without prejudice to Directive 96/8/EC, slimming or weight-control or a reduction in the sense of hunger or an increase in the sense of satiety or to the reduction of the available energy from the diet.

To be included in the Community list of permitted health claims, the claims shall be:

(i) based on generally accepted scientific evidence; and

(ii) well understood by the average consumer.

Member States provided the Commission with lists of claims as referred to in Article 13 (1) by 31 January 2008 accompanied by the conditions applying to them and by references to the relevant scientific justification. These lists have been consolidated into the list which forms the basis for the EFSA consultation in accordance with Article 13 (3).

ISSUES THAT NEED TO BE CONSIDERED

IMPORTANCE AND PERTINENCE OF THE FOOD[7]

Foods are commonly involved in many different functions[8] of the body, and for one single food many health claims may therefore be scientifically true. Therefore, the relative importance of food e.g. nutrients in relation to other nutrients for the expressed beneficial effect should be considered: for functions affected by a large number of dietary factors it should be considered whether a reference to a single food is scientifically pertinent.

[6] OJ L12, 18/01/2007
[7] The term 'food' when used in this Terms of Reference refers to a food constituent, the food or the food category.
[8] The term 'function' when used in this Terms of Reference refers to health claims in Article 13(1)(a), (b) and (c).

It should also be considered if the information on the characteristics of the food contains aspects pertinent to the beneficial effect.

SUBSTANTIATION OF CLAIMS BY GENERALLY ACCEPTABLE SCIENTIFIC EVIDENCE

Scientific substantiation is the main aspect to be taken into account to authorise health claims. Claims should be scientifically substantiated by taking into account the totality of the available scientific data, and by weighing the evidence, and shall demonstrate the extent to which:

(a) the claimed effect of the food is beneficial for human health,

(b) a cause and effect relationship is established between consumption of the food and the claimed effect in humans (such as: the strength, consistency, specificity, dose-response, and biological plausibility of the relationship),

(c) the quantity of the food and pattern of consumption required to obtain the claimed effect could reasonably be achieved as part of a balanced diet,

(d) the specific study group(s) in which the evidence was obtained is representative of the target population for which the claim is intended.

EFSA has mentioned in its scientific and technical guidance for the preparation and presentation of the application for authorisation of health claims consistent criteria for the potential sources of scientific data. Such sources may not be available for all health claims. Nevertheless it will be relevant and important that EFSA comments on the availability and quality of such data in order to allow the regulator to judge and make a risk management decision about the acceptability of health claims included in the submitted list.

The scientific evidence about the role of a food on a nutritional or physiological function is not enough to justify the claim. The beneficial effect of the dietary intake has also to be demonstrated. Moreover, the beneficial effect should be significant i.e. satisfactorily demonstrate to beneficially affect identified functions in the body in a way which is relevant to health. Although an appreciation of the beneficial effect in relation to the nutritional status of the European population may be of interest, the presence or absence of the actual need for a nutrient or other substance with nutritional or physiological effect for that population should not, however, condition such considerations.

Different types of effects can be claimed. Claims referring to the maintenance of a function may be distinct from claims referring to the improvement of a function. EFSA may wish to comment whether such different claims comply with the criteria laid down in the Regulation.

WORDING OF HEALTH CLAIMS

Scientific substantiation of health claims is the main aspect on which EFSA's opinion is requested. However, the wording of health claims should also be commented by EFSA in its opinion.

There is potentially a plethora of expressions that may be used to convey the relationship between the food and the function. This may be due to commercial practices, consumer perception and linguistic or cultural differences across the EU. Nevertheless, the wording used to make health claims should be truthful, clear, reliable and useful to the consumer in choosing a healthy diet.

In addition to fulfilling the general principles and conditions of the Regulation laid down in Article 3 and 5, Article 13(1)(a) stipulates that health claims shall describe or refer to "the role of a nutrient or other substance in growth, development and the functions of the body". Therefore, the requirement to

describe or refer to the 'role' of a nutrient or substance in growth, development and the functions of the body should be carefully considered.

The specificity of the wording is very important. Health claims such as "Substance X supports the function of the joints" may not sufficiently do so, whereas a claim such as "Substance X helps maintain the flexibility of the joints" would. In the first example of a claim it is unclear which of the various functions of the joints is described or referred to contrary to the latter example which specifies this by using the word "flexibility".

The clarity of the wording is very important. The guiding principle should be that the description or reference to the role of the nutrient or other substance shall be clear and unambiguous and therefore be specified to the extent possible i.e. descriptive words/ terms which can have multiple meanings should be avoided. To this end, wordings like "strengthens your natural defences" or "contain antioxidants" should be considered as well as "may" or "might" as opposed to words like "contributes", "aids" or "helps".

In addition, for functions affected by a large number of dietary factors it should be considered whether wordings such as "indispensable", "necessary", "essential" and "important" reflects the strength of the scientific evidence.

Similar alternative wordings as mentioned above are used for claims relating to different relationships between the various foods and health. It is not the intention of the regulator to adopt a detailed and rigid list of claims where all possible wordings for the different claims are approved. Therefore, it is not required that EFSA comments on each individual wording for each claim unless the wording is strictly pertinent to a specific claim. It would be appreciated though that EFSA may consider and comment generally on such elements relating to wording to ensure the compliance with the criteria laid down in the Regulation.

In doing so the explanation provided for in recital 16 of the Regulation on the notion of the average consumer should be recalled. In addition, such assessment should take into account the particular perspective and/or knowledge in the target group of the claim, if such is indicated or implied.

TERMS OF REFERENCE

HEALTH CLAIMS OTHER THAN THOSE REFERRING TO THE REDUCTION OF DISEASE RISK AND TO CHILDREN'S DEVELOPMENT AND HEALTH

EFSA should in particular consider, and provide advice on the following aspects:

- Whether adequate information is provided on the characteristics of the food pertinent to the beneficial effect.
- Whether the beneficial effect of the food on the function is substantiated by generally accepted scientific evidence by taking into account the totality of the available scientific data, and by weighing the evidence. In this context EFSA is invited to comment on the nature and quality of the totality of the evidence provided according to consistent criteria.
- The specific importance of the food for the claimed effect. For functions affected by a large number of dietary factors whether a reference to a single food is scientifically pertinent.

In addition, EFSA should consider the claimed effect on the function, and provide advice on the extent to which:

- the claimed effect of the food in the identified function is beneficial.
- a cause and effect relationship has been established between consumption of the food and the claimed effect in humans and whether the magnitude of the effect is related to the quantity

consumed.

- where appropriate, the effect on the function is significant in relation to the quantity of the food proposed to be consumed and if this quantity could reasonably be consumed as part of a balanced diet.
- the specific study group(s) in which the evidence was obtained is representative of the target population for which the claim is intended.
- the wordings used to express the claimed effect reflect the scientific evidence and complies with the criteria laid down in the Regulation.

When considering these elements EFSA should also provide advice, when appropriate:

- on the appropriate application of Article 10 (2) (c) and (d) in the Regulation, which provides for additional labelling requirements addressed to persons who should avoid using the food; and/or warnings for products that are likely to present a health risk if consumed to excess.

APPENDIX B

EFSA DISCLAIMER

The present opinion does not constitute, and cannot be construed as, an authorisation to the marketing of the food/food constituent, a positive assessment of its safety, nor a decision on whether the food/food constituent is, or is not, classified as foodstuffs. It should be noted that such an assessment is not foreseen in the framework of Regulation (EC) No 1924/2006.

It should also be highlighted that the scope, the proposed wordings of the claims and the conditions of use as proposed in the Consolidated List may be subject to changes, pending the outcome of the authorisation procedure foreseen in Article 13(3) of Regulation (EC) No 1924/2006.

APPENDIX C

Table 1. Main entry health claims related to chitosan, including conditions of use from similar claims, as proposed in the Consolidated List.

ID	Food or Food constituent	Health Relationship	Proposed wording
679	Chitosane Clarification provided Chitosan (D-glucosamin+ N-acétyl-D-glucosamin)	Combat l'obésité Clarification provided Weight management	Aide à combattre les excès de poids Aide dans le cadre d'un régime amincissant Soutient lors d'amincissement Clarification provided Contributes to management of weight control/can help in the reduction of body weight/can help to the control of weight by reducing the quantity of fat absorbed from the diet.
	Conditions of use - 6x250mg/jour		
ID	**Food or Food constituent**	**Health Relationship**	**Proposed wording**
1499	Chitosan	Weight Management	Contributes to management of weight control -can help in the reduction of body weight -can help to the control of weight by reducing the quantity of fat absorbed from the diet
	Conditions of use - 600 mg Chitosan—weiteres B-Vitamine, 150 µg Chrom. - 2-3 g /day, divided uniformly to the main meals a [?]. - 1-6 g per day, 30 minutes before the main meals.		
ID	**Food or Food constituent**	**Health Relationship**	**Proposed wording**
1985	Hydrolysat de chitosan	Réduit l'inflammation	Maintien de la flexibilité articulaire Aide au maintien de la santé articulaire Bien-être articulaire
	Conditions of use - Dose journalière recommandée: 1500 mg; A utiliser pendant 1 mois ; Destiné aux adultes, plus particulièrement aux seniors; A déconseiller aux femmes enceintes et allaitantes; Ne doit pas être consommé par des personnes allergiques à l'iode ou aux fruits de mer.		
	No clarification provided by Member States		
ID	**Food or Food constituent**	**Health Relationship**	**Proposed wording**
4663	Chitosan-Natural insoluble	Stimulates the regulation	Stimulates regulation of cholesterol.

	fibre from crustaceans shell.	of cholesterol levels due to O-carboxymethyl chitosan.	
	Conditions of use - Recommended dose: 1 - 6 g chitosan/day, 30 minutes before the main meals.		
ID	**Food or Food constituent**	**Health Relationship**	**Proposed wording**
4664	Chitosan - Natural insoluble fibre from crustaceans shell.	Stimulates the intestinal transit by volume effect.	Increases in volume in the interior of the digestive tube by hydration, launches laxation in non-irritative way.
	Conditions of use - Recommended dose: 1 - 6 g chitosan/day, 30 minutes before the main meals.		

GLOSSARY AND ABBREVIATIONS

RCT	Randomised Controlled Trial
LDL	Low-density lipoproteins
HDL	High-density lipoproteins

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UV–vis irradiation: An alternative to reduce SO_2 in white wines?

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ABSTRACT

Ultraviolet–visible irradiation was tested as an innovative technology that can help reducing the amount of sulfur dioxide used in white wine manufacturing. In addition, the effects of must freezing before processing were also investigated. Musts from Xarel·lo and Parellada varieties were vinified without any protective treatment, with a standard amount of SO_2 addition or after an irradiation step. The results showed that UV–vis irradiation of must was able to prevent wine from spoilage in the same degree as SO_2, without changing other quality parameters such as pH, tartaric acid and alcohol content. However, a residual addition of SO_2 would be required in order to completely inhibit polyphenol oxidase activity. Moreover, the obtained changes in wine color parameters led to the conclusion that further optimization of the irradiation process is required before its implementation. Must freezing was shown to have some influence on quality parameters of Xarel·lo wines, but not in those from Parellada.

1. Introduction

Winemaking (or vinification), i.e. the transformation of grape must into wine, has a long history dating back over 8000 years. Nowadays, the production techniques are essentially the same as the ones used by ancient civilizations, but elaborating a flavorsome and stable wine that does not spoil during storage requires considerable expertise on the part of the winemaker. In addition, this stability must be guaranteed in such a way that does not affect its organoleptic properties, which usually have to meet high quality standards.

A number of factors may affect wine stability. On the one hand, several secondary metabolites produced by spoiling microorganisms are volatile and potentially affect wine sensory qualities (Bartowsky, 2009). On the other hand, many constituents of wine, including phenolics, certain metals, tyrosine and aldehydes, are susceptible to oxidation during the winemaking process and lead to undesirable products that adversely affect its sensory and nutritional value (Li, Guo, & Wang, 2008). These oxidative processes can be classified in enzymatic and non-enzymatic. The former almost entirely occurs in grape must (having also consequences on wine quality), while the latter can happen both in grape must and wine (Es-Safi, Cheynier, & Moutounet, 2003; Oliveira, Ferreira, De Freitas, & Silva, 2011).

Regarding microorganisms, the natural microflora found in grape must includes several dozen species of yeasts (being *Saccharomyces cerevisiae* the main one), four genera of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*) and two genera of acetic acid bacteria (*Acetobacter* and *Gluconobacter*) (Bartowsky, 2009). Many of these microorganisms (especially yeasts) will not be able to grow in wine due to the ethanol content, acidity and limited nutrients, but certain species of bacteria (such as acetic acid ones) must be controlled in order to avoid the production of undesirable compounds like acetic acid, acetaldehyde and ethyl acetate. Spoilage defects are usually recognized by haze formation, increase in acetic acid or volatile acidity, ethanol concentration, volatile phenols, volatile sulfur and viscosity of wine (Fredericks, du Troit & Krügel, 2011).

As far as enzymatic oxidation is concerned, polyphenol oxidase (PPO) is the most important oxidoreductase responsible for browning during grape processing, followed by laccase and peroxidase (Li et al., 2008; Oliveira et al., 2011). PPO is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen and various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of a heterogeneous group of dark polymers called melanins (Falguera, Pagán, Garza, Garvín, & Ibarz, 2012).

Traditionally, sulfur dioxide (SO_2) has been used to control unwanted microorganisms and polyphenol oxidase activity during winemaking, being added to machine-harvested grapes and to wine after malolactic fermentation (Bartowsky, 2009; Oliveira et al., 2011). However, in recent times the wine industry is challenged to meet consumers' demands of reducing the amount of SO_2 added to

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wine, especially since it has been associated with some health risks such as allergic reactions incurred by sulphite-sensitive individuals (Fredericks et al., 2011; Threlfall & Morris, 2002). Moreover, its excessive use may affect the quality of wine, giving unpleasing flavors and aromas and making it turn cloudy during storage (Li et al., 2008). So far, the total substitution of SO_2 for another compound or technique that fulfill the same functions without its disadvantages remains unsuccessful. However, some studies have shown that a partial replacement may be possible if SO_2 is combined with another treatment to reduce enzyme activity and microbial load (Bartowsky, 2009; Fredericks et al., 2011; Li et al., 2008; Oliveira et al., 2011).

Some emerging technologies have been used to eliminate microorganisms in liquid food and beverages such as high hydrostatic pressure, pulsed electric fields, ultrasound and ultraviolet irradiation. In the particular case of wine, some of them may also be used to accelerate processes like oxidation or aging, developing a more rounded taste and flavor (Bhaskaracharya, Ashokkumar, & Kentish, 2009), or to enhance the extraction of some compounds before fermentation (Donsì, Ferrari, & Pataro, 2010; Vorobiev & Lebovka, 2010).

Ultraviolet–visible (UV–vis) irradiation is one of these technologies that can be used for eliminating or reducing the addition of SO_2 in winemaking, due to its proved effect in the pasteurization of beverages (Falguera, Pagán, Garza, Garvín, & Ibarz, 2011). However, the effects of the irradiation of must on the final quality of wine still remain unclear. This piece of work aims at the characterization of these effects on white wine production, comparing different parameters that may have influence on the product quality: the grape variety (Xarel·lo or Parellada), the use of SO_2 vs. UV–vis irradiation and must freezing before fermentation.

2. Materials and methods

2.1. Must preparation

Grapes from two white varieties, Xarel·lo and Parellada, were provided by local farmers (Raimat–Lleida and Sant Sadurní d'Anoia–Barcelona, respectively). The fruits were washed, squeezed with a household juicer and pressed. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 30 min at 26,000 *g*, so as to eliminate the solid impurity that remained in the juice. This process was done at 5 C to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. From every batch, after measuring the fresh juice properties the samples were split into different fractions of 800 mL, half of which were frozen.

The resulting samples were divided in three groups, one of which was vinified without further processing, while another group was vinified after SO_2 addition (50 mg/L of potassium metabisulphite, as suggested by the industry) and the last group was vinified after UV–vis irradiation. As a result, four samples (from different batches) for each combination variety (Xarel·lo/Parellada) – treatment (no treatment/SO_2/irradiation) – fresh/frozen must were vinified (12 possible factor combinations, as shown in Fig. 1).

2.2. UV processing

UV irradiation was carried out in a dark chamber containing the juice and the lamp. The must was placed in a methacrylate tank of 22 15 10 cm. 800 mL of juice were processed, reaching a height of 2.4 cm inside the tank. A refrigeration system consisting of a metallic coil fed with cold water was used to control temperature and avoid must heating. Juice temperature was maintained at 25 C 1 C in all experiments. A magnetic stirrer was used during irradiation to ensure that all the must was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 medium-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (Falguera, Pagán, & Ibarz, 2011; Ibarz, Garvín, Garza, & Pagán, 2009). The real incident energy, determined as described by Esplugas and Vicente (1991), was $3.88 \cdot 10^{7}$ E min^{1} (Ibarz et al., 2009). The distance between the juice surface and the lamp was 22.5 cm. The lamp was lit 10 min before putting the juice in the chamber. Irradiation was carried out for 3 h and 30 min. Samples were taken at 0, 60, 120, 180 and 210 min and placed in a refrigerator until its analysis.

2.3. Vinification

Both irradiated and non-irradiated samples were fermented in the Raimat winery (Codorníu Group, Lleida, Spain). Five hundred mL of each must were placed in a glass bottle. Once the must had reached 20 C, it was inoculated with 10 mL (2% v/v) premix of QA-23 commercial yeast (Lallemand Inc., Montréal, Canada), which was prepared with 10 g of active dry wine yeast and 100 mL of water at 40 C. 0.1 g of diammonium phosphate (DAP) were added as fermentation activator. Each bottle was covered with an S-shape airlock (*bubble*). During fermentation, temperature was kept at 20 1 C.

Temperature and density were measured every 24 h. It was considered that the fermentation had finished when density did not change for three consecutive days. The end of the fermentation was also checked with a sugar analysis by means of the method described by Rebelein (1973). Yeast and solid remains were separated by decantation. Then, 375 mL glass bottles were filled with the wine until 55 mm off the top. The remaining space was filled with CO_2–N_2 protective atmosphere and the cork was placed.

2.4. Physical, chemical and enzymatic analyses

The pH of the must (before and after irradiation) and the wine was measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed

Fig. 1. Variable combinations that describe the assayed samples.

Table 1
Initial properties of the grape musts from both varieties, fresh or frozen and thawed.

	Xarel·lo (fresh)		Xarel·lo (frozen/thawed)		Parellada (fresh)		Parellada (frozen/thawed)	
pH	3.25	0.14[a]	3.09	0.19[a]	3.36	0.11[a]	3.12	0.20[a]
Soluble solids (Brix)	20.6	0.6[a]	19.5	1.0[a]	17.5	0.9[b]	16.2	1.7[b]
Absorbance at 420 nm	0.090	0.02[a]	0.200	0.074[b]	0.269	0.044[b]	0.430	0.092[c]
PPO activity (U/mL)	0.0036	0.0008[a]	0.0032	0.0001[a]	0.0084	0.0032[c]	0.0043	0.0014[b]

Mean value standard deviation; different superscripts in a row indicate significant differences.

using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Japan). Must and wine color was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. Must and wine absorption spectra between 350 and 750 nm were measured with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width quartz cell. In wine samples, tartaric acid content, alcoholic degree and volatile acidity were measured by means of near infrared spectroscopy (NIR) with a Foss WineScan FT120 analyzer (FOSS Analytical, Hillerød, Denmark).

Polyphenol oxidase (PPO) activity in the juice was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as substrate, prepared in a concentration of 10 mmol in a McIlvaine buffer solution with a pH of 4.0. The reaction was carried out in a 1 cm light path quartz cell. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one absorbance unit (AU) at 420 nm in 1 min (Ülker-Yerlitürk, Arslan, Sinan, Gencer, & Özensoy, 2008). All determinations were carried out in duplicate.

2.5. Statistical analysis

Data means and standard deviations were calculated from experimental data by means of StatGraphics Plus v.5.1 software (STSC Inc., Rockville, MD, USA). The same software was used to carry out multiple range tests in order to find out significant differences between the means at a 95% confidence level. Furthermore, for PPO inactivation, experimental data was fitted to the first order kinetic model:

$$RA = RA_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where RA is the residual enzyme activity, RA_0 is the intercept of the curve, k is the first-order kinetic constant and t is the irradiation time (Giner, Gimeno, Barbosa-Cánovas, & Martín, 2001).

3. Results and discussion

3.1. Initial properties

Table 1 shows the main properties of the different batches of Xarel·lo and Parellada musts that were processed just after being obtained (in fresh) or after freezing and thawing. The main differences between Xarel·lo and Parellada musts are found in soluble solids content, being higher in the former. In addition, in both varieties freezing and thawing caused an increase in the absorbance at 420 nm (which was already higher in Parellada than in Xarel·lo) and a decrease in polyphenol oxidase activity that was only significant in Parellada must. Furthermore, as it can be seen in Table 2, Xarel·lo must is brighter, while Parellada juice is redder (has a higher a^* value) and more yellow (with a higher b^* value). Nevertheless, freezing and thawing do not cause any significant change in brightness (L^*), but an important increase in colorimetric parameters a^* and b^* in both musts. This darkening of thawed samples, as well as the increase in absorbance at 420 nm, was mainly attributed to the oxidation caused by PPO activity during this process.

Samples from Table 1 (fresh and frozen/thawed) were divided in three groups: the first one vinified without any protective treatment, the second one protected against spoilage and enzymatic activity with SO_2 and the third one processed by UV–vis irradiation. In the samples with added SO_2, polyphenol oxidase was completely inactivated.

3.2. Polyphenol oxidase inactivation in the irradiated samples

PPO was partially inactivated in all the samples that were treated by UV–vis irradiation. The residual activity of the enzyme depended mostly on the grape variety. In this way, Xarel·lo PPO still kept (18 1)% of its original activity after the treatment, while for Parellada samples this residual activity was (30 1)%. Moreover, in

Table 2
CIELab color parameters of all musts and their resulting wines.

Variety	Product	L^*		a^*		b^*	
Xarel·lo (fresh must)	Fresh must	41.79	2.81[a]	0.89	0.25[ab]	4.37	1.98[a]
	Irradiated must	41.11	4.30[a]	1.39	0.76[abc]	9.26	2.02[bc]
	Wine from fresh must	40.26	0.85[a]	0.76	0.03[a]	5.53	0.59[a]
	Wine from must with SO_2	40.11	3.15[a]	1.17	1.67[abc]	4.40	2.56[a]
	Wine from irradiated must	34.60	2.85[b]	2.29	0.52[de]	6.28	3.07[ab]
Xarel·lo (frozen/thawed must)	Frozen/thawed must	39.25	2.35[a]	4.95	1.69[e]	10.85	3.57[c]
	Irradiated must	38.16	3.24[ab]	3.06	0.60[de]	11.10	3.42[c]
	Wine from frozen/thawed must	32.08	2.21[c]	2.12	0.49[cd]	4.42	1.70[a]
	Wine from must with SO_2	33.90	2.00[c]	1.9	0.22[cd]	5.21	1.98[a]
	Wine from irradiated must	32.84	2.10[c]	3.44	0.49[e]	6.47	2.35[ab]
Parellada (fresh must)	Fresh must	37.78	3.74[r]	4.33	1.39[rst]	14.78	2.26[s]
	Irradiated must	37.34	2.10[r]	6.45	1.80[tu]	14.79	1.16[s]
	Wine from fresh must	30.63	1.59[rst]	4.74	0.55[st]	4.63	2.77[t]
	Wine from must with SO_2	35.76	3.21[rs]	4.04	0.60[rs]	13.33	4.07[s]
	Wine from irradiated must	29.33	1.52[stu]	8.55	1.56[uv]	5.15	2.49[t]
Parellada (frozen/thawed must)	Frozen/thawed must	35.08	1.98[rs]	10.01	2.28[v]	17.08	2.27[r]
	Irradiated must	29.38	3.30[stu]	10.50	1.91[v]	10.81	3.47[s]
	Wine from frozen/thawed must	22.89	15.04[u]	9.09	1.78[v]	4.21	2.47[t]
	Wine from must with SO_2	31.14	1.44[rst]	2.48	1.06[r]	3.19	1.94[t]
	Wine from irradiated must	26.42	1.04[tu]	6.24	0.69[t]	0.15	1.88[u]

Mean value standard deviation; different superscripts in a column (for each variety) indicate significant differences.

Table 3
Kinetic parameters for PPO residual activity data fitted to the first-order model.

Sample	RA_0 (−)		k (min ^{1})		R^2
Xarel·lo (fresh must)	1.00	0.05	0.0087	0.0009	0.9979
Xarel·lo (frozen/thawed must)	1.00	0.06	0.0088	0.0010	0.9976
Parellada (fresh must)	0.98	0.09	0.0055	0.0010	0.9909
Parellada (frozen/thawed must)	0.97	0.10	0.0060	0.0011	0.9887

Signification level: $\alpha = 0.05$.

a previous piece of work, the average residual PPO activity for two other white grape varieties (Victoria and Dauphine) after UV–vis irradiation was 20% (Falguera, Garza, Pagán, Garvín, & Ibarz, in press). These differences can be attributed to the fact that the must from the varieties with a lower achieved inactivation had a higher concentration of colored compounds (leading to a higher absorbance, a lower value of L^* and a higher value of a^* and b^*). These compounds absorb part of the UV–vis radiation, making the available dose to inactivate the enzyme to be lower (Falguera et al., in press). Fredericks et al. (2011) observed the same phenomenon in the reduction of spoilage in musts and wines with different optical properties (color and turbidity). No statistical differences were found in a single variety comparing the achieved PPO inactivation degree in fresh or frozen/thawed must.

Regarding PPO inactivation rate, Table 3 shows the results of fitting experimental data to the first-order kinetic model. Inactivation constants (k) for Xarel·lo were higher than those obtained for Parellada, reinforcing the idea of a faster denaturation of the enzyme. In addition, these values do not show any remarkable difference between fresh or frozen and thawed samples of both varieties. Although in the case of Parellada k is lower for fresh samples, this difference remains inside the 95% confidence interval.

3.3. Color parameters and absorbance spectra

First of all, it can be stated that irradiation itself did not cause a brightness decrease neither in Xarel·lo nor in Parellada musts. In Xarel·lo wines, fermentation only caused a decrease of L^* in fresh samples that had been previously irradiated, but not in SO_2-added ones. On the contrary, this decrease of brightness after vinification was found in frozen/thawed samples in untreated, irradiated and SO_2-added samples. In addition, comparing the wines from unfrozen and from frozen must, the later are significantly less bright regardless the treatment. In the case of Parellada, fermentation caused a decrease of L^* in fresh irradiated must and in untreated frozen/thawed must. In the samples from this variety, if no hurdle is applied against microorganisms or enzymes wine brightness decreases significantly if the must has been frozen.

When it comes to redness (a^*), freezing and thawing also led to a higher value of this parameter in untreated and irradiated musts. In addition, wines from irradiated musts were redder than those from SO_2-added ones, although irradiation itself did not cause any variation in a^*. Although it is known that sulfur dioxide can have a reversible bleaching effect on colored compounds such as anthocyanins (Jurd, 1964), in three of the four experimental series the values obtained for a^* in wines from musts with SO_2 were not significantly lower than those of wines from fresh (or frozen/thawed) musts.

Regarding yellowness (b^*), for untreated musts its values were higher in frozen samples, although this increase was not found in irradiated musts. In Xarel·lo wines, no differences were found between the different treatments or between fresh and frozen samples. However, fermentation of frozen/thawed musts caused an important decrease of yellowness that was not found in the vinification of the fresh ones. If the b^* values of the wines from irradiated and SO_2-added musts are compared, the former are less yellow than the later in Parellada, but no significant differences were found in the case of Xarel·lo.

No remarkable differences were found between the absorbance spectra of the wines from the musts with SO_2 and the wines from irradiated musts, neither in the case of Xarel·lo nor in Parellada, both in fresh and in frozen and thawed samples.

3.4. Fermentation process and wine quality parameters

Fig. 2 shows the evolution of density during the fermentation of frozen and thawed Xarel·lo musts with the different kinds of treatment. Parellada frozen must and fresh musts from both varieties (data not shown) behaved in the same way, leading to analogous graphs. Irradiated samples fermented slower than non-irradiated and SO_2-added ones, probably due to the elimination of the natural microflora of the musts during this process. These

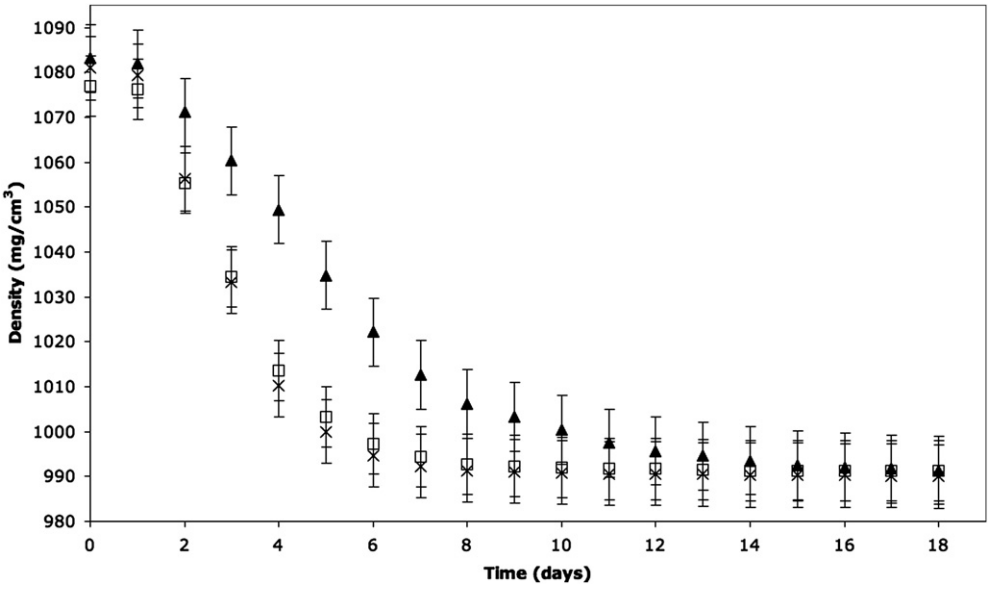

Fig. 2. Evolution of density during fermentation. Samples from frozen and thawed Xarel·lo musts: untreated. □ with added SO_2. ▲ irradiated.

Table 4
Wine quality parametes of samples from Xarel·lo and Parellada.

Variety	Must sample	pH (−)		Tartaric acid (g/L)		Alcohol (%v/v)		Volatile acidity (g/L)	
Xarel·lo (fresh must)	Fresh must	3.70	0.20^{b}	4.82	0.22^{a}	12.00	0.15^{ab}	0.18	0.05^{a}
	Must with SO_2	3.75	0.19^{bc}	5.02	0.20^{a}	12.44	0.44^{b}	0.19	0.07^{a}
	Irradiated must	3.98	0.14^{c}	4.48	0.16^{a}	12.19	0.33^{ab}	0.07	0.05^{a}
Xarel·lo (frozen/thawed must)	Frozen/thawed must	2.96	0.10^{a}	7.23	1.21^{c}	11.97	0.52^{ab}	3.52	1.11^{c}
	Must with SO_2	3.00	0.06^{a}	5.46	0.36^{b}	12.10	0.38^{ab}	1.44	0.31^{b}
	Irradiated must	2.92	0.02^{a}	7.47	0.40^{c}	11.63	0.47^{a}	1.73	0.84^{b}
Parellada (fresh must)	Fresh must	3.07	0.15^{r}	8.19	1.78^{r}	9.65	0.38^{s}	3.71	1.20^{r}
	Must with SO_2	3.23	0.13^{rs}	5.61	1.02^{s}	10.67	0.58^{t}	2.41	1.01^{s}
	Irradiated must	3.23	0.04^{rs}	5.29	0.26^{st}	10.52	0.53^{st}	2.20	0.26^{s}
Parellada (frozen/thawed must)	Frozen/thawed must	3.46	0.08^{t}	5.58	0.94^{s}	9.76	0.57^{st}	2.20	1.38^{s}
	Must with SO_2	3.25	0.12^{s}	5.03	0.70^{st}	9.82	0.83^{st}	1.18	0.93^{st}
	Irradiated must	3.56	0.09^{t}	3.97	0.24^{t}	8.24	0.71^{r}	0.62	0.14^{t}

Mean value standard deviation; different superscripts in a column (for each variety) indicate significant differences.

yeasts can also contribute in a definitive way to the fermentation, being able to start and develop a natural vinification (Bartowsky, 2009). No remarkable differences were found in density evolution between musts with SO_2 and untreated ones.

Quality parameters of the wines obtained from the musts with the different treatments are shown in Table 4. In the case of Xarel·lo, musts freezing and thawing led to wines with lower pH, higher tartaric acid content and higher volatile acidity, while no significant differences were found in alcohol content. However, none of these general trends were found in Parellada wines, where the pH was higher in wines from frozen/thawed musts and also in their irradiated counterparts, but not in those with SO_2. As far as the different treatments are concerned, in most of the studied cases wines from irradiated musts had a lower volatile acidity (an indirect measure of wine spoilage) than those from untreated musts, while no significant differences were found for this parameter between using UV–vis irradiation and SO_2. Although UV light has traditionally been reported to have detrimental effects on wine (Hartley, 2008), Rossi (1963) already found that acetification could be prevented in different kinds of wine through the application of UV irradiation at 253 nm and a residual amount of SO_2 gas, having negligible effects on quality (even when product exposure was exaggerated). However, in those experiments, irradiation was applied on wine (after fermentation), instead of must (before fermentation), and the effects on color parameters were not considered. Even though, this author (as well as Fredericks et al., 2011) also acknowledged the need for a residual use of SO_2 to ensure wine preservation.

Besides chemical parameters, assessing organoleptic quality is also essential to ensure the feasibility of applying this technology to winemaking. UV–vis irradiation is likely to affect aromatic compounds of musts, some of which are very important to the final profile of the resulting wine. Moreover, due to the long treatment time, taste could also be affected, and therefore new reactor configurations should be explored that allow reducing this time.

4. Conclusions

UV–vis irradiation has been proved to be effective in partially inactivating polyphenol oxidase and reducing volatile acidity (an indirect spoilage measure) in white wines from Xarel·lo and Parellada (when compared with untreated samples). No significant differences were found in quality parameters (pH, tartaric acid, alcohol content and volatile acidity) of wines obtained from musts that have been treated with SO_2 or with UV–vis irradiation. Meanwhile, wines from irradiated musts have a higher redness, a lower brightness and, in the case of Parellada, a lower yellowness than those with added SO_2. Must freezing before processing caused some variations in quality parameters in Xarel·lo wines, but not in those made from Parellada.

Since PPO residual activity is still important after UV–vis irradiation, an additional hurdle would be required to completely inactivate this enzyme and avoid color changes in the must during processing. In other words, this technology may be useful for reducing the amount of SO_2 used in winemaking, but a residual amount would be necessary. In addition, further research should be carried out in order to optimize the UV–vis irradiation process, in terms of time and dose, for each grape variety and used equipment.

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Replacement of SO_2 with an Unripe Grape Extract and Chitosan during Oak Aging: Case Study of a Sangiovese Wine

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Abstract: The aim of this study was to evaluate the chemical, microbiological and sensory characteristics of a Sangiovese wine aged in barrique with the addition of an unripe grape extract (UGE) as an alternative to sulfur dioxide. Three samples were considered: control wine (TQ) with free SO_2 of approximately 15 mg/L; sample A with chitosan (100 mg/L) and UGE (200 mg/L); and sample B with UGE (400 mg/L). The results achieved in this work demonstrated that the UGE, either alone or in combination with chitosan, was able to maintain the color characteristics of the Sangiovese wine and its sensory quality. Moreover, the addition of UGE contributed to an early and better stabilization of the color through the formation of polymeric pigments. The microbiological stabilization was comparable to SO_2 when UGE was used at 200 mg/L in combination with chitosan. The market survey conducted in the present study confirmed how the use of UGE as an alternative to sulfitation was positively accepted by consumers, who are increasingly attentive not only to the quality of the wines they select but also to the sustainability of the production processes from which they derive and to the fact that they are not harmful to human health.

Keywords: natural antioxidant; sulfur dioxide; chitosan; wine; color; copigmentation; yeasts; bacteria; economic sustainability

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1. Introduction

Sulfur dioxide is an additive traditionally used to preserve wine from oxidation and microbiological spoilage. Sulfur dioxide prevents the enzymatic and chemical oxidation of the product and inhibits both lactic acid and acetic acid bacteria, some spoilage yeasts such as *Brettanomyces* spp. and other non-*Saccharomyces* species that can be found in grapes and wine [1,2]. The set of these properties contributes to maintaining the sensory quality of wine. Furthermore, sulfur dioxide is an inexpensive chemical additive. For these reasons, it is difficult to find efficient alternatives to sulfur dioxide. However, sulfur dioxide has been associated with several human health risks, including headaches, dermatitis, hives, abdominal pain, bronchoconstriction and anaphylaxis [3]. Over the past few years, consumer demand for wines free of potentially harmful compounds has increased, and several studies investigated the effectiveness of alternative additives [4]. The attention to wines without added sulfur dioxide has increased with the significant rise in the global demand for organic wines. In some markets, such as the US, the product that wants to be claimed as "100% organic" must always also be "sulfite free"; moreover, the characteristic "no added sulfites" is no longer an extra distinctive element but an indispensable feature for wines of higher quality segments.

As alternatives to sulfur dioxide, in order to achieve microbiological stability of the wine, the OIV (Organisation Internationale de la Vigne et du Vin) and the EU have approved various physical and chemical methods [5]. Indeed, some oenological products

already available on the market have properties that can contribute to reducing the dose of sulfur dioxide added to wine. Oenological tannins are, for example, plant extracts from different botanical origins (oak gall, chestnut, quebracho, acacia, grape seeds and skins, tara, and mimosa) with interesting protecting properties against wine oxidation [6]. These compounds are extensively used due to the fact of their efficiency, mainly when they are added to the wine together with sulfur dioxide. However, the tannins doses normally used do not provide protection against pollutant microorganisms. Indeed, the phenolic compounds naturally occurring in grapes and wines, display various inhibiting or stimulating effects on the growth of lactic acid bacteria, depending on the polyphenols chemical structure and concentration, whereas the influences of phenolic compounds on yeasts growth have not been evaluated [5].

Recently, new phenolic extracts from various sources (black radish, almond peel and eucalyptus leaves) and from wine byproducts (vine shoots, skins and stems) have been proposed as potential substitutes of sulfur dioxide, with encouraging results [7–12]. The studies conducted with extracts obtained from the byproducts of the wine industry are particularly interesting, because they tend toward a model of regenerative production. However, few of these studies investigated the antimicrobial effect of the innovative extracts and their influence on wine sensory characteristics.

In the wine industry, cluster thinning is a green pruning practice carried out to improve grapes' quality at harvest. Thinning is often performed for high-quality wines according to the well-accepted idea that high crop yields delay ripening and reduce fruit and wine quality [13]. Unripe grapes (UGs) removed from the vine during thinning are usually abandoned in the field. Previous studies showed that UGs can be extracted and used as antioxidant and antibacterial additives or as fortifying agents in different types of food and beverages [14–20]. The properties of UGs are related to their richness of antioxidants, including phenols, stilbenes, glutathione, and vitamins. Organic acids are very concentrated in the UGs while sugars are low. In general, the concentration of these compounds depends on several factors, such as variety, vintage, climatic conditions, and stage of berry development [21].

At the market level, the use of UGE needs to be evaluated considering the growing demand for sustainable wines capable of safeguarding both the environment and consumer health. By reducing the inputs and waste occurring during wine production, the UGE offers an alternative that generates important process and product innovations, inspired by the principles of the circular economy [22]. The consumers could perceive wines with UGE addition as more sustainable [23], penetrating the niche of organic, biodynamic and natural wines leading them to be more willing to pay a higher price for such wines [24,25] as they perceive a better product both for the environment preservation and for their own health in terms of the so-called lifestyles of health and sustainability (LOHAS) [26].

In this work, an UGE was used as a stabilizing additive during the aging of a Sangiovese wine in comparison with sulfur dioxide. The chemical, microbiological and sensory characteristics of the wines were evaluated over twelve months of aging in barrique. Furthermore, an economic evaluation was also carried out for the entire UGE production and utilization activity, considering the costs that such innovation generates.

2. Materials and Methods

2.1. Unripe Grape Extracts

The extract was obtained from UGs (cv Sangiovese), following the method described in previous works [15,27]. The unripe grapes were handpicked, destemmed and crushed. Then, the grapes were extracted by maceration at 6 °C, for a period of 96 h, during which dry ice was added. The extract was racked and then decanted for 48 h, at 6 °C. Filtration was performed in order to remove large particles (i.e., 1 mm or more in diameter). The sugars were eliminated from the extract by ultrafiltration with the aid of a spiral wound configuration membrane with a molecular weight cut-off of 2500 Da (General Electric, Boston, MA, USA). After, the liquid extract was added with Arabic gum (2% w/v) (Nexira

Food, Rouen Cedex, France) and then lyophilized. The dried extract was stored under vacuum in polyethylene pouches at room temperature and protected from the light.

2.2. Winemaking Process

The Sangiovese grapes were hand-harvested from the vineyards of Castello di Gabbiano (Treasury Wine Estate group) in 2019 and vinified at the cellar of the same farm using a stainless-steel vat with a capacity of 25 hL. After destemming and crushing, the must was added with 30 mg/L SO_2. The must was inoculated with 20 g/hL of rehydrated active dried yeast (LALVEN ICV D254^{TM}, Lallemand, Verona, 37060, Italy). Alcoholic fermentation and maceration were conducted at 30 °C for 7 days, and two pump-overs were performed daily. On the second day of fermentation, 40 g/hL of yeast nutrient (Nutriferm Ultra, Enartis) were added. At the end of fermentation (residual sugar less than 2 g/L), the pomace was pressed with a pneumatic press, and the press wine up to 1.8 bars was added back to the free run wine. The wine was transferred to a stainless-steel vat until the spontaneous malolactic fermentation (FML) was completed. At the end of the FML, the chemical parameters of the wine were measured: total acidity, 5.15 g/L (expressed as tartaric acid); pH 3.60; volatile acidity, 0.47 g/L (expressed as acetic acid); sugars, 0.10 g/L; alcohol degree, 14.55%; dry extract, 29.40 g/L; free sulfur dioxide, 8 mg/L; total sulfur dioxide, 20 mg/L; and lactic acid, 0.8 g/L. Then, the wine was racked and transferred in barrique for aging. All the barriques (Tonnellerie Baron, Les Gondes, France) used for the study were made with French oak. The barriques were two years old and medium toasted. Three samples were set-up in duplicate: TQ (15 mg/L free SO_2, 140 mg/L Arabic gum); A (200 mg/L UGE, 100 mg/L chitosan); and B (400 mg/L UGE). After approximately 4 months of aging, the level of free sulfur dioxide of the TQ samples was checked and adjusted to approximately 15 mg/L, while 200 and 400 mg/L of UGE were added to samples A and B, respectively. Chemical, microbiological and sensory analyses were performed at the start (T0) and after 3 (T3), 6 (T6) and 12 (T12) months of aging in barrique.

2.3. Chemicals

All reagents and solvents were purchased from Sigma-Aldrich (Milan, Italy), except for methanol and ethanol, which were supplied by Carlo Erba (Milan, Italy). Potassium metabisulphite (Enartis), Chitosan Micro M (Enartis) and Oenogom instant (Laffort) were purchased from the market.

2.4. General Analysis

The total acidity, volatile acidity, pH, alcohol, total and free SO_2, lactic acid and dry extract were evaluated according to the methods recommended by the International Organization of Vine and Wine (OIV) [28].

2.5. Color Intensity and Hue

The color intensity (CI) and hue (Hue) of the wine were measured using a Perkin Elmer Lambda 10 spectrophotometer (Waltham, MA, USA), following the method described by other authors [29]. The absorbance (A) at 420, 520 and 620 nm was measured, and the CI and Hue were calculated as follows: CI = (A420 +A520 + A620) × 10 and H = A420/A520.

2.6. CIELab Chromatic Characteristics

The CIELab space was used to evaluate the color of the red wine. The following parameters were calculated: lightness from black to white (L*), color from blue to yellow (b*), color from red to green (a*), chroma or saturation (C*) and hue angle (H*), following the method OIV-MA-AS2-11: determination of chromatic characteristics according to CIELab [30].

2.7. Copigmentation

The copigmentation was evaluated according to the method proposed by Boulton et al. [31]. The following parameters were considered: color due to the fact of copigmented anthocyanins (AC); color fraction due to the fact of copigmentation (COP); color due to the presence of total anthocyanins (TAs); color fraction due to the presence of free anthocyanins (AL); color due to the fact of polymeric pigments (Eps); color fraction due to the fact of polymeric pigments (PPs); flavanol cofactor index (FC); and total phenol index (TPI280).

2.8. Total Phenols

The total phenols (TPs) were quantified according to the Folin–Ciocalteu method [32]. The phenolic compounds were removed from 1 mL of wine and a 10% UGE solution with a C18 Sep-pak cartridge (Waters, Milan, Italy) [33]. A volume of 4 mL of sodium carbonate (10%, *w*/*v*) was added to 1 mL of sample and left to stand for 5 min. A volume of 1 mL of diluted Folin–Ciocalteu reagent was added to the mixture. Then, the samples were left in the dark for 90 min at room temperature. After, the absorbance (700 nm) was measured with a Perkin Elmer Lambda 10 spectrophotometer (Waltham, MA, USA). Solutions of (+)-catechin ranging from 5 to 500 mg/L were used as references. The TPs content of the samples was expressed as mg of (+)-catechin equivalents (mg CAT eq)/g of UGE or (mg CAT eq)/L of wine.

2.9. DPPH

The antioxidant activity (AA) was determined by a 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [34]. For the reaction, 0.1 mL of the sample was mixed with 3.9 mL of the DPPH solution (6×10^{-5} M), prepared in methanol. A volume of 0.1 mL of the sample was added to 3.9 mL of methanol, as the reference. To obtain the maximum absorbance of DPPH, 0.1 mL of methanol was mixed with 3.9 mL of a 6×10^{-5} M DPPH solution. After 30 min at 30 °C in the dark, the decrease in the absorbance at 515 nm of the reaction mixture was measured against the methanol reference sample. Trolox standard solutions at concentrations ranging from 10 to 600 µmol/L were prepared in absolute ethanol. The antioxidant activity is expressed as µmol of Trolox/L of wine.

2.10. FRAP

The antioxidant activity was measured using the FRAP assay [35]. Trolox solutions, in the range of 0.05–1.18 mM, were prepared and assayed for the calibration curve. The FRAP reagent (acetate buffer: 300 mM:2,4,6-tris(2-pyridyl)-s-triazine 9.99 mM:FeCl3·6H2O 20 mM) (10:1:1)) was prepared. For the sample, 150 µL wine sample, diluted at 1:15 or 1:25 with methanol, or 150 µL of the Trolox standard solutions were mixed with 2.85 mL of FRAP reagent. All the mixtures were left for 30 min. Then, the absorbance was measured spectrophotometrically at 595 nm. The antioxidant activity is expressed as µmol of Trolox/L of wine.

2.11. ABTS

The antioxidant activity was measured by the ABTS method [36]. A 7 mM 2,2′-azinobis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution with the addition of potassium persulfate (2.45 mM) was prepared. The solution was left in the dark for 16 h. A calibration curve, ranging from 0.05 to 2.11 mM, was prepared from a 5 mM stock solution of Trolox. For sample, the wine, previously diluted at 1:7 or 1:15 with methanol, and 30 µL of the Trolox standard solutions were mixed with 2.97 mL of a ABTS•+ radical cation solution. After 30 min in the dark, the absorbance of the reaction mixture was spectrophotometrically measured at 734 nm. The antioxidant activity is expressed as µmol of Trolox/L of wine.

2.12. Microbiological Analysis

The quantification of yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) was carried out by plate counts and selective culture media. The yeasts were enumerated

on WL nutrient agar (Oxoid) [37], containing sodium propionate (2 g/L) and streptomycin (0.3 g/L). Lactic acid bacteria were quantified on MRS ISO agar (Oxoid) [38], with the addition of fructose (5 g/L), cysteine (0.5 g/L), tomato juice broth (2.5 g/L), agar (6 g/L) and pimaricin (0.05 g/L). The acetic bacteria were quantified on LF Agar Medium (glucose, 10 g/L; yeast extract, 5 g/L; peptone, 5 g/L; tomato juice broth, 2 g/L) with the addition of pimaricin (0.05 g/L) and penicillin (0.025 g/L). To enumerate *B. bruxellensis*, a representative number of colonies showing a green color, round morphology, and yellow halo were picked up and analyzed using RFLP-PCR of rITS for their identification [39].

2.13. Sensory Evaluation

A total of 30 subjects (56% women; aged between 22 and 50 years; mean age: 32.1 ± 7.8 years) were recruited among students and employees of the Faculty of Agriculture and Food Sciences of the University of Milan (Italy). Only subjects who like and regularly consume wine and were free of food intolerances and allergies were involved. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Milan (protocol code: 30/21; date of approval: 18 March 2021). Informed consent was obtained from all subjects involved in the study.

The wine samples were subjected to a triangle method [40]. The participants attended four sessions (T0, T3 T6 and T12) at the Sensory and Consumer Science Laboratory (SCS_Lab) of the Department of Food, Environmental and Nutritional Sciences of the University of Milan, designed according to ISO guidelines [41]. The evaluations were conducted between 11:30 am and 13:30 pm, and the judges were asked to consume only water and not smoke during the 2 h before the evaluation.

During the evaluations, the following comparisons were made: TQ vs. A and TQ vs. B at the four times of aging (T0, T3, T6 and T12). The judges were presented with three samples simultaneously (two equal) and were asked to identify within each triad which sample was perceived as different considering all the sensory characteristics related to sight, smell, taste, flavor and body. To minimize adaptation, a 3 min break occurred between triads and panelists were instructed to take additional breaks when they desired. The samples were swallowed and retasting was permitted. The evaluation was carried out in a single run, and the presentation sequences of the samples were randomized judge by judge. The samples were stored at room temperature away from light and heat sources and served in glass goblets encoded with three-digit numbers. Each glass was fitted with a top cap to avoid the dispersion of volatile compounds. The presentation order of the treatment comparisons was counterbalanced across panelists, and the sample presentation was randomized within triads.

Instructions were provided to the judges regarding the evaluation procedure in both written and verbal formats. They were instructed to focus on all sensory characteristics evaluating the samples one at a time, keep the samples capped when not being tasted, proceed at their own pace, and to cleanse the palate with natural mineral water and crackers to desaturate the sensory receptors between samples.

The session took approximately 30 min. The data acquisition was performed with Fizz v2.31 software (Biosystèmes, Couternon, France).

2.14. Economic Evaluation

An economic analysis was developed to evaluate how close to market is the use of the UGE. This economic analysis was carried out both at the company and market levels. The company analysis concerned the effects that the process innovation introduced had in terms of organization and the internal management of production costs. The cost analysis was carried out with the analytic accounting methodology of full costing, considering all expenses (fixed–variable, direct–indirect and explicit–figurative) sustained in the production and use of UGE. With regard to the market analysis, a direct survey was conducted on a significant number (1000) of wine consumers in Italy. The direct survey, conducted over the period 2020–2021, started from the analysis of consumer behavior

compliant with sustainable lifestyles in terms of LOHAS: subjects with a high level of LOHAS were recruited as targets for wines without added sulfites, testing their willingness to pay for such a product.

2.15. Statistical Analysis

All analyses were conducted in triplicate. The statistical analysis was carried out using the XLSTAT statistical and data analysis solution (2021) (Addinsoft, Paris, France). The analysis of variance (ANOVA) (least significant differences (LSD): 5% level) and the Tukey's least significant difference (LSD) test were conducted for each chemical and microbiological variable in order to assess the significant differences among wines (p-value < 0.05). For the sensory evaluation, the number of correct responses (identification of the different sample in the triad) was counted, and the significance was determined using a minimum number of correct responses (ISO 4120:2021). If the number of correct responses is greater than or equal to the tabulated number, a perceptible (p-value < 0.05) difference exists between the samples.

3. Results and Discussion

3.1. Chemical Analyses

The phenolic concentration of UGE was 20.4 mg CAT eq/g of powder and the antioxidant activity, evaluated using the DPPH method, was 33.8 µmol Trolox/g. In this work, the doses of phenols added to the wine were chosen on the basis of the results of previous studies in which the antioxidant effect of the phenolic extracts obtained from the viticultural byproducts was evaluated on the must and wine [9,16]. The UGE was added to the wine at doses of 200 and 400 mg/L providing approximately 4 (sample A) and 8 mg (sample B) of phenols/L of wine, respectively. Previous studies showed that UGEs contained high levels of organic acids, and their composition may include glutathione and water-soluble vitamins, which could contribute to the antioxidant activity of the extracts [15,16]. The same authors tested the UGEs as an alternative to sulfur dioxide to protect the color of red wine from oxidation during aging in steel tanks, with promising results [21]. However, microbiological wine spoilage during oak aging is particularly feared. For this reason, chitosan (100 mg/L) was added as a microbiological stabilizer in sample A, which received the lowest dose of UGE.

Table 1 shows the initial composition (T0) of each wine treated with different stabilizing agents. At the start (T0), immediately after the addition of SO_2, UGE and chitosan, some color parameters of the samples showed small but significant differences. In particular, samples A and B showed values of Abs520, Abs520% and CI that were significantly higher than that observed in the TQ. In addition, the CIELab analysis showed that samples A and B had the following characteristics: (i) the lowest L* values, related to the darkness of the color; (ii) the highest level of a*, related to the red shades of the color; (iii) the highest C* values, related to the saturation of the color. Hence, these results indicate that sample TQ was less colored, saturated, and lighter than the other samples. These results are compatible with the bleaching effect of sulphur dioxide on the anthocyanins of red wine [42,43]. Regarding the copigmentation parameters, the samples did not show significant differences except for the cofactor content (FC) that was the lowest in sample TQ. Previous results showed that extracts from unripe grapes are rich in flavonoids [14,15]. In the A and B samples, the addition of UGE could have contributed to cofactor content in terms of flavonoids which are cofactors in the copigmentation reaction [31]. At the start, the total phenol content (TP) evaluated by the Folin–Ciocalteu method was not significantly different among the samples studied (Table 1). The antioxidant activity of the different samples was measured by the DPPH, FRAP and ABTS methods (Table 1).

Table 1. Chemical parameters of the wine samples at the start (T0) after the addition of different stabilizing products.

	TQ	A	B	F-Value
Abs420	3.43 ± 0.01 c	3.68 ± 0.01 b	3.73 ± 0.02 a	749.65 ***
Abs520	4.88 ± 0.02 b	5.42 ± 0.01 a	5.43 ± 0.01 a	2067.66 ***
Abs620	1.03 ± 0.01 c	1.12 ± 0.01 b	1.14 ± 0.00 a	810.00 ***
ABS420%	36.70 ± 0.08 a	36.03 ± 0.07 c	36.22 ± 0.09 b	98.73 ***
Abs520%	52.26 ± 0.08 c	52.99 ± 0.10 a	52.66 ± 0.08 b	93.22***
Abs620%	11.02 ± 0.04 b	10.98 ± 0.03 b	11.11 ± 0.01 a	27.07 ***
CI	9.34 ± 0.03 c	10.23 ± 0.02 b	10.31 ± 0.03 a	1846.91 ***
Hue	0.702 ± 0.003 a	0.680 ± 0.003 c	0.682 ± 0.003 b	98.90 ***
L*	73.50 ± 0.38 a	70.99 ± 0.11 b	71.23 ± 0.21 b	164.18 ***
a*	27.92 ± 0.28 b	30.92 ± 0.15 a	30.90 ± 0.17 a	388.31 ***
b*	2.09 ± 0.09 a	2.23 ± 0.15 a	2.05 ± 0.11 a	3.27 (*)
C*	28.01 ± 0.28 b	31.02 ± 0.14 a	30.97 ± 0.16 a	404.00 ***
H*	13.31 ± 0.63 a	13.89 ± 1.08 a	13.77 ± 0.23 a	1.07
AC	0.79 ± 0.03 a	0.79 ± 0.06 a	0.83 ± 0.02 a	2.11
COP	18.43 ± 0.78 a	18.03 ± 1.19 a	18.73 ± 0.33 a	1.04
TA	1.85 ± 0.15 a	1.93 ± 0.01 a	1.97 ± 0.01 a	2.46
AL	43.06 ± 3.65 a	44.19 ± 0.74 a	44.18 ± 0.48 a	0.54
Ep	1.66 ± 0.14 a	1.651 ± 0.004 a	1.65 ± 0.01 a	0.02
PP	38.50 ± 3.32 a	37.77 ± 0.45 a	37.08 ± 0.20 a	0.80
FC	6.84 ± 0.11 b	6.93 ± 0.05 ab	7.00 ± 0.03 a	6.77 **
TPI280	54.50 ± 0.21 a	54.22 ± 0.11 b	53.99 ± 0.13 b	14.29 ***
TP	2.50 ± 0.13 a	2.55 ± 0.06 a	2.58 ± 0.07 a	0.95
DPPH	22,052 ± 2211 a	22,891 ± 1221 a	24,076 ± 1400 a	0.68
FRAP	23,677 ± 178 a	24,091 ± 2102 a	24,009 ± 2291 a	0.06
ABTS	31,842 ± 386 a	30,770 ± 3048 a	30734 ± 270 a	0.20

All parameters are given as the mean ± standard deviation. Treatments: TQ (SO2); A (UGE, 200 mg/L and chitosan); B (UGE, 400 mg/L). The different letters indicate significant differences among the samples according to the Tukey's least significative difference (LSD) test. Significant values are shown according to the following: (*) p-value < 0.1; ** p-value < 0.01; *** p-value < 0.001.

In agreement with the results obtained by other authors, the highest values of antioxidant activity were found when the ABTS method was used, followed by FRAP and DPPH [9]. However, when the samples were studied with the same method, there were no significant differences in the antioxidant activity among the samples studied.

Table 2 shows the final composition (after 12 months) of the different wine samples that were studied. After twelve months of aging, the wine samples showed differences in some color and copigmentation parameters, while the total phenol content and antioxidant activity were similar (Table 2). In particular, the TQ showed the lowest Abs520 and CI values and the highest L* value, indicating that TQ was slightly less colored and lighter than the other samples. According to the present results, other authors who analyzed the color parameters of red wines elaborated with stem or shoot extracts as alternative antioxidants to SO_2 found that wines treated with SO_2 had significantly higher luminosity values (L*) and lower color intensity than the wines elaborated with the alternative antioxidants [44,45]. The hue of color, b* and H* of the samples were similar at this stage of aging, highlighting that the wine color reached a similar level of oxidation.

A copigmentation analysis provides information concerning the evolution of wine color. Copigmentation is a complex phenomenon influenced by the composition of wine in term of anthocyanins, cofactors, metals, ethanol, and pH [46–48]. After twelve months (T12), the samples with the UGS showed significant differences for almost all copigmentation parameters when compared with sample TQ. Sample TQ showed the highest values of color due to the copigmented anthocyanins (C), color fraction due to the copigmentation (COP) and color fraction due to the fact of free anthocyanins (AL) and the lowest values of color due to the fact of polymeric pigment (Ep) and color fraction due to the fact of polymeric pigment (PP) and cofactor content (FC). There were some small but significant

differences between sample A and sample B. In particular, sample B, having received the highest dose of UGE, showed higher AC and COP and lower Ep and PP values than sample A. A similar effect of the natural antioxidant extracts used in comparison with SO_2 was highlighted by other authors [45]. These authors found that the red wine treated with stem or shoot extracts had significantly higher polymerization percentage values than the wine treated with SO_2. The formation of polymeric pigments, normally accompanied by a decrease in free anthocyanins, contributes to the stabilization of red wine color [6,31].

Table 2. Chemical parameters of the wine samples aged for twelve months (T12) in the presence of different stabilizing products.

	TQ	A	B	F-Value
Abs420	4.00 ± 0.09 b	4.32 ± 0.12 a	4.30 ± 0.11 a	11.60 **
Abs520	4.77 ± 0.08 b	4.99 ± 0.02 a	4.95 ± 0.05 a	16.81 ***
Abs620	1.20 ± 0.01 b	1.37 ± 0.05 a	1.34 ± 0.03 a	25.37 ***
ABS420%	40.09 ± 0.71 a	40.42 ± 0.41 a	40.58 ± 0.53 a	0.75
Abs520%	47.83 ± 0.81 a	46.71 ± 0.70 a	46.75 ± 0.70 a	3.10 (*)
Abs620%	12.07 ± 0.12 b	12.85 ± 0.30 a	12.66 ± 0.16 a	18.69 ***
CI	9.99 ± 0.11 b	10.69 ± 0.21 a	10.60 ± 0.16 a	28.84 ***
Hue	0.83 ± 0.03 a	0.86 ± 0.02 a	0.86 ± 0.02 a	1.75
L*	74.58 ± 0.02 a	73.75 ± 0.41 b	74.05 ± 0.47 ab	6.19 *
a*	21.55 ± 0.37 b	22.03 ± 0.05 a	22.22 ± 0.04 a	44.35 ***
b*	9.98 ± 0.47 a	9.80 ± 1.35 a	9.77 ± 0.61 a	0.09
C*	23.66 ± 0.15 b	24.40 ± 0.52 a	24.44 ± 0.29 a	20.96 ***
H*	2.11 ± 0.15 a	2.04 ± 0.33 a	2.03 ± 0.14 a	0.21
AC	0.63 ± 0.01 a	0.33 ± 0.03 c	0.47 ± 0.07 b	66.86 ***
COP	13.57 ± 0.11 a	10.01 ± 0.24 c	11.62 ± 0.32 b	251.60 ***
TA	1.54 ± 0.10 a	1.41 ± 0.09 a	1.42 ± 0.12 a	2.16
AL	35.88 ± 0.48 a	32.46 ± 0.33 b	32.72 ± 1.63 b	19.97 ***
Ep	2.265 ± 0.002 c	2.57 ± 0.01 a	2.528 ± 0.004 b	3383.12 ***
PP	48.88 ± 0.07 c	56.68 ± 0.35 a	54.70 ± 0.41 b	954.49 ***
FC	7.44 ± 0.04 b	7.93 ± 0.03 a	7.91 ± 0.03 a	309.03 ***
TPI280	59.11 ± 0.46 b	60.58 ± 0.66 a	60.37 ± 0.45 a	14.33 ***
TP	2.88 ± 0.09 a	2.89 ± 0.13 a	2.99 ± 0.15 a	1.17
DPPH	15,931 ± 312 a	15,812 ± 606 a	15,812 ± 606 a	1.54
FRAP	21,514 ± 534 a	21,050 ± 243 a	20,902 ± 145 a	0.04
ABTS	27,343 ± 457 a	27,255 ± 431 a	27,340 ± 843 a	1.00

All parameters are given as the mean ± standard deviation. Treatments: TQ (SO2); A (UGS, 200 mg/L and chitosan); B (UGS, 400 mg/L). Different letters indicate significant differences among the samples according to the Tukey's least significative difference (LSD) test. Significant values are shown according to the following: (*) p-value < 0.1; * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001.

The evolution of total phenols and antioxidant activity during aging is showed in Figure 1. Over the course of twelve months very few significant differences in the antioxidant activity of the samples were detected. In particular, it is possible to note that after three months, samples A and B had a significantly lower concentration of total phenols in comparison to that of sample TQ. Moreover, at the start (T0) and after three months, the values of ABTS were significantly lower in sample B.

Accordingly, Salaha et al. [12] did not find significant differences in the antioxidant activity between wines of different varieties treated with normal doses of SO_2 and the same wines treated with reduced doses of SO_2 combined with black radish extracts (Rafhanus niger) and ascorbic acid.

The evolution of the TP (Figure 1a) of all the wine samples showed a positive trend, mainly from the third month. In general, the TP content of the wines increased over aging due to the fact of contact with toasting oak [44]. The antioxidant activity evaluated by DPPH, FRAP and ABTS (Figure 1b–d), in general, decreased from the start to the end of the aging period. The highest decrease was observed in the antioxidant activity assayed by the DPPH method. The antioxidant properties of the wines, measured by the DPPH, FRAP

and ABTS methods, during the aging period (Figure 1) indicated that the addition of UGE (400 mg/L) or UGE (200 mg/L) and chitosan (100 mg/L) can provide a level of protection from oxidation similar to that exerted by SO_2. It is well documented that the antioxidant activity of phenol compounds can be related to different properties, including the capacity of scavenging superoxide radicals, consuming dissolved oxygen, reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) and chelating Fe^{2+} (6).

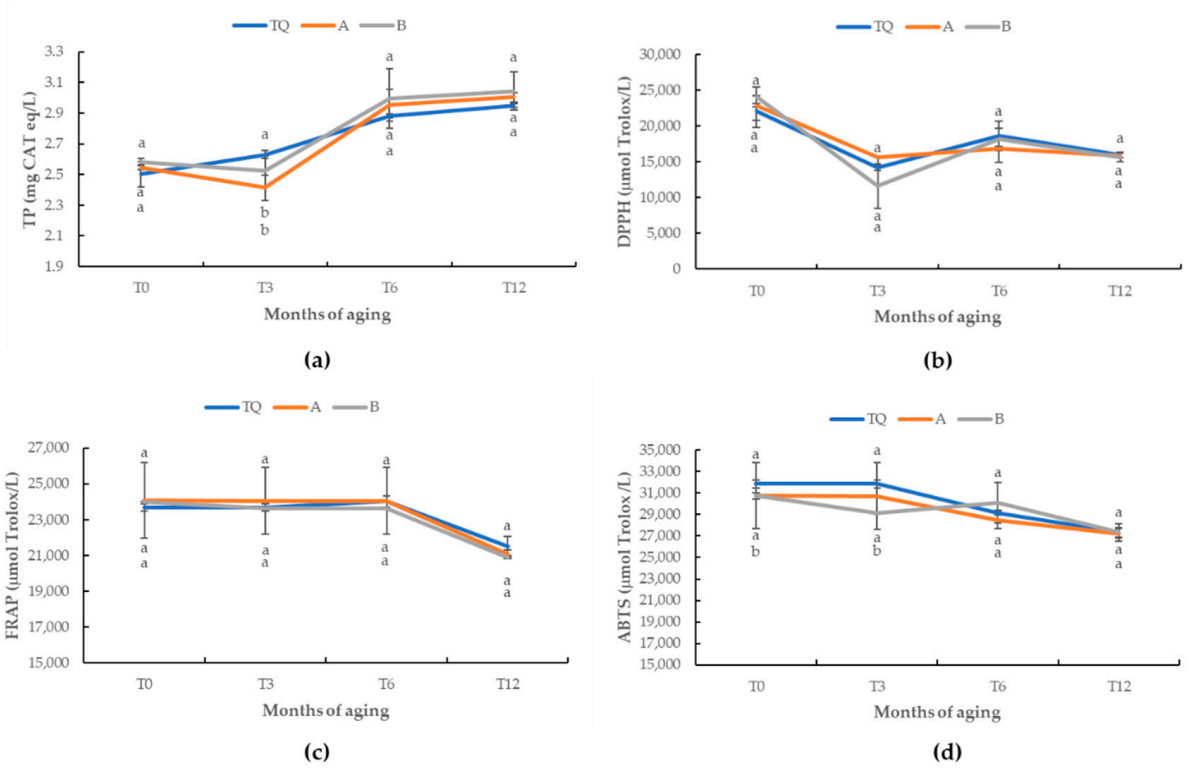

Figure 1. Evolution of antioxidant activity during the aging period (12 months) in the wine treated with different stabilizing products: (**a**) total phenols (TP); (**b**) DPPH values; (**c**) FRAP values; (**d**) ABTS values. Treatments: TQ (SO2); A (UGE, 200 mg/L and chitosan); B (UGE, 400 mg/L). Different letters indicate significant differences among the samples according to the Tukey's least significative difference (LSD) test.

The evolution of some parameters of the wine treated with different stabilizing products during the aging period is shown in Figure 2. The sample TQ had the significantly lowest CI and highest L* values at all stage of aging (Figure 2a,c). These results were a consequence consistent with the further addition of SO_2 made at the fourth month of aging, and its effect of bleaching on anthocyanins [42,43]. The differences in the AL, COP and PP content among the samples were detectable as early as the third month, and they were maintained until the end of aging (Figure 2). The TQ samples had the significantly highest AL and lowest PP values from T3 to T12 (Figure 1f,h). Moreover, the COP values of sample TQ were significantly the highest from the third month to the end of aging (Figure 1g). During this period, the rate of stable polymeric pigments (PP) formation in samples A and B was higher than that of TQ and was accompanied by a decrease in free anthocyanins (AL). These results highlighted that the addition of UGE could contribute to a faster stabilization of color. Hence, at the same stage of evolution, the wine aging with the addition of UGE contained more stable pigments towards oxidation phenomena in comparison to those of wine sample (TQ) aging with SO_2 [31,47].

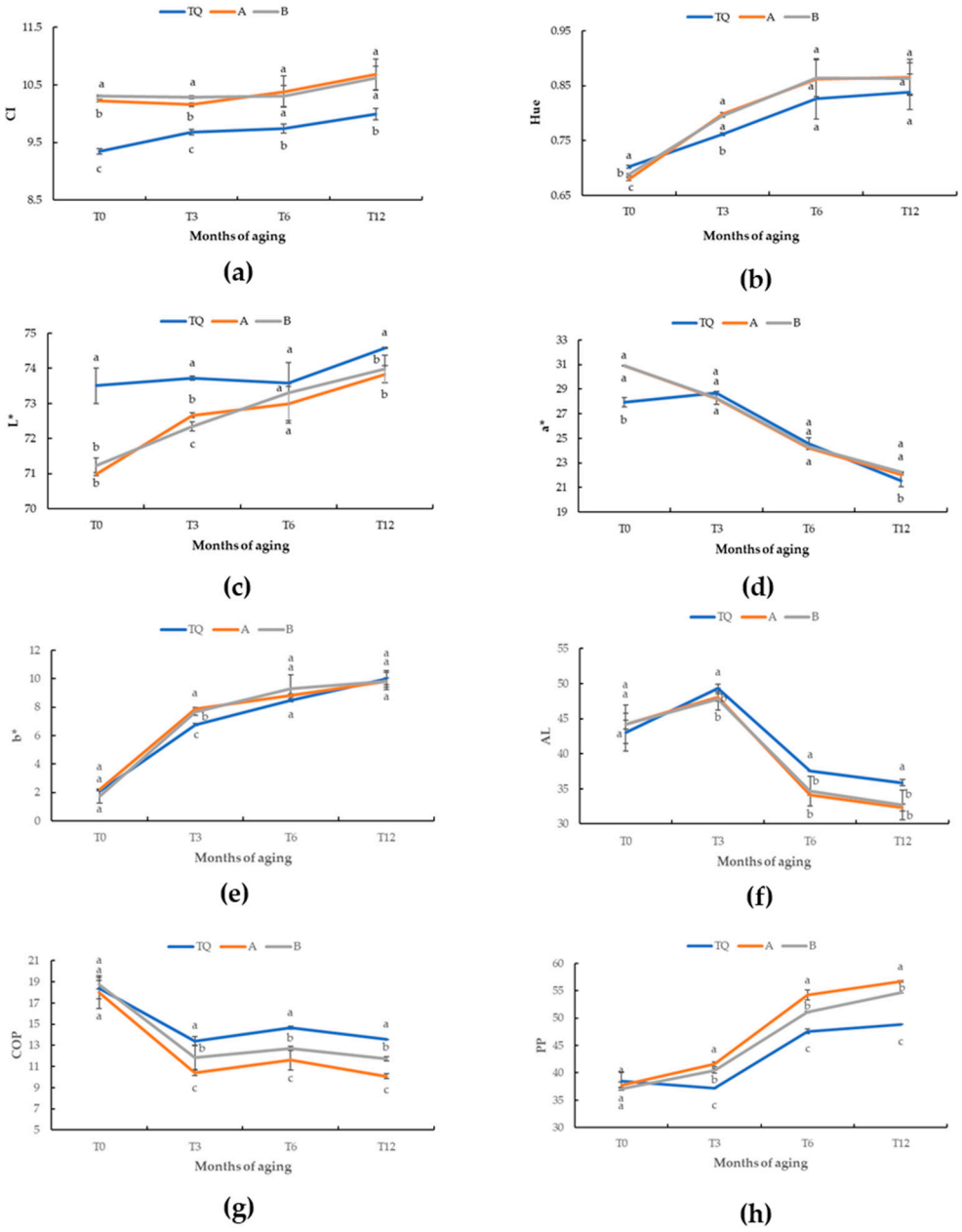

Figure 2. Evolution of several parameters during the aging period (12 months) in the wine treated with different stabilizing products: (**a**) color intensity (CI); (**b**) color hue (Hue); (**c**) lightness (L*); (**d**) red/green (a*); (**e**) blue/yellow (b*); (**f**) color fraction due to the fact of free anthocyanins (AL); (**g**) color fraction due to the fact of copigmentation (COP); (**h**) color fraction due to the fact of polymeric pigments (PP). Treatments: TQ (SO_2); A (UGE, 200 mg/L and chitosan); B (UGE, 400 mg/L). Different letters indicate significant differences among the samples according to the Tukey's least significative difference (LSD) test.

3.2. Microbiological Analysis

Microbial populations occurring in Sangiovese wines at the beginning and after 3, 6 and 12 months of aging are shown in Table 3. At the start, after the accomplishment of the malolactic fermentation, the concentrations of yeasts and acetic acid bacteria (AAB) were below the limit of detection, while lactic acid bacteria (LAB), belonging to the *Oenococcus oeni* species, attained concentrations between 10^4 and 10^5 UFC/mL that are values usually found at the end of malolactic fermentation [2]. During the aging period, in the control wine (TQ) treated with sulphur dioxide as well as in the A and B wines containing UGE at

different concentrations, LAB decreased to cell densities below the limit of detection except in the sample B after 12 months of aging in which they attained at 10^4 UFC/mL. On the contrary, after 3, 6 and 12 months of aging in barriques, the yeast populations increased both in the TQ wine and in the treated wines. In particular, after 3 months (T3) the concentration of yeasts in the TQ wine was significantly higher than in the A and B wines. However, in the TQ and A samples, the yeast population consisted exclusively of the *Saccharomyces cerevisiae* species, whereas in the B sample in which UGE was added at the highest concentration (400 mg/L) but not chitosan, *Brettanomyces bruxellensis* occurred, although at a very low concentration, corresponding, on average, to 36 CFU/mL. After 6 (T6) and 12 months (T12) of aging, the yeast concentrations showed no significant differences in the control wine and in two treated wines. The presence of *B. bruxellensis* was confirmed only in the B wine, where it reached a maximum cell density of 125 and 25 CFU/mL after 6 and 12 months of aging, respectively. Although these values were below the threshold of 10^3 UFC/mL that is considered critical for the production of ethyl phenols [49], they could represent a potential cause of spoilage. On the contrary, in the A sample containing UGE and chitosan, as well as in the TQ sample containing sulfur dioxide, *B. bruxellensis* was not detected. Hence, according to these findings, the addition of UGE at 400 mg/L contributed to the microbiological stability of Sangiovese wine by controlling the bacteria population growth until the sixth month of aging, similar to 15 mg/L sulphur dioxide. For a longer aging time, the use of UGE at 200 mg/L in combination with chitosan demonstrated its effectiveness in controlling both *B. bruxellensis* and bacteria populations.

Table 3. Yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) occurring in wines at 0, 3, 6 and 12 months of aging. Treatments: TQ (SO_2); A (UGE, 200 mg/L and chitosan); B (UGE, 400 mg/L). All parameters are given as the mean ± standard deviation. Different letters indicate significant differences among the samples (ANOVA; $p < 0.05$).

Aging (Months)	Sample	Yeasts (UFC/mL)		LAB (UFC/mL)		AAB (UFC/mL)
T0	TQ	<20		$(2.06 \pm 0.65) \times 10^5$	a	<10
	A	<20		$(2.16 \pm 0.55) \times 10^4$	b	<10
	B	<20		$(8.06 \pm 2.58) \times 10^4$	c	<10
T3	TQ	$(3.00 \pm 0.56) \times 10^2$	a	<10		<10
	A	$(1.00 \pm 0.50) \times 10$	b	<10		<10
	B	$(5.33 \pm 0.58) \times 10$ §	c	<10		<10
T6	TQ	$(1.30 \pm 0.28) \times 10^2$	a	<10		<10
	A	$(2.45 \pm 0.77) \times 10^2$	a	<10		<10
	B	$(7.75 \pm 4.86) \times 10^2$ †	a	<10		<10
T12	TQ	$(6.39 \pm 9.70) \times 10^3$	a	<10		<10
	A	$(6.35 \pm 5.66) \times 10^3$	a	<10		<10
	B	$(6.24 \pm 4.23) \times 10^3$ ‡	a	$(1.56 \pm 0.40) \times 10^4$		<10

§ 67% Brettanomyces bruxellensis; † 16% Brettanomyces bruxellensis; ‡ 0.4% Brettanomyces bruxellensis. Different letters indicate significant differences among the samples according to the Tukey's least significative difference (LSD) test.

3.3. Sensory Evaluations

The results of the sensory evaluations are shown in Table 4. As can be seen, at T0 no significant differences were observed between the pairs of samples that were considered. Therefore, both samples containing an increasing concentration of unripe grapes extract (A and B) were not perceived as different by the consumers compared to the wine sample containing sulfur dioxide (TQ). During aging, after three, six and twelve months, the differences between the wines were still not significant. This means that the panel of judges perceived these products as similar in terms of the sensory characteristics related to sight, smell, taste, flavor and body, suggesting a promising role in the substitution of sulfur dioxide in the winemaking process. On the contrary, previous results revealed that the use of

alternatives to SO_2 are responsible for important changes in sensory attributes. Among the possible natural alternatives, it has been recently suggested that natural extracts obtained from grape seeds and American oak wood by accelerated extraction with subcritical water did not cause sensory defects in wines and led to a greater aromatic complexity and were positively evaluated by consumers [45]. It has also been depicted that hydroxytyrosol, as an alternative to sulfur dioxide in Syrah red wines, led to significant differences in sensory analysis [7]. Indeed, the hydroxytyrosol addition affected the aroma intensity (black fruit and mature fruit) but without leading to any aromatic defects (such as yeast, chemical and rancid aromas). Accordingly, some authors described a greater intensity of the olfactory descriptor in red wines elaborated with a colloidal silver complex instead of SO_2 [50]. Similarly, Santos et al. [51] demonstrated that high-pressure treatments as an alternative to SO_2 of red wines significantly altered aroma and taste perception.

Table 4. Triangle method results for the pairs of Sangiovese wine samples analyzed at 0, 3, 6 and 12 months of aging.

Aging (Months)	Sample	Correct Responses	*p*-Value
T0	TQL–AL	11	0.41 n.s.
	TQL–BL	8	0.83 n.s.
T3	TQL–AL	12	0.20 n.s
	TQL–BL	8	0.75 n.s.
T6	TQL–AL	14	0.09 n.s.
	TQL–BL	8	0.83 n.s.
T12	TQL–AL	9	0.98 n.s.
	TQL–BL	5	0.71 n.s.

n.s., not significant.

3.4. Economic Evaluation

With regard to the economic evaluations, the results obtained at the farm level show that the extract can be easily implemented in a winery without any particular initial investment, relying on resources (i.e., capital and labor) normally available in any cellar. The exception is the drying process, for which external services must be used.

The full cost analysis carried out for the case study examined in this paper revealed that the farm, in order to produce the necessary UGE by itself, has to incur a total cost ranging from 0.79 to 1.18 per gram of obtained dried antioxidant extract. As explained in the Section 2.14, this cost was calculated by examining the entire process of producing the extract, starting from harvesting the unripe grapes to obtaining the final product; the process inputs (i.e., capital and labor) were considered for each operation, and the direct and indirect costs to be incurred by the entrepreneur for their use were quantified. The total cost range was mainly due to the dry extract yield, which can range from 20 to 30 g per liter of processed liquid extract.

The full cost analysis carried out for the case study revealed a total cost for the production of UGE ranging from EUR 0.79 to 1.18 per gram of dried antioxidant extract; the range was mainly due to the dry extract yield, which can range from 20 to 30 g per liter of processed liquid extract.

Given that 400 and 800 mg/L UGE were used for samples A and B, respectively, the cost of producing and adding UGE ranges from EUR 0.31–0.47 per liter of wine for sample A and EUR 0.63-0.94 per liter for sample B (Table 5).

These additional costs, which are minimal in absolute terms, are even smaller considering that the innovation examined in this study is mostly intended for medium–high quality wines, targeting consumers who select the product with a good willingness to pay, preferring sustainable and healthy wines. Regarding these last two aspects, the market survey carried out in the present study showed that more than 89% of respondents had a lifestyle particularly focused on personal health and sustainability (LOHAS); a wine

without added sulfites, perceived as "healthier" and environmentally friendly, is the perfect synthesis to respond to these market trends and satisfy this broad target.

Table 5. Total cost of the production and use of UGE per sample and the different dry extract yield per liter of liquid extract.

	Yield	
	20 g	**30 g**
	EUR/L	
Sample A	0.47	0.31
Sample B	0.94	0.63
	EUR/bottle (0.75 L)	
Sample A	0.36	0.24
Sample B	0.71	0.47

Respondents expressed a clear willingness to pay a premium price for a wine without added sulfites, especially if it is also organic: the direct survey reveals that this premium price is 8% for conventional NSA wines, while this value rises to over 13% if the wine is also organic.

4. Conclusions

The results of the present study suggest that UGE could be applied as promising alternatives to sulfur dioxide in the winemaking processes. Indeed, UGE either alone or in combination with chitosan was able to maintain the color characteristics and contributed to an early and better stabilization of the color through the formation of polymeric pigments of the Sangiovese wine. No significant differences in the antioxidant activity after 3, 6 and 12 months of aging were found, and the samples added with different UGE concentrations were perceived as comparable to the wine sample containing sulfur dioxide (TQ) in terms of sensory quality. The microbiological stabilization was comparable to SO_2 when UGE was used at 200 mg/L in combination with chitosan. The market survey conducted in the present study confirmed how the use of UGE as an alternative to sulfitation was positively accepted by consumers, who are increasingly attentive not only to the quality of the wines they select but also to the sustainability of the production processes from which they derive and to the fact that they are not harmful to personal health. The substitution of SO_2 by UGE should be carried out in future studies taking into consideration the vintage effect in wines.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data is contained within the article.

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Suitability of ultraviolet-C irradiation for white grape must

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Abstract

Background and Aims: Ultraviolet (UV)-C irradiation is used for the reduction of microbial spoilage in food. Its effectiveness for the treatment of white grape must has been investigated considering different microbial species and modification of must composition.

Methods and Results: Static and dynamic laboratory-scale systems emitting at 254 nm were used for the treatment of *Vitis vinifera* cv. Chardonnay and cv. Crimson grape juices of variable turbidity. The must samples were singularly inoculated with a pure culture of wine spoilage strains belonging to three species, *Dekkera bruxellensis*, *Acetobacter aceti* and *Lactobacillus brevis*, and treated with UV-C ranging from 300 to 1800 J/L. Cell counts and microbial reduction (derived from α values, according to the Weibull model), colour change, polyphenol oxidase (PPO) inactivation and formation of α-dicarbonyl compounds were evaluated. After treatment at the maximum UV-C dosage under static conditions, reductions of yeast counts of 4.91–5.99 $\log_{10}$ colony-forming units (CFU)/mL and bacterial counts of 4.12–5.05 $\log_{10}$ CFU/mL were achieved, depending on the strain being tested. Polyphenol oxidase activity decreased by up to 7.8 1.4% with variation attributed to must turbidity, which indicated that clarification of must before UV-C treatment can lead to PPO inactivation. No significant variation in the colour or the concentration of α-dicarbonyl compounds was detected. Results were confirmed for grape juice with the continuous flow apparatus.

Conclusions: The UV-C systems achieved microbial reduction without formation of oxidative compounds, suggesting that the experimental conditions employed did not cause any noticeable oxidative phenomena.

Significance of the Study: The UV-C treatment of grape juice can be considered a potential alternative to sulfur dioxide addition for processing of white must. Improved efficacy of treatment can be achieved by increasing the flow rate and by clarification of must, making the system potentially applicable under industrial conditions.

Keywords: *chemical oxidative markers, microbial reduction, polyphenol oxidase activity, UV-C radiation, white grape must*

Introduction

Winemaking is a well-known process where the fermentative capabilities of yeast and bacteria influence the quality of the end product (Ribéreau-Gayon et al. 2006). Grapes and must are contaminated naturally by different microbial populations that can be responsible for undesirable secondary transformations; in particular, non-*Saccharomyces* yeasts, acetic acid bacteria (AAB), lactic acid bacteria (LAB) and moulds. The metabolic activities of these microorganisms can negatively affect wine quality as a consequence of increased volatile acidity, the release of off-flavours, changes to viscosity or colour and/or the formation of haze or precipitation (Du Toit et al. 2006, Fugelsang and Edwards 2007, Bartowsky and Henschke 2008). Beside the growth of undesirable microorganisms, white must can undergo oxidation due to the activity of grape-derived polyphenol oxidase (PPO). This enzyme can oxidise hydroxycinnamoyltartaric acids to *o*-quinones, which are highly reactive compounds and known precursors of the polymers responsible for browning of must (Cheynier and Ricardo da Silva 1991, Macheix et al. 1991).

To counteract the proliferation of spoilage species, the addition of sulfur dioxide (SO_2) is frequently carried out to inhibit microbial growth (Boulton et al. 1996, Vigentini et al. 2013). Moreover, this routine winemaking practice also prevents enzymatic and chemical oxidation (Fugelsang and Edwards 2007). Sulfur dioxide can, however, be detrimental to human health (Vally and Thompson 2001, Pozo-Bayón et al. 2012). As such, SO_2 up to a concentration of 200 mg/L is permitted in white wines, but it is mandatory for 'contains sulfites' to be specified on labelling when the concentration exceeds 10 mg/L (European Commission 2009). In recent years, there has been increasing interest from wine producers to find alternative strategies for managing both microbial spoilage and oxidation, so as to limit the use of SO_2. UV radiation is widely used in food processing for reducing microbial contaminants (Bintsis et al. 2000). Irradiation wavelengths in the range of 100–280 nm (UV-C) have been proven to have microbicide power when applied to surfaces or clear liquid foods (Guerrero-Beltrán and Barbosa-Cánovas 2006, Hadjock et al. 2008, Keyser et al. 2008, Koutchma et al. 2016). Dispersed solids can limit the efficacy of UV-C treatment because they act as a shielding material. Microbial reduction is achieved by photolysis, resulting in DNA modification being induced by UV-C exposure, which prevents cell duplication (Thompson 2003).

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Irradiation with UV-C has also been used during processing of coloured and turbid liquids, such as apple juice (Falguera et al. 2011), grape must (Fredericks et al. 2011, Falguera et al. 2013) and wine (Matias et al. 2016, Mijowska et al. 2017). The impact of UV treatment, however, needs further investigation, especially the resistance of different microbial strains and the potential for activation of oxidative reactions. Nevertheless, Rizzotti et al. (2015) reported that UV-C irradiation was effective at reducing the microbial counts present in wine by up to five log colony-forming units (CFU)/mL.

This study aimed to evaluate laboratory-scale irradiation systems allowing the treatment of grape must through measured UV-C dosage in order to investigate the inactivation rate of different wine spoilage microorganisms present in the wine environment. In addition, the influence of UV-C irradiation on chemical oxidative markers known to affect wine quality (Müller et al. 2014) were evaluated.

Materials and methods

Samples

Grape juice samples were obtained from *Vitis vinifera* cv. Chardonnay and cv. Crimson, both harvested in 2013 and 2015 vintages. Chardonnay must (50 L), free from treatment with SO_2, was obtained from a private company located in Franciacorta area (Lombardia, Italy) using industrial grape pressing conditions. The must was allowed to settle for 12 h at 4 C before being stored at −18 1 C until usage.

A laboratory prepared Crimson grape juice (20 L) was obtained by manual pressing of grapes under N_2 flow and by clarification under N_2 flow by storage overnight at 4 C. The clarified must was then stored at −18 1 C until usage. Grape lees were frozen and later used to adjust the turbidity of must samples. The chemical composition of the must samples was determined in an accredited laboratory (Enoconsulting, Erbusco, Brescia, Italy) using official methods of the Organisation Internationale de la Vigne et du Vin (OIV) (Table 1) (Organisation Internationale de la Vigne et du Vin 2010, 2015).

Microbial strains and culture media

Two yeast strains belonging to *Dekkera bruxellensis* (CBS2499, CBS74^T) and four bacterial strains ascribed to *Acetobacter aceti* (DSM3508^T and DSM2002) and *Lactobacillus brevis* (DSM20054^T, UMB7308) were selected for investigation, with the aim being to calculate their survival rate under different UV-C irradiation conditions. Yeast cells were cultured in yeast extract peptone dextrose (YPD) medium according to previous methodology (Vigentini et al. 2016); while AAB were grown in yeast peptone mannitol (YPM) medium (5 g/L yeast extract, 3 g/L peptone, 25 g/L mannitol, pH 6.2) and LAB were grown in Lactobacilli de Man, Rogosa and Sharpe (MRS) broth (Difco, Becton Dickinson, Franklin Lakes, NJ, USA). All strains were maintained as pure concentrated cultures and were stored at −80 C in their corresponding medium with the addition of 20% (v/v) glycerol.

Preparation of the inoculum and microbiological analyses

Fresh cells were prepared for the controlled contamination of must samples. They were revitalised from −80 C glycerol stocks by inoculating at 1% (v/v) in a suitable medium dependent on the strain being tested. Briefly, *D. bruxellensis* and *A. aceti* strains were grown under aerobic conditions at 25 C for 72 and 96 h, respectively. *Lactobacillus brevis* strains were cultivated under anaerobic conditions (GasPak System, BBL, Becton Dickinson) at 30 C for 48 h. The must samples were inoculated at a starting concentration of approximately 1×10^7 CFU/mL, with cells in late exponential phase. The optical density of fresh cell cultures was measured at 600 nm with a spectrophotometer (Jenway, UV–visible spectrophotometer, model 7315, Bibby Scientific, Stone, England). Plate counts were carried out in the aforementioned media, with the addition of 18 g/L (w/v) agar (Merck, Darmstadt, Germany). Aliquots (100 µL) of appropriate decimal dilutions were prepared in sterile Peptone Water (peptone 10 g/L, pH 6.5) and spread on agar plates; colonies were counted following incubation as outlined in OIV-MA-AS4-01 (Organisation Internationale de la Vigne et du Vin 2010).

Equipment used for UV-C irradiation

Grape must was treated with two UV-C systems: one involving static conditions and one employing dynamic conditions. The first apparatus comprised a 20 W low-pressure mercury vapour lamp (HER 357 model, ADP, Peschiera Borromeo, Milan, Italy) emitting at 254 nm. The emission intensity of the applied radiation was constantly and accurately monitored with an HD2021T.4 sensor (DeltaOhm Caselle, Selvazzano, Padova, Italy). The sensor had a detection range from 2 to 200 W/m^2, with a linear output of 4–20 mA, recorded by means of an HP 34970A data logger (Agilent Technologies, Santa Clara, CA, USA), interfaced with software that recorded the energy emitted during treatment (Benchlink data logger, Agilent Technologies). The apparatus also comprised a sanitising chamber (200 mm in length, with a 20 mm radius) positioned between the cylindrical quartz sleeve (5 mm radius) of the lamp and an external coaxial cylinder made from polymethylmethacrylate, with an internal volume of 80 mL and a thickness of 5 mm; the must was loaded by means of a duct (Figure 1). Part of the lamp remained external to the chamber for the continuous monitoring of UV-C dosage by the sensor connected to the data acquisition device. As the UV-C energy used during treatment was known and the current intensity that penetrated

Table 1. Mean values (SD) of the composition for must samples treated with UV-C irradiation under static and dynamic conditions.

Grape cultivar	Vintage†	Sugars (g/L)‡		pH		TA (g/L tartaric acid)§		Turbidity (NTU)¶		OD_{254nm}	
Chardonnay	2013	187	19	3.20	0.05	6.8	0.4	76	4	1.28	0.08
	2015	193	19	2.80	0.05	7.5	0.3	17	1	1.67	0.1
Crimson	2013	180	9	3.60	0.05	2.1	0.2	21	2	2.75	0.15
	2015	207	12	4.10	0.05	1.7	0.1	101	4	4.18	0.18

†Must samples prepared from fruit harvested in 2013 were treated using apparatus operating under static conditions, while samples prepared from fruit harvested in 2015 were treated under dynamic conditions. ‡MA-AS311-01A (Organisation Internationale de la Vigne et du Vin 2010). §MA-AS313-01 (Organisation Internationale de la Vigne et du Vin 2010). ¶MA-AS2-08 (Organisation Internationale de la Vigne et du Vin 2010). OD, optical density.

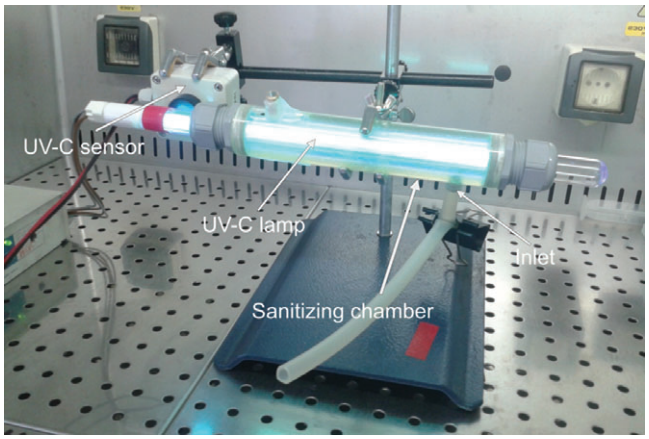

Figure 1. UV-C irradiation apparatus developed for laboratory scale treatment of wine under static conditions.

through the section profile could be measured, it was possible to set the UV-C irradiation according to the volume of the medium, instead of by area, as previously reported by other authors (Fredericks et al. 2011, Rizzotti et al. 2015, Matias et al. 2016). The output current was transformed into the relative UV-C power (W) and then divided by the volume of sample being treated (W/L). The dosage was calculated in real-time considering the applied time for the treatment (1 W*s/L = 1 J/L).

The second apparatus consisted of a sanitising chamber positioned between a quartz glass (10 mm radius) which contained a 70 W cylindrical quartz lamp (HER 668 model, ADP, Peschiera Borromeo, Milan, Italy) emitting at 254 nm and with an external coaxial stainless steel cylinder of 14.5 mm radius; the internal thickness was of 4.5 mm. The flow of liquid inside the chamber was achieved with a peristatic pump (MasterFlex L/S, Cole-Parmer, Vernon Hills, IL, USA) so as to avoid contact with air.

Microbial inactivation by UV-C treatment

For the treatment of grape juice under static conditions, 500 mL samples of must were first heat treated in a water bath at 85 C for 1 min, to inactivate any residual microbial populations. Samples were immediately chilled in ice and maintained at 4 C until inoculation. Microorganisms were inoculated, one at a time, immediately before the UV-C irradiation. Microbial suspensions were then subjected to UV-C irradiation at 0, 300, 600, 900, 1200, 1500 or 1800 J/L, with each treatment in triplicate. Microbial counts were also made on untreated must. Microbiological data were analysed with SPSS software (Win 12.0 program, SPSS, Chicago, IL, USA) to perform linear and non-linear regression analysis and modelling. The mathematical model of inactivation that gave the best interpolation of experimental data points was evaluated using the adjusted determination coefficient (R^2 adj) and mean square error (MSE) values, according to the approach outlined by Taze et al. (2015). Plate count values (CFU/mL) were transformed in decimal logarithm to match the normal distribution of data. The UV-C dosage required to attain a decimal reduction in viable count was calculated as the reciprocal of the slope of regression curve in case of the linear model (D value), and as the α value in the case of the Weibull model.

For UV-C treatment under dynamic conditions, must samples were first centrifuged at 5000 × *g* for 30 min at 4 C, to settle grape solids and most microorganisms. The level of residual microbial contamination in the supernatants was assessed by plate counts. The efficacy of microbial inactivation achieved using the prototype operating under dynamic conditions was evaluated as follows. Must samples were co-inoculated with three microbial strains (one for each species), with the strains chosen having shown the highest resistance to UV-C radiation in the experiments carried out with the apparatus operating under static conditions. The inoculated musts were then irradiated at 1800 J/L, with each treatment in triplicate. To determine the effect of must turbidity on microbial inactivation, the turbidity of samples was increased from 17 1 to 212 4 Nephelometric Turbidity Units (NTU), by adding the grape lees collected after settling, prior to inoculation.

Evaluation of colour changes in must due to UV-C treatment

The colour of must samples was evaluated using the CIE L*a*b* system by measuring reflectance with a Lambda 650 spectrophotometer (PerkinElmer, Waltham, MA, USA), with white used as the reference colour. The L*, a* and b* values were determined by the software Color (PerkinElmer), setting the observer at 10 and the light source D65. The values of the Chroma (Equation 1), hue angle (Equation 2) and ΔE (difference between two colours) (Equation 3) were also calculated. Colour measurements were carried out in triplicate on both untreated and UV-C (1800 J/L) treated must samples.

$$Chroma = \sqrt{a^{*2} \times b^{*2}} \quad (1)$$

$$Hue\ angle = arctg\ \frac{a^*}{b^*} \quad (2)$$

$$\Delta E = \sqrt{(L^*_{UM} - L^*_{TM})^2 + (a^*_{UM} - a^*_{TM})^2 + (b^*_{UM} - b^*_{TM})^2} \quad (3)$$

where *UM* and *TM* denote untreated and UV-C treated must, respectively.

Assessment of PPO activity in must

The inactivation of the PPO enzyme was evaluated by measuring the increase in absorbance at 400 nm, using catechol (Sigma-Aldrich, St Louis, MO, USA) as a substrate. One unit (U) of PPO was defined as the amount of enzyme leading to an increase of one absorbance unit at 400 nm in 10 min (Rapeanu et al. 2005, Ülker-Yerlitürk et al. 2008, Falguera et al. 2011). The determination of PPO was carried out in triplicate on both untreated and UV-C (1800 J/L) treated must samples, prepared from 2013 Chardonnay grapes. To determine the effect of UV-C treatment on PPO inactivation, the laboratory must prepared in the laboratory from 2013 Crimson grapes was subjected to UV-C irradiation at 0, 300, 600, 900, 1200, 1500 or 1800 J/L, and PPO activity determined, with each treatment in triplicate.

The inactivation of PPO was also determined after treatment of must prepared from 2015 Chardonny and Crimson grapes under dynamic conditions. Must samples were also treated with UV-C irradiation after turbidity was increased to 252 6 NTU following addition of grape lees, in a manner similar to that outlined above for the microbial reduction assays.

Quantification α-dicarbonyl compounds in must

Chemicals and reagents, such as 2,3-pentanedione, 2,3-diaminobenzene, sodium hydroxide, barium chloride

and methanol, were purchased from Sigma-Aldrich. The concentration of α-dicarbonyl compounds was determined spectrophotometrically (Lambda 25, PerkinElmer) after their reaction with 2,3-diaminobenzene under basic conditions, as described by the OIV-MA-AS315-20:R2010 method (Organisation Internationale de la Vigne et du Vin 2010), with some modifications. Barium chloride was added to 100 mL grape must to a final concentration 3.2 g/L, which was then centrifuged at 5000 × *g* for 10 min (Sorvall Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA). A 20 mL aliquot of the supernatant was then eluted through a solid phase extraction cartridge packed with 1 g of C18 (Strata C18-T, Phenomenex, Torrance, CA, USA). The eluted must (10 mL) was placed in a tube, to which 2,3-diaminobenzene (5 mg) was added, after pH adjustment to 8.0 by addition of 1 mol sodium hydroxide. The tube was closed using a screw-cap fitted with a Teflon-faced seal and mixed until complete dissolution of the reagent was achieved. The mixture was then stored at 60 C for 3 h, before being cooled in an ice bath. The cooled mixture was filtered (0.22 µm polyvinylidene difluoride (PVDF) membrane Millipore, Bedford, MA, USA) and the total amount of α-dicarbonyl compounds was determined spectrophotometrically by measuring the absorbance at 313 nm (Lambda 25, PerkinElmer). The spectrophotometric data were acquired and processed with a UV-Winlab data processor and viewer (PerkinElmer). The total amount of α-dicarbonyl compounds was quantified using an external standard method at 313 nm. The calibration curve was obtained by spiking grape must with 2,3-pentanedione at 5.0–30.0 mg/L. The method showed a linear response and gave a high correlation coefficient (r = 0.959). The limit of detection andquantification was 0.75 and 2.5 mg/L, respectively. Recovery was assessed in triplicate in grape must spiked with 15 mg/L 2,3-pentanedione and was found to be 99%.

Grape must was not heat-treated prior to quantification of α-dicarbonyl compounds. The determinations were carried out in triplicate on both untreated and UV-C (1800 J/L) treated must samples prepared in triplicate.

Statistical analysis

The SPSS software was used for all statistical analysis. Statistical differences between untreated and UV-C treated musts in terms of colour change, PPO inactivation and of the concentration of α-dicarbonyl compound in both static and dynamic prototypes, and the microbial reductions obtained with the static versus dynamic prototypes were determined by the *F* test, and were considered significant at $P < 0.05$. The same software was used to perform linear and non-linear regression analysis and modelling of microbiological data, in particular, to determine values of α and β parameters from the Weibull model.

Results and discussion

Development of laboratory-scale prototypes for the UV-C treatment of grape must

First, an apparatus operating under static conditions for treatment of liquid was developed by optimising size, lamp power and the minimum thickness allowing penetration of UV-C radiation through the entire must sample. The penetration profile of UV rays in Chardonnay grape must (76 4 NTU) was determined using quartz cuvettes, with an optical path length of 1, 5 and 10 mm (Snowball and Hornsey 1988, Lodi et al. 1996) and the relevant attenuation of current was monitored with the HD2021T.4 sensor connected to the data acquisition device. As expected, the signal decreased as the optical length increased, and no UV-C radiation could be recorded when the optical length exceeded 5 mm. For this reason, a maximum thickness of 5 mm was established. Furthermore, as shown in Figure 2, no variation in lamp emission was found throughout five replicated cycles of irradiation, except during the first test, when the lamp was cold. The lamp emission can be affected by its temperature, which can increase during usage; consequently, the UV-C radiation cannot be accurately predicted (Lau et al. 2009). A rise in temperature might influence the efficacy of UV-C treatment. As such, temperature was measured, to identify any effect related to variation in temperature during UV-C treatment. Temperature changes were negligible (+2 C) and, consequently, temperature was not expected to influence any of the parameters investigated, that is microbial reduction, inactivation of PPO, change in colour or the formation of α-dicarbonyl compounds.

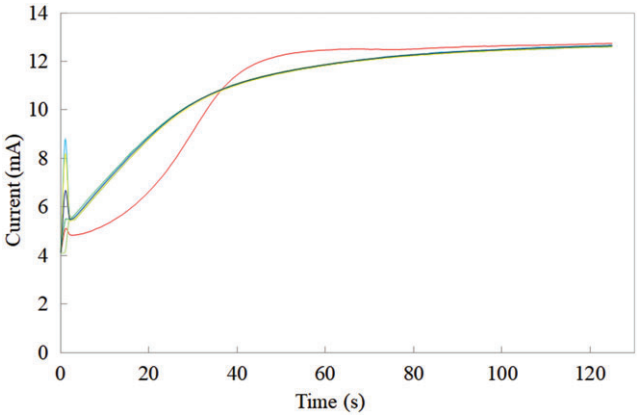

Figure 2. Preliminary tests, Test 1 (——), Test 2 (——), Test 3 (——), Test 4 (——) and Test 5 (——), showing the kinetics of the lamp radiation intensity over time; the average value, which excluded Test 1 (i.e. when the lamp was cool), is also shown (——).

Microbicide effectiveness of the UV-C treatments

The fungal and bacterial counts found in the 2013 Chardonnay grape must following heat treatment were both <1 $\log_{10}$ CFU/mL, demonstrating that preliminary processing operations were effective.

Must samples with a turbidity level of 76 4 NTU were inoculated with one of two yeast strains or four bacterial strains, and subjected to UV-C treatment using the static prototype, at six dosage values, ranging from 300 to 1800 J/L. A non-linear, inverse relationship was observed between the supplied energy and the number of surviving cells. Convex curves were shaped in the inactivation diagrams obtained by interpolation of experimental points expressed as $\log_{10}$ CFU/mL plate counts (Figure 3). The values of adjusted determination coefficients and mean square errors were higher when the Weibull model was used in comparison to the linear model, indicating that the former better described the data trends (Table 2). Consequently, α values obtained with the Weibull model were considered to estimate the log microbial reduction. Figure 3 also shows the fit of the Weibull and linear first-order models.

As expected, the greatest decrease in viability was observed at the dosage of 1800 J/L, where the calculated

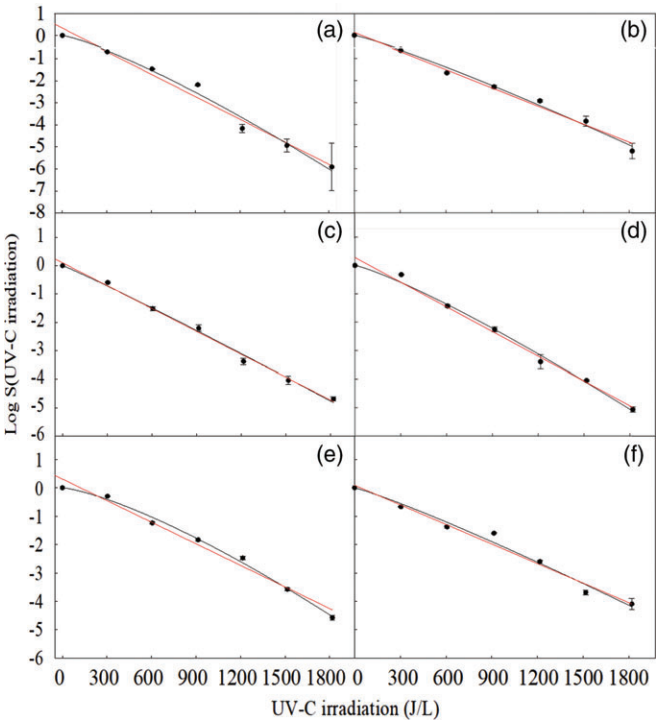

Figure 3. Microbial inactivation of (a) *Brettanomyces bruxellensis* CBS2499, (b) *Brettanomyces bruxellensis* CBS74^T, (c) *Acetobacter aceti* DSM3508^T, (d) *Acetobacter aceti* DSM2002, (e) *Lactobacillus brevis* DSM20054^T and (f) *Lactobacillus brevis* DSM7308, in white must treated with UV-C irradiation at variable dosage (0–1800 J/L). The black line was plotted by the Weibull model (——); the red line was plotted by linear model (——).

reduction in microbial counts reached 4.91–5.99 $\log_{10}$ CFU/mL and 4.12–5.05 $\log_{10}$ CFU/mL for yeast and bacteria, respectively (Table 3). Taking into account that the microbial count in must after grape crushing is usually 3–4 $\log_{10}$ CFU/mL (Fugelsang and Edwards 2007, Vigentini et al. 2016), these results indicate that the species studied can be successfully inactivated by UV-C treatment at 1800 J/L, under real conditions. As deduced by the calculated D values, the yeast strain that was most sensitive to UV-C irradiation was *D. bruxellensis* CBS2499, while *A. aceti* DSM3508^T strain proved to be less resistant to UV-C treatment among the bacterial species (Table 2). These results confirm that the detrimental effect of UV-C exposure is species-related, as previously reported (Fredericks et al. 2011, Mijowska et al. 2017).

In order to compare the microbial reduction achieved in the current study with that observed by Fredericks et al. (2011), the $\log_{10}$ CFU/mL reduction of some microorganisms was calculated for the same UV-C dosages reported by Fredericks et al., using data obtained from the Weibull model (α values) we obtained (Table 3). For treatments on yeast: UV-C irradiation of *B. bruxellensis* ISA 1649 at 459 J/L gave 0.47 $\log_{10}$CFU/mL in unclarified Chenin Blanc (Fredericks et al. 2011), whereas in the current study, irradiation of *D. bruxellensis* CBS2499 and CBS74^T at the same UV-C dosage, gave 1.10 and 1.03 $\log_{10}$ CFU/mL, respectively. When UV-C irradiation was applied at 918 J/L microbial reduction for *B. bruxellensis* ISA 1649 was 1.24 $\log_{10}$ CFU/mL (Fredericks et al. 2011), compared with 2.59 and 2.27 $\log_{10}$ CFU/mL for *D. bruxellensis* CBS2499 and CBS74^T in the current study. The highest UV-C treatment of *B. bruxellensis* ISA 1649, being 1377 J/L, employed by Fredericks et al. (2011), achieved 1.83 $\log_{10}$ CFU/mL reduction, whereas that calculated for *D. bruxellensis* CBS2499 and CBS74^T in the current study was 4.29 and 3.61 $\log_{10}$ CFU/mL, respectively. These outcomes and the relevant α values indicate a strain-specific resistance to UV-C exposure by yeast cells (Table 2). Indeed, a significantly different dosage of UV-C radiation was required to induce a

Table 2. Mean values (SD) for parameters from the Weibull and linear models†.

Species	Strain	Weibull model						Linear model			
		α (J/L)		β (shape)		R^2 adj‡	MSE	D (J/L)		R^2 adj	MSE
Dekkera bruxellensis	CBS2499	427	50a	1.25	0.12	0.986	0.297	294	7a	0.973	0.370
	CBS74^T	449	41b	1.15	0.09	0.988	0.196	357	22c	0.983	0.233
Acetobacter aceti	DSM3508^T	412	30a	1.06	0.06	0.993	0.145	345	20b	0.993	0.145
	DSM2002	462	34c	1.19	0.07	0.992	0.169	370	12 cd	0.989	0.201
Lactobacillus brevis	DSM20054^T	588	31e	1.34	0.07	0.994	0.130	385	30d	0.977	0.253
	DSM7308	511	55d	1.13	0.11	0.978	0.218	435	21e	0.976	0.238

†Values followed by different letters within columns indicate a significant difference among strains ($P < 0.05$). ‡Adjusted determination coefficient. α, slope of regression curve in case of the linear model; β, shape parameter; D, slope of regression curve in case of the linear model; MSE, mean square error.

Table 3. Mean microbial counts obtained for selected strains following UV-C treatment under static conditions†.

Species	Strain	Initial counts ($\log_{10}$ CFU/mL)		$\log_{10}$ reduction (300 J/L)		$\log_{10}$ reduction (600 J/L)		$\log_{10}$ reduction (900 J/L)		$\log_{10}$ reduction (1200 J/L)		$\log_{10}$ reduction (1500 J/L)		$\log_{10}$ reduction (1800 J/L)	
Dekkera bruxellensis	CBS2499	7.27	0.07	0.65	0.12	1.53	0.16	2.53	0.15	3.61	0.10	4.77	0.02	5.99	0.12
	CBS74^T	7.29	0.05	0.63	0.09	1.40	0.11	2.22	0.10	3.08	0.06	3.98	0.01	4.91	0.08
Acetobacter aceti	DSM3508^T	7.25	0.15	0.72	0.07	1.49	0.08	2.29	0.07	3.10	0.04	3.92	0.00	4.76	0.06
	DSM2002	7.19	0.05	0.60	0.07	1.37	0.09	2.21	0.09	3.12	0.05	4.07	0.01	5.05	0.07
Lactobacillus brevis	DSM20054^T	6.97	0.11	0.41	0.05	1.03	0.07	1.77	0.07	2.60	0.05	3.50	0.01	4.47	0.05
	DSM7308	7.15	0.11	0.55	0.10	1.20	0.12	1.89	0.11	2.61	0.07	3.36	0.01	4.12	0.08

†Values are means of three replicates ($n = 3$) SD. CFU, colony-forming unit.

90% reduction in the populations of *D. bruxellensis* CBS2499 (α = 427 50 J/L) and CBS74^T (α = 449 41 J/L) strains (P = 0.030). Despite differences in experimental conditions, the variation in resistance to UV-C irradiation observed by *D. bruxellensis* strains may be associated with the recognised genetic and physiological intraspecific polymorphism found in this species (Agnolucci et al. 2009, Hellborg and Piškur 2009, Vigentini et al. 2012).

Similar findings were observed for treatments on bacteria (Table 3); *L. brevis* species showed $\log_{10}$ CFU/mL reduction comparable to that reported for irradiation of *L. plantarum* 130 inoculated into Shiraz juice by Fredericks et al. (2011). A UV-C dosage of *L. plantarum* at 918 J/L resulted in a $\log_{10}$ CFU/mL reduction of 1.86, while the estimated reduction for *L. brevis* DSM20054^T and DSM7308 was 1.82 and 1.93 $\log_{10}$ CFU/mL, respectively. After UV-C irradiation at 1377 J/L, however, *L. plantarum* 130 gave a 3.38 $\log_{10}$ CFU/mL reduction, which was significantly different (P = 0.005) from the reduction calculated for *L. brevis* DSM20054^T and DSM7308, being 3.12 and 3.05 $\log_{10}$ CFU/mL, respectively.

The AAB and LAB species showed similar strain-related behaviour, with significant variations in α values being detected (P = 0.001 for *A. aceti* species and P = 0.015 for *L. brevis* species) (Table 2). In this study and according to data published in the literature, Gram positive bacterial cells exhibit greater resistance to UV-C radiation than Gram negative bacteria (Thompson 2003, Guerrero-Beltrán and Barbosa-Cánovas 2006). *Lactobacillus brevis* DSM20054^T proved to be the least sensitive to UV-C treatment among the microorganisms studied, and required a UV-C dosage of 588 31 J/L to reduce cell concentration by one log cycle.

The mould, yeast, LAB and AAB counts for the 2015 Chardonnay grape must used in trials carried out with the dynamic protoype, before inoculation with test strains, were respectively <1, 3.18 0.22, 2.30 1.22 and <1 $\log_{10}$ CFU/mL, respectively. These levels of contamination were considered negligible if compared to the cell concentration of the inoculum. The results obtained with the UV-C irradiation system working in a continuous flow, confirmed the microbial inactivation levels found with the static one. Indeed, in the case of more turbid must sample (212 4 NTU), mean values of $\log_{10}$ CFU reduction were 4.80 0.86, 4.60 0.63 and 3.97 1.29 for *D. bruxellensis* CBS74^T, *A. aceti* DSM2002 and *L. brevis* DSM20054^T, respectively. Even if the microbial count reductions were slightly lower for the must with higher turbidity (212 4 NTU); however, they were not significantly different from those obtained with the less turbid must (17 1 NTU), as well as with the static prototype (Table 4). The only significant difference observed was for treatment of the *D. bruxellensis* CBS74^T strain, which gave a higher $\log_{10}$ reduction in experiments involving more clarified must (17 1 NTU) under dynamic conditions. These data suggest the efficacy of UV-C treatment is affected by both the strain and the must turbidity, but increased flow rates can achieve more effective microbial inactivation.

Collectively, these findings confirm the effectiveness of UV-C treatment in killing fungal and bacterial cells in an oenological matrix, in agreement with results reported previously (Fredericks et al. 2011, Falguera et al. 2013, Rizzotti et al. 2015).

Effect of UV treatment on PPO inactivation and must composition

The effect of UV-C irradiation on white must was evaluated in terms of inactivation of the PPO enzyme, changes in colour and the formation of α-dicarbonyl compounds.

Although the UV-C treatment was found to have suitable microbicide activity, to provide an effective alternative to SO_2 for treatment of grape must, UV-C treatment should also impede PPO activity. Irradiation with UV-C can break the disulfide bonds present in cystine and induce either direct or indirect oxidation of sulfur-containing amino acids and aromatic amino acids (Augenstein and Riley 1964, Davies and Truscott 2001). The UV-induced PPO inactivation in grape must, as well as in other fruit, has been reported in the literature, but the treatment conditions employed were either not clearly described or not comparable to one another (Guerrero-Beltrán and Barbosa-Cánovas 2006, Falguera et al. 2013, Müller et al. 2014). Moreover, it is not clear whether the treatment conditions that yield microbicide effects also achieve enzyme degradation. Therefore, the UV-C treatment conditions applied previously were evaluated for PPO inactivation. Grape must (prepared from 2015 Crimson grapes under laboratory conditions) was clarified (21 2 NTU) before UV-C irradiation (at up to 1800 J/L) under static conditions. The starting PPO activity (1.04 U PPO/L) linearly decreased as the irradiation energy increased (Figure 4), with PPO activity reduced by 30% (0.7 U PPO/L) following irradiation at 1800 J/L. This level of inactivation is useful for protecting grape must from oxidation, but is not comparable to the inactivation level achieved with SO_2 addition (Valero et al. 1992). A high level of

Table 4. Comparison of microbial counts obtained for selected strains following UV-C treatment at 1800 J/L, under static versus dynamic conditions†.

Flow condition	Microbial count ($\log_{10}$ CFU reduction)					
	Static		Dynamic		Dynamic	
Turbidity (NTU)	76	4	17	1	212	4
Dekkera bruxellensis CBS74^T	4.91	0.08a	6.30	0.00b	4.80	0.86a
Acetobacter aceti DSM2002	5.05	0.07a	5.19	0.51a	4.60	0.63a
Lactobacillus brevis DSM20054^T	4.47	0.05a	4.42	0.21a	3.97	1.29a

†Values are means of three replicates (n = 3) SD. Values followed by different letters within rows indicate a significant difference among treatments (P < 0.05). CFU, colony-forming unit.

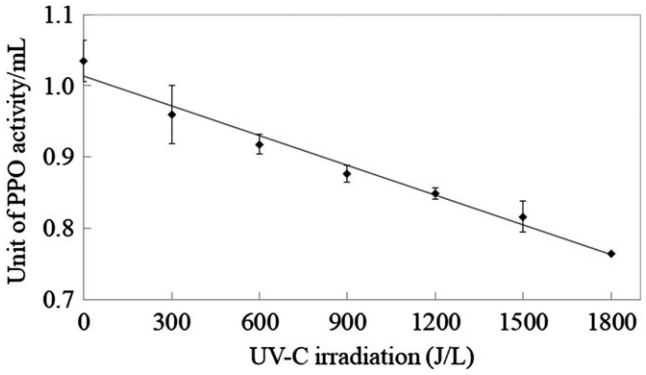

Figure 4. Residual polyphenol oxidase (PPO) activity determined in laboratory-prepared must treated with UV-C irradiation at variable dosage (0–1800 J/L). $y = -0.0001x + 1.0155$, $R^2 = 0.9844$.

Table 5. Colour measurements determined by CIE L*a*b* for must with variable turbidity, before and after UV-C treatment at 1800 J/L, under static and dynamic conditions†.

Must sample	Turbidity (NTU)		L*		a*		b*		Chroma		Hue angle		ΔE	
Untreated	17	1	34.95	0.09	0.35	0.08	1.10	0.18	0.30	0.05	1.16	0.15		
Treated (static)			34.99	0.19	0.19	0.07	0.73	0.11	0.22	0.05	1.34	0.18	0.66	0.09
Untreated	252	6	30.86	0.10	1.23	0.03	1.81	0.08	2.23	0.05	0.97	0.15		
Treated (dynamic)			31.10	0.16	1.17	0.03	1.75	0.07	2.04	0.09	0.98	0.02	0.35	0.09

†Values are means of three (n = 3) and nine (n = 9) replicates for untreated and treated must, respectively, SD.

irradiation energy can also induce either direct or indirect oxidation of many flavour-related grape precursors, such as carotenoids and cysteinylated compounds (Davies and Truscott 2001); therefore, low energy treatments are preferable. The efficacy of dynamic UV-C treatment (at 1800 J/L) on PPO activity was also assessed. Clarified grape must was spiked with grape lees to achieve a turbidity level comparable to vinification conditions (252 6 NTU). Under these conditions, the loss of PPO activity was considerably lower (7.8 1.4%) than that achieved under static conditions. This was likely due to the turbidity level being 12-fold higher than that for the grape must used in UV-C treatment under static conditions, as well as an optical density value at 254 nm being twofold lower in the must used in the latter experiments (Table 1). The results obtained were comparable to data reported by Müller et al. (2014), despite a higher turbidity value being reported in treated grape juice. The significant shielding effect exerted by the suspended grape solids, expressed as absorbance at 254 nm, is well known. To confirm the influence of flow rate on PPO inactivation, experiments were repeated with the flow rate being lowered from 0.44 to 0.072 L/min, using dynamic treatment conditions (laminar flow) but maintaining UV-C irradiation at 1800 J/L. Under these conditions, negligible variation in PPO activity was observed after the treatment. The low inactivation of the PPO enzyme can be responsible for must oxidation if no antioxidant is added. Even though PPO inactivation was lower using the dynamic apparatus, these results suggest that increasing flow rate and/or decreasing turbidity, could improve PPO inactivation. In this way, the oxidation of must could be limited, preventing browning and preserving the desired yellow colour.

No significant difference ($P > 0.05$) was detected between any of the colour parameters measured for untreated and treated must samples, under either static or dynamic conditions (Table 5). A decrease in a* and b*, however, was observed for must samples treated with UV-C irradition under static versus dynamic conditions; although the turbidity of the must samples treated using the static and dynamic apparatus (being 17 1 vs 252 6 NTU, respectively) may explain the variation of a* and b* values. The lightness (L*) and hue (a*, b*) values did not significantly change following UV-C treatment at increasing intensity, up to 1800 J/L. Similar results were obtained by Falguera et al. (2013) and Unluturk and Atilgan (2015) when white grape must was considered. Further confirmation that colour was not affected by UV-C treatment was obtained by calculating ΔE; the ΔE values of 0.66 and 0.35 calculated for must treated under static conditions, respectively, reflect small and very small differences in colour (Ford and Roberts 1998). This was in contrast to the major degradative effects of UV-C irradiation on the free anthocyanins observed in red grape musts reported by Falguera et al. (2013).

α-Dicarbonyl compounds are normal by-products of chemical oxidation in wine (Elias et al. 2008) and their concentration is often higher in sweet wines (Da Silva Ferreira et al. 2007). This is because the Maillard reaction can occur, even at room temperature, and it can be facilitated by the presence of metal ions, especially iron and copper, in both wine and grape must (Jackson 2000, Danilewicz 2003). The most abundant constituents of must are represented by aldo- and keto-sugars, compounds which are easily oxidised. The potential for radicals formed during UV-C irradiation to oxidise sugars to generate α-dicarbonyl compounds should be taken into account (Danilewicz 2003). The formation of α-dicarbonyl compounds after UV-C treatment of must was therefore considered in the current study. The concentration of α-dicarbonyl compounds present in must samples before UV-C treatment at 1800 J/L was 123.0 22.4 mg/L, whereas after treatment, the concentration, 124.4 22.5 mg/L, was not significantly different. Must samples treated with UV-C did not show the formation of oxidative molecules that could negatively impact wine quality. Similar results were obtained following compositional analysis of the laboratory-prepared must; no significant difference in the concentration of α-dicarbonyl compounds was observed before and after UV-C treatment, being 192.5 27.0 and 212.0 28.2 mg/L, respectively.

Conclusions

This study suggests that UV-C irradiation shows promise as an alternative to SO_2 in winemaking. A relationship between UV-C exposure (J/L) and decimal reduction values (α) was established for several microbial strains involved in wine spoilage. Despite increasing interest from the wine industry, there are few studies in the literature concerning the inactivation of the species investigated here, by UV-C treatment, and so this study addresses a key knowledge gap. The UV-C irradiation applied to white must effectively reduces microbial counts, without promoting the formation of chemical compounds associated with oxidation and therefore, negatively affecting must composition. UV irradiation can lead to the inactivation of PPO if a suitable flow rate can be applied to prevent any shielding effect imparted by suspended materials. The application of UV-C radiation might therefore offer winemakers an alternative to the SO_2 additions currently employed at different stages of must processing, thereby responding to the consumer demand for high quality, SO_2-free wines.

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Sources, production and commercial applications of fungal chitosan: A review

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ABSTRACT

Chitosan is a type of biopolymer that can be obtained from animal/marine sources, and it can also be extracted or produced from agriculture waste products like mushroom or different fungal sources after the chitin deacetylation. Depending on the size of mushroom farm, the amount of waste ranges between 5% and 20% of the production volume. The cell wall of the filamentous fungi, a good source of chitin, offers an easy way to extract chitin. The physicochemical characteristics such as molecular weight and degree of deacetylation of fungal chitosan can be controlled compared to chitosan obtained from crustacean sources. Fungal sourced chitosan can be used in food, pharmaceutical or biomedical applications for different applications, for example, as an antimicrobial agent, coating material, water purification or bio-pesticide. This review mainly focused on the extraction of chitin from mushroom or different fungal sources and also showed some applications of commercial chitosan products.

1. Introduction

Chitin is a linear biopolymer formed by N-acetyl-D-glucosamine units linked by glycosidic β (1,4) bonds. Chitin is the second most abundant polysaccharide in nature after cellulose, and it can be found in the exoskeletons of crustaceans and molluscs and in insect cuticles. Fungal biomass is another major source of chitin. Recent advances in fermentation technology for the production of biopolymers from fungal source have been getting worldwide attention. The biomass used for the fermentation of chitosan is an inexpensive biowaste from a plentiful and economical source, and these bio-wastes would have to be managed/post-treated any way. Extraction of highly functional value-added products, such as chitosan may provide a lucrative solution to these bio-wastes.

Fungal biomass can be produced by solid-state fermentation (SSF) and submerged fermentation (SmF). The SmF has a specific advantage as this fermentation method provides easier control of fermentation parameters such as pH, temperature, and nutrient concentration in the fermentation medium. However, the SSF is known to produce larger quantities of biomass than SmF, therefore a higher potential for chitosan production. Generally, chitosan production from fungi requires various ingredients such as, yeast extract, D-glucose, and peptone. Recently, studies have been focused on the utilization of inexpensive carbon sources, such as biowastes for culturing fungi for chitosan production (Kannan et al., 2010). The mycelial cell walls of fungi are consisted with polysaccharides (e.g., chitin and glucan) and glycoproteins. Polysaccharides are the structural components, and glycoproteins (i.e., mannoproteins, galactoproteins, xylomannoproteins and glucuronoproteins) are the interstitial components of the cell walls of fungi. Chitin makes up

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Table 1
Advantages and limitations of source materials for chitosan production.

Source material	Advantages	Limitations
Crustacean shell	Industrially established method for the production of chitosan, and commercially available chitosan in market.	(1) Seasonal and limited raw material supply; (2) Chemical required with high quantities such as alkali and acids. The process is also time-consuming and requires large amounts of energy. High alkali concentration of 30%–50% (w/V) and temperature over 100 °C is required; (3) Demineralisation treatment is required because of the high volume of calcium carbonate; (4) Difficult to get highly deacetylated chitosan, which makes a limitation in biomedical applications.
Fungi	(1) There is no seasonal variation. Fungal source chitosan can be produced anytime in the year; (2) Free of heavy metals like nickel, copper; (3) Free of allergenic animal source protein; (4) Molecular weight and degree of deacetylation can be controlled during fungal chitosan production, which is useful for highly technical applications; (5) Low polydispersity index and homogeneous preparation; (6) Biocompatible; (7) Fungal chitosan can be a by-product of fungi-based industries.	(1) The availability and quantity of fungal raw material is not comparable to marine or animal sources; (2) Production cost is higher than that of crustacean based production; (3) Limited numbers of commercial producers.

Sources: Aranaz et al., 2009a; Nwe et al., 2010; Kumari and Rath, 2014; Ghormade et al., 2017.

to 45% of the cell wall of *Aspergillus niger* and *Mucor rouxii* and 20% of the cell wall of *Penicillium notatum* (Kaur and Dhillon, 2014). Chitin can be converted to chitosan by a deacetylation process. Chitosan is soluble in aqueous acid such as acetic acid, lactic acid, and its solubility depends on the deacetylation degree (DD) and molecular weight, a high DD and low molecular weight increase the solubility (Mourya et al., 2011). More recently, a "shell biorefinery" concept has been proposed in a number of publications for fractioning and upgrading exoskeleton of crustaceans and fungal cell wall to functional biomaterials (Chen et al., 2016; Hülsey 2018; Yang et al., 2019; Aneesh et al., 2020; Suryawanshi and Eswari, 2021).

The applications of chitosan in different areas (Fatehi et al., 2010; Shen et al., 2014; Abdel-Gawad et al 2017; Duan et al., 2019; Dai et al., 2020; Saeed et al., 2011; Yu et al., 2021) have been growing rapidly due to its unique properties. Chitosan has a relatively stable chemical structure with polycationic, innocuous, non-toxic and biodegradable properties, and it is biocompatible with many organs, tissues and cells. The molecules of chitosan are physically and biologically active, which can be chemically or enzymatically modified for specific applications. Chitosan can be processed into flakes, beads, powders, membranes, gels and sponge forms for different applications. Chitosan has been used as food additives and wastewater treatment agents, live cell encapsulation, enzyme immobilization, and anti-cholesterolemic agents, as well as wound healing or dressing for biomedical, and drug excipients in the pharmaceutical industry (Khan et al., 2019; Khan et al., 2020). Particularly, fungal chitosan is an excellent scaffolding material to construct a biodegradable tissue regeneration template (Nitar Nwe, Furuike, & Tamura, 2010).

In this review, we have highlighted various advantages and limitations of chitosan extraction from fungal sources, with references to chitosan from crustacean/shrimp. The production and application of chitosan from fungal sources have been discussed in detail. A comprehensive analysis of global chitosan market size has also been carried out, with focus on the commercially available chitosan-based products and their applications.

2. Different sources of chitosan: advantages and limitations

Chitosan can be produced from different sources. The most traditional source of chitosan is from waste crustaceans' shells from the seafood processing industry, such as crab or shrimp shells. The exoskeleton of insects such as beetles is another source. Another natural source with growing interest and popularity is from the fungal kingdom of molds and macro-mushrooms. In Table 1, the advantages and limitations of chitosan production from crustacean shells and fungi are listed.

Chitin is found naturally in shellfish exoskeletons, insect cuticles, and fungal cell walls. The raw materials for bulk production of chitin and chitosan are usually from marine sources because of the abundance of the raw materials in the form of shellfish waste from the seafood industry. However, alternative sources such as insect biomass, mushroom and fungal bodies, and microbial biomass have gained interest in recent years, as marine sources may have seasonal variations in availability, and the heavily mineralized exoskeletons make for an arduous extraction process. These various sources present unique advantages and disadvantages and yield chitin/chitosan products of with various properties. Insect-derived chitin/chitosan generally have properties similar to those obtained from crustaceans (Philibert et al., 2017), while fungal chitin/chitosan has superior particle size uniformity and lower molecular weight compared with those derived from insects and crustaceans (Darwesh et al., 2018). More importantly, the shellfish chitin/chitosan generally has evenly distributed acetyl groups. In contrast, the acetylation pattern of mushroom chitosan tends to be in clusters.

3. Chitosan production from crustacean seafood waste

The seafood waste from shrimp (roughly 45% of the production) is composed of exoskeleton and cephalothoraxes. This kind of waste represents about 50%–70% of the weight of the raw material, and it contains valuable proteins (30%–40%), calcium carbonate (30%–50%) and chitin (20%–30%). Also, this waste contains pigments of a lipidic nature such as carotenoids (astaxanthin, astathin,

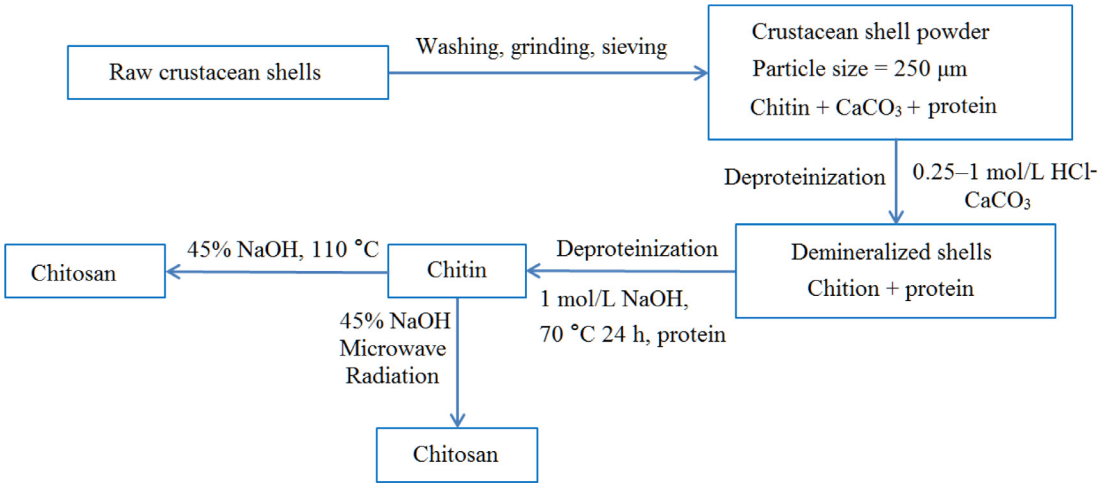

Fig. 1. Preparation of chitin and chitosan from crustacean sea waste (Sagheer et al., 2009).

canthaxanthin, lutein and β-carotene). Chitin, next to cellulose, is the second most common polysaccharide on the earth, with a yearly production of approximately 1×10^{10}–1×10^{12} tons. This polymer consists of a linear chain of linked 2-acetoamido-2-deoxy-β-D-glucopyranose unit (Aranaz et al., 2009b; Sagheer et al., 2009; Duan et al., 2019).

Chitin is extracted by acid treatment to dissolve the calcium carbonate, followed by alkaline treatment to dissolve the proteins and by a depigmentation step to obtain a colourless product mainly by removing the astaxantine (Acosta et al., 1993). Chitosan is prepared by hydrolysis of acetamide groups of chitins. This is normally conducted by strong alkaline hydrolysis treatment under a high temperature due to the resistance of such groups imposed by the trans arrangement of the C_2–C_3 substituents in the sugar ring. Usually, sodium or potassium hydroxides are used at a concentration of 30%–50% (w/V) and at high temperature (100 ˚C) (Kumari and Rath, 2014). A simplified flow diagram is shown in Fig. 1. for chitin and chitosan production from crustacean sea waste.

More novel processing methods have been reported in recent studies to process crustacean materials in greener means. These new processes include hot water treatment (Margoutidis et al., 2018; Yang et al., 2019), solid state mechanochemical treatment (Chen et al., 2017) and glycerol treatment (Devi and Dhamodharan, 2018). These treatments can greatly reduce the amounts of chemical consumption and waste water generation, which may also benefit the fungal chitin/ chitosan extraction processes.

4. Chitin and chitosan in fungi

Chitin can be found in many microbes like fungi, protists and algae. The mycelium of several fungi, such as *Mucor rouxii*, *Absidia glauca*, *Aspergillus niger*, *Gongronella butleri*, *Pleurotus sajor-caju, Rhizopus oryzae, Lentinus edodes,* and *Trichoderma reesei* have been considered as possible sources of chitin and chitosan due to its abundance in the cell walls. Zygomycetes (fungi) are good sources of chitin and chitosan. Their cell walls contain larger amounts of chitin when compared with other classes of fungi. Chitin found in the inner fungal cell wall as a microfibril serve in compensating the cells' turgor pressure (Merzendorfer, 2011; Dhillon et al., 2013). Chitin can be synthesized from different types of cells, such as vegetative and sporulating cells (Lesage and Bussey, 2006).

For the most of fungi, chitin can be synthesized from the sites of the polarized growth cell wall. A period of isotropic growth starts in large budded cells where material is deposited over the entire bud surface. Following nuclear division, a repolarisation phase begins where material is directed towards the mother-bud neck to prepare for cytokinesis. In hyphal or filamentous forms, cell extension is a continuous and indefinite process of apical (Fig. 2) (Fischer et al., 2008; Lenardon et al., 2010).

Chitin synthase (CHS), with different classes, plays critical rule in the chitin synthesis, although the functional importance of all CHS is not clear yet. The general pathway of chitin synthesis involves a defined number of enzymatic reactions to form N-acetylglucosamine (GlcNAc) polymer. The sugar source is glucose or its storage compounds glycogen or trehalose. The pathway can be divided into three sets of sub-reactions. The first set of sub-reactions leads to the formation of the amino sugar GlcNAc, the second set follows a variant of the Leloir pathway yielding the activated amino sugar UDP-GlcNAc, and the last sub-reaction involves the polymerization of chitin by using UDP-GlcNAc as the activated sugar donor. The first two sub-reactions occur in the cytoplasm and the third one at specialized microdomains of the plasma membrane. These microdomains include plasma membrane regions at the emerging bud of yeast cells and at the hyphal tips and cross-walls of filamentous fungi (Watanabe et al., 2005; Dhillon et al., 2013).

The molecular weight (M_w) of chitin/chitosan is determined by the chain length that is affected by the CHS activities. The degree of acetylation (DA) of chitosan (i.e., the content of GlcNAc groups in the chitosan chain) is impacted by chitin deacetylase (CDA). Therefore, the properties of chitosan (DA and M_w) largely depend on the activities of these two enzymes that are largely influenced by the environment, i.e., the concentrations of organic and inorganic compounds and metal ions in the surrounding areas. The CDA plays a crucial role in the bio-synthesis of chitosan of fungi. The deacetylases of many fungi (e.g., *Colletotrichum lindemuthianum*, *Aspergillus nidulans*, *Rhizopus oryzae*have and *Mucor rouxii*) have been isolated for research. However, the insolubility of the chitin

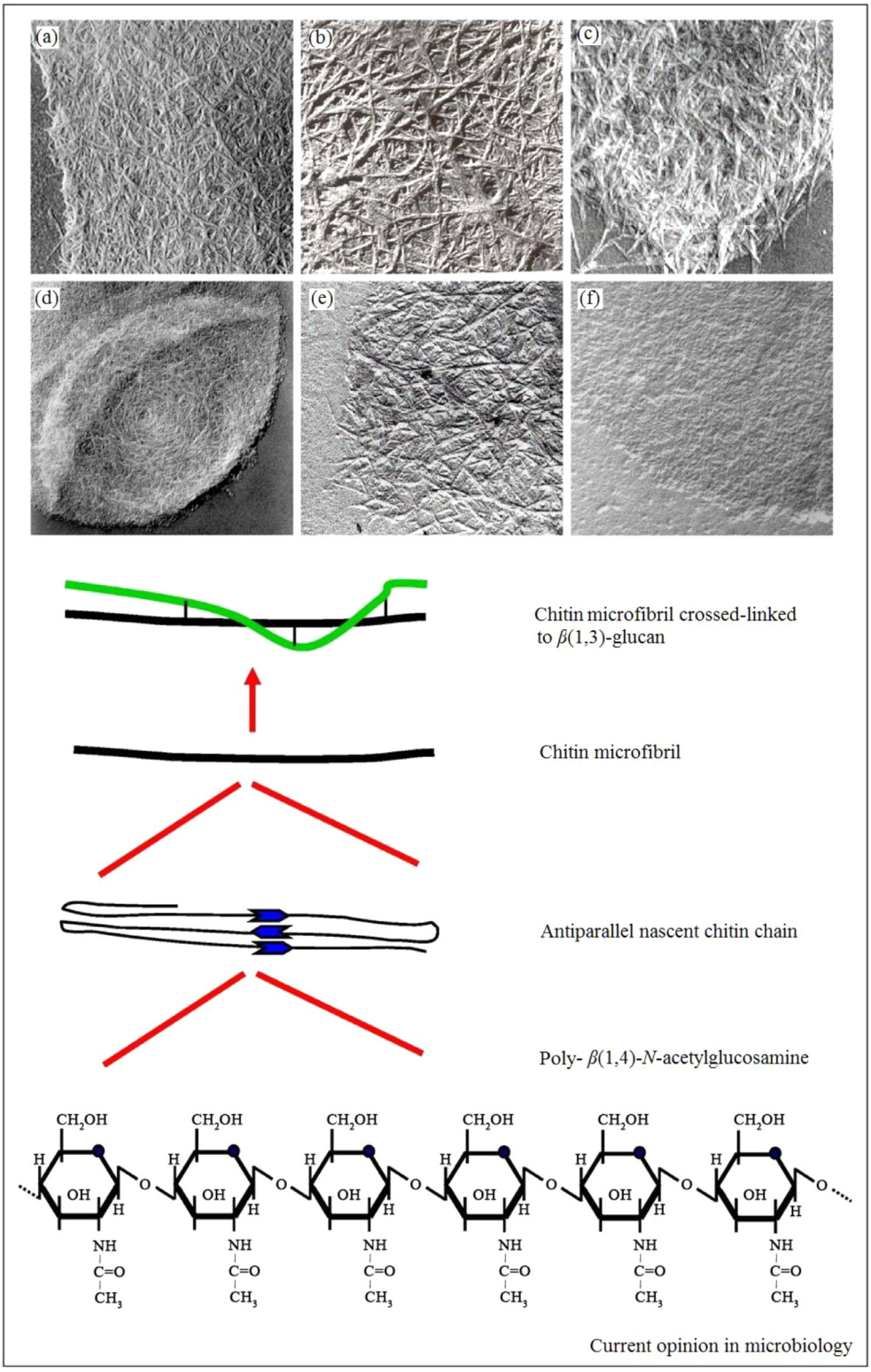

Fig. 2. Chitin structure and diversity in fungi. Chitin is a $\beta(1,4)$-homopolymer of *N*-acetylglucosamine that folds in an anti-parallel manner forming intra-chain hydrogen bonds. Chitin chains are cross-linked covalently to $\beta(1,3)$-glucan (green) to form the inner skeleton of most fungi. Examples of shadow cast electron microscopy images of chitin from (a) *Neurospora crassa*; (b) *Coprinus cinereus*; (c) chitin-chitosan from *Mucor mucedo*; and (d) *Candida albicans*. In (e) and (f), the structure of chitin from *C. albicans* is shown in a *chs*3Δ and *chs*8Δ mutant, respectively, demonstrating that the architecture of chitin is genetically determined (Lenardon et al., 2010).

substrate hindered the activities of the deacetylases severely. Many attempts have been made to study the deacetylase enzyme in vitro using high DA amorphous chitin as the substrate, but the isolation of acid-soluble chitosan failed. The insolubility of high-DA chitin in aqueous solvents remains a barrier for the synthesis of chitosan with deacetylases. Nevertheless, some success has been achieved with in-vivo activity studies. Enzymatic deacetylation with enzyme CDA can produce products with more consistent properties in an environmentally friendly manner (Chatterjee et al., 2008; Dhillon et al., 2013).

5. Production of chitosan from fungal mycelia: fermentation, extraction and purification

In fermentation technology, there are a range of possibilities for the production and extraction of chitosan using different fungi at industrial scale. Each fungal biomass has different chitosan yield. Moreover, recent advances in fermentation technology have made possible the large-scale controlled production of chitosan by culturing the microorganism, such as fungi, which contain chitosan in their cell walls. The yield of chitosan from several *Zygomycetes* fungi including *Absidia*, *Gongronella*, *Mucor*, *Rhizopus* was studied and *G. butleri* gave the highest yield of chitosan. At the same time, it has been found that the yield of chitosan produced from SSF is higher than that in SmF. Solid substrate fermentation has been found as the best fermentation method to produce chitosan by fungus *Gongronella butleri* (Nwe and Stevens, 2008).

Chitin/chitosan and glucan are the main fungal skeletal polysaccharides. In the fungal cell wall, chitin/chitosan presents in two forms, as free amino glucoside and covalently bonded to β-glucan. Initially chitin and β-glucan chains accumulate individually in the fungal cell wall and after that it forms the inter polymer linkage. The formation of the chitin/chitosan-glucan chain complex results in a rigid cross-linked network in the cell wall and causes a considerable problem for the extraction of intact chitosan and glucan, which does not break down easily under mild extraction conditions. That is why strong alkali treatment is required to break down the chitin-glucan linkage. In order to get the high quality and quantity of chitosan, an enzymatic digestion has been performed for the fungal wall (chitin-glucan complex) by glucanase, chitinase, and amylase (Nwe et al., 2010).

Traditionally the production of chitosan is performed by a two-step extraction process (acid and base treatment) from the cell wall of the fungi after fermentation. By treating the mycelia under alkaline conditions (such as 2%–4% NaOH) at elevated temperature (90–121 ˚C for 15–120 min), the remaining degraded cell wall with proteins and chitosan are obtained as an alkali insoluble material (AIM). After this step, the chitin-glucan complex is dissociated releasing chitin, which is converted to chitosan by a deacetylation process. The AIM is then treated with an acid such as hydrochloric, lactic or acetic acid, specifically 2%–10% acetic acid at 25–95 ˚C for 1–24 h to remove the phosphates or insoluble materials from the cell wall and separated from the remaining material in the cell wall. This cell wall material is generally considered to be rich in “fungal chitosan” and it can be precipitated by raising the pH to 9–10 followed by centrifugation and washing with acetone and ethanol (Tasar et al., 2016). This procedure does not include the deproteinization nor the demineralization processes required in the extraction of chitosan from crustacean sources. It has been shown that fungal chitosan has been found to be free of proteins that could cause an allergic reaction such as tropomyosin, myosin light chain and arginine kinase, which are present in crustaceans (Li et al., 2012).

Various factors, such as acid-base concentration, incubation time and temperature, and particle size have been found to affect the physico-chemical properties of chitosan. However, the possibility of manipulating the different physicochemical properties of chitosan through the regulation of factors, such as growth media composition and processing parameters in the extraction protocols have made fungi an ideal research candidate.

6. Effect of alkali and acid treatment

The concentration of base used in the extraction process significantly affects the DD. It has been demonstrated that increasing the concentration of base along with increased incubation time and temperature significantly resulted in increased DD of the chitosan extracted (Kannan et al., 2010). But it has also been reported that alkali concentration and temperature showed a significant negative effect on DD of chitosan (Abdel-Gawad et al., 2017). To obtain a higher chitosan extraction yield, longer incubation time and higher temperature are required to ensure effective interactions of the fungal cell wall constituents with the base. Using a lower alkali concentration in combination with a higher temperature and longer incubation time can maximize the chitosan yield. Chitin is probably converted to chitosan in the process. On one hand, high alkali concentration can cause chitosan oxidation and chain degradation, particularly when both temperature and incubation time of the deproteinization process are increased. Therefore, alkali concentration had a synergistic effect with temperature and time on the oxidation and degradation of chitin/chitosan macromolecules. It has also been reported that chitosan yield increased when increasing caustic soda concentration from 1 mol/L to 11mol/L, probably due to the conversion of chitin into chitosan in the process. It is noted that sodium borohydride was added in the process to inhibit chitosan oxidation (Nwe et al., 2010).

On the other hand, the nature of acid used can also affect the final yield of chitosan during the extraction process. Different acids such as lactic acid, acetic acid or formic acid and hydrochloric or sulphuric acid have been used for the extraction process. The utilization of lactic acid instead of hot sulphuric acid during chitosan extraction process has been found to give a higher yield of chitosan. This process used a lower temperature compared with the sulphuric acid method (Tasar et al., 2016). It was also found that using formic acid (6% V/V) as the extracting solution gave a higher yield of chitosan compared with acetic acid followed by hydrochloric acid (Kannan et al., 2010). The authors also observed that during chitosan extraction, various acids produced different effects, and interactions at the same incubation time and concentration. Chitosan extraction with hydrochloric acid showed a significantly higher DD compared with acetic and formic acid. However, hydrochloric acid caused a greater extent of hydrolysis of the acetyl moieties along with hydrolysis within the network of monomers in the chitosan polymer due to its strong acid characteristics. Increasing the

Table 2
Chitosan based commercial hemostatic dressings.

Commercial name	Company	Material	Function
HemCon	HemCon	Freeze-dried chitosan acetate salt	Stops bleeding in emergency situations
Chitoflex	HemCon	Chitosan based biocompatible and antibacterial wound dressing	To control moderate to severe bleeding by stuffing in the wound
Chitoseal	Abbott	Chitosan based dressing with cellulose coating	For major wounds with bleeding
Clo-sur	Scion	Chitosan based pressure pad	Accelerates wound healing when applied topically
TraumaStat	Ore-Medix	Freeze-dried chitosan containing highly porous silica	To stop bleeding of superficial wounds, minor cuts, and abrasions
BST-CarGel	Biosyntech Company	Chitosan-glycerophosphate hydrogels	Cartilage repair

Note: data referred to Allied market research in January 2017.

concentration of different acids also resulted in the increase of DD. High acid concentration and high temperature produced a darker coloured chitosan, whereas milder treatments gave a lighter coloured chitosan (Dhillon et al., 2013).

7. Market overview and commercial chitosan products

Global chitosan market size is expected to reach 2.55×10^9 US dollar by 2022 from 1.205×10^9 in 2015 according to Allied market research in January 2017. Market research report by Technavio on the global chitosan market predicts a compound annual growth rate (CAGR) of above 18% by 2021. Advanced biopolymers, Heppe Medical Chitosan GmbH, Golden-Shell Biochemical and G.T.C. Bio-corporation are the leading players in the market. Usually, chitosan market is segmented according to region like North-America, Europe, Asia-Pacific and LAMEA (Latin America, Middle East, Africa). Asia Pacific is expected to lead the chitosan market due to the abundant availability of the raw materials, especially in China, Japan, India and Thailand. Shrimp shell source chitosan occupied almost 80% of the total chitosan market. Due to the limited fungal source, it was unable to get the information of the market research for fungal source chitosan (according to Allied market research in January 2017).

Fungal-based chitosan products are still limited in market. First fungal based chitosan has been patented by Cargill, an US based company, in 2005. Cargill's chitosan was highly deacetylated and obtained from microbials biomass. In Europe, a Belgium based company, KitoZyme first brought fungal based product (KitoZyme) to market. In June 2011, KitoZyme obtained Generally Recognized as Safe (GRAS) status approval from Food and Drug Administration (FDA) for their vegetal chitosan product named KiOnutrime-CsG® to be used in beverage applications. The FDA issued a notice confirming that the panel of experts has no objection on the use of chitosan, as processing aids in the manufacture of alcoholic beverage. The KiOnutrime-CsG® is also registered in European Union (EU) under the novel food regulations and is approved by European Food Safety Authority (EFSA). Kitozyme also has other non-animal source chitosan products on market. These products are: 1) KiOfine®-B, which is a new tool for winemakers that helps to prevent and cure contamination by *Brettanomyces bruxellensis*; 2) KiOfine®-CsG, which is used for several applications as an anti-oxidant and in different stages of the winemaking process (clarification, fining etc.) and 3) Slim MED® ADVANCED, which is a dietary fiber for weight loss.

KitoZyme have a spin off company named Synolyne Pharma with University of Liège to develop a biomedical device and implants named "Vegetech Inside". The product is mainly a chitosan-based microbeads hydrogel for the treatment of Osteoarthritis. Synolyne Pharma has changed the name in 2016 to KiOmed Pharma in order to reflect a better growth aspiration in the area of the joint health (Fan et al., 2005).

In Canada, it was Mycodev (in 2013) who brought fungal based chitosan to public attention in order to commercialize a new fermentation-based product in the Canadian market, with the focus on biomedical and pharmaceutical applications. Another Canadian based company Chinova Bioworks started to commercialize their first mushroom-based chitosan in 2016. Chinova is developing mushroom chitosan based formulations to replace synthetic preservative from food and beverage applications. Chinova Bioworks' mushroom chitosan, CHIBER™ has already entered the market place. Apart from Chinova Bioworks, mushroom based chitosan is also getting interest in Asia. Chibio is a China-based biotech company, which has non-animal source chitosan for food and pharmaceutical applications.

Chitosan wound dressing commercial products, which are currently available on the market are mostly obtained from crustaceans or animal source chitosan. Some of the United States Food and Drug Administration (USFDA) approved wound dressing products have been presented in Table 2.

The production cost for the traditional crustaceans based chitosan is cheap compared with fungal based chitosan. Crustaceans raw materials are readily available and cheap whereas the cost of raw materials is the main bottleneck for fungal chitosan production. Crustaceans chitosan can be found from 10 US dollar per kg to 1000 US dollar per kg. It also depends on product quality and application. In recent years, there have been a number of statistics around the world for vegan and vegetarian populations. There are about 5 out of 100 persons getting affected by crustacean allergy, which means that about 300 million persons need non-crustacean-ingredients food. This is the reason that people are getting interested in replacing the animal source products with non-animal source products. The estimated price is shown in Table 3 for fungal based chitosan in Canada, Europe and China (the information has been collected by contacting individual companies).

Table 3
Estimated price chart for fungal based chitosan

Region	US dollar per kg
Canada	250–5000
Europe	200–2000
China	50–500

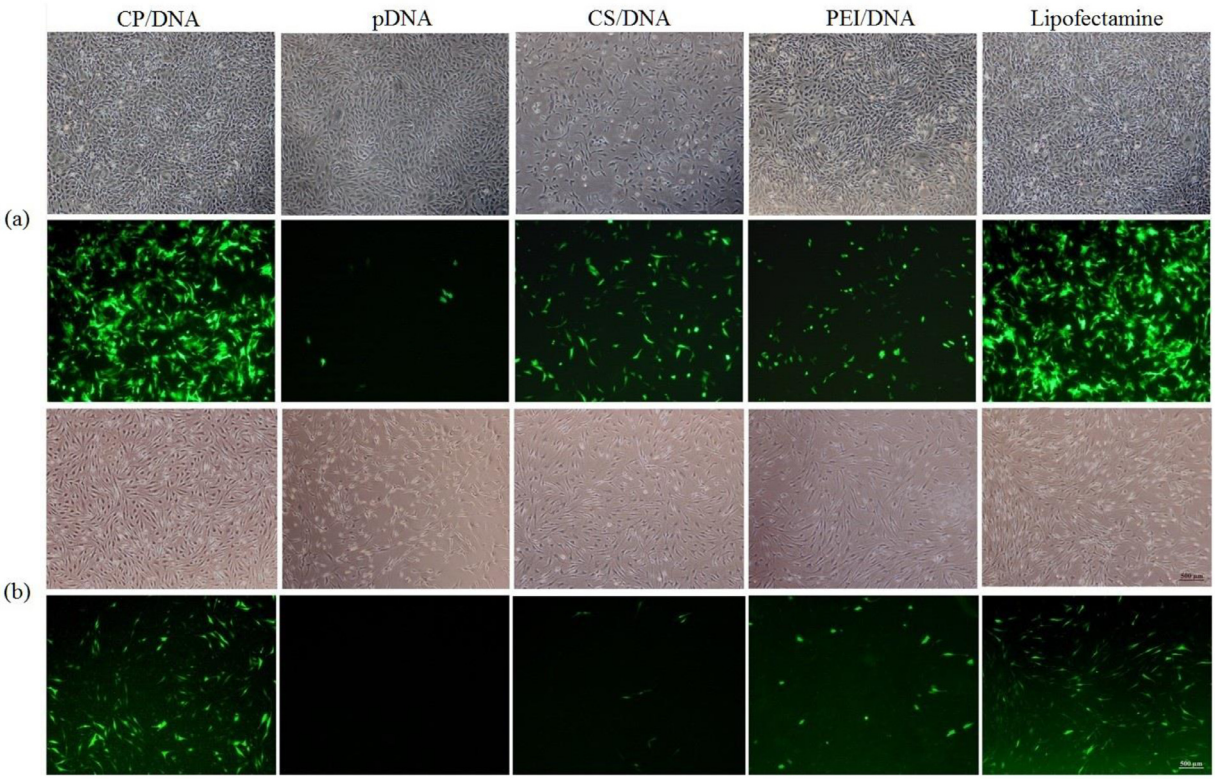

Fig. 3. Images of chondrocytes (a) or synoviocytes (b) transfected with polyethylenimine (CP)/DNA nanoparticles, naked pDNA, chitosan (CS)/DNA nanoparticles, polyethylenimine (PEI) (25 ku)/DNA nanoparticles, and LipofectamineTM 2000 as observed under fluorescence microscope or inverted phase contrast microscope (Lu et al., 2014).

8. Application of chitosan in biomedical-tissue engineering and food

Fungal chitosan has some great advantages for biomedical application due to molecular characteristics. It is more readily possible to make different M_w (very low to high) from fungal chitosan compared with crustacean chitosan. Fungal chitosan is soluble in physiological pH ranges together with its poly-cationic characteristics and lower antigen effect, and it can be used as a potential drug carrier and non-viral gene delivery system. The application of fungal based chitosan is still under development. The chitosan-based applications are mainly focused on biomedical/tissue engineering and food applications (Ghosh and Urban, 2009; Duan et al., 2019; Khan et al., 2019; Wang et al., 2019; Khan et al., 2020; Tang et al., 2020).

Chitosan-graft-polyethylenimine (CP)/DNA nanoparticles used as novel non-viral gene vectors for gene therapy of osteoarthritis, where the chitosan had a M_w of 50 kDa with 90% DD. The CP/DNA nanoparticles (5–20 mg/mL) showed a safer cytotoxicity carrier than other nanoparticle carriers such as polyethylenimine (PEI)/DNA, Lipofectamine^{TM} 2000, for joint tissue/chondrocytes or synoviocytes. It has also been reported that the transfection efficiency of CP/DNA complex was similar to that of the Lipofectamine^{TM} 2000 at CP:DNA (w/w) ratio = 3, and significantly higher than that of chitosan (CS)/DNA nanoparticles, PEI (25 kDa)/DNA nanoparticles, and naked pDNA (Fig. 3) (Lu et al., 2014).

8.1. Wound dressing application

Additionally, fungal chitosan can also be used as a membrane in many medical applications such as wound dressings due to its wound healing properties. Wound healing is a particular biological route allied to the general phenomenon of growth and tissue regeneration. It progresses through a series of inter-reliant and corresponding stages in which a variety of cellular and matrix com-

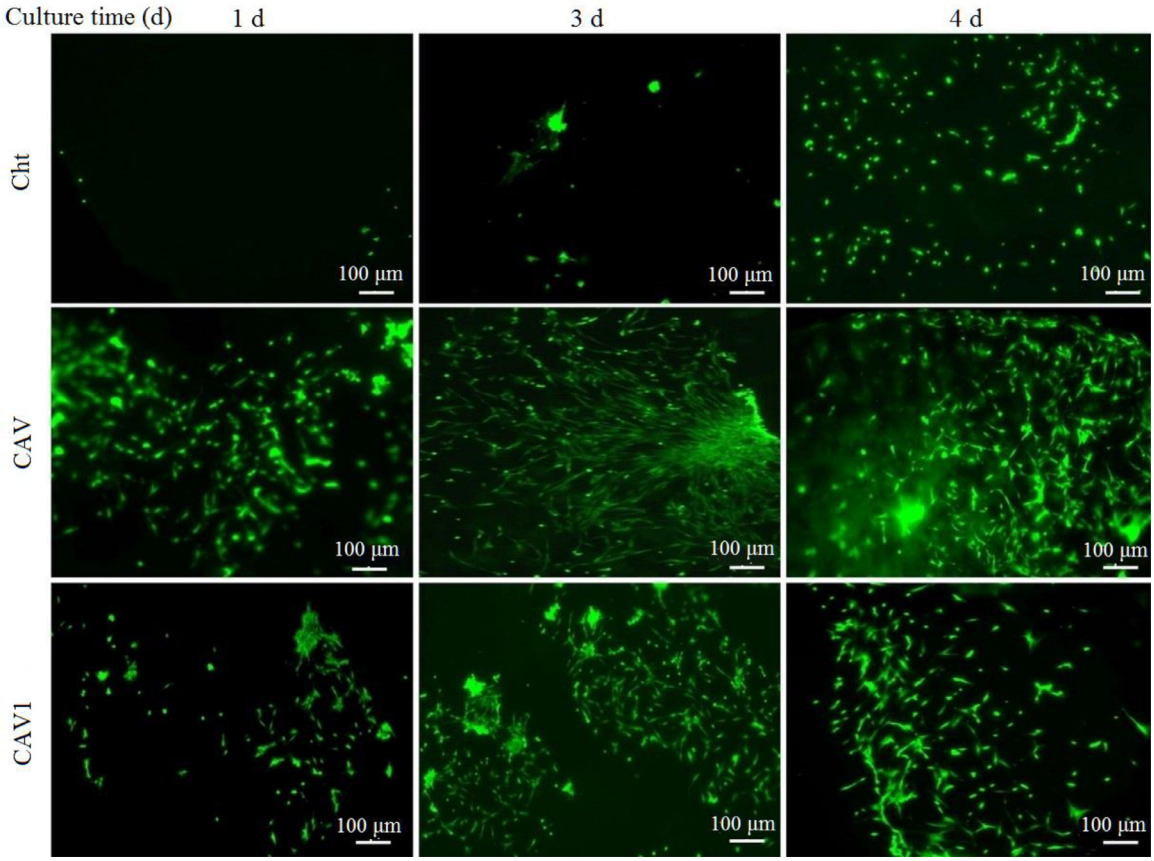

Fig. 4. Calcein-AM (acetoxymethyl) staining of hDFs cells cultured for 1, 3 and 7 days on the membranes (chitosan (Cht), chitosan aloe-vera (CAV) and CAV1); permission obtained from (Silva et al., 2013).

ponents act together to regenerate the integrity of damaged tissue and replacement of lost tissue. Wound healing is a complex and dynamic regenerative process that is divided into four phases: haemostasis, inflammation, proliferation, and remodelling (Sinno and Prakash, 2013). Chitosan was applied in wound dressing application in two main directions, first as coated-textile materials and second in a combination with polyvinyl alcohol (PVA) composite membranes using a freeze-thawing (F-T) cycles method. Chitosan was first used as coating martial-based textiles for wound dressings. Cotton textile has been layered with chitosan and polyethylene glycol (PEG) afterwards freeze-dried, where chitosan-PEG formed a very porous thin dressing film. It has been found that an addition of chitosan controlled the film surface morphology, while PEG used as pores forming agent. A composite hydrogel membrane from polyvinyl alcohol-poly (N-vinyl pyrrolidone)-chitosan containing antibiotic showed a quick release at the beginning. Owing to the controlled time, the release becomes slower which has been recommended for the healing management use (Yu et al., 2006).

Wounds can be contaminated by harmful pathogenic bacteria, and the risk of infection can be high under the conditions. To reduce the risk of wound infection, it is needed to develop wound dressings that have antimicrobial effects by incorporating antimicrobial agents in the dressing. It has been shown that the therapeutic properties of aloe vera (AV) are effective for active wound dressing materials. Chitosan and aloe-vera membranes have been developed for wound dressing applications. The antimicrobial capability of the chitosan aloe-vera (CAV) membranes was improved by the addition of aloe-vera. The CAV1 membrane with the ratio of CS:AV = 1:1 (V/w) had the highest antibacterial capacity. The greater bactericidal effect and inhibitory potency of the CAV1 was caused by the higher content of AV in the membranes. Figure 4 shows that on the chitosan membrane, human dermal fibroblasts (hDFs) exhibited a round morphology with cell agglomeration throughout the whole study period. When the cells were seeded on the CAV membrane, better spreading of cells was observed with a higher number of cells attached to the surface. On the surface from 1 to 7 days, it was observed that the cells were uniformly distributed on the CAV membrane with a spindle-like shape that was a typical morphology of fibroblasts. This observation suggested good adherence of cells to the membrane surface. Furthermore, on day 7, hDFs were well spread on the CAV membrane surface and adhered to both sides of the membrane. However, the CAV1 membrane with a higher AV content did not result in better cell spreading and proliferation compared with the CAV membrane with a lower AV content. The conclusion is that chitosan/aloe vera membranes can be promising wound dressing materials (Silva et al., 2013; Ahmed and Ikram, 2016; Kamoun et al., 2017).

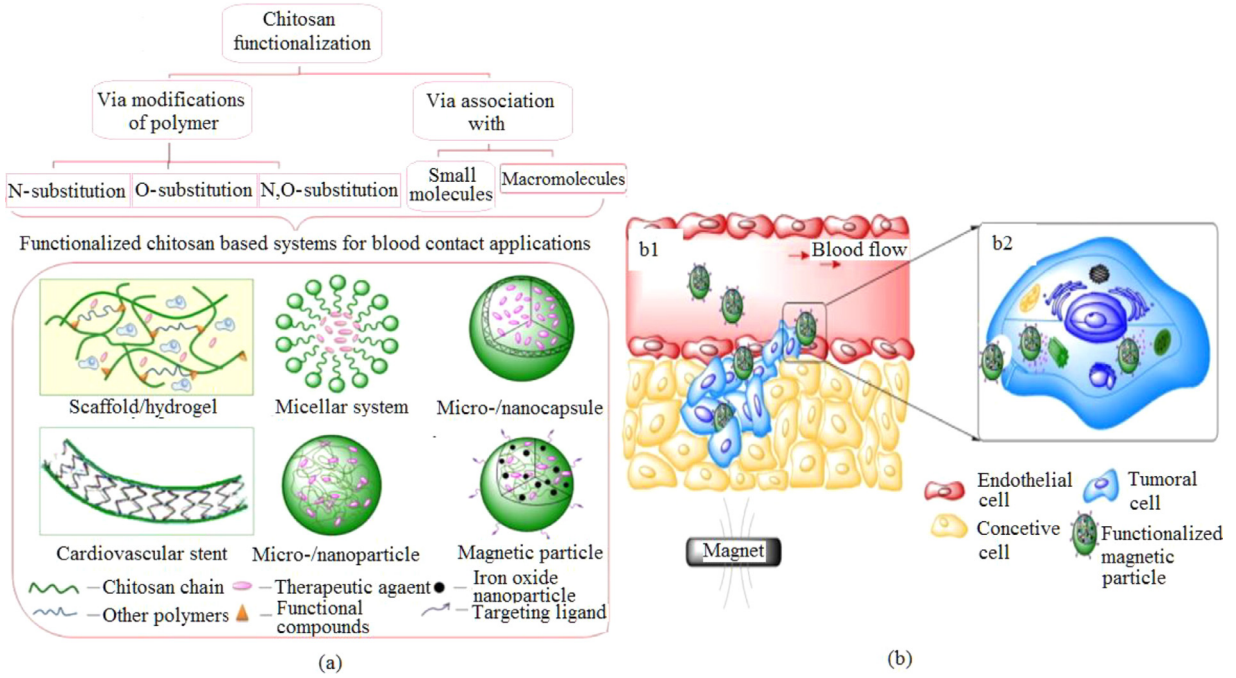

Fig. 5. (a) Strategies used to improve the blood compatibility of the chitosan; and (b) Schematic representation of theragnosis concept using functionalized magnetic particles: (b1) guidance in magnetic field and (b2) tumoral cells internalization; permission obtained from (Balan and Verestiuc, 2014).

8.2. Chitosan as a hemo-compatible biomaterial

The use of chitosan is growing in medical and pharmaceutical fields; it is important to study its interactions with blood. In the last couple of years, the hemostatic effect of chitosan has been extensively explored for wound healing. A hemo-compatible polymer should be non-thrombogenic with plasma proteins or/and blood cells that could induce a thrombogenic or/and a hemolytic response. It has been reported that chitosan is highly thrombogenic because it can activate and complement the blood coagulation system (Hirano et al., 2000). In order to improve the blood compatibility of chitosan, it is important to use chitosan derivatives by further modification. Functional chitosan is used to improve biopolymers blood compatibility.

The strategies can be classified in two categories to improve the blood compatibility of chitosan by the modification manner (Fig. 5a): 1) Chemical modifications of chitosan; 2) Association of chitosan with complementary compounds.

N-succinyl chitosan (N-SCs) has been found to be a remarkable member of the group of N-acyl chitosans that have good hemo-compatibility. It has been shown recently that N-succinyl chitosan has antitumor effect and can stimulate the immune reconstitution in the case of umbilical cord blood transplantation (Luo et al., 2010). Micro particles or micelles based on chitosan derivative such as amphipathic N-succinyl-N-octyl chitosan covalently modified with folate demonstrated good drug loading, entrapment efficiency and sustained release behavior. This kind of chitosan can be a promising carrier for hydrophobic therapeutic drug or diagnostic agents targeting tumor cells (Wu et al., 2014). Chitosan and its derivatives are playing a remarkable role against cancer therapy and exploiting to inhibit the tumor angiogenesis. The theragnosis using functionalized magnetic particles can also be based on chitosan (Fig. 5b). Functional magnetic particles carrying loads of imaging or therapeutic agents will travel to the tumor cells following the magnetic field, at which they will be internalized and the bio-agents are released to the targets (Li et al., 2016).

The blood brain barrier (BBB) separates the blood from the cerebral parenchyma and limits the delivery of drugs including antibiotics, anti-Alzheimer, neuroleptic drugs, and antineoplastic agents. Proper delivery of these drugs into the brain could improve treatment effectiveness for central nervous system diseases (e.g., multiple sclerosis, Alzheimer's and brain tumors). It has been proposed that chitosan micro or nano particles are more efficient in enhancing the transferring process across the blood brain barrier compared to chitosan solutions; a series of chitosan derivatives have been designed for the formulation of nanoparticles with the capacity of targeting drug delivery (Ouyang et al., 2017). Chitosan derivative nanoparticles, for example, N-Trimethyl chitosan nanoparticles can enhance the bio-distribution of an anti-neuroexcitation peptide (especially for peptides or proteins) in the brain by the mechanism of absorption-mediated transcytosis, which is a promising brain targeting delivery. Surface modified trimethylated chitosan poly (D,L-lactide-co-glycolide) nanoparticles (a mean diameter around 150 nm) had the capability to cross the BBB and showed a low toxicity (Zubareva et al., 2013).

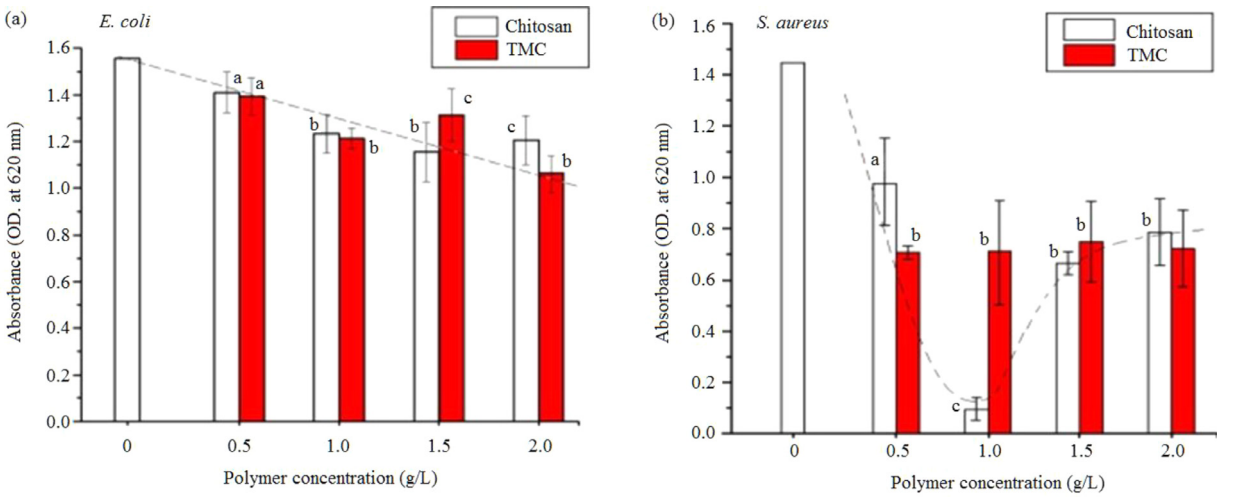

Fig. 6. Absorbance, as measured at 620 nm, as a function of chitosan and chitosan and trimethyl chitosan (TMC) concentration added in the medium with *Escherichia coli* (a) *and Staphylococcus aureus* (b), according to turbidity method after 12 h interaction (Goy et al., 2016).

8.3. Chitosan as a natural antimicrobial in food industry

Food safety is a global issue with significant implications for human health. The World Health Organization (WHO) reports that foodborne illnesses are usually infectious or toxic in nature and caused by bacteria, viruses, parasites or chemical substances entering the body through contaminated food or water. Contaminated food causes more than 200 diseases ranging from diarrhoea to cancers. Almost 1 in 10 persons in the world fall ill after eating contaminated foods and 420 000 die every year (WHO, Food safety, 2017). In the United States, the Centers for Disease Control and Prevention (CDC) estimate that each year about 1 in 6 Americans becomes ill and thousands die of foodborne diseases (Scallan et al., 2011). In Canada, foodborne illness affect approximately 4 million persons each year (Thomas et al., 2013). The Public Health Agency of Canada (PHAC) announced an outbreak for *Escherichia coli* contamination on romaine lettuce on December 12, 2017 where seventeen individuals were hospitalized, and one individual died. The majority of cases were female (74%) (Public Health Notice—Outbreak of *Escherichia coli* infections linked to romaine lettuce-Canada.ca). In September 2013, there was an *E. coli* outbreak in the province of British Columbia from a cheese farm which caused the hospitalizations of several persons across multiple provinces including the tragic death of a woman. The *Listeria monocytogenes* contamination in a popular ready-to-eat (RTE) meat product sent shockwaves across Canada. The listeriosis outbreak was responsible for several hospitalizations and claimed the lives of 20 Canadians (Khan et al., 2015).

Chitosan has antimicrobial activity, which depends on its molecular weight, degree of deacetylation, and the method used to obtain the polymer. It has been reported that chitosan has antimicrobial action against various microorganisms, including Gram-positive bacteria, Gram negative bacteria, and fungus including yeast. Moreover, chitosan has numerous advantages over other synthetic antimicrobials as it exhibits a high efficiency against harmful micro-organisms at low concentrations but low toxicity towards mammalian cells than other molecules. There is an increasing interest to use chitosan as a natural preservative in food, cosmetics and pharmaceutical industries (Moussa et al., 2013).

There is a growing interest towards the use of natural preservatives as antimicrobial agents in food. Chitosan and its derivatives show a broad spectrum (bacteria, yeast and fungi) antimicrobial activity. It can be applied as a form of powder or solution, coating or film on different types of foods. Chitosan can be applied to extend the storage life of different categories of food products. The antimicrobial activity of chitosan is based on its cationic nature. The electrostatic interaction between positively charged R $N(CH_3)^{3+}$ sites and negatively charged microbial cell membranes is proposed to be responsible for cellular lysis and assumed as the main antimicrobial mechanism. This interaction suggests the occurrence of a hydrolysis of the peptidoglycans in the microorganism wall, provoking the leakage of intracellular electrolytes, leading to the death of microorganisms. The charges present in chitosan chains are generated by protonation of amino groups when in acid medium or they may be introduced via structural modification (Hafdani and Sadeghinia, 2011; Severino et al., 2014; Goy et al., 2016).

Chitosan and its derivatives such as trimethyl chitosan, showed antibacterial effect against (*Escherichia coli and Staphylococcus aureus*) *E. coli* and *S. aureus* (Fig. 6a and 6b). According to Fig. 6a, chitosan (0.5–2.0 g/L) and its charged derivative showed a similar effect against *E. coli* with an apparent linear tendency in reducing bacterial population as the concentration increases (dashed lines, Fig. 6a).

However, a different behavior was observed when these polymers were tested against the Gram-positive bacteria *S. aureus* (Fig. 6b). For this strain, both chitosan and its derivatives showed a reduction on the growth colonies, where the chitosan concentration played an important role in the antimicrobial activity. For solutions at a concentration of 1 g/L of chitosan, the absorbance is significantly reduced indicating this as an efficient concentration for inhibiting the *S. aureus* growth in liquid medium. The trimethyl chitosan

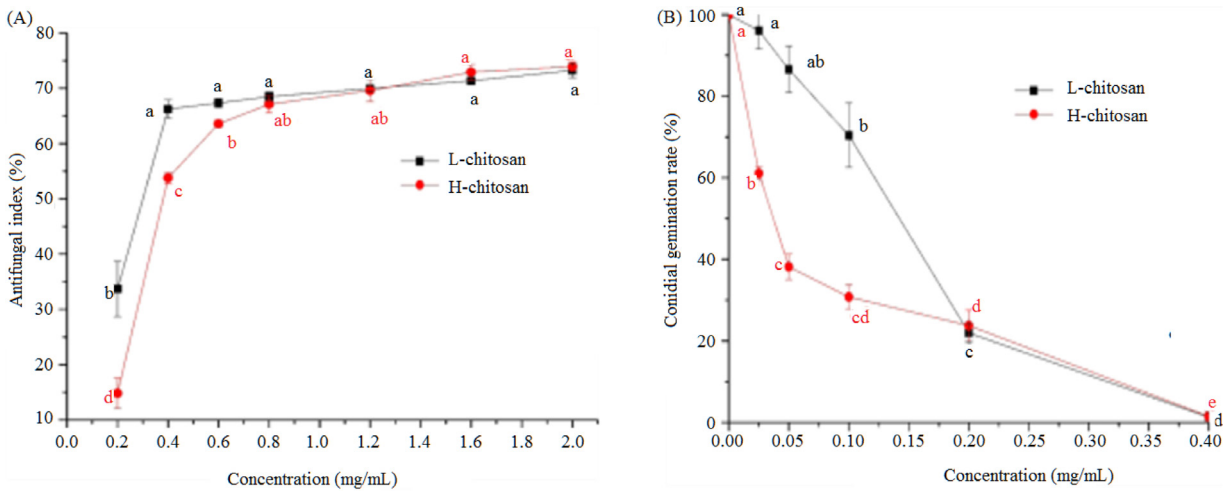

Fig. 7. Effect of L-chitosan and H-chitosan on (A) Antifungal index and (B) Conidia germination of *Botryotinia fuckeliana* (Jiang et al., 2016). Permission obtained from Wiley Periodicals.

(TMC) is efficient in reducing the number of colonies, but it does not show a quantitative dependence on the concentration (Goy et al., 2016).

Chitosan can be applied to extend the storage life of different categories of food products. Addition of chitosan in unpasteurised orange juice at concentrations up to 1 g/L extended the quality and preserved the ascorbic acid and carotenoids during storage time without impacting the nutritional value of the food products (Martín-Diana et al., 2009). In another study, it has been found that incorporation of chitosan in palm sap could be used as an alternative way to extend shelf life of pasteurized palm sap. Combination of pasteurization with chitosan (0.50 g/L) and low temperature storage could preserve palm sap for approximately six weeks (Naknean et al., 2015). Chitosan showed its antibacterial activity against *E. coli*, *Salmonella enterica*, *Enterobacter aerogenes*, *Bacillus cereus*, *Staphylococcus aureus* at 160–5120 mg/L in raw tomato juices (Giner et al., 2012). Chitosan is also useful as a natural preservative in rice-based products. It could retard the growth of *B. cereus* and total aerobic counts (TAC) in cooked rice stored at 37 °C up to 1 day. Under refrigeration (4 °C), there was no apparent growth of TAC or *B. cereus* in any cooked rice. It has also been demonstrated that the addition of chitosan does not have any impact on physicochemical properties of rice (Rachtanapun et al., 2015; Klinmalai et al., 2017).

Chitosan and its derivatives are also getting interests for micro/nano particles or emulsions for use as an antimicrobial coating or bioactive food packaging applications. One of the most common causes of food spoilage is fungal contamination. Chitosan could act on fungal cells causing the molecular disorganization and structural alterations of the cytoplasm and plasma membrane, as well as swelling and damage of the hyphae. Antifungal activities of low M_w (L)-chitosan and high M_w (H)-chitosan against *Botryotinia fuckeliana* were concentration-dependent. When the concentration was lower than 0.4 mg/mL, the antifungal indexes were up to 66% and 54% for L- and H-chitosan, respectively (Fig. 7A).

It has been found that the rate of conidia germination was also concentration dependant (Fig. 7B). Similar inhibitory activity has been found for Candida species and *Aspergillus niger* (Plascencia-Jatomea et al., 2003; *Jiang et al., 2016*). Chitosan coating with combined treatment showed a 3.3 log reduction on green beans after 14 days of storage against *Listeria innocua* (Severino et al., 2014).

Chitosan also finds application in active packaging, which is one of the latest innovative preservation techniques in food industry to increase the shelf life of food products. Chitosan film and cross-linked chitosan film exhibited antimicrobial activity alone and also when blended with plant extracts in ready to eat food applications. It had no impact on the texture, color and pH of the food products during storage, which demonstrates the useful antioxidant properties of chitosan (Quesada et al., 2016; Kaya et al., 2018).

Genipin crosslinked chitosan film coated with nisin showed an antimicrobial effect on five strains of *Listeria monocytogenes* on ready- to- eat meat applications. The growth rate for the films with 18.65 $\mu g/cm^2$ of nisin (both the un-cross-linked and cross-linked) were 0 log CFU/g per day as they completely inhibited the growth of *L. monocytogenes* (Fig. 8) (Khan et al., 2014).

9. Summary and Future Perspective

Chitosan-based products have immense commercial potential for the biomedical/tissue engineering and food applications. Several strategies have been proposed to modulate or functionalize chitosan for these applications. However, the commercial application of chitosan is still somewhat limited. The allergic concern of chitosan that are extracted from crustaceans is a significant one. The major allergen present in different crustaceans is the muscle protein tropomyosin, which can cause mild to severe allergic reactions. As a result, the demand for the production of chitosan from fungal source is getting more and more traction. Chitosan extraction from fungi cell wall by fermentation is economically feasible and is considered as a green synthesis process.

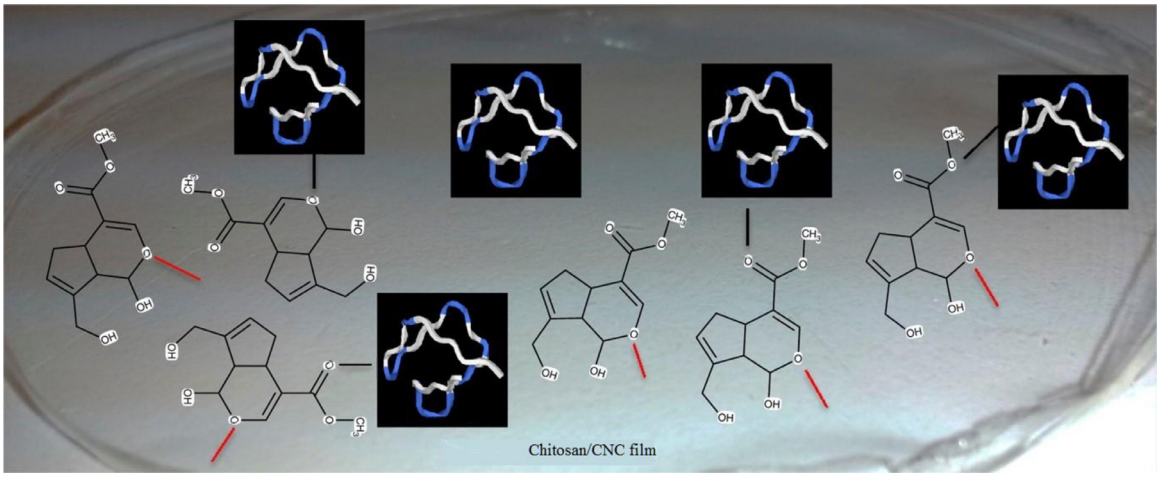

Fig. 8. Schematic representation of immobilization of nisin on the surface of the chitosan/CNC (cellulose nano crystal) films due to genipin cross-linking. Black lines indicate possible linkage between nisin and genipin; red lines indicate possible linkage between genipin and chitosan. Permission obtained from (Khan et al., 2014).

Fungal-based chitosan products are still limited at commercial scale. At present, there are a number of companies at the world stage, including Chinova Bioworks in Canada, have been making great efforts in developing new markets for fungal chitosan. Due to some unique advantages, it makes sense that the applications of fungal chitosan have been focused on biomedical/tissue engineering and food products, such as carriers for drug/gene delivery, wound dressing, hemo-compatible biomaterials, preservatives as antimicrobial agents in food, antibacterial food packaging.

For future research and development activities, more efforts should be directed to improve the fungal based chitosan production process, for example, in decreasing the use of harsh alkaline treatment conditions so that the process is eco-friendlier. Fungal- based chitosan is an excellent candidate for biomedical and food products. However, a great deal of research will be still needed to unlock the full potential of fungal- based chitosan. New methods such as hot water, mechanochemical and glycerol treatments for processing crustacean materials have been demonstrated to be greener compared with traditional methods in terms of chemical consumption and wastewater production. These novel greener methods may be used for fungal chitin/chitosan extraction and processing. There is no doubt that over the next few years, we should expect to see a lot more commercial chitosan-based products in the market.

Declaration of Competing Interest

There are no conflicts to declare.

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Review article

Chitosan nanoparticles (ChNPs): A versatile growth promoter in modern agricultural production☆

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Plant growth promoter

ABSTRACT

Agriculture is a backbone of global economy and most of the population relies on this sector for their livelihood. Chitosan as a biodegradable material thus can be explored for in various fields in its nano form to replace non-biodegradable and toxic compounds. The chitosan has appealing properties like biocompatibility, non-toxicity, biodegradability, and low allergenic, making it useful in several applications including in agriculture sector. Because of their unique properties, chitosan nanoparticles (ChNPs) are extensively applied as a bioagent in various biological and biomedical processes, including wastewater treatment, plant growth promoter, fungicidal agent, wound healing, and scaffold for tissue engineering.

Furthermore, the biocompatibility of chitosan nanoparticles (ChNPs) is reported to have other biological properties such as anti-cancerous, antifungal, antioxidant activities, even induces an immune response in the plant, and helps manage biotic and abiotic stresses. Chitosan can also find its application in wastewater treatment, hydrating agents in cosmetics, the food industry, paper, and the textile industry as adhesive, drug-delivering agent in medical as well as for bioimaging. Since chitosan has low toxicity, the nano-formulation of chitosan can be used for the controlled release of fertilizers, pesticides, and plant growth promoters in agriculture fields. The ChNPs applications in precision farming being a novel approach in recent developments. Here we have comprehensively reviewed the major points in this review are; the synthesis of ChNPs by biological resources, their modification and formulation for increasing its applicability, their modified types, and the different agricultural applications of ChNPs.

1. Introduction

After cellulose, chitin is the major natural polymer in the world. The primary sources exploited are two marine crustaceans, viz. shrimp, crabs, lobster, and crawfish (in general 20–30% on a dry basis), and are sufficient to support a commercial chitin/chitosan industry [1]. Chitin, a cellulose-like polysaccharide, is a linear, poly-β-(1,4)-N-acetyl-D glucosamine [2]. Chitin occurs in nature as ordered crystalline microfibrils. It is found in three polymorphic forms: α chitin, β-chitin, and γ-chitin. Chitosan, also known as deacetylated chitin, is a naturally occurring polycationic polysaccharide derived from partial deacetylation of chitin (as shown in Figure 1). Estimates of the global annual production of shell wastes from crab, lobster, shrimp, krill, and clam/oyster, on a dry basis, are as much as 1.44 million metric tons.

The degree of deacetylation, described by the molar fraction of deacetylated units or percentage of deacetylation, and the molecular weight of chitosan, were found to affect these properties. Due to its unique properties, chitosan is being extensively applied as a bioagent in various

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biological and biomedical processes like water treatment, wound-healing materials, as a drug carrier, and scaffold for tissue engineering. The cationic nature of chitosan is unique. The current world supply of chitinous wastes could support 50 to 100 million pounds [1]. Primary U.S. sources of crustaceans that are processed into chitin and chitosan are Dungeness crab (*Cancer magister*) and the Pacific shrimp (*Pandalus borealis*) [3]. Formerly, King crab (*Paralithodes camtschaticus*) was proposed as a chitin/chitosan resource; however, it is no longer available in sufficient quantity.

In agriculture sector, chitosan nanoparticles (ChNPs) by themselves can act as growth enhancers and potent antimicrobial agent against pathogenic fungi and bacteria [4]. Alternatively, they can also act as a nanocarriers for existing agrochemicals, hence are referred to as chitosan-based agronanochemicals [5, 6, 7]. In plant pathogen control and disease management, chitosan with or without the amalgamation of macronutrients could act as a substituted sustainable potent biocide agent against crop pathogens like bacteria, fungi and viruses [8]. Chitosan possesses a sustainable choice to be used as conventional fungicides against various diseases such as *Fusarium* wilt and head blight in chickpea and wheat, leaf blast in rice, stalk rot and leaf spot in maize as well as blast in finger millet [9]. Foliar application of oligo-chitosan and oligo-chitosan nano-silica demonstrated that soybean seed yield increased 10.5 and 17.0% for oligo-chitosan and oligo-chitosan nano--silica [10]. Systematic analysis of application of ChNPs (1–100 μg/mL) and chitosan showed adsorption of ChNPs on the surface of wheat seeds was higher than that of chitosan. Chitosan NPs application (5 μg/mL) induced the auxin-related gene expression [11]. The appealing properties of chitosan include biocompatibility, non-toxicity, biodegradability, and low allergenicity, making the chitosan valuable in several applications.

Chitosan is well known bio-stimulant used for promotion of growth and manage the stresses including pest and diseases [12]. Nanoparticles are more efficient with lesser molecular weights, have improved bioavailability, increased half-life and greater surface area to volume ratio. Therefore, present review is based on synthesis and characterization of ChNPs which holds the potent alternative for chemical pesticides and as bio-stimulant used in plant disease management. The latest researches on the application of ChNPs clearly indicate its benefit on plant productivity, plant protection against the attack of pathogens, and extension of the commercialization. Chitosan is a biodegradable material thus can be explored for various fields in its nano form to replace non-biodegradable and toxic compounds.

2. Definition, sources, and synthesis of chitosan nanoparticles (ChNPs)

Recently, ChNPs has developed a lot of attention for a wide range of applications in the agricultural, biomedical, and pharmaceutical industries. Chitosan NPs are synthesized through numerous methods by "bottom-up" approaches such as polymerization or a reverse micelle medium or microemulsion methods, and top-down techniques like milling, high-pressure homogenization, and ultra-sonication are also applied [13]. Various methods of ChNPs synthesis and their applications are represented in Figure 2.

The ionotropic gelation technique utilizes the electrostatic interaction between the amine group of chitosan and a negatively charged group of polyanion like tripolyphosphate. Chitosan can be dissolved in acetic acid, and NPs were formed spontaneously under mechanical stirring at room temperature. Changing the ratio of chitosan to the stabilizer can be modified the size and surface charge of particles [14]. Polyelectrolyte complex (PEC) formed by self-assembly of the cationic charged polymer and plasmid DNA due to fall in hydrophilicity because of charge neutralization between cationic polymer and DNA. The ChNPs can be synthesized spontaneously upon adding DNA into chitosan (in acetic acid) solution, under continuous stirring at room temperature. Polymer Grafting is a process of modifying polymer by attaching an active functional group. Examples are thiolation, esterification, and carboxylation, etc., these processes are used for active or passive targeting [15].

The pH-sensitive carboxymethyl chitosan (CMCS) NPs with fluorinated surface modification were prepared for efficient drug delivery. N-(3-Aminopropyl)-imidazole was pre-grafted onto CMCS to fabricate the pH-sensitive NPs, and then was surface-modified with perfluorobutyric anhydride to give the fluorinated NPs. The results suggested that the CMCS NPs had great potential to be efficient drug carriers for cancer chemotherapy [16].

2.1. Biologically synthesized chitosan nanoparticles (ChNPs)

Chitosan NPs can be synthesized by biological method with the help of different biomolecules. Sathiyabama and Parthasarathy [17] prepared ChNPs by adding anionic proteins isolated from *Penicillium oxalicum* culture to chitosan solutions. ChNPs with high antifungal activities are obtained through biological processes [17].

Anitha et al. [18] worked on nanoformulation of curcumin using dextran sulfate and chitosan. The result showed the preferential killing of cancer cells compared to normal cells by the curcumin-loaded NPs. Thus, the developed curcumin-loaded nanoformulation could be a promising candidate in cancer therapy. Figure 3 illustrated the graphical representation of biological synthesis and formulation of ChNPs.

3. Role of biological chitosan NPs as a plant growth promoter

Modern agriculture has the primary concern regarding the production of food with good quality and sufficient quantity to meet the demand of population rise in the world, restraining environmental impacts. Hence, scientists started to think about nanotechnology in the field of agriculture [19]. Although various studies reported the use of chitosan in agriculture, the application of ChNPs has yet to be explored (Table 1). The oppositely charged polymers and the amine group of chitosan form several complexes that could be beneficial in the agriculture sector [20]. Different forms of chitosan are applied in the field for plant growth promotion in detail described in later sections.

3.1. Free chitosan nanoparticles (ChNPs)

Chitosan is a non-hazardous, biocompatible, biodegradable, and natural biopolymer having a broad application. Chitosan on application to plant induces photosynthetic rate, stomatal closure, enhance antioxidant enzymes by signaling pathways of nitric oxide and hydrogen peroxide. It also stimulates the biosynthesis of amino acids, sugars,

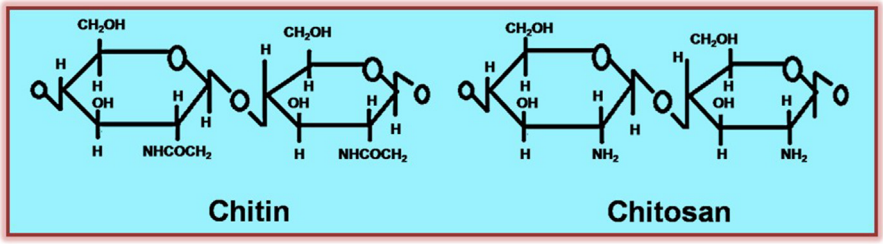

Figure 1. Structure of chitin and chitosan.

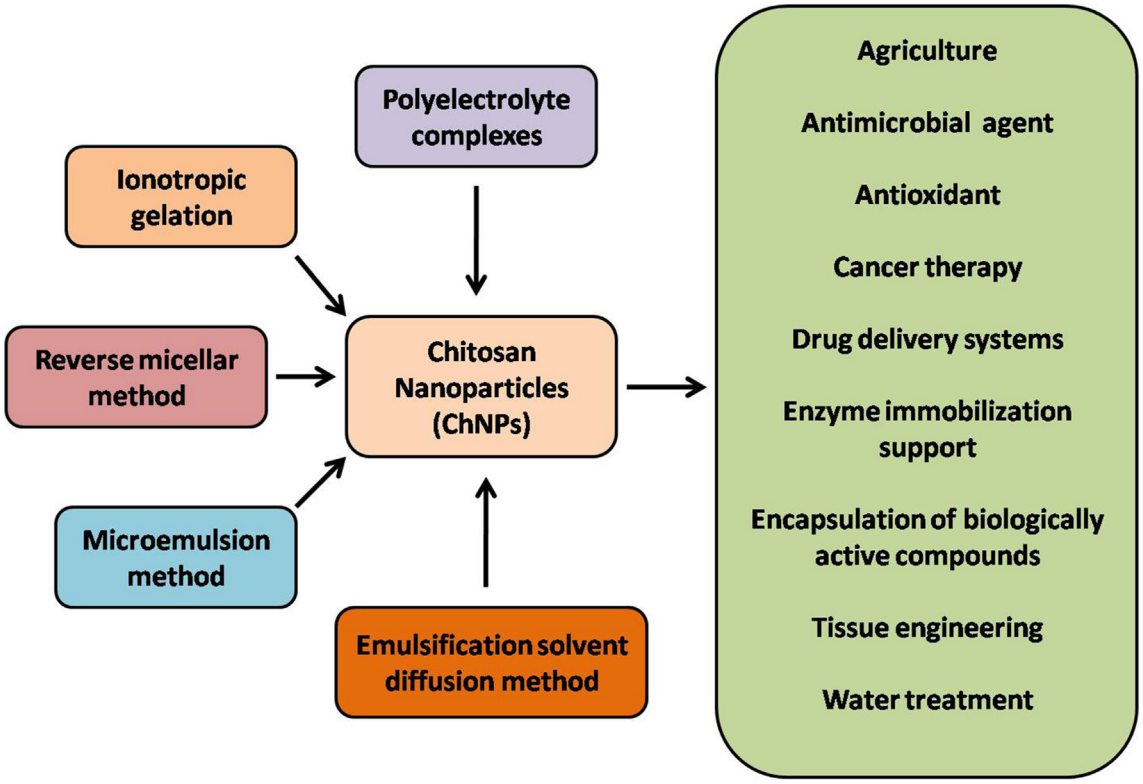

Figure 2. Methods of Chitosan Nanoparticles (ChNPs) synthesis and their applications in different fields.

organic acids, and other metabolites, an essential component of stress tolerance and energy metabolism pathways [21].

Many researchers worked on ChNPs as plant growth promoters [20]. Chitosan enters the seeds through the imbibition and, as a result of seed/chitosan interaction, positively affects the seeds germination index, reduced germination and flowering time, increased plant growth, and biomass production [21]. Behboudi et al. [22] experimented with the effect of ChNPs under drought stress on *Triticum aestivum* L. seedlings. The wheat seeds were sown in soil after treatment with ChNPs. The ChNPs, at a concentration of 90 ppm, augmented relative water content, leaf area, photosynthesis rate, chlorophyll content, superoxide dismutase, and catalase activities, biomass, and yield compared to the control. Lastly, their outcome shows that using ChNPs at a concentration of 90 ppm could mitigate the unfavorable effects of drought on the wheat seedling growth under drought stress [22].

3.2. Conjugated chitosan nanoparticles (ChNPs)

Chitosan composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine (acetylated unit) as a linear hetero-polysaccharide. Chitosan is one of the few essential polysaccharides widely used in agriculture, biotechnology, food, chemical, medicine, feed, and environmental protection [9, 23, 24]. As a new drug delivery, conjugated NPs have been widely concerned by researchers in recent years. It can easily be conjugated with other moieties due to having an amine group.

In pot experiments, Cu-ChNPs demonstrated the growth-promoting effect in plant height, stem diameter, chlorophyll content, root length, and number. The defense response during NPs treatment showed higher antioxidant and defense enzymes [25].

The oligo-chitosan was prepared through degradation of chitosan solution (4%) having 0.5% H_2O_2 by gamma Co-60 radiation and the silica NPs by calcinations of acid-treated rice husk at 700 C for 2 h. These oligo-chitosan and oligo-chitosan-silica NPs were employed on soybean seed yield in the experimental field by foliar application. The exciting results indicated an increased soybean seed yield by 10.5 and 17.0% for oligo-chitosan and oligo-chitosan silica NPs, respectively, over the control [37].

Somayyeh and Masouleh [38] evaluated the effect of chitosan and magnetism in lily yearling bulblets to synthesize photosynthetic compounds. Carboxymethyl chitosan (CM) and Magnetic nanocomposite (MN) were used during the production of the yearling bulblet. The results indicated that MN highly affected the photosynthetic pigments and the amount of starch in lily bulbs. The highest amount of CM showed soluble carbohydrates and amylase [38].

3.3. Polymeric chitosan nanoparticles (ChNPs)

Polymeric ChNPs could be synthesised from synthetic as well as natural polymers. These NPs could be used due to their simplicity to modify its surface and stability. Biopolymeric NPs have additional advantages such as accessibility from marine (for e.g. chitin and chitosan) or agricultural (such as starch, cellulose and pectin) resources, biocompatibility, biodegradability, and non-toxicity. Chitosan NPs are biodegradable polymers hence these are mainly studied as delivery systems for slow and controlled release of active ingredients, stabilization of biomolecules such as proteins, genetics materials, and peptides [39, 40]. In another research study, Pereira et al. [41] developed the two systems of polymeric ChNPs with the alginate/chitosan and chitosan/tripolyphosphate NPs for the delivery of plant hormone gibberellic acid (GA). These systems showed efficient changes on both morphological and biochemical parameters, which resulting in increasing the leaf area and root length, and the chlorophylls level and carotenoids content in *Phaseolus vulgaris* (French beans) [41]. There are several systems have been developed that demonstrated the good potential by providing excellent stability and effectiveness of this plant hormone like GA in agriculture applications [42].

3.4. Encapsulated chitosan nanoparticles (ChNPs)

Encapsulation technique is essential for food processing, bioengineering industries, and agriculture fields. For the encapsulation of active

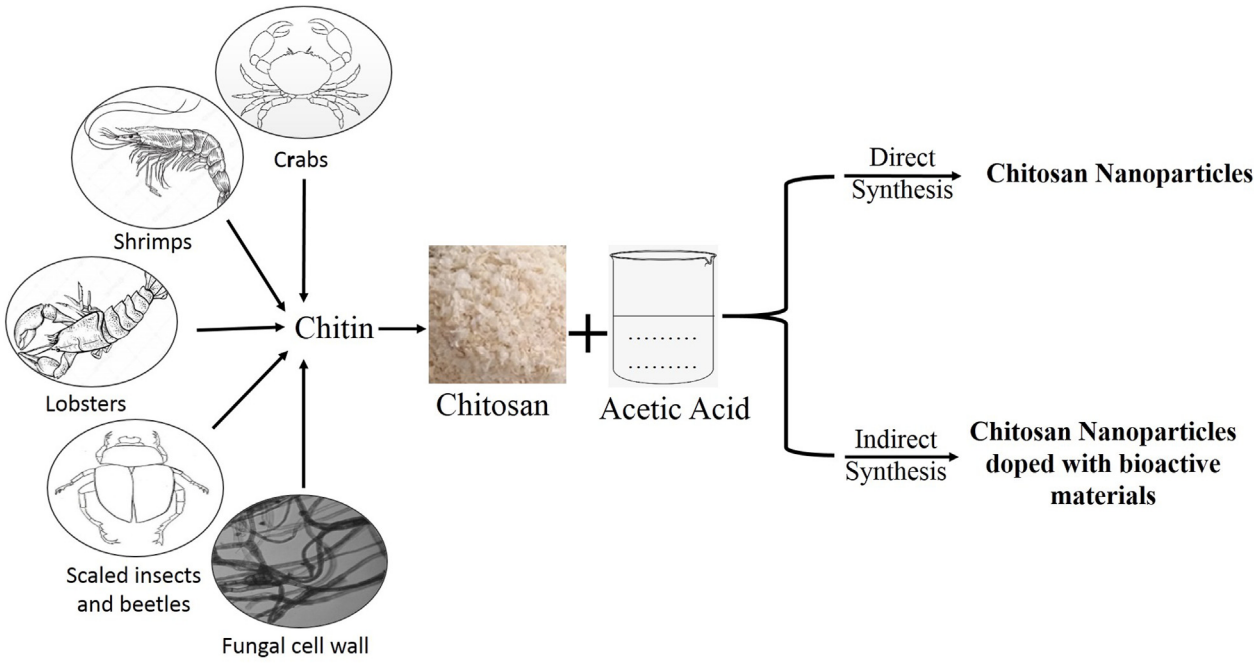

Figure 3. Biological synthesis and formulation of Chitosan Nanoparticles (ChNPs).

food ingredients, immobilization of enzymes and as carriers of different molecules used in agriculture and fertilizers, chitosan has been widely employed in industries. In recent years, metal encapsulated chitosan-NMs have to pay more attention because of their dual activity as a plant protection agent and plant growth promoter [24].

The delivery of ChNPs loaded with nitrogen, phosphorus, and potassium (NPK) by foliar application on the wheat seedlings was investigated by Abdel-Aziz et al. [23]. Chitosan-NPK-NPs were quickly applied onto leaf surfaces and penetrated the stomata by gas uptake, evading direct interaction with soil systems. The results discovered that the NPs be taken up and transported via phloem tissue. When treated with nano chitosan-NPK fertilizer, wheat seedlings provoked a significant increase in wheat yield variables than respective control seedlings [23]. In another study, Choudhary et al. [25] synthesized Cu/Zn ChNPs and tested them against crop pathogenic fungal species like *Culvularia lunata*.

Furthermore, Cu/Zn ChNPs are involved in inducing enzymes amylase and protease related to the mobilization of food for seed germination [25]. To develop an effective nano delivery system, plant growth regulators may be encapsulated in the chitosan nanocarriers that slowly release the hormones with higher bioavailability. In another study, Pereira et al. [41] reported the growth-promoting effect of chitosan-gibberellic acid NPs in French beans that exhibited a 37% and 82% boosting in root development and leaf area, respectively, as compared to the free hormone gibberellic acid.

4. Importance of biologically synthesized chitosan NPs over other nanoparticles

Sathiyabama and Parthasarathy [17] biologically synthesized the ChNPs and evaluated their antifungal activity against some phytopathogenic fungi for e.g. *Alternaria solani*, *F. oxysporum*, and *Pyricularia grisea*. Whereas in another work, chitosan and ChNPs had been used to induce the biotic stress tolerance in tomato (*Solanum lycopersicum*) plant against bacteria such as gram positive and gram negative, fungi like *Fusarium solani*, and viruses like potato spindle tuber viroid (PSTV), bean/tomato bushy stunt virus (TBSV), and tobacco/TNV and also to initiate the immune response against them where ChNPs are used, which are sensed by plant PRR (pattern recognization receptor) which then induces the immune response in the plant [43].

Chitosan has an advantage over other NPs as they are very versatile and biocompatible, have low toxicity, and can be degraded easily, while other NPs can cause toxicity [44]. Chitosan NPs act as antimicrobial agents that show antibacterial, antifungal, antiviral, antioxidant activity, induce an immune response in the plant and helps in managing abiotic and biotic stresses. Since chitosan has low toxicity, nano-formulation of chitosan can be applied for the controlled-release of fertilizers, pesticides, and plant growth promoters in agriculture fields. Chitosan could also be employed for wastewater treatment, hydrating agents in cosmetics, in the food industry, in paper and textile industry as adhesive, as a drug-delivering agent in medical, and bioimaging [9].

The ChNPs and the chitosan-based agronanochemicals applied in agriculture could be organized by several methods, including emulsion cross-linking, precipitation, spray drying, ionic gelation, and sieving and reverse micellar processes [45]. The techniques mentioned are chemical and physical, which are having some demerits such as sieving method has been documented to fabricate NPs with irregular shape and size; however, the emulsion cross-linking process is relatively tedious and require cross-linking agents like alginate, formaldehyde, and glutaraldehyde, that may reason the impediments because of its incongruity with the active ingredients used as agrochemicals. The resultant particles size mostly depends upon the droplet size of the emulsion, which consecutively relies on the degree of cross-linking, surfactant type, the molecular weight of chitosan, and stirring speed [9]. The reverse micellar method is thermodynamically stable produced the uniformly distributed, small-sized chitosan nanoparticulate system. But this method needs a specialized surfactant solution like cetyl trimethyl ammonium bromide (CTAB), which is somewhat toxic and expensive; also, the process is quite laborious. Precipitation methods developed the ChNPs with no stability, irregular shape, and lower mechanical strength. The spray drying processes have been broadly employed to fabricate dry granules, pellet, and powder forms of chitosan. The techniques use the sequential addition of active ingredients and cross-linking agents to the chitosan solution dispersed in acetic acid. The precursor solution then underwent an evaporation method under hot air steam that ultimately forms the desired NPs.

The physical and chemical synthesis methods have several disadvantages, as described earlier, which leads to applying biological resources in the fabrication of ChNPs. The biologically synthesized ChNPs

Table 1. Chitosan Nanoparticles (ChNPs) formulations and their effect on agriculture crops.

Sr. no.	Type of Chitosan Nanoparticle formulation applied	Type of Application	Crop used	Effects on crop plants	Reference
1	Chitosan NPs	Seed treatment	*Pennisetum glaucum*	Leads to activation of early defense responses and elevation in nitric oxide accumulation against Downy mildew	[20, 26]
2	Chitosan-coated mesoporous silica NPs	Fruit surface treatment	*Citrullus lanatus*	Improved suppression during the fungal disease and altered expression of stress-related genes	[27]
3	ChNPs	Media enrichment	*Capsicum annum*	Increased plant heights, chlorophyll content, leaf numbers, leaf width as well as length	[28]
4	ChNPs	Foliar application	*Camellia sinensis* (L.) O. Kuntze	Induced the defense response in a nitric oxide dependent manner against Blister blight disease	[29, 30]
5	ChNPs + rhizobacteria (PS2 and PS10)	Stem Treatment	*Zea mays*	Improved seed germination, plant height, leaf area, internodes number and chlorophyll content in maize; enhanced dehydrogenase, alkaline phosphatase activity and fluorescein diacetate hydrolysis; increased stress tolerance mechanism	[31]
6	ChNPs	Foliar and soil application	*Hordeum vulgare* L.	Considerably augmented the leaf area and color, the number of grains per spike, the grain yield and the harvest index, reduced effect of drought stress	[21]
7	Chitosan-aloe vera gel coating	Post-Harvest Application	*Mangifera indica* L.	Coating mango fruit with chitosan will reduce the rate of rotting	[32]
8	Cu-ChNPs	Seed treatment and *in vitro* antifungal assay	*Solanum lycopersicum* Mill.	Significant growth promoting effect on germination of tomato seed, seedling length, fresh and dry weight; also *in vitro* antifungal activity against *Alternaria solani* and *Fusarium oxysporum*	[33]
9	Cu-ChNPs	Seed treatment	*Z. mays*	Higher percent germination, root and shoot length, root number, seedling length, fresh and dry weight and seedling vigor index; encouraged the activities of enzymes α-amylase and protease and increased the total protein content in germinating seeds	[34]
10	Cu-ChNPs	–	*Z. mays*	Defense against *Curvularia* leaf spot disease by inducing antioxidant and defense enzymes such as phenylalanine ammonia-lyses and polyphenol oxidase	[24, 25]
11	Chitosan–PVA and Cu NPs	Seed treatment	*Solanum lycopersicum* Mill	Increased tomato growth and chlorophylls 'a' and 'b', carotenoids, total chlorophylls, and superoxide dismutase content; activated synthesis of vitamin C and lycopene	[35]
12	ChNPs	Seed treatment	*Triticum aestivum* L.	Growth promoter, induces auxin-related gene expression, hastened indole-3-acetic acid (IAA) biosynthesis and transport, reduced IAA oxidase activity	[36]

have several advantages: stability, regularity in shape and size, bioavailability, biocompatibility as the biologically active compounds taken part in the capping and the reducing process, non-toxic, and no added instrumentation or labor requirement, and so on. Therefore, the scientific community in recent years focuses on the green or biogenic synthesis of NPs using biological resources. In agriculture, nano-formulations are mainly aimed at enhancing the benefits of agrochemicals and chitosan though concurrently diminishing the undesirable results. Because of the amphiphilic nature, the encapsulation of chitosan may conquer the deprived solubility of several agrochemicals in water, provided that unusual use of inert chemicals in conventional agrochemicals, thus, tumbling their toxicity level [46]. Besides these, chitosan has provided excellent protection to the encapsulated agrochemicals because of their bioadhesive properties that enhance the stability and bioavailability in the plant. The efficacy of chitosan-based agro-nanochemicals, compared to conventional agrochemical to embark upon the actual tribulations faced by the agriculture industry, must be appraised by checking all the above parameters.

The biologically synthesized chitosan and chitosan-based agro-nanochemicals could be applied in different functions and play various roles as slow or controlled discharge formulations, plant growth enhancement, and biocidal or antimicrobial activity against plant pathogens and pests are described in detail in the sections below.

5. Other roles/functions of chitosan nanoparticles (ChNPs) in agriculture

In the agriculture sector, ChNPs by themselves could act as an antimicrobial agent against the crop pathogenic microorganisms like fungi for eg. *Pyricularia grisea*, *Alternaria solani*, and *Fusarium oxysporum* and bacteria like gram positive and gram negative, and other insect pests like *Aphis gossypii*, *Callosobruchus chinensis*, and *Callosobruchus maculatus* and as a plant growth promoter [8, 9]. The formulations of ChNPs have the capability to enhance the plant defense mechanisms by obtaining the defense enzyme functions upon its application. Xing et al. [47] reported significant antifungal effect of oleoyl-chitosan nano-formulation against several pathogens. The comparative antimicrobial activity of ChNPs and bulk chitosan counterpart on *A. solani*, *P. grisea*, and *F. oxysporum* was investigated by Sathiyabama and Parthasarathy [48]. Treatment of seeds followed by foliar application with chitosan induces the resistance of tomato plants to *Phytophtora infestans* and *A. solani* [49]. Chitosan NPs application at anthesis (1000–5000 ppm, molecular mass 161–810 kDa, deacetylation degree 75–90%) is effective to control *Fusarium* head blight of wheat caused by *Fusarium graminearum* [50]. The anthracnose disease of cucumber caused by *Colletotrichum* spp. was efficiently controlled through foliar application of 0.05% and 0.1% chitosan [51]. Further the life cycle of the nano-fertilized wheat plants was shorter than normal-fertilized wheat plants (130 days compared with 170 days for yield production) [23]. Chitosan combined with waste silica may allow farmers to reduce the use of NKP fertilizers to improve corn production in Indonesia with environmental and economic advantages [52]. Maize seeds treated with Cu-ChNPs for 4 h (0.04–0.16%, Chitosan 50–190 kDa, deacetylation degree 80%), followed by spraying of plants every day for 35 days show enhanced plant height, stem diameter, root length and number, chlorophyll content, ear length and weight/plot, grain yield/-plot and weight [26]. Besides this, they could also act as nanocarriers for some accessible agrochemicals that are generally referred to as chitosan-based agronanochemicals [7, 46]. The nanocarrier system facilitates the agriculturally active ingredients encapsulated by covalent or ionic inter or intramolecular bonds or entrapment in a chitosan polymeric matrix to develop an adequate formulation nano delivery system [7]. The recently reported studies on ChNPs have proven the various

Figure 4. Roles of chitosan nanoparticles (ChNPs) in agriculture.

efficient ways of applying ChNPs in agriculture crops and fields as shown in Figure 4.

As these ChNPs have shown their ability as a potent plant growth promoter [33], antimicrobial activity through various modes against pathogens of bacterial and fungal origin [53] and also induces an immune response in plants against viruses [43]. They may be employed directly to the soil as a soil applicant or a foliar applicant to show the systemic effect on the plant body [54]. The nanoformulations of chitosan are also applied for post-harvest uses as fruit nanocoatings to improve shelf life and prevent any damage due to microbes [47, 55]. In the above Table 1 the different types of ChNPs and their effect on the various crops has been presented.

5.1. Plant growth enhancement and increased productivity

In recent years, ChNPs and their nanoformulations have been extensively researched as a plant growth enhancer. The positively charged rich protonated chitosan demonstrates enhanced affinity towards the cell membranes, ensuing in increased reactivity in the plant system. In addition, chitosan has a nitrogen content of around 9–10% that serves as a macronutrient for the plant [45]. Alternatively, chitosan could be integrated with plant nutrients phosphorus (P), nitrogen (N), magnesium (Mg), potassium (K), sulfur (S), calcium (Ca), boron (B), iron (Fe), copper (Cu^{2+}), manganese (Mn), zinc (Zn) and nickel (Ni). The application of ChNPs and micro/macronutrients nanocarrier in plant growth promotion in wheat, maize, French beans, and Robusta coffee, has been documented by several researchers [23, 24, 34, 56, 57, 58, 59, 60, 61, 62].

The nanoformulations of chitosan have been extensively applied as an unconventional method in seed treatment to promote the germination rate and enhance biomass accumulation. Furthermore, chitosan nanoformulations have been employed as a growth promoter by improving the nutrients uptake, chlorophyll content, and photosynthesis rate. For instance, chitosan oligomer with a high molecular weight and ChNPs of three variable average diameter sizes by High Resolution Transmission Electron Microscopy (HRTEM), i.e., small with 420 nm, medium with 750 nm, and 970 nm (large) size, when sprayed on the Robusta coffee seedlings leaves, demonstrated better nutrient uptake of Ca, K, N, Mg, P, etc., by ChNPs at all sizes, than that of chitosan oligomer. The nutrient uptake was affected; with the size was insignificant [56]. Alternatively, an impact of length of ChNPs could be observed on the content of chlorophyll and photosynthesis rate. The enhancement in the chlorophyll content and photosynthesis rate have been reported up to 61% and 29% for small, 81% and 59% for medium and 61% and 72% for large-sized NPs, respectively, by treating ChNPs. The treatment of ChNPs has also improved the vegetative growth of the seedlings compared to the chitosan oligomer treated and the untreated control seedlings [56]. In another study, Zayed et al. [60] demonstrated the abiotic stress (salinity stress) tolerance by *Phaseolus vulgaris* seedlings when supplementation of ChNPs was given to them.

Furthermore, chitosan-polymethacrylic acid-NPK NPs nanoformulation has been developed and applied for the wheat crop [23]. The efficacy of the nanoformulation was evaluated with the conventional bulk NPK fertilizer. The nanoformulation with 500 mg/mL of N, 60 mg/mL of P, and 400 mg/mL of K applied to wheat, evidenced for the plant height 41.29 cm, main spike weight 0.178 g, crop yield 6.95 g/plant, and harvesting index 26.94. At a similar quantity, the bulk NPK fertilizer evidenced for the same parameters were found the plant height 38.85 cm, main spike weight 0.136 g, crop yield 6.13 g/plant, and harvesting index 21.64, which showed the superior nanoformulation potential as plant growth and crop yield improvers of wheat [23]. The efficacy of copper sulfate ($CuSO_4$), bulk chitosan, and Cu-ChNPs was investigated on the growth of maize seedlings by Saharan et al. [34] that showed the significant impact of the nanoformulations on the development of maize seedlings, α-amylase and protease activity, and total protein content. Depending upon the finding, it was assumed that the nanoformulations could facilitate penetration into seeds and, consequently, enhance seed metabolism; most probably, bulk chitosan may extend a film coating on the seed surface, which, therefore, prohibited their entree to water as well as nutrients.

The development of an efficient nano delivery system of hormones could be possible by encapsulating the plant growth regulators into chitosan nanocarriers for slow release and with greater bioavailability. The plant growth regulators are nothing but the plant hormones, like auxins, cytokinins, gibberellins, ethylene, and abscisic acid, which are chemicals responsible for plant cell development and growth. Gibberellic acid-ChNPs demonstrated an increase of root development by 37% and in leaf area by 82% in French beans than free gibberellic acid [59]. Pereira et al. [59] highlighted the beneficial effects of the nanoparticulate systems by reporting the formation of the more lateral roots in the *Phaseolus vulgaris* seedlings supplemented with the γ-polyglutamic acid-gibberellic acid-ChNPs compared to the free hormones. The seeds of chickpea, when undergone the treatment of thiamine-ChNPs, showed a more significant germination percentage (90%) compared to the combination of Thiamine-chitosan (84%) and water control (75%) [62]. The seedlings treated with a nanoparticulate system demonstrated a 10-fold increase in auxin levels and more defense enzymes than the untreated control seedlings.

5.2. Antimicrobial for crop pathogens and pests to manage diseases in plants

In plant-pathogen control and disease management, chitosan alone or in combination with macronutrients could substitute effective, sustainable biocide agents against crop pathogens like bacteria, fungi, and viruses. Chitosan alone or in integration with other active agents demonstrated promising perspective as a sustainable choice to the conventional fungicide application against *Fusarium* wilt and head blight disease in chickpea and wheat, blast leaf of rice, stalk rot after flowering and leaf spot in maize, blast disease of finger millet, and other [9]. The formulations of ChNPs integrated by polyacrylic acid provide the tremendous potential to manage the attack of some common pests such as cotton aphids (*Aphis gossypii*) and beetles during soybean cultivation [8]. Many studies have discovered the ChNPs formulation to enhance the plant defense mechanisms by obtaining the defense enzyme functions. Additionally, Xing et al. [47] demonstrated the in-vitro antifungal activity of oleoyl-chitosan nanoformulation against some crop pathogenic fungi like *Alternaria tenuissima*, *Botryosphaeria dothidea*, *Fusarium culmorum*, *Gibberella zeae*, *Nigrospora oryzae*, and *Nigrospora spaerica*. From which *A. tenuissima*, *N. oryzae*, *N. spaerica*, and *B. dothidea* showed significant antifungal effects categorized as chitosan-sensitive fungi, while *G. zeae* and *F. culmorum* could be classified as chitosan-resistance fungi [47]. Chitosan (CS)-g-poly (acrylic acid) (PAA) NPs was found to be sensitive to the fungi like *Aspergillus flavus* (75%), *F. oxysporum* (30%), *Aspergillus terreus* (40%), *Fusarium solani* (41%), *Alternaria tenuis* (40%), and *Sclerotium rolfsii* (36%) [8]. Furthermore, the *in-vitro* spore germination and mycelial growth of *Alternaria alternata* (90%), *Rhizoctonia solani* (60%), and *Macrophomina phaseolina* (63%) was efficiently inhibited during Cu-ChNPs treatment [63]. The comparative antimicrobial activity of ChNPs and bulk chitosan counterparts on *A. solani*, *P. grisea*, and *F. oxysporum* was investigated by Sathiyabama and Parthasarathy [17]. The ChNPs exhibited a more significant percentage of mycelia growth inhibition than that of bulk chitosan. It has been accounted that the smaller size, higher porosity, and more significant zeta potential of ChNPs make it highly stable, which ultimately affects the tested fungal pathogens.

The bulk chitosan (BCS), ChNPs, and ChNPs supplemented by ethanolic blueberry extract (ChNPs-EBE) showed inhibitory effect on *A. alternata*; in this case, the trend was found to be ChNPs-EBE (83.3%), ChNPs (83.1%) > BCS (6%) only [64]. Their inhibitory effect on *Colletotrichum gloeosporioides* showed the trend as ChNPs-methanol extract (79.6%) > ChNPs (57%) > BCS (9.4%). In another study, Kheiri et al. [50] used chitosan with three variable molecular weights (MW), i.e., the

lowest 161 kDa MW, medium 300 kDa, and highest 810 kDa, the fabrication of the nanoparticulate system. The formed NPs demonstrate lower zeta potential and a larger mean size with the MW increase and, therefore, lowered the in-vitro antifungal activity on *Fusarium graminearum*. The ChNPs of lower MW demonstrates 2-fold greater antifungal activity than NPs of medium and higher MW chitosan. This is because the smaller size facilitates the easy cell penetration and greater charge makes the ChNPs of lower MW more stable.

Furthermore, the formation of chitosan-agrochemical NPs, the chitosan nano delivery system was loaded with agrochemicals as the active agent that offer the proscribed release properties with higher efficiency and potency, as the active constituent could arrive at the target cell or parts of the plant more efficiently within a definite time [65]. The essential parameters considered in the design and development of chitosan-agrochemicals NPs involves active agent's loading, encapsulation competence, discharge profile, particle size, and shape. Several studies reported and considered all these mentioned parameters during the design and development of these nanoformulations. Ye et al. [66] developed an herbicide (diuron) nanocarrier system as a photosynthetic inhibitor using cross-linking 2-nitro benzyl and carboxymethyl chitosan with the mean HRTEM diameter size of 140 nm to control the weed growth. A mechanism of photo-controlled release developed these nanoformulations. In another study, Kumar et al. [67] developed an intelligent formulation of alginate-chitosan nanocapsules with the size 30–40 nm diameter by HRTEM for the proscribed release of acetamiprid. In the present system, the proscribed release properties were accomplished at three different pHs, where a 50% of release of insecticide was observed at pH 10 after 24 h, and after 24 h at pH 7 and 4, compared to merely about 6 h for the conventional insecticide release at all pHs. Maluin and Hussein [9] reviewed the applications of chitosan-based agronanochemicals as a sustainable choice for the protection of crops, in which the authors have discussed the variable chitosan-based agronanochemicals as a controlled release formulation, as a plant growth enhancer, and as a biocide against the crop pathogens and pests.

5.3. Seed treatment

Seed treatment is a better and advanced approach as compared to soil amendment. It is a targeted and controlled delivery approach of active plant ingredients that reduces their overuse and decomposition in soil. A most recent approach is based upon maintaining the integrity of seed coat and simultaneously reducing water solubility using biodegradable hybrid coats of chitosan for seeds [68]. The strategy of chitosan application in seed treatment is considered as the primary artificial defense activation in plants against the different infectious agents. Differential characteristic features of chitosan at various molecular weights make it an excellent seed treating agent. The biopolymer with a high molecular weight of chitosan could be applied as a covering film around the seeds to protect the infection by pathogens [69]. Chitosan seed coating can also be used as a deliverance system for different products used in plant protection, fertilizers, and plant growth-promoting micronutrients [70]. Chitosan is used as a film for seed, which helps deliver fertilizers, micronutrients, and plant protection products such as essential oils and others. It helps elicit systemic confrontation in the plants [20].

5.4. Soil application

Although chemical-based fertilizers and pesticides have high and immediate impacts on crop yield, they also negatively affect the environment and consumers. Less than 0.1% of agrochemicals are delivered to plant systems, and the rest are washed off into the atmosphere [71]. Chitosan NPs are studied for their utilization in agriculture as a soil applicant to manage various fungal and bacterial diseases, as a nanofertilizer, and as an efficient delivery system for agrochemicals. The agrochemicals encapsulation in chitosan nanoformulations can offer a controlled release system for agrochemicals. This has helped to comparatively evaluate the effect of chitosan-based agronanochemicals and their counterparts on soil microbial populations. There is no significant effect of ChNPs on the soil enzyme activity and microbial population compared to the chemical fertilizers [9, 72]. Maruyama et al. [73] have shown an improved effect on microbial population after applying chitosan-alginate-herbicide NPs to soil [54, 73].

5.5. Foliar application

Numerous synthetic chemical fertilizers and pesticides prevent crops from attacking pests and diseases and provide nutrients such as nitrogen, phosphates, and minerals to increase agriculture productivity. But the use of synthetic fertilizers, pesticides cause damage to the quality and fertility of the soil. Moreover, the applied biomolecules are fully absorbed by the plants and the large quantity of these biomolecules' runoff into water bodies or leach in soil due to rain and irrigation. Nanotechnology has been demonstrated to help minimize the loss and enhanced the nutrient uptake by the plant. Due to its small size, this nanoformulation can reach deep into the soil [74]. These nutrients, growth hormones, and fertilizers are encapsulated in NPs and spray in the soil. The nanoformulation acts as a delivering agent and prevents the nutrient from coming in contact with soil microbes. They slowly release their content in the soil, which plants effectively take up. Due to its exceptional advantageous property, scientists employ chitosan in the agriculture field [75, 76].

Foliar application of ChNPs is used to increase the growth and production in the plant. Chitosan NPs get easily absorbed by leaves, penetrate the plant through stomata, travel down into the plant through the phloem, and provide nutrient to a different part [77]. Van et al. [78] worked on the biophysical characteristics of ChNPs and the greenhouse growth study of Robusta coffee. The ChNPs increased the nutrient uptake of nitrogen by 9.8–27.4%, phosphorous by 17.3–30.4%, and that potassium by 30–45%, and it also impacts coffee seeding growth. Abdel-Aziz et al. [77] demonstrated the foliar application of ChNPs-NPK fertilizer to improve wheat yield, developed on the two different soils. The foliar application increased the output of the wheat plant, and it also reduced the life cycle of the crop. Whereas in another work where they studied the foliar application of ChNPs-NPK fertilizer affected the chemical composition of wheat grains found a change in the composition of wheat grain with increased element content such as potassium and phosphorous while a decrease in nitrogen and protein content also accumulation of carbohydrates [77].

6. Conclusions and future perspectives

It can be concluded from the available literature that the ChNPs could be a versatile, biodegradable, and biocompatible, low toxicity, and easy degrading alternative to presently available agrochemicals. In combination with the other metallic and metal oxide NPs, they show a considerable range of activities. Chitosan NPs can be applied in various fields based on their properties such as antibacterial, antifungal, antiviral, antioxidant activity, inducing an immune response in the plant, and helps in managing biotic, and abiotic stress so they could be implicated in wastewater treatment, hydrating agents in cosmetics, food industries, the paper, and textile industries as adhesive, drug-delivering agent in medical, and bioimaging. But the thorough studies on product development and method optimization are required before commercial production and *in vivo* use. Various methods are used for the application of ChNPs in agriculture crops and fields. Chitosan NPs could be applied on crops by methods like dust and foliar sprays, soil application, and seeds treatment. Similarly, ChNPs explored in the ever-growing field of agriculture and sustainable agricultural practices. Chitosan NPs can be a potential substitute for toxic and non-degradable compounds with biocatalytic activity. As ChNPs are discovered to have the ability to encapsulate the various agro-supplements, they could be employed for the controlled deliberations of fertilizers, pesticides, and plant growth promoters in

agriculture fields. The research community working in the area of crop protection and improvement will be the targeted beneficiaries. The ChNPs will be a cost-effective alternative for the use of toxic chemicals in the field of agriculture. Hence, green synthesised ChNPs would be a boon for the agriculture sector.

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Author contribution statement

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Review

A Review of the Preparation, Characterization, and Applications of Chitosan Nanoparticles in Nanomedicine

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Abstract: Chitosan is a fibrous compound derived from chitin, which is the second most abundant natural polysaccharide and is produced by crustaceans, including crabs, shrimps, and lobsters. Chitosan has all of the important medicinal properties, including biocompatibility, biodegradability, and hydrophilicity, and it is relatively nontoxic and cationic in nature. Chitosan nanoparticles are particularly useful due to their small size, providing a large surface-to-volume ratio, and physicochemical properties that may differ from that of their bulk counterparts; thus, chitosan nanoparticles (CNPs) are widely used in biomedical applications and, particularly, as contrast agents for medical imaging and as vehicles for drug and gene delivery into tumors. Because CNPs are formed from a natural biopolymer, they can readily be functionalized with drugs, RNA, DNA, and other molecules to target a desired result in vivo. Furthermore, chitosan is approved by the United States Food and Drug Administration as being Generally Recognized as Safe (GRAS). This paper reviews the structural characteristics and various synthesis methods used to produce chitosan nanoparticles and nanostructures, such as ionic gelation, microemulsion, polyelectrolyte complexing, emulsification solvent diffusion, and the reverse micellar method. Various characterization techniques and analyses are also discussed. In addition, we review drug delivery applications of chitosan nanoparticles, including for ocular, oral, pulmonary, nasal, and vaginal methodologies, and applications in cancer therapy and tissue engineering.

Keywords: chitosan; chitin; chitosan nanoparticle; drug delivery; cancer therapy; tissue engineering

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1. Introduction

Significant developments have been made in the recent past in medical imaging, drug delivery systems, advanced therapy, and the treatment of fatal diseases. In particular, the discovery of nanomaterials and nanomedicine has dramatically improved the precision and efficacy of a significant number of medical procedures and treatments. Nanoparticles (NPs) are nano-sized (on a scale of ~10^{-9} m) particles of matter that may have very unusual mechanical, physical, optical, and chemical properties compared to their larger-sized or bulk counterparts. Due to our increased capability of tuning desired characteristics and properties at various sizes and dimensions, the uses of nanomaterials have widened across a wide extent of industrial applications, including for medicine, cosmetics, air purification, agriculture, and environmental remediation. In recent years, the application of nanoparticles has increased significantly to the point of creating a nascent field of medicine, generally termed nanomedicine. Nanoparticles are widely used as contrast agents for medical imaging applications and as transport agents for drug and gene delivery in vivo [1]. Due to their small size, nanoparticles can enter the body and reach the specific tissue more efficiently and in a more direct fashion. Nanoparticles also have the capability to deliver molecules, such as from drugs, in less time and with lower pain to detect and cure diseases [2]. For nanomaterials to be used in the treatment of disease, many factors need to be considered, such as biodegradability, biocompatibility, size, hydrophilicity,

and conjugation power with various drugs. Among the water-soluble materials that are currently available, chitosan has all of the properties mentioned above. Furthermore, in part due to being inexpensive, chitosan nanoparticles are widely investigated for their implementation as drug delivery systems to cure various fatal diseases.

In this review, we discuss the synthesis, characterization, drug conjugation, and in vivo treatment of various diseases using chitosan nanoparticles. Chitosan is a polysaccharide or fibrous compound prepared by the N-deacetylation of chitin. Chitin is a biopolymer naturally produced within crustaceans' shells, such as those of shrimps, lobsters, and crabs. Chitosan also occurs in microorganisms, such as fungi and yeast [1]. The molecular structure of chitosan consists of glucosamine and N-acetyl-glucosamine units: The repeatability of the units is determined by the degree of deacetylation (DD) (see Figure 1). Having an equilibrium acid association constant pK_a value of ~6.5 on the amine groups makes chitosan insoluble at neutral pH values; however, chitosan is soluble at acidic pH values < 6.5, whereas the chitosan molecule is positively charged [3]. Notably, compounds in their un-ionized form tend to be less soluble, but can more easily penetrate lipophilic barriers between them, and are a biological target of interest [4]. The degree of deacetylation directly affects the occurrence of amine groups in the chitosan molecular structure, which can be protonated [3]. This has a direct bearing on the solubility, the degree of hydrophilicity vs. hydrophobicity, and the nature of interactions of chitosan with various polyanions. Chitosan is soluble in acetic, formic, citric, tartaric, and other organic acids [5]. Chitosan, however, is insoluble in some inorganic acids, including phosphoric and sulfuric acids [6]. Chitosan can be obtained in a broad range of molecular weights and degrees of deacetylation [1]. For considerations of the synthesis of chitosan nanoparticles, molecular weight and the degree of deacetylation have a direct bearing on their particle size, the nature of particle formation, and their degree aggregation in solution [1].

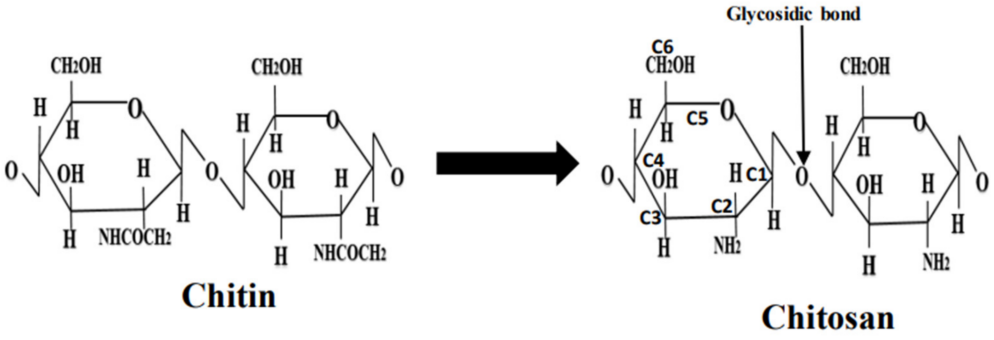


Figure 1. Molecular conversion of chitin to chitosan.

Chitosan nanoparticles (CNPs) can be produced with the desirable nano-scale characteristics, such as a small size, certain surface and interface effects, and quantum size effects [7]. Owing to the enormous potential of CNPs in biomedical applications, including for drug delivery, gene delivery, the treatment of various diseases such as cancer therapy, and tissue engineering, this review covers the various synthesis methods and the characterization of structural and related properties of CNPs. In addition, we review the applications of CNPs in the field of nanomedicine.

2. Structural and Physiochemical Characteristics of Chitosan

The structural and physicochemical properties of chitosan have been investigated in detail by various researchers. The discovery of chitin was first made by Henri Braconnot in 1811 during his investigations on mushrooms [8]. In 1859, Charles Rouget discovered that the alkali treatment of chitin could form a very different organic polysaccharide that can be dissolved in acids [7]. This organic polysaccharide was termed chitosan by Hoppe Seiler [9].

Chitin is among the (i.e., second) most abundant polysaccharides in nature. It is present in the crustacean shells of shrimps, lobsters, and crabs [10]. It is also the primary constituent

of insects' cuticles, fungal cell walls, yeasts, and green algae [11]. Unlike chitin, chitosan has a much lower occurrence in nature; however, it has been discovered to occur within the cell wall of certain types of fungi [12]. Chitin is a polymer that comprises [β-(1-4)-2-acetamido-2deoxy-D-glucopyranose] units. The idealized structure of chitin is similar to that of cellulose with the exception that an acetamido group substitutes for the C(2) hydroxyl group [13]. There are three types of chitin: α, β, and γ [7]. Specifically, α-chitin has an antiparallel chain, β-chitin has intrasheet hydrogen bonding within parallel chains, and γ-chitin is the combination of both α- and β- chitin [14]. Chitosan is predominantly a derivative from chitin [13].

Chitosan contains 60% or more glucosamine (D units) [15]. The D unit content (and the free amine groups) in chitosan enables its solubility in aqueous acidic solution. The degree of deacetylation (DD) value is a reflection of the fractional molar content of D units in chitosan [16]. Thus, the DD value has a direct bearing on the performance of chitosan in a wide variety of applications [17]. The DD value of chitosan can be determined by using infrared radiation (IR) spectroscopy [18], UV-visible spectrophotometry [19], potentiometric titration [20], H-liquid-state nuclear magnetic resonance (NMR), and solid-state NMR spectroscopy [21]. Chitin is a comparatively intractable polymer that has sufficient structural dissimilarity to cellulose so that it is insoluble in solvents used to dissolve cellulose, including cuprammonium hydroxide (Scheweizer's reagent), cadoxe, and cupriethylene diamine [22].

The molecular structures of chitin and chitosan are shown in Figure 1. The solubility of chitosan depends on various factors such as DD, pH, temperature, polymer crystallinity, and the type of solvent. Chitosan solubility in aqueous media is determined by the extent of protonated NH_2 groups: for example, chitosan is soluble in aqueous solutions when ~50% of the protonation of amino groups occurs [23]. If DD is ~28%, chitosan is soluble in an acetic acid solution. Thus, for all other factors remaining the same, chitosan solubility is directly impacted to the degree of deacetylation since this determines the extent of glucosamine units and modifies its crystal structure [23].

The molecular weight of chitosan and its viscosity in aqueous media also have a determinative effect in the biochemical, nanomedicinal, and pharmacological applications of the polymer. Additional determinative factors include the degree of crystallinity, crystal size, ash content, moisture content, and the presence of heavy metals [24]. An additional benefit of its industrial use is that chitosan harvesting leads to ameliorating the pollution of the environment caused by the disposal of crustacians' shells by the seafood industry [7]. Every year millions of tons of crustacean shells are produced as waste, which can degrade slowly and pollute the environment. The conversion of these shells into chitin and chitosan is one of the best solutions to combat this pollution, as chitin and chitosan have many applications in a variety of fields.

3. Chemistry of Chitosan

Chitosan has three reactive or functional groups: an amino group (-NH_2) situated at C_2-NH_2, and two hydroxyl groups located at C_3-OH, and C_6-OH (Figure 1). The C_6-OH hydroxyl group is more chemically active than the one at C_3-OH. The glycosidic bond can also be considered a functional group that allows for chemical modifications, producing a polymer with new properties and behavior [23]. Through the use of suitable reagents, chemical modification at the amino group results in N-modified chitosan derivatives, whereas chemical modification at the hydroxyl groups results in O-modified chitosan derivatives, thus providing improved physicochemical properties [25]. Although the chemical modification of chitosan may occur at all of its functional groups, it is the reactions of side groups at the hydroxyl group sites that result in minimal or no change in its biophysical properties [7].

Chitosan functionalized by the N-cinnamyl substituting O-amine group is substantially more hydrophobic and is a substantial antimicrobial agent against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [26]. Chitosan-based glycopolymer modifying C_6 N-quaternary ammonium-O-sulfobetaine is soluble in aqueous

media and has a good affinity for binding with lectins [27]. In addition, this modified chitosan has proven to poses antimicrobial activity [28]. O-acylated chitosan nanofibers with fatty acids anhydrate side groups have been shown to poses varying degrees of hydrophobic and hydrophilic values that directly correlate with the chain length of the substituted acyl group [29]. Chitosan quaternary ammonium salt can act as a coagulant and flocculant agent that is effective against *Microcystis aeruginosa* cyanobacteria [30]. The quaternary ammonium salts of chitosan combined with Fe_3O_4 nanoparticles can act as a bioadsorbent for methyl orange and chromium (VI) [31]. Similarly, a hydrogel produced by the cross-linkage of chitosan with glyoxal, glutaraldehyde, and terepathaldehyde is effective for organ transplant purposes and for the restoration of organ function [32]. Ho-166, Sm-153, and Lu-166 radionuclides cross-linked with chitosan have been used for targeted radiation therapy [33].

4. Synthesis of Chitosan Nanoparticles

Various procedures are used to synthesize CNPs due to chitosan's ability to form a gel and to form beads [7]. The most prevalent methods used to prepare CNPs include ionic gelation, microemulsion, emulsion-based solvent evaporation, and emulsification solvent diffusion [34]. These processes generally involve non-complex procedures, utilizing less organic solvents during preparation. The main characteristics that have a direct bearing on the particle size, crystallinity, and surface charge of the CNPs obtained using these preparation techniques are molecular weight, the concentration of chitosan used, and the degree of deacetylation of the chitosan. A short synopsis of the procedures used to synthesize CNPs that are discussed in this review is shown in Table 1.

Table 1. Synthesis methods used to make chitosan nanoparticles.

Methods	Process	Advantages	Disadvantages
Ionic gelation	Ionic cross-linking activated by mixing an aqueous solution containing chitosan and another containing TPP, thus resulting in a complex coacervate aqueous phase.	Straightforward procedure using mild chemicals. NP size easily regulated by altering the concentration of chitosan and TPP.	Difficult to produce uniformly sized NPs.
Microemulsion/reverse micelles	Based on covalent cross-linking where reverse micelle is formed upon introducing a surfactant into an organic solvent and then adding the mixture to an appropriate acidic solution containing chitosan.	Straightforward procedure achieving greater uniformity of size of NPs.	Use of harmful chemicals and a time intensive process.
Emulsification solvent diffusion method	Polymeric precipitation resulting in the formation of nanoparticles.	Straightforward procedure.	Substantial shear forces occur during the formation of CNPs.
Polyelectrol yte complex method	A self-assembly occuring due to the electrostatic interaction between the oppositely charged chitosan and the added polymer or counter ion, resulting in charge neutralization.	NP size can be regulated by pH of the solution, molecular weight (MW), and concentration of the constituents.	Due to the neutralization of charge, the PEC is self-assembled, leading to a substantial reduction in hydrophilicity.

4.1. Ionic Gelation Method

Ionic gelation involves dissolving chitosan, which is positively charged, in an acetic acid solution at room temperature and magnetically stirring for an hour. A second solution is made using polyanion tripolyphosphate (TPP) dissolved in deionized water (DI). Generally, TPP is used as an ionic cross-linker [35]. The ionic gelation synthesis involves mixing an aqueous solution containing chitosan and another containing TPP, thus resulting in a complex coacervate aqueous phase [36]. The TPP–chitosan mixture needs to be magnetically stirred at room temperature [37]. The solution results in three individual phases depending upon the stage of the procedure, starting with clear (chitosan solution),

followed by opalescent or milky (after adding TPP to the chitosan solution), and, finally, aggregated (after adding more TPP to a milky solution), whereby the milky appearance is the sign of the formation of CNPs. The harvested CNPs are highly suitable for drug delivery applications either in vitro or in vivo [35].

There are a number of examples of drug delivery applications using CNPs formed via the ionic gelation process. Gulati [38] used the ionic gelation process to form and evaluate the sumatriptan succinate-loaded CNPs, which were delivered through an intranasal system for migraine therapy. The goal of the study was to investigate the application's therapeutic efficacy and whether this approach may lead to a reduction in dosing frequency. The formulation of thymoquinone (TQ)-encapsulated CNPs for Alzheimer's disease using ionic gelation, targeting through nose-to-brain, was studied by Alam et al. [39]. During synthesis, the authors used a variable ratio of TQ to chitosan in the solution prior to the growth of the nanoparticles [39]. Alishahi et al. investigated CNPs loaded with vitamin C to test their effect on the immune system of trout [40]. The authors used ionic gelation with TPP to synthesize the CNPs, followed by ionotropic gelation to load the CNPs with vitamin C. The authors purport that their in vivo studies show that the vitamin C-encapsulated CNPs are effective in producing immune-induced activity in rainbow trout. Several researchers have used modified versions of the ionic gelation method to prepare drug delivery systems for various purposes. Saha et al. prepared chitosan nanoparticles loaded with ampicillin trihydrate via ionotropic gelation [41]. In this process, TPP was added to an aqueous solution containing the CNPs and ampicillin trihydrate via magnetic stirring at room temperature, resulting in the loading of the nanoparticles with the drug. The antimicrobial efficacy of the ampicillin trihydrate-loaded CNPs was tested on the *Staphylococcus aureus* strain and was determined to be considerably higher than that of CNPs alone. Trapani et al. [42] prepared dopamine-loaded CNPs via the modified ionic gelation method in order to test their potential for the treatment of Parkinson's disease. In vivo exaperiments on a rat brain showed that the dopamine-loaded CNPs induce increased levels of striatal dopamine output that depend upon dosing.

4.2. Microemulsion Method

The microemulsion method used for the synthesis of CNPs has been reviewed by Yanat and Schroën [43]. In this method, the CNPs are synthesized using a suitable reverse micelle. The reverse micelle is formed upon introducing a surfactant into an organic solvent and then adding the mixture to an appropriate acidic solution containing chitosan. Banerjee et al. used the microemulsion method to prepare chitosan nanoparticles in a 1,4-bis-2-ethylhexylsulfosuccinate (AOT) and N-hexane reverse micelle mixed with chitosan in an acetic solution. [44]. Glutaraldehyde was added to the solution at room temperature, and the solution was then stirred overnight to accomplish the reaction with the free amine group of chitosan. The glutaraldehyde in this method acts as a cross-linker for chitosan [45]. Excess surfactant is removed by precipitation with $CaCl_2$ and then centrifuged. The reverse miscellar method is well known for achieving a greater uniformity of size of nanoparticles. Banerjee et al. reported that the CNPs' size varied from 30 to 110 nm with the size depending upon the degree of cross-linking of the chitosan that was accomplished using glutaraldehyde [44].

Hu et al. used 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) to promote a coupling reaction between stearic acid (SA) and chitosan oligosaccharide (CSO), resulting in a micelle-like nanoparticle formation between the two constituents [46]. The CSO-SA micelle-type nanoparticles were loaded with plasmid DNA in order to test its efficacy as a gene delivery system. Manchanda and Nimesh [47] used the same microemulsion method as Banerjee et al. [44] to prepare CNPs cross-linked with glutaraldehyde. The CNPs were loaded with synthetic oligonucleotides (ODNs) to study their release in vitro under varying pH conditions. The authors concluded that the release of ODNs was higher under basic conditions than at neutral or acidic conditions.

Brunel et al. [48] used the reverse micellar method to prepare CNPs and concluded that control over the particle size and size distribution is increased with a decrease in the MW value of chitosan. The authors attributed this to either a lowering of the viscosity of the aqueous droplets or the fact that chitosan polymer chains become increasingly disentangled as the MW is lowered. Fang et al. used the reverse micellar method, whereby a reverse phase suspension from the Span-80 emulsifier and glutaraldehyde was formulated, to prepare Fe_3O_4 magnetic CNPs [49]. In addition, CNPs loaded with bovine serum albumin (BSA) were prepared via the reverse micellar method by Kafshgari et al. [50]. From this work, the authors concluded that decreasing the chitosan concentration increased the release of BSA.

4.3. Emulsification Solvent Diffusion Method

In emulsification solvent diffusion, an emulsion is created by adding an organic phase to a chitosan-bearing solution that contains a stabilizing agent (e.g., poloxamer): the entire mixture needs to be mechanically stirred and then homogenized under pressure. Niwa et al. [51] used a modified emulsification method, which was first reported by El-Shabouri [52], in which they employed a D,L-lactide/glycolide copolymer (PLGA). In this method, polymeric precipitation and nanoparticle formation occur when the emulsion is diluted with liberal amounts of water. The diffusion of the organic solvent in the aqeous media is the governing mechanism of the emulsification solvent diffusion method. The disadvantages of this method include the substantial shear forces that occur during nanoparticle formation and organic solvent use.

4.4. Polyelectrolyte Complex Method

The polyelectrolyte complex (PEC) method involves the formation of CNPs by adding oppositely charged polymers or counter ions to chitosan in solution. The oppositely charged polymer or counter ion is typically dissolved with chitosan in an acetic acid solution while stirring under ambient conditions [53]. The PEC formulation occurs on account of the electrostatic interaction between the oppositely charged chitosan and the additional polymer or counter ion, resulting in charge neutralization. Thus, because of the charge neutralization, the PEC is self-assembled, leading to a substantial reduction in hydrophilicity [35]. The nanoparticles formed by this technique were reported to be from 50 to 700 nm in size [35]. These PEC-complexed CNPs have been used as delivery systems for drugs, proteins, peptides, and plasmid DNA. In addition, Liu and coworkers [54] used the PEC method to prepare CNPs loaded with heparin. They considered the effect of the pH of the solution, molecular weight (MW), and the concentration of the constituents on the yield size of nanoparticles. Their conclusions were that lower pH conditions and moderate MW values resulted in greater nanoparticle complexation. Sharma et al. [55] concluded that the IgA-loaded chitosan–dextran nanoparticles formed using the PEC method provide a simple and effective way to create a drug delivery system. The CNPs prepared by the authors had a size range of 300–500 nm with Zeta potential (see Section 5.7) values of +40 to +50 mV.

5. Characterization of Chitosan Nanoparticles

Characterization is critical for developing a full understanding of the formation mechanism, as well as the physicochemical and medicinal properties, of CNPs. The characterization of CNPs for the elucidation of their physicochemical properties has been considered in a number of studies. Herein, we provide a brief summary of the prevalent characterization techniques utilized for the interrogation of the physicochemical properties of CNPs.

5.1. Measurement of the Size of Chitosan Nanoparticles

The size of CNPs plays a critical role in regulating its various properties, such as conjugation power with drugs, crystallinity, and overall charge. The size determination of CNPs is generally made using either transmission electron microscopy (TEM) or dynamic light scattering (DLS). The use of DLS for size determination is often complicated because

of the high polydispersity of CNPs. Characterization for information pertinent to the morphological, optical, and structural properties of CNPs can be obtained using various complementary techniques. This includes X-ray diffraction (XRD), Raman spectroscopy, TEM, scanning electron microscopy (SEM), and atomic force microscopy (AFM). UV-vis spectroscopy is occasionally used to determine concentrations of solutions containing CNPs, but will not be considered here.

5.2. X-ray Diffraction

The nature and degree of crystallinity of CNPs can be studied using X-ray diffraction (XRD). XRD is the foremost analytical tool used to identify the crystalline phase(s) of materials. In addition to determining structural properties, XRD can also be used to measure the mean diameter of the nanoparticles [56]. When measured in powder X-ray diffraction, the Scherrer equation [57] may be used to determine the mean crystallite size of the material after corrections in the broadening of the diffraction peaks due to instrumental, strain, and lattice imperfections. The Scherrer equation relates the full width with the half-maximum (FWHM) of a given XRD peak of a specific crystalline phase (after the aforementioned corrections) to the mean size of the nanoparticles, assuming the typical size of a nanoparticle is the same as the size of a crystallite [58]. The XRD patterns of chitosan nanoparticles synthesized using ionic gelation in the study by Ali et al. [59] are shown in Figure 2. XRD analysis revealed characteristic broad peaks occurring at $2\theta \approx 11^\circ$ and 19.6° for chitosan, which are essentially extinguished upon the formation of the CNPs due to cross-linking with TPP [59]. The lack of distinct peaks in the XRD pattern measured from the CNPs is indicative of their amorphous structure, which is in agreement with a study carried out by Sivakami et al. [60]. Karavelidis et al. discovered that higher drug release rates were observed in nanoparticles produced from aliphatic polyesters that have a low degree of crystallinity, which were used to encapsulate ropinirole HCl [61]. Vaezifar et al. attributed the decrease in crystallinity of CNPs to the interpenetration of dense counter ions of TPP inside chitosan nanoparticles and the disruption of the complex network formed from long-chain polymers [62].

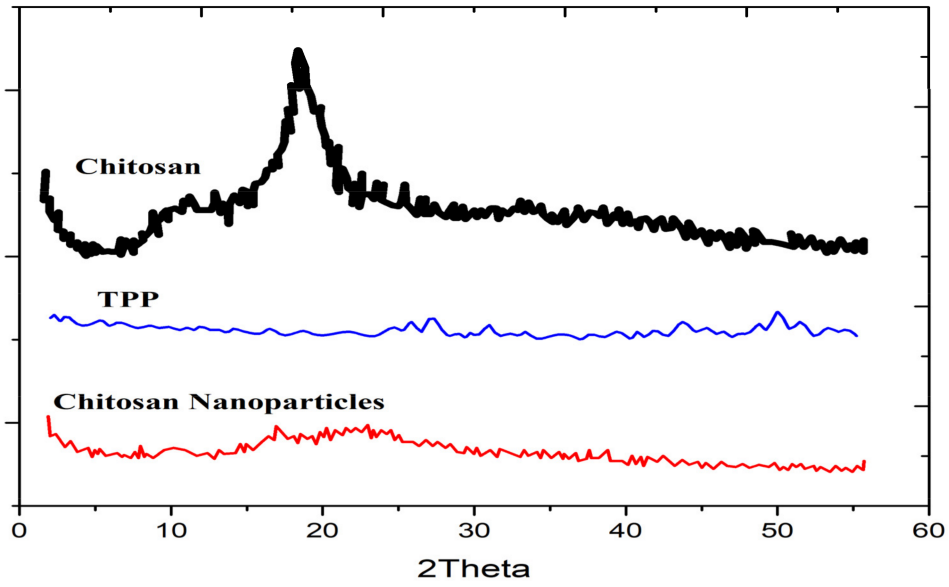


Figure 2. XRD patterns of pure chitosan, TPP, and chitosan nanoparticles reproduced from Ali et al. [59].

5.3. Raman Spectroscopy

Raman spectroscopy is a sensitive technique used to detect the structural modifications in macromolecular complexes, as deduced from the excitation of the associated Raman-active vibrational modes [63]. The Raman effect is based on the scattering of light, which

includes both elastic scattering at the same wavelength as the incident light and inelastic scattering at a shifted wavelength, which is due to the excitation of a specific molecular vibration. In a study by Gordon et al. [64], Raman spectroscopy was used to test whether the adsorption of ovalbumin (OVA) to CNPs caused conformational changes to the protein. As shown in Figure 3, the Raman spectrum measured from OVA-loaded CNPs differs either from the spectra of the two individual components or from the spectrum of a simple physical mixture of the two components, demonstrating a conformational change in the OVA protein upon loading. The resultant hydrogen bonding, ion–ion, and ion–dipole interactions account for the conformational alteration of OVA. As demonstrated by Yamasaki et al., more subtle effects can be observed in the Raman spectra as a result of conformational changes, including frequency shifts, intensity changes, and line broadening of the pertinent Raman bands [65]. There is evidence for conformational modifications to vaccines having a direct bearing on the immune response of a host [66].

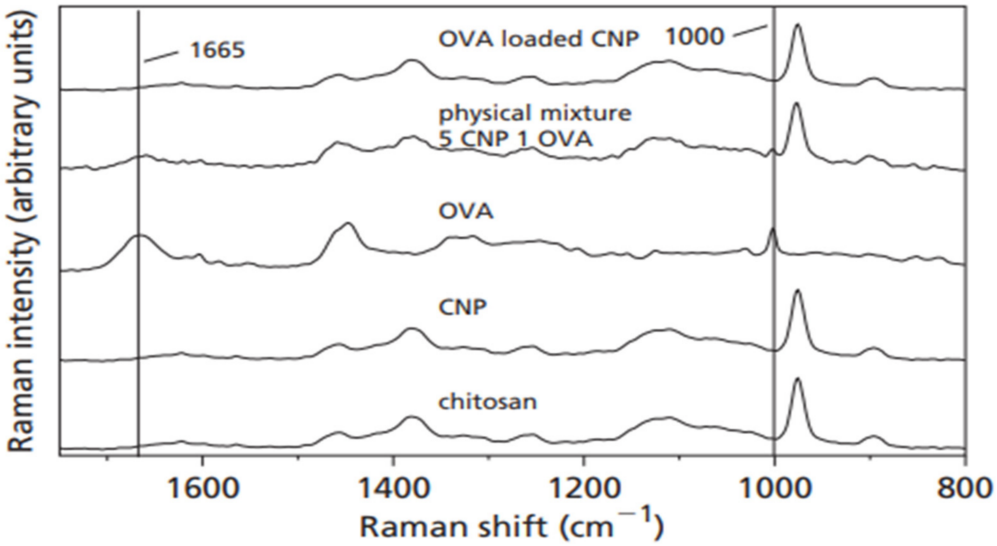


Figure 3. Raman spectra of chitosan, chitosan nanoparticles (CNPs), OVA, a physical mixture of 5:1 ratio of CNPs to OVA, and OVA-loaded CNPs (5:1 ratio of OVA to CNPs) [64].

5.4. Transmission Electron Microscopy (TEM)

TEM can be used to study the degree of crystallinity, size, and morphology of nanoparticles. TEM uses a beam of focused high-energy electrons that transmits the specimen, whereby the resulting image can be viewed on an imaging system (i.e., phosphor screen) situated on the opposite side of the impinging electron beam. The image results from the interference between the electron beam that is transmitted through the sample and the beam that is diffracted from the sample. Typically, high resolution TEM imaging is possible to a length scale on the order of 2 Å. For TEM analysis, the sample needs to be very thin (~500 Å or less) to produce a high-resolution image [35]. TEM specimens can be prepared by using a negative staining material (e.g., uranyl acetate) or by the simple deposition of a dilute suspension of the sample on a carbon-coated copper grid. In a study by Ghadi et al. [67], TEM was used to analyze the magnetic chitosan nanoparticle's shape and particle size. As shown in Figure 4, the Fe_3O_4 particles used in the study were coated well with chitosan and the diameter of CNPs ranged from 10 to 80 nm [67].

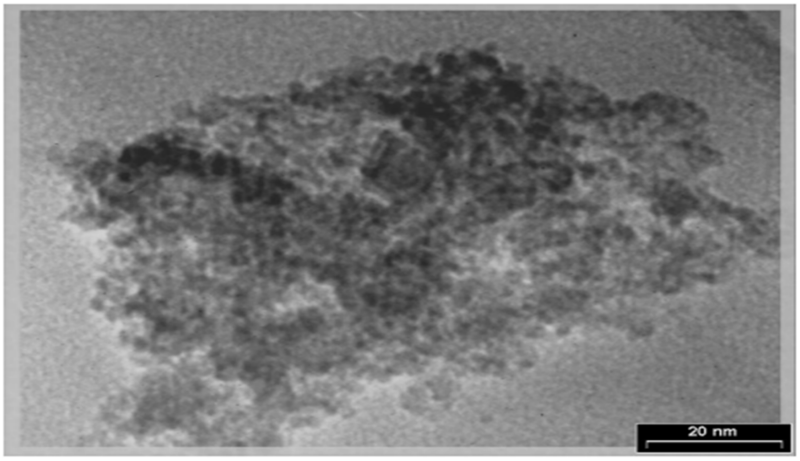
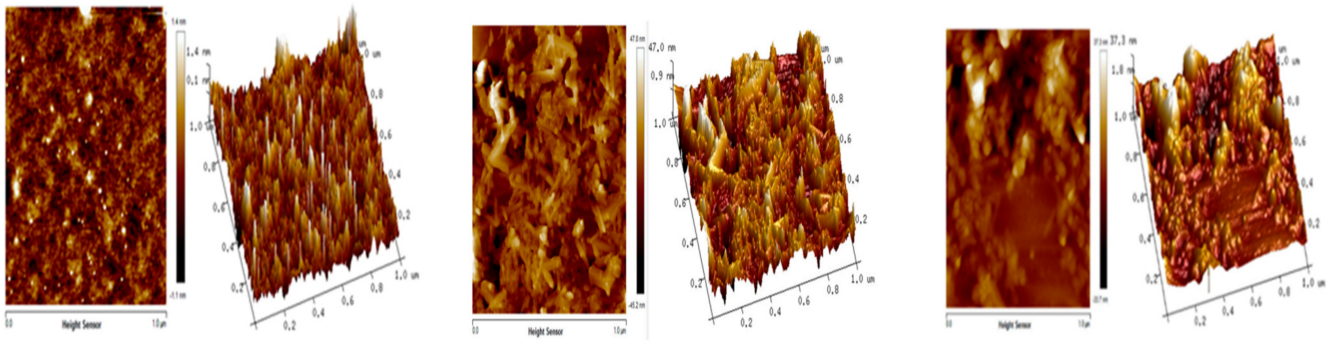


Figure 4. A TEM image of chitosan nanoparticles [67].

5.5. Atomic Force Microscopy (AFM)

The AFM can be used to image the nanoparticles with atomic resolution in a three-dimensional surface profile and to measure the force exerted by the sample surface on the AFM tip at the nano-Newton scale. Fauzi et al. [68] used the AFM to study the surface morphology of chitosan/maghemite nano-composite thin films with the aim of potentially applying the films for the optical detection of Hg^{2+} ions via surface plasmon resonance. Figure 5 shows the AFM three-dimensional surface profile of chitosan, maghemite, and chitosan/maghemite nano-composite thin films. The surface morphology can be quantified using the root-mean-square (RMS) roughness value [68], which is calculated either using the cross-sectional or a two-dimensional profile [69]. The RMS roughness of the chitosan, γ-Fe_2O_3, and chitosan/γ-Fe_2O_3 was found to be 1.4 nm, 47 nm, and 37.3 nm, respectively. The authors concluded that a smoothening of the meghamite surface was induced by the deposition of chitosan, whereby the smoothening mechanism was attributed to surface diffusion [70]. The resulting surface morphology introduced in the nanostructured chitosan/maghemite composite thin film was deemed to be appropriate to enhance the sensing of Hg^{2+} [70]. Almalik et al. [71] used AFM to study chitosan nanoparticles that were coated with hyaluronic acid (HA). In this study, AFM was used to determine the presence of an HA on CNPs. The thickness of the HA coverage on the CNPs was estimated to be approximately 20–30 nm when dry.



(**a**) (**b**) (**c**)

Figure 5. Atomic force microscopy image of (**a**) chitosan thin film, (**b**) Fe_2O_3 thin film, (**c**) and a composite chitosan/Fe_2O_3 thin film [68].

5.6. Scanning Electron Microscopy (SEM)

SEM utilizes a focused high-energy beam of electrons to image the surface of a specimen. Other than in an environmental SEM that will not be discussed herein, samples

are analyzed upon drying. Non-conducting samples need to be sputter-coated with a thin layer of carbon or metal. SEM provides a direct image of the surface morphology and size of nanoparticles. For SEM's equipped with an element dispersive spectroscopy (EDS) detection capability, the elemental composition of samples can be studied from the collected emission X-ray spectra. Saharan et al. [72] prepared chitosan nanoparticles, chitosan–saponin nanoparticles, and Cu-CNPs using ionic gelation to test their effect individually as antifungal agents. In their study, SEM was used to confirm the organization of their chitosan nanoparticles on the nanometer scale. In addition, SEM imaging showed the chitosan–saponin nanoparticles to be spherical in shape, whereas Cu-CNPs had the shape of a compact polyhedron [72]. Jingou et al. [73] used SEM for a morphological study of CNPs that were cross-linked with TPP and loaded with a combination of salicylic acid and gentamicin. Their investigations showed that these CNPs were nearly spherical in shape with an average size of ~200 nm [73].

5.7. Dynamic Light Scattering (DLS)

Through detection of the scattered light intensity, the DLS technique enables the measurement of the dynamic fluctuations of particles stemming from their Brownian motion in a solution. The light source is monochromatic and is typically from a laser, and the particles in the solution produce a dynamic diffraction of light in a speckel pattern. The average particle size within a sample can be determined using the Stokes–Einstein equation. In addition, DLS enables the determination of the polydispersity of nanoparticles within a sample (i.e., the polydispersity index (PDI)), or the dispersity in the new convention as recommended by the IUPAC, and their Zeta potential. The use of the DLS technique for nanoparticle size determination typically requires monitoring intensity variations at various detector angles for several polarizations relative to the incident light [74,75]. In a study by Fan et al. [76], DLS was performed on CNPs, saponin-CNPs, and Cu-CNPs at a light-scattering angle of 90°. The size distribution profiles are shown in Figure 6a–c: the authors report that, based on these measurements, the mean diameter and the PDI or dispersity values of CNPs, saponin-loaded CNPs, and Cu-loaded CNPs were ~192 nm and 0.6, ~374 nm and 1.0, and ~196 nm and 0.5, respectively [72]. The lower PDI values show that the CNPs and Cu-CNP nanoparticles have narrower size distributions than the saponin-CNPs. The authors reported that the CNP and Cu-CNP samples had larger Zeta potential values, of +45.33 and +88 mV, respectively, compared to the value reported for saponin-CNPs (+31 mV) [73]. The Zeta potential, which is a measure of the electric potential between the attached and non-attached fluid with respect to the nanoparticle surface, is a measure of the stability of nanoparticles in the fluid. Therefore, the CNP and Cu-CNPs, having larger Zeta potential values, exhibit higher stability than saponin-CNPs in an aqueous media [77]. In a study by Lu et al. [78], CNPs loaded with DNA and grafted with polyethylenimine (PEI) were prepared for gene therapy for osteoarthritis. Using DLS to measure the dispersity and Zeta potential, the authors reported that particle size decreased, whereas surface charge increased (i.e., larger Zeta potential), as the CNP:DNA weight-to-weight ratio increased.

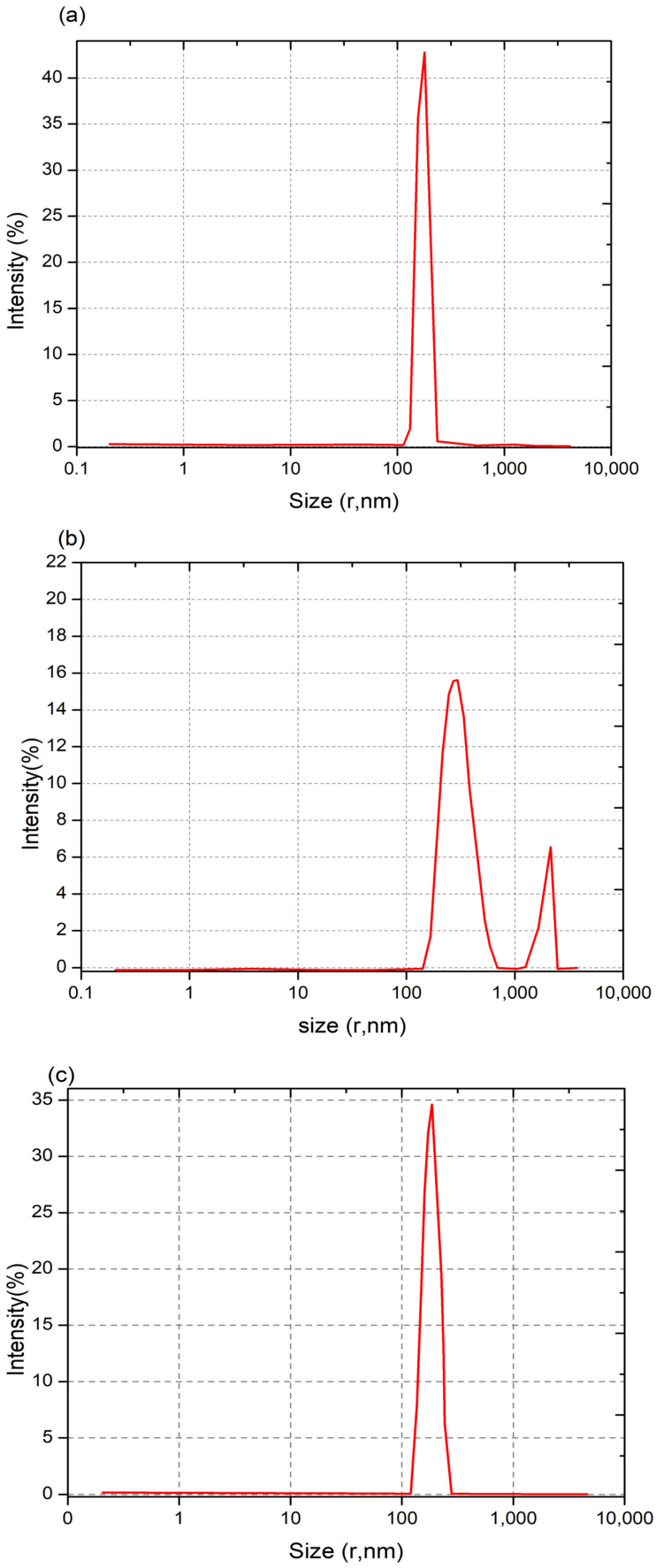


Figure 6. The size distributions as measured using DLS of (**a**) CNPs, (**b**) saponin-CNPs, and (**c**) Cu-CNP nanoparticles reproduced from Saharan et al. [72].

6. Nanomedicinal Application of Chitosan Nanoparticles

Based on current trends, it is conceivable that nanomedicine will help to bring the next leap in developing advanced therapy, imaging, drug delivery, and the treatment of fatal diseases. Chitosan nanoparticles are natural materials that are widely used in medicinal applications due to their hydrophilic, nontoxic, biocompatible, and bio-degradable nature. Because CNPs have these properties, they are highly suitable for a wide range of drug delivery, gene therapy, and tissue engineering applications. Some of these applications are discussed below.

6.1. Drug Delivery

The demonstrated potential use of CNPs as drug delivery systems has provided opportunities for the development of a largely expanded range of CNP-based delivery vehicles [79]. Due to its biocompatibility, chitosan is classified by the United States Food and Drug Administration as GRAS (Generally Recognized as Safe) [80]. As noted above, the presence of the amino and hydroxyl functional groups, as well as the glycosidic bond, enables the loading of CNPs with drug molecules and DNA. Because CNPs are soluble in acidic aqeous solutions, sustainable chemistry may be employed in their synthesis without the use of harmful organic solvents [81]. An additional advantage of using CNPs is that, through mucoadhesion, they enable the controlled release of drugs in vivo [82]. CNPs have several important potential applications for the delivery of drugs, namely, parenteral, ocular, oral, pulmonary, nasal, buccal, and vaginal, in addition to applications in cancer therapy, tissue engineering, etc. [34]. A few of these applications of CNPs for drug delivery are discussed below.

6.1.1. Ocular Drug Delivery

Because chitosan has mucoadhesive properties, the use of CNPs for controlled drug delivery is favorable via mucosal membranes [83]. CNPs undergo surface gel layer formation when in contact with near-neutral aqueous fluids, which may improve residence time on the mucosal surface and the efficacy of drug delivery to ocular tissue [84]. CNPs cross-linked using sulfobutylether-β-cyclodextrin (SBE-β-CD) were utilized for investigations of their potential for ocular drug delivery by Mahmoud et al. [85]. These authors used econazole nitrate (ECO) to test for ocular antifungal efficacy in albino rabbits. Their results showed that the prepared CNPs were predominantly pseudospherically shaped with average particle sizes ranging from 90 to 673 nm and Zeta potential values ranging from 22 to 33 mV. The ECO drug loading percent values ranged from 13 to 45% [85]. The authors performed in vivo studies, which showed that the ECO-loaded CNPs had better antifungal ocular efficacy than an ECO solution, thus confirming that chitosan nanoparticles are a promising ECO drug delivery vehicle for antifungal ocular treatment [86]. Santhi et al. [86] used the emulsification preparation technique to synthesize fluconazole-loaded CNPs with an average particle size of 152.85 $\pm$ 13.7 nm. The authors used the cup-plate method to test the efficacy of antifungal treatment using fluconazole-loaded CNPs as compared to that of fluconazole eye drops [86]. The fluconazole-loading capacity of their CNPs was found to be optimal at $\leq$ 50%. The authors concluded that the CNPs exhibited promising characteristics, including drug loading capacity, antifungal activity, and prolonged drug release, for fluconazole drug delivery for antifungal treatment [86].

6.1.2. Oral Drug Delivery

Oral drug delivery is widely used because of several factors, including convenient drug administration, controlled delivery, low production cost, and patient compliance: However, challenges in conventional oral drug delivery include drug solubility issues in low-pH gastric fluids, the degradation and reduced activity of drugs due to the presence of enzymes, and the lack of adequate membrane permeability [3]. Nanomedicine offers potential opportunities to overcome such challenges in oral drug delivery [87,88]. The various physicochemical properties of CNPs noted above, including mucoadhesion, bio-

compatibility, large surface-to-volume ratios, and drug conjugation versatility, make them suitable candidates for improving oral drug delivery. Pan et al. [36] conjugated insulin to CNPs, which ranged 250–400 nm in size and were positively charged, and performed oral drug administration to diabetic rats. The authors reported that the CNP-assisted oral drug delivery resulted in the enhanced intestinal absorption of insulin in diabetic rats. By modulating the dose of insulin loaded on the CNPs, the authors found that glucose levels in diabetic rats could be brought to normal levels for an extended period of time. Although the precise mechanism is not known, the authors conjectured that the CNPs improve the stability of insulin by providing protection in the gastrointestinal environment, thereby helping to increase the drug uptake [36]. In order to study the potential improvement in the bioavailability of lipophilic drugs, such as cyclosporine, by nanoparticle encapsulation, El-Shabouri [49] used cyclosporine-A-loaded CNPs for oral administration in dogs, whose blood samples were analyzed at predetermined intervals after administration for drug uptake. The mean size of the chitosan HCl nanoparticles was 148 nm with a Zeta potential of +31 mV. The results from this study showed that cyclosporine A bioavailability was increased by 73% when administered via CNP encapsulation compared to oral delivery using the commercial microemulsion Neoral® [49]. El-Shabouri conjectured that the positively charged CNPs interact more strongly with negatively charged epithelial cells of the gastrointestinal tract than neutral or negatively charged carriers, thereby resulting in greater permeability and bioavailability of the drug [49].

6.1.3. Pulmonary Drug Delivery

There are several physiological properties of the lungs that may enable enhanced drug delivery, including a relatively thin absorption barrier for drug uptake, a large surface area, and their extensive vascularity [89]. As with oral drug delivery, pulmonary drug delivery is conjectured to benefit from the physicochemical properties of CNPs, including a large loading capacity, mucoadhesion, a positive charge, an antibacterial property, and sustained drug delivery. Islam and Ferro [90] have made an extensive review of CNP-based vehicles for pulmonary drug delivery. Several noteworthy studies have been made to investigate the efficacy of using CNPs for pulmonary drug delivery. Yamamoto et al. used a poly(DL-lactide-co-glycolide) (PLGA) copolymer for the synthesis of CNPs that were loaded with elcatonin (used for lowering blood calcium) and aerolized for pulmonary drug delivery [91]. The results from their studies demonstrated the efficacy of the drug delivery, along with sustained drug release (up to 24 h), using CNP-PLGAs loaded with elcatonin when compared to the use of unmodified CNPs. The authors conjectured that the positively charged properties of the PLGA-modified CNPs enabled the opening of the tight junctions in the epithelial cells of the lungs, thus improving drug uptake. Jafarinejad et al. [92] prepared aerosolized CNPs loaded with itraconazole, which is an antifungal drug, with in vitro testing for pulmonary administration. The study was made to test whether the low solubility of itraconazole in the gastrointestinal tract upon oral administration can be overcome by pulmonary drug delivery using CNPs. The authors reported an increased uptake of itraconazole using their aerosolized CNPs, particularly when leucine was added for aerosolization of the nanoparticles [92]. Rawal et al. prepared CNPs loaded with rifampicin for pulmonary administration in rats in order to test for the efficacy of drug delivery in the treatment of tuberculosis [93]. The primary advantage in pulmonary drug delivery is the potential of eliminating the considerable adverse effects to the drug when administered orally. The in vitro study showed sustained release of the drug for up to 24 h and negligible toxicity.

6.1.4. Nasal Drug Delivery

The effective administration of peptides, nucleic acids, vaccines, and other drugs encapsulated in nanoparticles via nasal delivery is highly desirable because this route may induce a substantially more potent immune response; however, the nasal epithelium presents low permeability to hydrophilic molecules, whereas mucosal clearance and the

mucus gel inhibit drug uptake in nasal passageways [81]. Due to their mucoadhesion, biocompatibility, low toxicity, and other properties, chitosan nanoparticles are postulated to be good candidates for an effective nasal delivery of drugs [94]. Shahnaz et al. synthesized thiolated CNPs (chitosan conjugated with thioglycolic acid) loaded with leuprolide, which is used to treat prostate cancer, the lining of the uterus, and uterine fibroids, to test whether this formulation could improve the bioavailability of the drug via nasal delivery [95]. Their results showed substantially improved bioavailability of leuprolide from nasal administration using thiolated CNPs in rats compared to the administration of leuprolide solution alone. One of the main complications in the conventional oral delivery of anti-epileptic drugs is the prevention of drug uptake in the brain due to the blood–brain barrier, resulting in drug resistance. Liu and coworkers [96] used carboxymethyl-CNPs loaded with carbamazepine, which is an anti-epileptic drug, to study the bioavailability of the drug when administered intranasally. The authors used CNPs that were found to have a particle size of ~219 nm with high entrapment efficiency (80%) [96]. From in vivo tests in mice, the authors concluded that carboxymethyl-CNPs carriers caused substantially improved bioavailability and enhanced brain-targeting of carbamazepine, when compared to the nasal administration of a carbamazepine solution [96]. However, the authors report that the CNP-carrier administration is limited by the volume of the drug that can be delivered intranasally.

6.1.5. Buccal Drug Delivery

Buccal drug delivery is a preferred route for the delivery of drugs, particulary ones with high molecular weights, that cannot be administrated by the oral route [97]. This is a transmucosal delivery mechanism that has advantages over the oral route, including overcoming drug degradation in the gastrointestinal tract and the first-pass metabolism effect. Thus, the buccal drug delivery method may lead to enhanced drug bioavailability and lower required doses of the drug [98,99]. Mazzarino et al. [100] prepared films containing CNPs coated with polycaprolactone and loaded with curcumin, which has potential uses for the treatment of periodontal disease, for administration via the buccal mucosa route. AFM and SEM characterization of the films showed the confirmed presence of CNPs in the films and that they were uniformly distributed throughout the films. In vitro studies conducted by the authors in simulated saliva solutions showed maximum swelling of the films due to a hydration of ~80% and the sustained delivery of curcumin, which are required for the successful treatment of periodontal disease.

6.1.6. Vaginal Drug Delivery

As with buccal drug delivery, the vaginal mucosa offers another transmucosal route for drug administration. Drugs are administered vaginally for two approaches: either for local treatment or for systemic effects whereby the drug passes through the vaginal mucosa and enters the bloodstream [101–103]. Primarily due to their mucoadhesive and conjugation properties, chitosan nanoparticles may be ideally suited as drug carriers in vaginal drug administration for systemic effects. Nevertheless, there are several challenges for vaginal drug delivery including a low pH (3.8–4.5) vaginal environment, considerable fluid discharge, and extensive epithelial tissue folding. Martínez-Pérez et al. [104] prepared CNPs that were surface modified with PLGA and loaded with clotrimazole for vaginal drug administration. In vitro studies revealed that delivery via CNP-PLGAs improved antifungal activity in relation to delivery without the use of CNPs, thus making CNP-PLGAs potentially useful for the treatment of fungal infections of the vagina [104]. Similarly, in a separate study, Perineli et al. [105] developed a hydrogel system containing hydroxypropyl methylcellulose (HPMC) and chitosan to treat fungal infection of the vagina caused by *Candida albicans* and non-albicans strains. The HPMC-chitosan hydrogel was found to possess either CNPs or monomolecular chitosan and was loaded with metronidazole. In vitro studies and mucoadhesive tests revealed that both types of HPMC-chitosan hydrogel, whether containing CNPs or monomolecular chitosan, exhibited improved anti-*Candida* activity of all strains and enhanced mucoadhesive properties [105].

6.2. Cancer Therapy

Chemotherapy remains an important therapy route for the treatment of cancers; however, because cytotoxic chemotherapeutic drugs cause chemical damage to both cancerous and noncancerous cells, this option produces considerable adverse effects in patients. Chitosan-based nanostructures (i.e., nanoparticles, nanocomposites, nanorods, etc.) are one class of polymer-based nanomaterials that are projected to play an important role in providing cancer-targeted therapies under controlled drug release that will minimize adverse effects. Mathew et al. [106] prepared CNPs decorated with Mn-doped ZnS quantum dots as drug carriers and as a cancer cell imaging agent using fluorescent microscopy. In vitro studies using the CNPs loaded with 5-Fluorouracil on the MCF-7 breast cancer cells revealed that these carriers are useful for controlled and targeted drug delivery. Sekar et al. [107] synthesized CNPs loaded with ascorbic acid for tests of efficacy in potential targeted drug delivery in the treatment of cervical cancer. The authors found from in vitro studies that the ascorbate-CNPs are effective in targeting HeLa cervical cells with no effect on human-diploid fibroblast (WI-38 strain) normal cells, thus demonstrating the potential use of these carriers as cancer drug delivery systems. Nascimento et al. [108] developed chitosan-polyethylene glycol (PEG) nanoparticles loaded with silencing RNA (siRNA) that were specifically designed to target epidermal growth factor receptor (EGFR) proteins and silence the overexpressing Mad2 gene in tumor cells. The authors report that the use of CNP-PEG-siRNA nanoparticles loaded with cisplatin, in comparison to CNP-PEG-siRNA alone, had dramatically stronger inhibiting effects against cisplatin-resistant tumors in the lung. In addition, the CNP-PEG-siRNA-cisplatin nanoparticles enabled considerably reduced drug dosage with negligible adverse effects. Another approach to targeting tumor cells involves using glycol chitosan, which, due to its surfactant properties, can be used for the self-assembly of glycol-CNPs encapsulating tumor-targeting drugs [109]. Hydrophobic camptothecin (CPT) (a topoisomerase I inhibitor) was encapsulated in glycol-CNP drug carriers by Min et al. [110] for studies of anticancer efficacy. Tests on MDA-MB231 human breast cancer xenograft models indicated that the CPT-loaded glycol-CNPs had much higher antitumor efficacy than glycol-CNPs alone.

6.3. Tissue Engineering

Due to their biocompatibility, biodegradability, non-toxicity, antibacterial activity, functionalizability, and other properties mentioned above, chitosan-based nanostructures are increasingly being used in and investigated for tissue engineering applications [111–114]. Chitosan-based nanostructures have found applications in bone, periodontal, blood vessel, skin, corneal, and cartilage tissue engineering [111]. Chitosan is typically combined with other biopolymers or with bioactive nanoscale ceramic materials to formulate scaffolding for use in tissue engineering. One of the more active areas in the application of chitosan-based scaffolds is for periodontal tissue engineering [111,114]. Studies made on chitosan-based scaffolds generally show good efficacy in periodontal tissue engineering, but have poor mechanical strength. Investigations in utilizing bioactive bioceramics (e.g., hydroxyapatite, Bioglass, etc.) in composite form with chitosan in scaffolding may lead to improved mechanical strength properties [112]. Another noteworthy area of application of chitosan-based scaffolds is for corneal tissue engineering. Tayebi et al. [115] recently developed a transparent composite scaffolding containing CNPs and polycaprolactone (PCL). Culture studies showed that human corneal endothelial cells attached appropriately to the CNP-PCL scaffolding in monolayer formation, indicating significant potential for corneal tissue engineering applications [115].

7. Conclusions

In this review paper, we have made a summary of the types of synthesis and characterization techniques used in the study of chitosan nanoparticles, along with their various nanomedicinal applications. As one of the most abundant polysaccharides with all of the essential characteristics, such as biodegradability, non-toxicity, biocompatibility, hydrophilicity, antibacterial ability, etc., CNPs are one of the best options for nanomedicinal

application. CNPs show excellent potential as carriers for the encapsulation and incorporation of drug molecules. Thus, in association with their mucoadhesive properties, CNPs show good potential for effective drug delivery, controlled drug release, and the enhanced therapeutic efficacy of the drugs. The fact that CNPs demonstrate favorable in situ gel formation ability and mucoadhesive characteristics, and are positively charged, makes them effective for pulmonary and ocular drug delivery. Similarly, their ability to open tight junctions of the mucosal membrane and enhance drug absorption properties makes CNPs promising oral and pulmonary drug delivery carriers, whereas the ability to increase the permeability of various drugs makes them suitable for nasal drug delivery. Investigations made on CNP-based nanostructures loaded with appropriate anticancer drugs show that these can be highly effective in targeted cancer therapy at lower dosing levels that result in considerably less adverse effects than in conventional chemotherapy. Chitosan-based nanostructures in composite formulation with other polymers and bioactive bioceramics for scaffolding show considerable promise for applications in tissue engineering. However, further investigations of the potential toxicity of CNPs, dependent upon the precise nature of anticipated application, need to be performed to ensure complete safety prior to industrial application. Our hope is that more CNP-based applications can be developed for use in treatment, therapy, imaging, and drug delivery to cure cancers and other fatal diseases.

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Chapter Six - Environmental and toxicological implications of chitosan nanostructures

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Abstract

Amidst agriculture, biomedicine, and environmental remediation, chitosan is widely lauded for its quick biodegradability and security, leading to a growing appeal of this sustainable option. Specifically, chitosan's nanostructures, including nanoparticles and nanofibers, have fueled its increasing popularity. That said, chitosan nanostructures' potential environmental harm and toxicity mandate thoughtful consideration to ensure their safe and sustainable presence. This chapter examines the fate and toxicity of chitosan nanostructures in natural settings. Chitosan nanostructures and their effects on aquatic life, human health, and the environment are explored in this chapter. Additionally, we investigate the contributing factors that affect how these structures behave. Examining how these nanostructures impact their surroundings, we consider their movements and ultimate outcomes in diverse environmental contexts. Our research reviews the current understanding of the toxicity and ecological implications of chitosan nanostructures and outlines potential problems and future areas of exploration. By addressing the eco- and toxicological impact of chitosan nanostructures, this chapter aims to provide valuable insights into their potential risks and

benefits, facilitating informed decision-making for their safe and sustainable utilization in various fields.

Introduction

Chitin is present abundantly in the exoskeletons of crustaceans such as prawns or crabs; through industrial processes used by specific industries like fisheries, this residual waste can be utilized in producing chitosan as a natural polysaccharide which can replace artificial resources while being both biodegradable and bio-friendly. Nano-sized materials composed of chitosan-like nanoparticles and nanofibers offer exciting potentials for various fields, including biomedicine and agriculture. However, at an early developmental stage, much work is needed before commercial use is plausible. The environmental impacts on our planet must be carefully evaluated before unveiling major all-encompassing processes in production.[1]

Chitosan nanoparticles have proven helpful in various environmental applications, including water and soil remediation. However, their release into the environment must be well managed and monitored to overcome potential risks.[1] Chitosan nanoparticles might affect the development, reproduction as well as rate of survival of aquatic life.[2] According to research, chitosan nanoparticles can pose varying toxicity levels towards marine life that must be mainly dependent upon concentration and duration of exposure. Irrespectively the risk remains low.[3]

Chitosan is a safe and natural biopolymer that humans can ingest without harmful effects. It's commonly used in drugs, wound dressings, and dietary supplements. However, caution should be taken when dealing with chitosan nanoparticles as they may cause toxicity and oxidative stress to cells if the utilized concentration is too high. The severity of such side effects varies depending on the chitosan employed.[4]

Chitosan nanoparticles are tiny enough to interact with cellular components and even cross biological barriers like the blood-brain barrier.[5] However, their size raises concerns about potential accumulation and long-term effects. Accelerated disintegration and changes in chitosan nanostructures can occur due to various factors such as pH, temperature, and the presence of enzymes.[6] These changes ultimately affect how fast the decomposition occurs. Chitosan is an environmentally friendly material that readily biodegrades. When decomposed, Chito oligosaccharides are produced as a byproduct that does not harm microorganisms, but it may even benefit them.[7] The impact of chitosan nanoparticles on the

environment is intricate and can be influenced by different factors.[8] To evaluate their potential bioaccumulation, stability, and aggregation, one must closely analyze them.

Chitosan is a naturally occurring biopolymer composed of D-glucosamine and *N*-acetyl-D-glucosamine units, which are linked together by β-1,4-glycosidic bonds. It is found in the exoskeletons of crustaceans like prawns, crabs, lobster, and fungal cell walls.[9] Due to its numerous advantages, such as biocompatibility, biodegradability, antibacterial activity and nontoxicity, it finds applications across industries, including pharmaceuticals and biomedical products.[10]

Nanostructured chitosan is highly valuable due to its desirable qualities obtained through physical or chemical techniques. With a larger surface area than bulk chitosan and unique physical and chemical properties, nanostructured forms are more effective in various applications. They outperform their bulk counterparts.[11] Fig. 1 showcases several commonly used types of nanostructured chitosan.

The creation of chitosan nanoparticles can be achieved using several approaches like ionotropic gelation, coacervation, emulsion or precipitation. These processes allow the customization of materials with chosen physical and chemical properties, making them highly advantageous.[12] Moreover, using specific-sized particles ranging from 10 to 1000 nm has led to effective drug targeting as they can safely deliver biomedical agents, including drugs and proteins, without harm.[13]

Fragile strands merely measuring at the nanometer scale sets chitosan nanofibers apart. Through methods like electro-spinning or self-assembly techniques, they can be manufactured.[14] Chitosan nanofibers are widely employed due to their expansive surface area, resilience and porosity, which has made them optimal materials for tissue regeneration endeavors alongside wound management solutions and filtration procedures.[15]

Utilizing either chemical or physical cross-linking methods, chitosan nanoparticles are bound together to fabricate an expansive, intricately entwined matrix known as chitosan nanogels. This gel-based nanostructure has been utilized scientifically as a confinement system for medication or bioactive chemical substances that necessitate regulated release patterns. The application of these versatile particles is vast, ranging from wound healing and tissue engineering to targeted drug transport for effective treatment.[16]

Increasing the quality of rigid natural polymer-based materials such as chitosan matrices requires techniques such as adding minute-scale filler materials like metallic nanoparticles over time.[17] Further research has shown that this technique can produce excellent results on

mechanical resilience with added heat stability and an additional boost in antibacterial properties types compared with untreated natural polymers alone.[18] It has therefore been concluded that when applied appropriately by professionals within the industry is capable of multiple deployments, including but not limited to biomedical device production, significantly improved packaging material alongside advanced sensors.[19]

Certain characteristics of chitosan-based nanomaterials, including greater bioavailability, improved stability and appropriate release of encapsulated chemicals and their interaction with cells, have prompted their use for various applications in different fields, including health, agriculture, and environmental sciences.
Environmental researchers are keen on exploring chitosan's full potential from chitin found within crustaceans. Consequently, chitin biotransformation yields chitosan—a versatile natural biopolymer prized for its unique features: becoming an ‘excellent’ candidate with potential applications in myriad fields like agriculture, food tech or environmental reclamation, amongst others.[20] As scientific advancements push towards practical solutions-based launch platforms—creating viable technology models for society—researchers must pause to consider the broader implications of using these nanostructures.[21]

Indeed, their potential raises many questions concerning how they might impact the ecosystem and toxicology aspects attributable to these ‘nanostructures.’ Researchers have then shifted focus onto studying variables such as accumulating ability or disintegration besides trying to determine if they interact with surrounding water-air-soil compartments in any way harmful.

Aholistic approach is critical for discerning appropriate preventive measures by gauging such parameters. According to initial findings, part of our understanding is deciphering whether it persists or dissipates while interacting within different environmental compartments affecting ecosystems beyond.[22]

Additionally, it becomes paramount that scientists proceed ever more cautiously, having first studied how these nanostructures affect marine-life terrestrial beings, crucially essential beneficial organisms like pollinators who serve a vital purpose.[2] Understanding chitosan nanomaterials' toxicology properties then takes on an elevated sense of importance, preventing any undesired long-term implications.

Using chitosan nanostructures in biomedicine and food technology[23] necessitates thoroughly assessing its possible influence on human health. This calls for investigating its

toxicity levels, bioaccumulation potential and any undesirable effects resulting from ingestion, inhalation or skin contact with humans[24], as depicted in Fig. 2.

The prolonged existence of chitosan nanoparticles within the human body depends upon multiple factors, including size, surface properties, and route of administration. At the same time, continuous research is being carried out in this area as the human body's exact life span is unknown.

Chitosan nanoparticles can be eliminated from the body via various routes after delivery. Renal excretion (through urine) and hepatobiliary excretion (by bile) are the principal clearance routes. The rate of clearance of chitosan nanoparticles can be affected by their size, surface charge, and surface changes.[26]

Chitosan nanoparticles may be distributed to many tissues and organs throughout the body. The extent of their existence within the human body depends upon various factors. Some studies have found chitosan nanoparticles in organs such as the liver and spleen, while others have seen them in the kidneys, lungs, and lymphatic system.[26]

The breakdown of chitosan nanoparticles is carried out by enzymes such as lysozymes and chitinases. Such nanoparticles are biodegradable and thus can be degraded within the body with any particular route for removal. At the same time, factors such as deacetylation level, molecular weight and susceptibility to enzymatic activity might affect the degradation rate and metabolism.[27]

The degree to which chitosan nanoparticles persist or are cleared from the body can be influenced by their physicochemical qualities and specific clearance mechanisms. Some research has revealed that chitosan nanoparticles have long circulation durations, while others have demonstrated that they are quickly eliminated.[22]

The critical issues related to chitosan nanoparticles nowadays are safety and biocompatibility. Although chitosan nanoparticles are considered biocompatible, research is still being carried out to determine their consequences on human health. Regulatory bodies such as the United States Food and Drug Administration (FDA) must ensure the safety of chitosan nanoparticles before licensing their use.

The evaluation of the environmental and toxicological impact of chitosan nanostructures might help to gather data for establishing legislation guidelines and help to determine the safety levels of exposure for its safe handling and disposal.[12] Despite their biocompatibility assurance, they are immunogenic and are widely tested for safety in biological systems.[28]

Evaluating chitosan nanostructures' environmental and toxicological impact is crucial for determining safe levels of exposure, establishing guidelines, and developing best practices for handling and disposal. This information helps ensure their appropriate usage in a sustainable manner while minimizing associated risks.[12]
Extensive research is being conducted on the potential of chitosan-based nanoparticles for medication delivery.[29] Due to their small size and large surface area, they are highly effective at encapsulating and releasing a wide range of medications, including proteins, nucleic acids, and small molecules. These tiny particles can also preserve and stabilize medicines while offering targeted delivery with sustained release capabilities. See Fig. 3 for more details on their action mechanism.[30]

Chitosan-based nanoparticles have been investigated as gene delivery vectors because of their capacity to compress and protect DNA or RNA molecules. These nanoparticles can form stable compounds with nucleic acids, preventing enzymes from degrading them and increasing their uptake by target cells. Chitosan-based nanoparticles have demonstrated promise in gene therapy applications such as gene editing and silencing.[31]

The chitosan nanoparticles are now extensively used in tissue engineering and regenerative medicine as scaffolds or hydrogels to enhance adhesion and proliferation. Chitosan nanoparticles can also act as delivery systems for growth factors and other bioactive compounds, supporting tissue regeneration and wound healing.[24] Chitosan-based nanoparticles can be functionalized with fluorescent dyes or contrast agents for fluorescence imaging, magnetic resonance imaging (MRI), and computed tomography (CT).

They've also been used in biosensing and diagnostic applications to detect and quantify biomarkers and analytes.[32] The chitosan nanoparticles can be used for antimicrobial coatings, wound dressings, and infection control applications depending upon their antibacterial characteristics, making them the material of choice.[33]

Rigorous eco-toxicological studies must be carried out to make educated decisions by scientists and industry stakeholders regarding the safe use of chitosan nanoparticles in various domains.

Section snippets

Environmental behavior of chitosan nanostructures

Chitosan nanoparticles' fate depends on their characteristics, such as stability, degradation and possible impact on ecosystems.[2] Due to the remarkable properties of chitosan nanoparticles in environmental sciences, they have gained significant importance in the fields such as wastewater treatment and agriculture.[34]

The chitosan nanoparticles are biodegradable and can degrade naturally over time by enzymes such as lysozyme and chitinases in various organisms such as bacteria and fungi.[35] The …

Eco-toxicological impact of chitosan nanostructures

The assessment of the eco-toxicological impact of chitosan nanostructures is to ensure their safe and sustainable use. It aims to assess such nanostructures' toxicity, bioaccumulation and ecological risks.[50] Their effects on various organisms, such as aquatic, terrestrial, and even microorganisms, are being observed, and different parameters, such as acute and toxic toxicities, reproductive effects, growth inhibition, and developmental changes, are being assessed.[51] The properties of chitosan …

The toxicological impact of chitosan nanostructures in humans

The study of the human toxicological impact of chitosan nanostructures has been a topic of great interest for the last few years. Chitosan, derived from chitin found in the exoskeleton of crustaceans, has gained immense potential in various fields due to its biocompatibility and biodegradability. However, assessing the potential risks chitosan nanostructures might pose to humans is crucial to ensure their safety for human health.[57] One of the primary concerns is the capability to induce adverse …

Risk assessment and regulation

Risk assessment and regulation of chitosan nanostructures are essential aspects in ensuring their safe and responsible use in various applications.[75] The following points discuss the different approaches involved in risk assessment and regulation of chitosan nanostructures.

Hazard identification: To identify and characterize the hazards associated with chitosan nanostructures, risk assessment is essential, and this can be done by evaluation of their physicochemical properties, toxicity profiles …

Conclusion

The ecological and toxicological impact of chitosan nanostructures is a complex and multidimensional approach, as they have shown promising results in various fields and exhibit relatively low toxicity compared to other nanoparticle types. The studies suggest that chitosan nanostructures can be degraded in natural environments, reducing their potential for persistent use. Their ability to bioaccumulate and aggregate in soil and water environments can vary depending on particle size, surface …

Volume chapters Recommended articles

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S. Zhang *et al.*
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Role of nanoparticles in fish disease management: A review
2024, Biocatalysis and Agricultural Biotechnology

Citation Excerpt :

> …Several research works proved the role of different nanoparticles in fish-pathogen interactions (Shaalan et al., 2016). Rapid development of metal and metal-oxide nanoparticles and their usage in several fields including electronics, energy, biomedicine etc., is a frequent practice in recent times besides understanding their nature (Liu et al., 2016; Khan et al., 2024). In general, metal-oxide nanoparticles are more popular due to their high reactivity and effectivity than metal NPs.…

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Environmental toxicity and biodegradation of chitosan derivatives: A comprehensive review

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The Studies on Chitosan for Sustainable Development: A Bibliometric Analysis

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* Correspondence: whlam@utar.edu.my

Abstract: Chitosan is a biocompatible polymer with vast applications in pharmacology, medicine, paper making, agriculture, and the food industry due to its low toxicity. Chitosan also plays an important role in the sustainable environment since chitosan is able to absorb greenhouse gases, harmful organic matter, and heavy ions. Therefore, this paper conducts a bibliometric analysis of chitosan for sustainable development using the Scopus database from 1976 to 2023. A performance analysis on the 8002 documents was performed with Harzing's Publish or Perish. Science mapping was conducted using VOSviewer. The annual publication on chitosan for sustainable development showed an upward trend in recent years as the annual publication peaked in 2022 with 1178 documents with most of the documents being articles and published in journals. Material science, chemistry, and engineering are tightly related subject areas. China had the highest publication of 1560 total documents while the United States had the most impactful publication with 55,019 total citations, 68.77 citations per document, 77.6 citations per cited document, *h*-index 110, and *g*-index of 211. India had the largest international collaboration with 572 total link strength. "International Journal of Biological Macromolecules", "Carbohydrate Polymers", and "Polymers" have been identified as the top three source titles that publish the most documents on chitosan for sustainable development. The emerging trends in chitosan on sustainable development focus on the application of chitosan as an antibacterial agent and biosorbent for contaminants, especially in water treatment.

Keywords: chitosan; bibliometric analysis; biopolymer; sustainability; VOSviewer

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1. Introduction

Derived from chitin, chitosan is a polysaccharide produced from the skeletons of crustaceans or the walls of fungi [1]. Chitin (β-(1→4)-*N*-acetyl-D-glucosamine) is abundantly available, however, its application is less favored due to its hydrophobic nature [2]. Pure chitin is translucent, resilient, and tough. Chitin exists in the α and β allomorphs. α-Chitin is compact with strong hydrogen bonding while β-chitin has weak intermolecular hydrogen bonding. Chitin, which is white and hard, can be obtained naturally as ordered crystalline microfibrils. Through deacetylation, chitosan, which is a β-1,4-D-glucosamine, can be derived from chitin, an *N*-acetyl-D-glucosamine [3,4]. Since chitosan is obtained from renewable resources, it is biodegradable with a low toxicity. The United States, Europe, Korea, and Japan have granted approval for the use of chitosan for consumption [5]. Moreover, when the amino groups in chitosan undergo protonation, chitosan becomes cationic and hydrophilic in an acidic aqueous solution, which is of great interest for biomedical and pharmaceutical applications [6]. This is contributed by the presence of a free amino group in chitosan, which is absent in chitin. Chitosan is easily available and relatively cheap with good biocompatibility, biodegradability, and ease of chemical modification.

Chitosan has a high degree of deacetylation (DD), which is normally above 40%. Hence, its solubility, crystallinity, and antimicrobial activity can be modified through its reactive

sites such as the hydroxy (-OH) and amino (NH_2) groups to suit various applications [7]. Chitosan, with amino acid modification, is biocompatible for wound healing and tissue generation [8,9]. Chitosan can also be modified for antibacterial and antifungal properties to inhibit the growth of *E. coli* and *B. cinerea* [10]. Chitosan-based sprays and aerogels provide a significant reduction in bacterial growth and are good for food packaging [11,12]. Amino acid-modified chitosan with folic acid is also hemocompatible to target cancer cells and decrease tumor spheroid volume [13].

Quaternized chitosans serve as absorbents to capture higher uptakes of greenhouse gases [14]. In addition, phosphorylated chitosan can be used as a flame retardant and is highly investigated for heat insulation [15,16]. Carboxymethyl chitosan is also favored in the cosmetics industry as a source of antioxidants for hydration and protection of the skin [17]. Moreover, chitosan serves as a scaffolding material for cell growth and tissue engineering, as the cationic property of chitosan allows interaction with glycoproteins and other structural molecules [18]. Chitosan is also used as a stabilizer in emulsions for the food industry [19].

Based on the concept of circular economy and the United Nation's Sustainable Development Goals (SDGs), companies are encouraged to always consider the present and the future in their production and consumption. Chitosan is a biopolymer that contributes to sustainability in industry. Chitosan can replace harmful chemicals in agriculture while protecting crops from diseases due to its antimicrobial activity [20,21]. Chitosan composites are also good absorbents of pollutants and metal ions such as tartrazine, tetracycline hydrochloride, arsenic, uranium, Pb^{2+}, Cu^{2+}, and Hg^{2+} ions [22–26]. Chitosan can also be used to manufacture paper so that the use of chemical additives can be reduced [27,28]. Moreover, chitosan helps to capture harmful gases such as formaldehyde and greenhouse gases [29,30].

One great advantage of chitosan is that chitosan can act as a biocompatible and biodegradable substrate for electronics. A maleic–chitosan proton conducting layer has good field-effect proton mobility and is a breakthrough discovery in green electronics [31]. Starch–chitosan substrate-based transparent electrodes can also be used in wearable electronics [32]. Silver nanowire–chitosan substrate can be used as the bottom electrode for perovskite solar cells with good stability [33]. Optimized chitosan electrostatic layer-by-layer films acting as cathode interlayers for inverted organic solar cells show high power conversion efficiency and could reduce the work function of electrodes to improve device performances [34]. Chitosan with yttrium oxide (Y_2O_3) can act as gate dielectric thin films in organic thin-film transistors with improved dielectric characteristics and pinholes and could operate in low voltage situations for various curvature radii [35].

Based on the vast applications of chitosan that have garnered the interest of many researchers, this paper aims to conduct a bibliometric analysis of chitosan for sustainable development. Aranaz [1] reviewed chitosan for the green synthesis of metallic nanoparticles and biocatalysts. Salgado-Cruz [2] performed a bibliometric review on the application of chitosan for coating in postharvest products using the Scopus database for a period of 10 years. Kou et al. [4] reviewed ways to produce chitosan using chemical and biological methods. Maliki et al. [36] performed a minireview on chitosan for green applications. Hameed et al. [37] reviewed the applications of chitosan for filtration, metal removal, antibacterial properties, wound dressing, food preservation, agriculture, and drug delivery. Kostag and Seoud [38] reviewed the molecular structures of chitin and chitosan and studied the dissolution mechanism for these biopolymers. Klongthong [39] conducted a bibliometric analysis on the treatment of viral diseases with chitosan. Martău et al. [40] reviewed the applications of chitosan in the biomedical and food sectors. Ranjan [41] did a bibliometric analysis of the biomedical applications of chitosan. The top-cited paper by Crini [42] reviewed chitosan as one of the environmentally friendly absorbents that can be used for purification. The third-most-cited paper by Boateng et al. [43] reviewed wound-healing dressings using various polymers, including chitosan which helps in accelerating granulation.

Based on our search, a bibliometric analysis of chitosan for sustainable development has not been performed in past studies. Sustainable development is an important topic for the safety and wellbeing of all living organisms. Chitosan, which is a biopolymer, is cost effective with high sustainability and wide functionality. Therefore, this paper shall conduct a bibliometric analysis of chitosan for sustainable development using the Scopus database from 1976 to 2023 (as of 26 January 2023) [44–46]. A bibliometric analysis was conducted for performance analysis and scientific mapping of a research domain [47,48]. The outcome of a bibliometric analysis explains the development of the research domain over time. A bibliometric analysis also highlights the emerging trends in the domain to allow researchers to better identify the research gaps [49,50]. This analysis also aims to contribute by highlighting the sustainable applications of chitosan and pointing out the possible sustainable areas of research in chitosan. Section 2 explains the methodology used in this bibliometric analysis. Section 3 discusses the results of the bibliometric analysis. Section 4 concludes the paper with the highlights of the outcomes of the analysis.

2. Methodology

This paper intends to conduct a bibliometric analysis of chitosan for sustainable development. The search for scientific literature was performed on the Scopus database, which is a globally trusted database for high-quality peer-reviewed materials [51,52]. The Scopus database also has the most extensive coverage and widest citation records among all the scientific databases [53,54]. Figure 1 explains the process of the bibliometric analysis of chitosan for sustainable development [55,56].

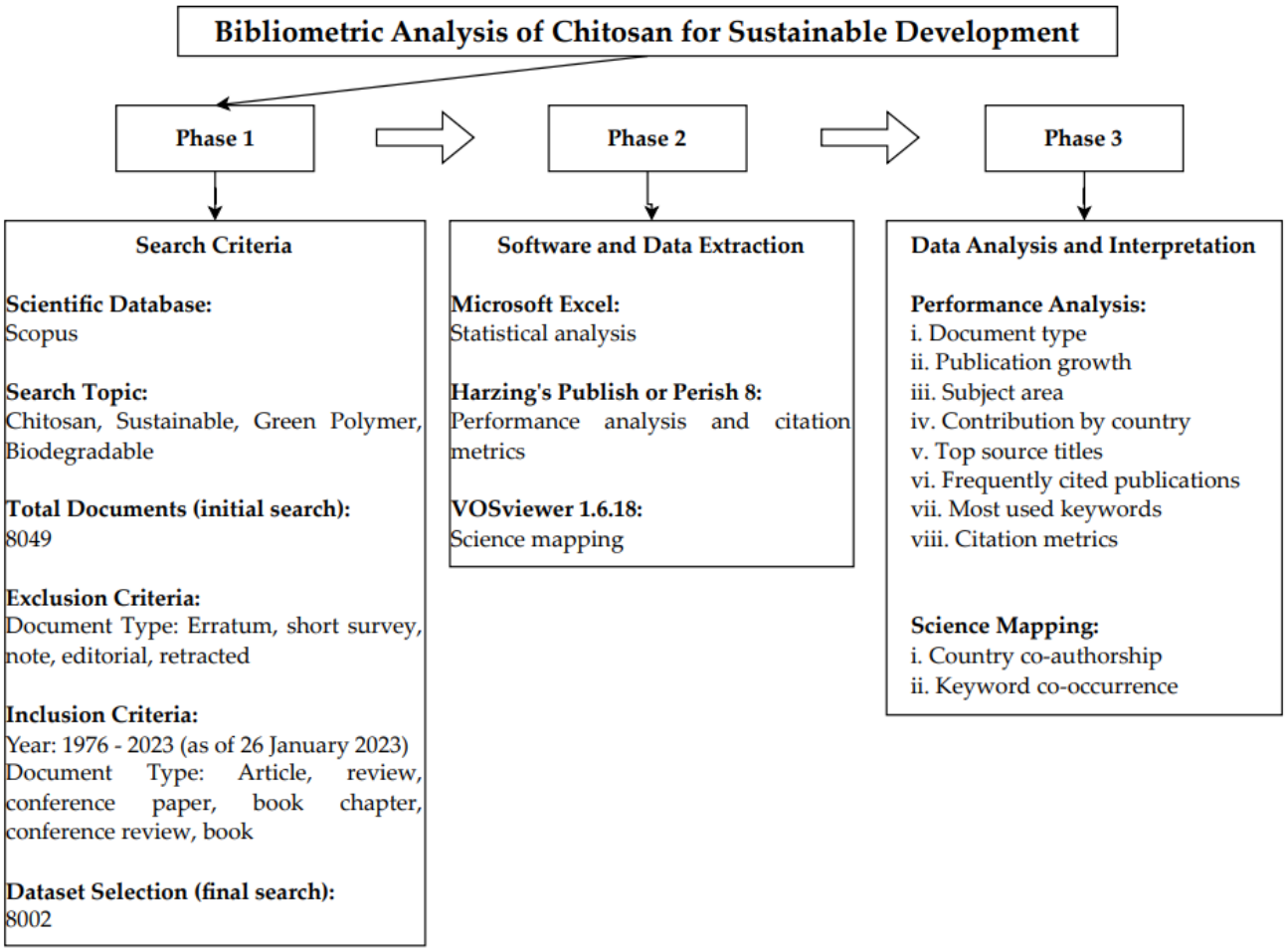

Figure 1. Process of bibliometric analysis of chitosan for sustainable development.

In the first step, "chitosan" was identified as a biopolymer with wide applications and benefits to the present and future needs of the industry. Data were retrieved from the Scopus database on 26 January 2023. The following query was applied: TITLE-ABS-KEY ("chitosan" and ("sustainable*" or "green polymer" or "biodegradable*" or "ecofriendly*")). From 1976 to 2023, 8049 documents were displayed. However, erratum, short survey, note, editorial, and retracted documents were excluded from this analysis [57–59]. A total of 8002 articles, reviews, conference papers, book chapters, conference reviews, and books were included in this bibliometric analysis.

In the second step, data were downloaded from the Scopus database for statistical analysis of bibliometric indicators. In the third step, further bibliometric analysis was conducted with Harzing's Publish or Perish 8 and VOSviewer version 1.6.18. Harzing's Publish or Perish was used for performance analyses with citation metrics for annual publication, documents by country, source titles, and highly cited documents [60]. In citation metrics, total document (TD) quantifies the number of documents, total citation (TC) shows the citations received for a document or author, *h*-index (*h* number of documents with minimum *h* number of citations) and *g*-index (*g* number of documents with minimum g^2 average citations) show the research achievements [61]. VOSviewer was used for scientific mapping, which maps the country coauthorship and keyword co-occurrence [46,62].

3. Results

The results of the bibliometric analysis of chitosan for sustainable development are discussed in this section. The results include document types, source types, annual publication, subject areas, country contribution, highly cited documents, and analysis of keywords. The summary of bibliometric analysis will be explained in the citation metrics for all 8002 documents from 1976 to 2023, as of 26 January 2023.

3.1. Document Type and Source Type

The 8002 documents are under six document types. Articles and reviews make up about 85% of the total documents. Of these, 5736 documents (71.68%) are articles while 1107 documents (13.83%) are reviews. Other document types are conference papers (560 documents or 7.00%), book chapters (509 documents or 6.36%), conference reviews (67 documents or 0.84%), and books (23 documents or 0.29%). Table 1 presents the document types of the 8002 papers on chitosan in sustainable development.

Table 1. Document type.

Document Type	Total Documents	Percentage (%)
Article	5736	71.68
Review	1107	13.83
Conference Paper	560	7.00
Book Chapter	509	6.36
Conference Review	67	0.84
Book	23	0.29
Total	**8002**	**100**

Documents can be published in various sources, and 6874 documents (85.90%) were published in journals, 460 documents (5.75%) were published in books, 413 documents (5.16%) were published in conference proceedings, 228 documents (2.85%) were published in book series, while less than 0.35% of the documents were published in trade journals or were undefined. Figure 2 shows the source types of the documents on chitosan for sustainable development.

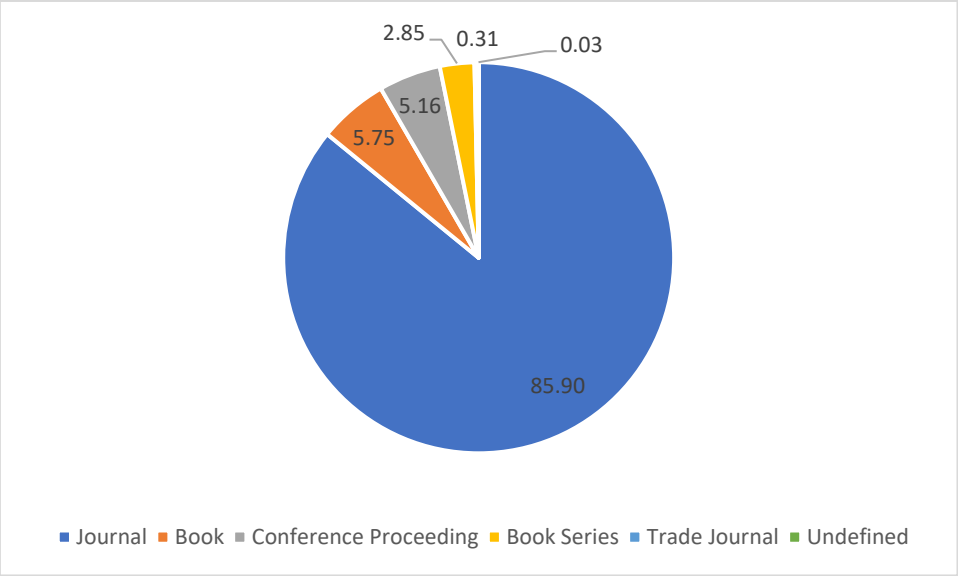

Figure 2. Source type.

3.2. Annual Publication

The first paper listed on the Scopus database was published in 1976 and authored by Kohn [63]. This paper, titled "Shellfish wastes vie for cpi role", noted that the shells of shrimps and crabs have chitin to derive chitosan, which is nontoxic and biodegradable. Chitosan can be used as flocculants, coagulants, food thickeners, or coatings, which are less toxic and less harmful than other chemicals [63]. This first paper has received two citations to date. The second and third papers listed on the Scopus database were published in 1986 by Koh et al. [64] and Machida et al. [65] which received 8 citations and 52 citations to date, respectively. The paper by Koh et al. [64] found that ground mixtures with chitosan offered a quicker dissolution rate of piroxicam than with chitin. Thus, chitosan can be used as a drug carrier. This study also noted that chitosan does not present a biological hazard. Machida et al. [65] performed an experiment to study the enzymatic degradation of chitosan and hydroxypropylchitosan on uracil. This paper then concluded that chitosan and hydroxypropylchitosan can be used in anticancer drugs.

Figure 3 describes the annual publication on chitosan for sustainable development and the total citation (TC) received by the documents. The focus on sustainability in chitosan was less prominent before the year 1995, as the total number of documents (TD) each year is below 10. An upward trend is observed from 1997 as the total number of documents (TD) peaked in 2022, with 1178 documents. As of 26 January 2023, 139 documents have been published and indexed in the Scopus database for the year 2023. This shows that researchers are increasingly interested in chitosan for sustainable development.

Table 2 presents the citation metrics of the annual publication of chitosan for sustainable development. The citation metrics include a number of cited documents (NCD), total citation (TC), citation per document (C/D), citation per cited document (C/CD), *h*-index, and *g*-index. The highest total citation was recorded in 2010 with 20,161 citations. This was largely contributed by the second and fourth highly cited papers by Kumari et al. [66] and Bhattarai et al. [67], respectively. Kumari et al. [66] received 2789 citations while Bhattarai et al. [67] received 1796 citations. The highest C/D and C/CD were recorded by the document published in 1988 by Hirano et al. [68]. This paper showed that chitosan can be used in oral and intravenous drug carriers.

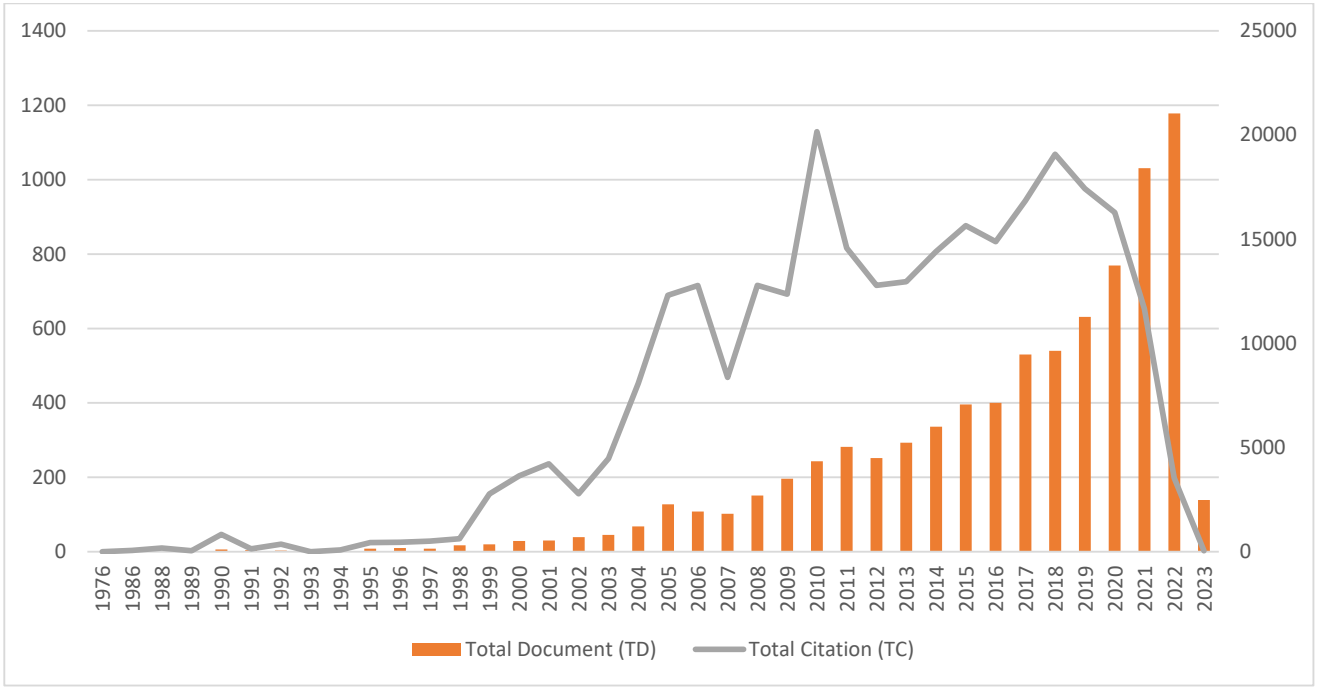

Figure 3. Total document and total citation.

Table 2. Annual Publication.

Year	TD [1]	Percentage (%)	Cumulative Percentage (%)	NCD [2]	TC [3]	C/D [4]	C/CD [5]	*h*-Index	*g*-Index
1976	1	0.01	0.01	1	2	2	2	1	1
1986	2	0.02	0.04	2	60	30	30	2	2
1988	1	0.01	0.05	1	175	175	175	1	1
1989	2	0.02	0.07	2	48	24	24	2	2
1990	6	0.07	0.15	5	830	138.33	166	3	6
1991	4	0.05	0.20	4	142	35.5	35.5	4	4
1992	3	0.04	0.24	3	363	121	121	3	3
1993	2	0.02	0.26	0	0	0	0	0	0
1994	3	0.04	0.30	3	90	30	30	3	3
1995	8	0.10	0.40	5	439	54.88	87.8	5	8
1996	10	0.12	0.52	9	451	45.1	50.11	7	10
1997	8	0.10	0.62	8	509	63.63	63.63	5	8
1998	17	0.21	0.84	16	620	36.47	38.75	9	17
1999	20	0.25	1.09	18	2774	138.7	154.11	14	20
2000	29	0.36	1.45	28	3642	125.59	130.07	21	29
2001	30	0.37	1.82	29	4219	140.63	145.48	24	30
2002	39	0.49	2.31	36	2787	71.46	77.42	24	39
2003	45	0.56	2.87	44	4469	99.31	101.57	24	45
2004	68	0.85	3.72	62	8086	118.91	130.42	38	68
2005	127	1.59	5.31	109	12,313	96.95	112.96	52	110
2006	108	1.35	6.66	96	12,771	118.25	133.03	47	108
2007	102	1.27	7.94	95	8362	81.98	88.02	45	91
2008	151	1.89	9.82	137	12,787	84.68	93.34	53	112
2009	196	2.45	12.27	182	12,359	63.06	67.91	60	107
2010	243	3.04	15.31	229	20,161	82.97	88.04	62	139
2011	282	3.52	18.83	256	14,576	51.69	56.94	67	113
2012	252	3.15	21.98	227	12,785	50.73	56.32	59	106
2013	293	3.66	25.64	262	12,965	44.25	49.48	61	105
2014	336	4.20	29.84	301	14,408	42.88	47.87	60	107
2015	396	4.95	34.79	363	15,649	39.52	43.11	64	109

Table 2. *Cont.*

Year	TD [1]	Percentage (%)	Cumulative Percentage (%)	NCD [2]	TC [3]	C/D [4]	C/CD [5]	*h*-Index	*g*-Index
2016	400	5.00	39.79	368	14,882	37.21	40.44	62	104
2017	530	6.62	46.41	458	16,842	31.78	36.77	63	107
2018	540	6.75	53.16	508	19,074	35.32	37.55	66	110
2019	631	7.89	61.05	591	17,423	27.61	29.48	63	96
2020	769	9.61	70.66	709	16,277	21.17	22.96	59	86
2021	1031	12.88	83.54	887	11,654	11.3	13.14	43	61
2022	1178	14.72	98.26	725	3541	3.01	4.88	22	31
2023	139	1.74	100.00	20	43	0.31	2.15	3	5
Total	8002	100			278,578				

[1] Total Document; [2] Number of Cited Documents; [3] Total Citation; [4] Citations per Document; [5] Citations per Cited Document.

3.3. Subject Area

In Scopus, documents are categorized under four main subjects including life sciences, physical sciences, health sciences, and social sciences and humanities. Then, there were 27 major subject areas and more than 300 minor subject areas. Every document may be categorized into more than one subject area based on Scopus classification [69,70]. The 8002 documents are grouped into several subject areas, with 18.56% of the documents grouped under materials science, followed by chemistry (14.46%), engineering (11.49%), chemical engineering (10.66%), and biochemistry, genetics, and molecular biology (10.18%). Pharmacology, toxicology, and pharmaceutics (6.33%), environmental science (5.23%), physics and astronomy (4.69%), agricultural and biological science (4.49%), and medicine (4.09%) are also under the top 10 subject areas. The complete list of subject areas is tabulated in Table 3.

Table 3. Subject Area.

Subject Area	Total Document	Percentage (%)
Materials Science	3249	18.56
Chemistry	2531	14.46
Engineering	2010	11.49
Chemical Engineering	1865	10.66
Biochemistry, Genetics, and Molecular Biology	1782	10.18
Pharmacology, Toxicology, and Pharmaceutics	1108	6.33
Environmental Science	915	5.23
Physics and Astronomy	821	4.69
Agricultural and Biological Sciences	786	4.49
Medicine	716	4.09
Energy	540	3.09
Economics, Econometrics, and Finance	276	1.58
Immunology and Microbiology	234	1.34
Computer Science	131	0.75
Business, Management, and Accounting	100	0.57
Multidisciplinary	91	0.52
Earth and Planetary Sciences	90	0.51
Social Sciences	56	0.32
Health Professions	49	0.28
Veterinary	34	0.19
Mathematics	32	0.18
Neuroscience	30	0.17
Dentistry	21	0.12
Nursing	20	0.11
Arts and Humanities	7	0.04
Psychology	4	0.02
Decision Sciences	3	0.02
Total	17,501	100

3.4. Country Contribution

There were about 120 countries that contribute to the publication of chitosan on sustainable development. China (1560 documents), India (1400 documents), and the United States (800 documents) were the top three countries contributing to this domain. Iran, Brazil, Italy, South Korea, Egypt, Spain, and Malaysia also provided high contributions in this domain with 470, 347, 311, 305, 300, 269, and 251 documents, respectively. The United States received the highest total citation (TC) of 55,019, citation per document (C/D) of 68.77, and citation per cited document (C/CD) of 77.6%. The United States also had the highest publication impact with an *h*-index of 110 and a *g*-index of 211, and 110 documents published by researchers in the United States received at least 110 citations, respectively. At least 44,521 total citations were also received from 211 documents from the United States. Table 4 presents the top 10 country contribution.

Table 4. Country Contribution.

Country	TD [1]	NCD [2]	TC [3]	C/D [4]	C/CD [5]	*h*-Index	*g*-Index
China	1560	1354	50,477	32.36	37.28	100	163
India	1400	1197	48,529	34.66	40.54	101	183
United States	800	709	55,019	68.77	77.6	110	211
Iran	470	402	13,940	29.66	34.68	60	98
Brazil	347	307	8471	24.41	27.59	48	78
Italy	311	277	10,406	33.46	37.57	51	92
South Korea	305	267	10,931	35.84	40.94	49	95
Egypt	300	256	8301	27.67	32.43	46	83
Spain	269	242	11,411	42.42	47.15	49	100
Malaysia	251	212	5487	21.77	25.88	39	66

[1] Total Document; [2] Number of Cited Documents; [3] Total Citations; [4] Citations per Document; [5] Citations per Cited Document.

VOSviewer maps the authors' collaborations across countries. This feature allows a deeper understanding of the scientific collaboration among the authors in different countries [71]. Link strength explains the magnitude of collaboration. The larger the link strength, the higher the number of collaborations [72]. Table 5 shows the top 10 countries with the highest link strengths. India had the highest total link strength of 572 with 1400 total documents. This shows that India had the highest collaboration with authors from other countries. The United States had a 557 total link strength with 800 documents. Even though China had the highest document total of 1560 papers, China was only in the third position in international collaboration with a 527 total link strength. Saudi Arabia (263), Iran (248), the United Kingdom (228), Egypt (220), Italy (201), Malaysia (201), and South Korea (200) were also among the top 10 for total link strength in international author collaboration. Figure 4 maps the country's coauthorship of the publications on chitosan for sustainable development.

Table 5. Top 10 countries for author collaborations.

Country	Total Document	Total Link Strength
India	1400	572
United States	800	557
China	1560	527
Saudi Arabia	164	263
Iran	470	248
United Kingdom	204	228
Egypt	300	220
Italy	311	201
Malaysia	251	201
South Korea	305	200

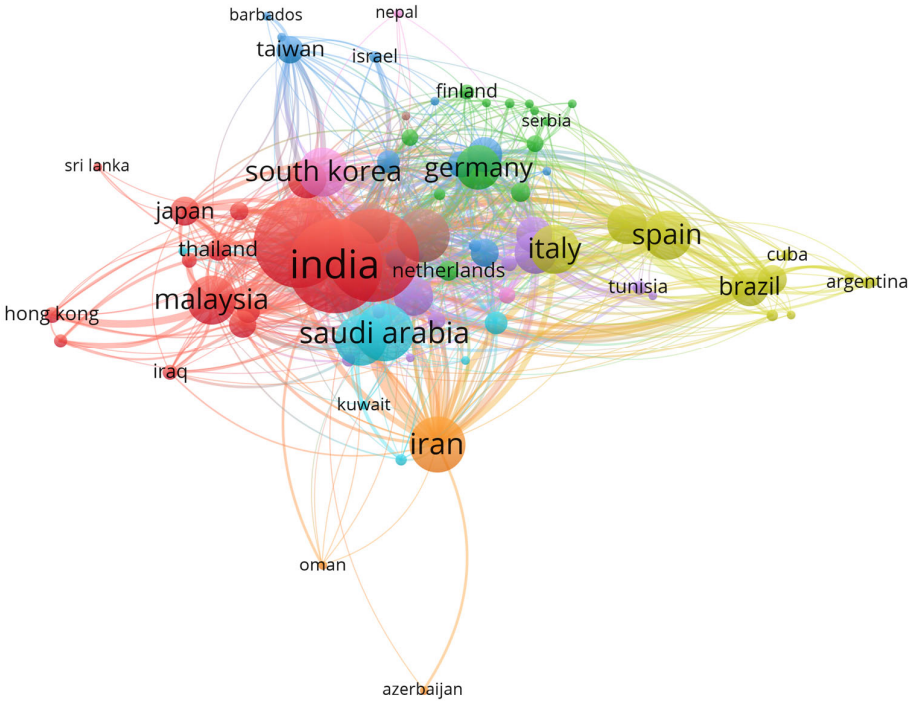

Figure 4. Country coauthorship.

Based on Figure 4, India has the largest node because of the highest total link strength. The thickest line spans from China to the United States with a link strength of 120. China and the United States collaborate the most in this domain. The colors of the nodes and lines represent the clustering of the countries [73]. There are nine clusters in total. The first cluster, which is red, has 15 countries made up of Australia, China, Hong Kong, India, Indonesia, Iraq, Japan, Malaysia, Nigeria, Singapore, South Africa, Sri Lanka, Thailand, the United States, and Vietnam. The second cluster, which is green, consists of Austria, Bangladesh, Bulgaria, Croatia, Finland, Germany, Hungary, Jordan, Lithuania, the Netherlands, Serbia, Slovenia, and Switzerland. The dark blue cluster consists of Barbados, Belgium, the Czech Republic, Denmark, Ethiopia, Israel, Latvia, the Philippines, Poland, the Russian Federation, Sweden, Taiwan, and Ukraine. The yellow cluster has Argentina, Brazil, Chile, Columbia, Cuba, Ecuador, Italy, Mexico, Peru, Portugal, Spain, and Venezuela. The next cluster, which is purple, is made up of Algeria, Canada, France, Greece, Lebanon, Morocco, Norway, Qatar, Romania, Tunisia, and Turkey. Egypt, Ireland, Kuwait, New Zealand, Saudi Arabia, Slovakia, and Yemen make up the light blue cluster. The orange cluster has Azerbaijan, Iran, and Oman. Kazakhstan, Pakistan, and the United Kingdom make up the brown cluster. The final cluster has Nepal, South Korea, and the United Arab Emirates.

3.5. Source Title

There were more than 150 source titles published on chitosan for sustainable development. Table 6 highlights the top 10 source titles. The source title that published the most documents on chitosan for sustainable development was "International Journal of Biological Macromolecules" (361), followed by "Carbohydrate Polymers" (271), "Polymers" (135), "Journal of Applied Polymer Science" (128), "Materials Science and Engineering C" (74), "Journal of Polymers and the Environment" (70), "Biomaterials" (69), "IOP Conference Series: Materials Science and Engineering" (69), "ACS Sustainable Chemistry and Engineering" (68) and "Molecules" (65). Documents published in "Biomaterials" received the highest total citation of 17,999. The top 10 source titles are also listed on Web of Science (WoS), except "Materials Science and Engineering C" and "IOP Conference Series: Materials Science and Engineering".

Table 6. Source Title.

Source Title	TD [1]	Percentage (%)	TC [2]	Publisher	Cite Score	SJR [3]	SNIP [4]	*h*-Index	JIF [5]	JCI [6]
International Journal of Biological Macromolecules	361	4.51	16,073	Elsevier	11.6	1.100	1.449	144	8.025	1.42
Carbohydrate Polymers	271	3.39	14,519	Elsevier	16.0	1.612	1.821	228	10.723	2.19
Polymers	135	1.69	2286	Multidisciplinary Digital Publishing Institute (MDPI)	5.7	0.726	1.170	89	4.967	0.88
Journal Of Applied Polymer Science	128	1.60	2968	Wiley-Blackwell	5.0	0.528	0.793	175	3.057	0.61
Materials Science and Engineering C	74	0.92	4613	Elsevier	12.6	1.191	1.417	145	NIL [7]	NIL [7]
Journal Of Polymers and The Environment	70	0.87	1411	Springer Nature	6.8	0.648	1.038	80	4.705	0.65
Biomaterials	69	0.86	17,999	Elsevier	21.5	2.678	2.045	397	15.304	2.68
IOP Conference Series: Materials Science and Engineering	69	0.86	138	IOP Publishing Ltd.	1.1	0.249	0.344	48	NIL [7]	NIL [7]
ACS Sustainable Chemistry and Engineering	68	0.85	2513	American Chemical Society	14.5	1.743	1.361	132	9.224	1.44
Molecules	65	0.81	1822	Multidisciplinary Digital Publishing Institute (MDPI)	5.9	0.705	1.267	171	4.927	0.64

[1] Total Document; [2] Total Citation; [3] SCImago Journal Rank 2021; [4] Source Normalized Impact per Paper 2021; [5] Journal Impact Factor 2021; [6] Journal Citation Indicator 2021, [7] Data Not Available.

3.6. Highly Cited Documents

Table 7 presents the top 10 highly cited documents on chitosan for sustainable development. The most-cited document, "Non-conventional low-cost adsorbents for dye removal: A review" by Crini [42], received 3590 citations. The review paper presented a critical analysis and the characteristics, advantages, limitations, and mechanisms of sorbents. Chitosan was identified as a promising adsorbent for environmental and purification purposes. The second-most-cited document, with 2789 citations, was by Kumari et al. [66] titled "Biodegradable polymeric nanoparticles based drug delivery systems". The review paper discussed the impact of nanoencapsulation of various disease-related drugs on biodegradable nanoparticles such as chitosan and gelatin. The third-most-cited document, by Boateng et al. [43], discussed the common wound management dressings and novel polymers used for the delivery of drugs to various types of wounds. These included chitosan, hydrocolloids, hydrogels, alginates, collagen, polyurethane, hyaluronic acid, and pectin. The paper by Bhattarai et al. [67], titled "Chitosan-based hydrogels for controlled, localized drug delivery", received 1796 citations and was the fourth highly-cited document. The authors investigated the developments in chitosan hydrogel preparation and defined the design parameters in the development of chemically and physically cross-linked hydrogels. The following most-cited paper by Khor and Lim [74] discussed the works of key groups in Asia developing chitosan and chitin materials for implantable biomedical applications.

Madihally and Matthew [75] authored the paper titled "Porous chitosan scaffolds for tissue engineering" which received 1338 citations. The authors studied the application of chitosan for the formation of porous scaffolds of controlled microstructures in tissue-relevant geometries. The seventh-most-cited paper by Chenite et al. [76] studied the use of polymer salt aqueous solutions as gelling systems and proposed the discovery of a prototype for a new family of thermosetting gels highly compatible with biological compounds. The following most-cited paper by Rieux et al. [77] discussed the influence of size and surface properties on the nanoparticles' nonspecific uptake or their targeted uptake by enterocytes and M cells. Li et al. [78] obtained 1037 citations for their paper titled "Injectable and biodegradable hydrogels: gelation, biodegradation, and biomedical

applications". The authors presented the progress on biodegradable and injectable hydrogels fabricated from natural polymers such as chitosan and biodegradable synthetic polymers. The 10th-most-cited document was achieved by Klouda [79] for the paper "Thermoresponsive hydrogels in biomedical applications A seven-year update". The author reviewed the literature on thermosensitive hydrogels by focusing on natural polymers as well as synthetic polymers.

Table 7. Highly Cited Documents.

Author	Title	Year	Cites	Cites Per Year	Source Title
G. Crini [42]	Non-conventional low-cost adsorbents for dye removal: A review	2006	3590	211.18	Bioresource Technology
A. Kumari, S.K. Yadav, S.C. Yadav [66]	Biodegradable polymeric nanoparticles based drug delivery systems	2010	2789	214.54	Colloids and Surfaces B: Biointerfaces
J.S. Boateng, K.H. Matthews, H.N.E. Stevens, G.M. Eccleston [43]	Wound healing dressings and drug delivery systems: A review	2008	1945	129.67	Journal of Pharmaceutical Sciences
N. Bhattarai, J. Gunn, M. Zhang [67]	Chitosan-based hydrogels for controlled, localized drug delivery	2010	1796	138.15	Advanced Drug Delivery Reviews
E. Khor, L.Y. Lim [74]	Implantable applications of chitin and chitosan	2003	1468	73.4	Biomaterials
S.V. Madihally, H.W.T. Matthew [75]	Porous chitosan scaffolds for tissue engineering	1999	1338	55.75	Biomaterials
A. Chenite, C. Chaput, D. Wang, C. Combes, M.D. Buschmann, C.D. Hoemann, J.C. Leroux, B.L. Atkinson, F. Binette, A. Selmani [76]	Novel injectable neutral solutions of chitosan form biodegradable gels in situ	2000	1199	52.13	Biomaterials
A. des Rieux, V. Fievez, M. Garinot, Y.-J. Schneider, V. Préat [77]	Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach	2006	1068	62.82	Journal of Controlled Release
Y. Li, J. Rodrigues, H. Tomás [78]	Injectable and biodegradable hydrogels: Gelation, biodegradation and biomedical applications	2012	1037	94.27	Chemical Society Reviews
L. Klouda [79]	Thermoresponsive hydrogels in biomedical applications	2008	989	65.93	European Journal of Pharmaceutics and Biopharmaceutics

3.7. Keyword Analysis

VOSviewer provides a feature to map the keyword co-occurrence map to detect the research clusters and how these clusters are linked to form a subdomain [80]. Table 8 shows the 20 most frequently used keywords with the respective total link strengths. "Chitosan" (6201) was the most-used keyword with the highest total link strength of 93,328. The second-most-used keyword was "biocompatibility" with 1525 occurrences. The third-most-used keyword was "nonhuman" with 1492 occurrences. Figure 5 depicts the keyword co-occurrence map.

Based on Figure 5, the keywords can be categorized into three clusters. The first cluster is red and contains keywords such as acetylation, antibacterial activity, antiinfective agent, antimicrobial activity, antioxidants, bioactivity, biodegradable, biodegradation, biopolymer, bioremediation, carbon dioxide, carboxymethyl chitosan, catalysis, cellulose, chitin, chitosan, chitosan derivatives, chlorine compounds, coagulation, coating, crystallinity, deacetylation, differential scanning calorime, ecofriendly, electrolytes, environmental im-

pact, *Escherichia coli*, ethylene, flocculation, food packaging, functional polymers, glycerol, green chemistry, heavy metals, hydrogen bond, hydrolysis, II-VI semiconductors, infrared spectroscopy, ions, lignin, microbial sensitivity test, minimum inhibitory concentration, morphology, nanofiber, plant extract, polysaccharides, temperature, tensil strength, thermodynamics, ultraviolet radiation, wastewater treatment, water vapor permeability, wound dressings, and X-ray diffraction.

Table 8. Top 20 Keywords with Total Link Strengths.

Keywords	TD [1]	Total Link Strength
Chitosan	6201	93,328
Nonhuman	1492	38,976
Chemistry	1468	37,602
Human	1287	34,247
Biocompatibility	1525	31,074
Drug delivery system	1143	28,912
Animals	1051	28,575
Humans	944	26,310
Biodegradability	1122	23,721
Scanning electron microscopy	1103	23,341
Nanoparticles	1060	21,317
Polymer	900	21,023
Animal	750	20,874
Nanoparticle	820	20,062
Particle size	767	19,536
Tissue Engineering	795	19,081
In vitro study	620	18,166
Chitin	1163	17,624
Biodegradable polymers	1146	17,262
Biomaterial	611	16,379

[1] Total Document.

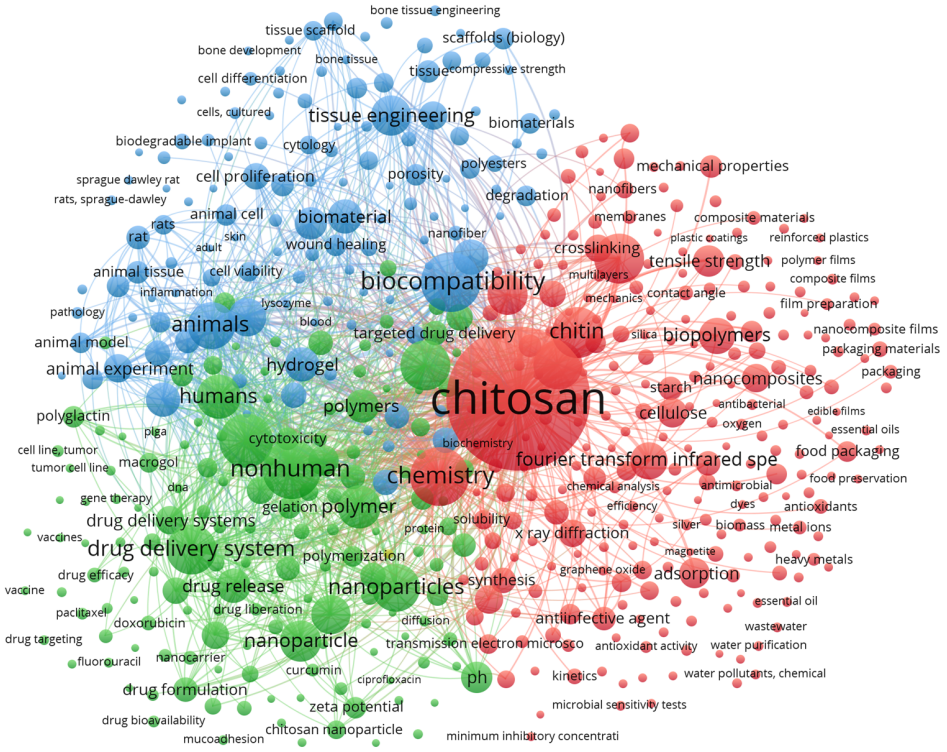

Figure 5. Keyword co-occurrence map.

The second cluster, which is green, contains alginates, antineoplastic agents, apoptosis, bovine serum albumin, cancer therapy, chondroitin sulfate, cyclodextrin, cytotoxicity, DNA, drug carrier, drug delivery, emulsion, encapsulation, gel, gelatin, gene therapy, hexuronic acids, human, hyaluronic acid, hydrophilicity, hydrophobicity, immunogenicity, liposome, macrogol, molecular structure, nanotechnology, nonhuman, paclitaxel, pH, polyethyleneimine, polyvinyl alcohol, vaccine, and zeta potential. The third cluster is blue and has adhesion, animal, angiogenesis, biomimetics, bone regeneration, calcium phosphate, cartilage, cell proliferation, cytology, fibroblast, flow kinetics, freeze drying, hydrogel, in vitro study, in vivo study, lysozyme, pharmacology, scaffold, tissue engineering, and Young modulus.

Figure 6 shows the overlay visualization map of keywords, which explains the trends of the publications on chitosan for sustainable development. The yellow keywords are the recent trends and the current focus of researchers. They include sustainability, graphene oxide, antibacterial agents, field emission scanning electron microscopy, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, water management, water pollutant, heavy metal, recycling, food packaging, *Escherichia coli*, lignin, contact angle, II-VI semiconductors, and microbial sensitivity test.

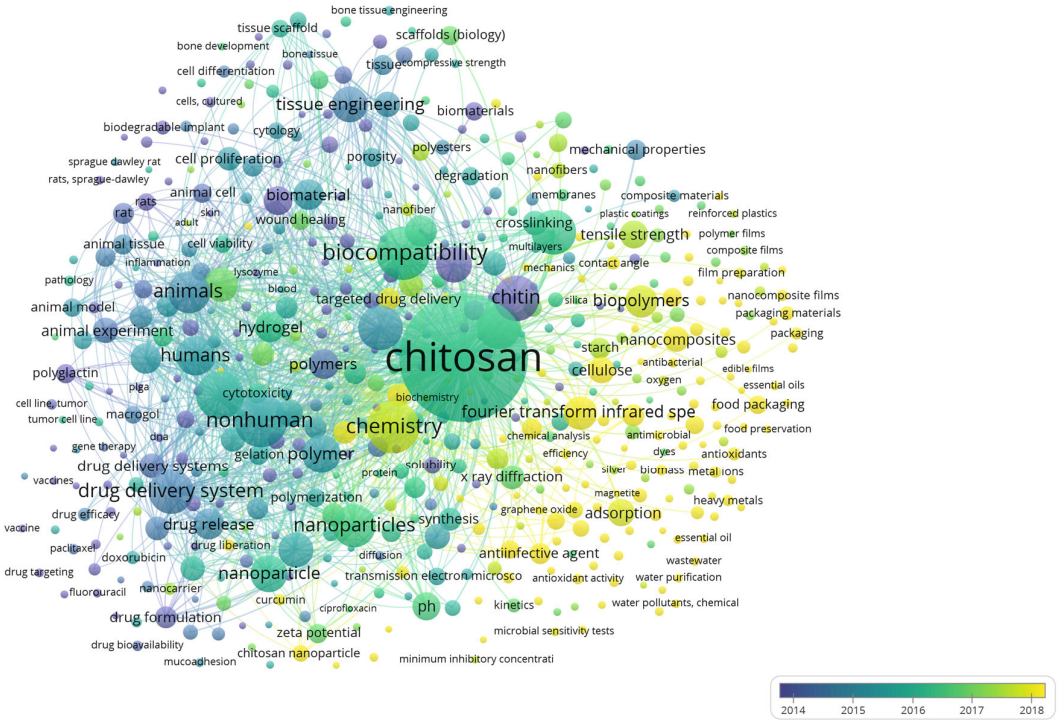

Figure 6. Keyword overlay visualization map.

Green electronics are also significant in sustainable development. Based on our query, there were several papers that adopt chitosan in green electronics and are impactful to current and future research. Chitosan and polyvinylpyrrolidone substrates produced using a solution casting process have great optical transmittance, high temperature stability, high biodegradation rate, and excellent mechanical stability to be used in flexible electronics [81]. Miao et al. [32] have also experimented with a starch–chitosan substrate for wearable green electronics. Du et al. [35] used chitosan with Y_2O_3 in organic thin-film transistors with superior dielectric properties. Li et al. [82] performed a review on the use of chitosan in electronic devices such as solar cells, organic field-effect transistors, and light-emitting diodes (LED). In addition, chitosan-based solid carbon dots can also be used for white LED and 3D printing [83]. Chitosan-mediated LED illumination also has better antibacterial treatment on *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* spp. than LED illumination only [84]. Chitosan nanoparticles with LEDs of different spectra can

also modify *Eleutherococcus senticosus* for the treatment of diseases [85]. Chitosan-based asymmetric electrodes also have high selectivity and absorption of oxidized hexavalent uranium, strontium cation, and cesium [86]. Therefore, the applications of chitosan in green electronics are beneficial in food science, nutrition, medicine, technology, and water treatment. Chitosan is also the current trend based on the keyword overlay visualization.

The research on water purification and the management of water pollutants are insightful areas with broad prospects. There are more than 100 articles discussing the removal of impurities and purification. Liu et al. [87] experimented with the usage of chitosan-modified cellulose fibers and ferric chloride to remove *Microcystis aeruginosa* and microcystin-LR. In their book, Ahankari et al. [88] studied water remediation and purification and found that nanochitosan-based materials have a better absorption capacity than microsized chitosan when removing heavy metals due to a larger surface area and reactivity. Magnetic chitosan nanoparticles also have great superparamagnetism and are low cost with high efficiency [88]. Chitosan-$AgIO_3$ can also treat *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Escherichia coli*, and *Staphylococcus aureus* which are helpful in water purification [89]. Photothermal chitosan–cellulose nanofiber is also a promising solar-driven cost-effective water purification technique [90]. Water treatment and heavy metal removal for purification have also been studied in various pieces of research [91–97].

However, there are also some challenges to using chitosan. Mujtaba et al. [98] stated that chitosan has low hydrophobicity and low mechanical and thermal strength which inhibits its use as a barrier enhancer in food packaging. Therefore, chitosan has been combined with other biopolymers, plant or animal proteins, waxes, and minerals while other methods such as cross-linking, graft copolymerization, and enzymatic treatments are used to improve the barrier properties of chitosan. Meanwhile, the stability of chitosan is also a challenge in pharmaceutical and biomedical fields [99]. The purity of chitosan affects the drug delivery process. Chitosan may not be able to dissolve due to the presence of ash or residual proteins for drug delivery [100–102]. Chitosan may also degrade through enzymatic hydrolysis if there is microbiological contamination. Centrifugation and extensive shearing could also reduce the molecular weight of chitosan and cause fluctuation in the polydisperity index in biomedical applications [103,104]. A low degree of deacetylation (DD) also causes acute inflammation and low affinity to enzymes in vitro. On the other hand, chitosan with high DD is less porous with low water uptake which slows down the rate of acidic hydrolysis [105,106].

During storage, the physiochemical and mechanical characteristics of chitosan may be altered due to the change in moisture level. Prolonged storage can dehydrate chitosan which then reduces its crushing strength and causes a spike in friability and disintegration [107]. Excessive moisture in the chitosan structure will increase the damage level due to hydrolysis. When the storage environment has a high relative humidity, the mechanical properties of chitosan will be decreased, as there is more swelling of the chitosan to induce a quicker release of the active compounds [108]. This will also bring down the adhesiveness of chitosan carriers with mucin [109–111]. The application of chitosan in hydrogels may be unstable as dissolution may happen, has poor mechanical resistance, and its pore size is hard to control, while chemical crosslinking may alter its intrinsic properties. Chitosan sponge may shrivel and is less porous [112]. Since there is vast potential to use chitosan, especially in the pharmaceutical and biomedical industries, researchers may identify these suitable research gaps, which may be studied to overcome the limitations of chitosan.

3.8. Citation Metric

The citation metric of the 8002 documents on chitosan for sustainable development from 1976 to 2023 as of 26 January 2023 is shown in Table 9. Based on the 8002 documents, 278,578 citations have been received with an *h*-index of 215 and a *g*-index of 343.

Table 9. Citation Metric.

Items	Metrics
Date of Extraction	26 January 2023
Papers	8002
Citations	278,578
Years	47
Citation per Year	5927.19
Citation per Document	34.81
Citation per Author	80,267.14
Papers per Author	2135.23
Authors per Paper	4.86
h-index	215
g-index	343

4. Conclusions

The research on chitosan for sustainability has received notable attention in recent years. This can be seen in the upward trend of annual publications where the number of documents exceeded 1000 in the year 2021 and continued to rise in 2022. Most of the documents were articles published in journals. The first paper indexed in Scopus was "Shellfish wastes vie for cpi role" by Kohn [63] which was published in 1976. The highest cited document is "Non-conventional low-cost adsorbents for dye removal: A review" by Crini [42] which received 3590 citations since its publication in 2006. China (1560) produced the highest number of documents on chitosan for sustainable development. The United States received the highest total citation of 55,019, 68.77 citations per document and 77.6 citations per cited document. The top three source titles which publish documents on chitosan for sustainable development were the "International Journal of Biological Macromolecules", "Carbohydrate Polymer" and "Polymers". All these three source titles were also indexed in WoS.

Most of the documents were under materials science, chemistry, and engineering. India had the highest international collaboration with 572 total link strength on the 1400 documents. The biggest link strength (120) was found between China and the United States. From the keyword co-occurrence map, it can be highlighted that the research trend is moving toward the application of chitosan for sustainable development. Increased research interest has been placed on the antibacterial functionality of chitosan on bacteria such as *staphylococcus aureus*, *pseudomonas aeruginosa*, and *Escherichia coli*. Lately, chitosan has also been studied for its flocculation in water treatment to remove organic matter, suspended solids, and heavy ions for a sustained environment.

There are several limitations to this study. Firstly, the Scopus database is constantly updating from time to time. Therefore, this bibliometric analysis may be repeated in the future for an intensive understanding of the evolving trends. Secondly, the first indexed paper in the Scopus database was published in 1976. Documents published before 1976 which were not indexed in the Scopus database were not considered in this study.

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Purification of chitosan by using sol–gel immobilized pepsin deproteinization

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Abstract

The residual proteins in commercially available chitosan products potentially induce immunological responses, thus compromising their clinical usage. Conventional deproteinization processes use diluted base and heat. However this heterogeneous hydrolysis is inefficient. In the present study, pepsin was selected and immobilized with tetramethoxysilane (TMOS) and 3-aminopropyltriethoxysilane (APTES). The immobilized pepsin was utilized in an alternative approach for the purification of chitosan. Optimum deproteinization was carried out at pH 4.5 and 45 °C. Amino acid analysis proved a removal of 53.8–80.4% protein in chitosan after 160 min incubation, which was more efficient than conventional sodium hydroxide deproteinization. When chitosan was deproteinizated by immobilized pepsin, its molecular weight decreased, but in a much milder manner than the free pepsin. The study revealed that immobilized pepsin was an efficient method for deproteinizing chitosan.

Highlights

► Pepsin was immobilized and was used for deproteinization of chitosan. ► Optimum deproteinization was carried out at pH 4.5 and 45 °C. ► Protein removal more effective than NaOH deproteinization. ► The decrease of molecular weight was much milder than free pepsin.

Introduction

Chitosan, the N-deacetylated derivate of chitin, is a heteropolysaccharide consisting of linear β-1,4-linked glucosamine (GlcN) and N-acetyl-glucosamine (GlcNAc) units (Franca, Lins, Freitas, & Straatsm, 2008). Both chitin and chitosan, either in their pure or modified forms, are used in a wide range of applications, such as in food, biotechnology, material science, pharmaceuticals, and recently in gene therapy (Muzzarelli, 2009, Prashanth and Tharanathan, 2007).

For the production of medical grade chitosan, an indispensible step is to remove protein residue from chitosan (Zhang et al., 2010), particularly tropomyosin the potent allergen from crustacean flesh, as they induce immunological responses (Muzzarelli, 2010). Under certain circumstances, the inflammatory response can have deleterious effects, resulting in significant tissue damage or even death (Goldsby, 2006, Chapter 16). Many studies also have reported that residual proteins are bound by covalent bonds to chitin, forming stable complexes (Iconomidou et al., 2005, Kasaai, 2008, Kurita, 2006, No et al., 1989, Vincent and Wegst, 2004). Besides covalent bonds, hydrogen-bonding and hydrophobic interactions might also be involved (Percot, Viton, & Domard, 2003).

Conventional processes for chitin and chitosan production from crustacean shells involve deproteinization of the crustacean shells with a dilute base (e.g. sodium hydroxide) and heat (Khor, 2001, Chapter 5; No et al., 1989). However, deproteinization with dilute base is a heterogeneous reaction, so interactions between chitin/chitosan and the protein can prevent the protein hydrolyzing during alkaline treatment (Chaussard and Domard, 2004, Percot et al., 2003a). In addition, basic deproteinization inevitably induces the degradation of chitosan. For example, the molecular weight (MW) of chitosan decreased from 813 kDa to 549 kDa after a 30-min deproteinization in 3% NaOH at 15 psi/121 °C (No, Lee, Park, & Meyers, 2003). NaOH deproteinization is also time-and-energy-consuming and produces voluminous waste base.

Enzymatic hydrolysis is an effective method to reduce the protein molecular weight, peptide size, and consequently the allergenicity (Chandra, 1997, Hays and Wood, 2005), thus it has already become a standard procedure for the production of hypoallergenic

hydrolyzates (Monaci, Tregoat, Hengel, & Anklam, 2006). However, its application to remove protein in chitin and chitosan was limited since the enzyme usage might induce protein contamination. Moreover, proteases such as lysozyme (Vårum, Holme, Izume, Stokke, & Smidsrød, 1996), papain (Terbojevich, Cosani, & Muzzarelli, 1996), bromelain (Hung, Chang, & Sung, 2002), and pepsin (Kumar et al., 2007, Roncal et al., 2007) was proved to retain chitosanase activity, further restrained the application of enzymatic deproteinization in chitosan purification.

Fortunately, immobilized enzymes can be easily separated from the final product, thereby minimizing or eliminating protein contamination of the product (Duran et al., 2002, Sheldon, 2007). Furthermore, the substrate selectivity of immobilized enzymes can also be manipulated by tailoring the immobilization condition (Fernandez-Lorente et al., 2007, Marin-Zamora et al., 2007, Park et al., 2010). Through immobilization, enzymes can also be reused and enzyme stability can also be enhanced (Brady and Jordaan, 2009, Hanefeld et al., 2009, Sheldon, 2007).

In the present study, pepsin was immobilized and utilized for chitosan purification. Hybrid silane, tetramethoxysilane (TMOS) and 3-aminopropyltriethoxysilane (APTES) were applied to entrap pepsin. The deproteinization by immobilized pepsin was studied. Purification of chitosan was performed with the immobilized product. Meanwhile, amino acid analysis was carried out to evaluate the protein removal efficiency.

Section snippets

Materials

Commercially available pepsin (porcine, EC 3.4.23.1, 1:3000) was obtained from Amresco (Solon, United States). Tetramethoxysilane (TMOS, >98.0%), 3-aminopropyltriethoxysilane (APTES, >98.0%) and polyvinyl alcohol (PVA, MW 22,000) were from Shanghai Jingchun Industry (Shanghai, China). Glucosamine hydrochloride (>99.0%) and amino acids (>98.0%) were purchased from Sinopharm Chemical Reagent (Shanghai, China). Hemoglobin (porcine) was obtained from Sigma (St. Louis, United States), and chitosan …

Selection of enzyme

Some commercial available enzymes, including pepsin, bromelain (1000U/mg, Green Chemical, China), trypsin (3000U/mg, Shanghai Jingchun, China), and chymotrypsin (800U/mg, Shanghai Jingchun, China), were studied to select proper enzyme for further immobilization and deproteinization. Their protease activity was determined in pH 4.5 acetate buffer. Chitosanase activities were also determined and the protein selectivity (protease activity/chitosanase activity) was studied because the …

Conclusions

The study revealed that purification of chitosan by immobilized pepsin entrapped with a hybrid silane (TMOS and APTES) was more efficient than traditional NaOH deproteinization. Amino acid analysis showed that the removal of Tyr, Val and Met was most efficient. The enzyme deproteinization was energy efficient and environmental friendly. Only slightly degradation of chitosan was observed after immobilized pepsin deproteinization. …

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> …Additionally, reduced activities related to protein function can be caused by homogeneous-to-heterogeneous condition transitions, which are dependent on Brownian motion surface limitations. Of the numerous methods for immobilizing proteins on a substrate surface, chemical crosslinking [9–11], specific/non-specific adsorption [12–14], and sol–gel processing [15–17] methods have been widely used for conventional applications; however, the chemical crosslinking and the non-specific adsorption methods make it difficult to control protein orientation [2,14,18,19] with any sort of precision on the substrate as there are always strong immobilization points present, e.g., through an amino group, carboxyl group, or hydrophobic site. In addition, chemical crosslinking often requires several preparatory steps to modify the protein, the substrate, or both [20,21].…

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Research Article

Effects of the pre-fermentative addition of chitosan on the nitrogenous fraction and the secondary fermentation products of SO_2-free red wines

Lourdes Marchante, Adela Mena, Pedro M Izquierdo-Cañas, Esteban García-Romero, María Soledad Pérez-Coello, María Consuelo Díaz-Maroto

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Abstract

BACKGROUND

Different red winemaking were carried out to evaluate the effects of the prefermentative addition of chitosan, as an alternative to the use of SO_2, on the secondary products of alcoholic fermentation, yeast available nitrogen (YAN), biogenic amines and ethyl carbamate.

RESULTS

The wines made with chitosan presented higher total acidity and higher content of tartaric and succinic acids than those made only with SO_2. The use of chitosan in winemaking resulted in wines with higher glycerol and diacetyl content without increasing the concentration of ethanol, acetic acid, acetaldehyde or butanediol. YAN was lower in wines made with chitosan, which may mean an advantage for the microbial stability of the wines. Furthermore, the use of chitosan at the beginning of alcoholic fermentation did not increase the concentration of biogenic amines or the formation of ethyl carbamate in SO_2-free red wines.

CONCLUSION

fraction or their very important secondary fermentation products such as acetic acid or acetaldehyde. © 2020 Society of Chemical Industry

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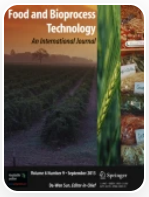

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Antonio Morata, Iris Loira, Ricardo Vejarano, Maria Antonia Bañuelos, Pedro D. Sanz, Laura Otero & Jose Antonio Suárez-Lepe

Abstract

Vitis vinifera (variety Tempranillo) grapes were subjected to high hydrostatic pressure (HHP) treatments of 200, 400 and 550 MPa for 10 min, and its effect on microbial populations, phenol extraction and wine quality was examined. At ≥400 MPa, the wild yeast population was strongly reduced from 10^4 to <10 cfu/ml. Bacteria showed greater resistance, and a residual load remained even after the treatment at 550 MPa. The extraction of phenolic compounds from the HHP-treated grapes was improved, with higher concentrations of total

phenols obtained compared to crushing alone. Anthocyanin extraction was also increased, producing wines with better colour intensity. These wines also had higher methanol and ethanol contents and returned higher aromatic quality and colour scores. The HHP treatment of grapes may assist in the use of yeast starters, increase phenol extraction from grape skins and improve wine quality.

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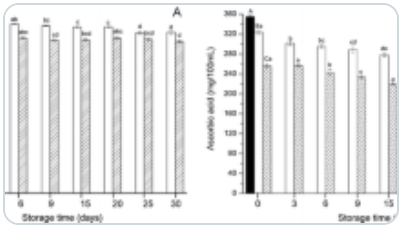

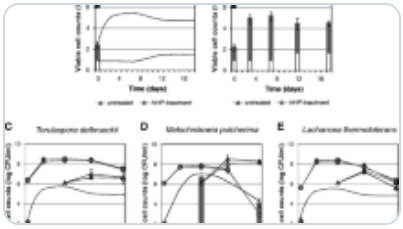

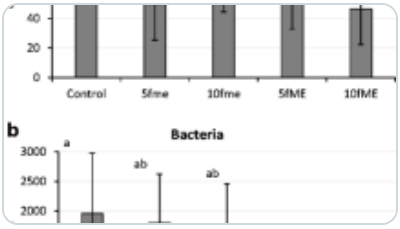

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Applications of *Metschnikowia pulcherrima* in Wine Biotechnology

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Abstract: *Metschnikowia pulcherrima* (*Mp*) is a ubiquitous yeast that frequently appears in spontaneous fermentations. The current interest in *Mp* is supported by the expression of many extracellular activities, some of which enhance the release of varietal aromatic compounds. The low fermentative power of *Mp* makes necessary the sequential or mixed use with *Saccharomyces cerevisiae* (*Sc*) to completely ferment grape musts. *Mp* has a respiratory metabolism that can help to lower ethanol content when used under aerobic conditions. Also, *Mp* shows good compatibility with *Sc* in producing a low-to-moderate global volatile acidity and, with suitable strains, a reduced level of H_2S. The excretion of pulcherrimin gives *Mp* some competitive advantages over other non-*Saccharomyces* yeasts as well as providing some antifungal properties.

Keywords: *Metschnikowia pulcherrima*; oenological uses; enzymes; stable pigments; pulcherrimin

1. Ecology and Physiology

Metschnikowia pulcherrima (*Mp*) is a globous/elliptical yeast that cannot be distinguished from *Saccharomyces cerevisiae* (Sc) by microscopy (Figure 1). Sometimes, it can be observed a single large, highly refractive oil droplet inside the cell. *Mp* is a teleomorph yeast belonging to an ascomycetous genus [1]. Its anamorph form is called *Candida pulcherrima*. *Mp* is a ubiquitous yeast that has been found in grapes, fruits (fresh and spoiled), flowers, nectars and tree sap fluxes. Several insects can work as vectors for this yeast. *Mp* strains can be identified through the use of selective and differential substrates; *Mp* strains showed both positive β-glucosidase enzyme activity and proteolytic activity [2]. *Mp* grows properly in either YPD or L-lysine media, and it can also can use arbutin as a carbon source in agar plates, indicating the expression of β-glucosidase activity (Figure 2) [3]. Recently, its nitrogen requirement was evaluated and slower consumption rates of ammonium were observed in *Mp* in comparison to other yeast genera [4]. This slow nitrogen uptake is indicative of its low fermentative ability [5].

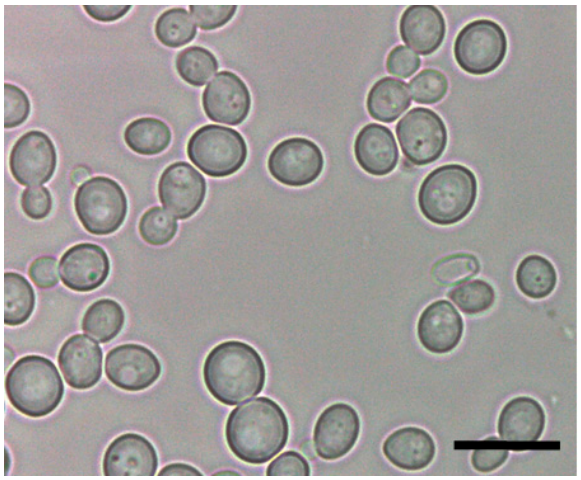

Figure 1. Cell morphology and shape of *Metschnikowia pulcherrima*. Graphical scale 10 μm.

The β-glucosidase activity related to *Mp* has been associated with different intracellular β-glucosidases, with the identification of three different bands observed when using fluorogenic substrates via an electrophoretic technique [6]. Of these three bands, the major band has similar physicochemical properties to those found in other studied yeasts, with high activity in ethanol and glucose concentrations often found in wines but low stability below pH 4. *Mp* is unable to develop in YPD at 37 °C and shows very weak or no growth in nitrate agar (Figure 2). It is able to use glucose, sucrose, fructose, galactose and maltose as carbon sources but shows weak or inexistent development in lactose [7]. It can grow properly under low temperature (15–20 °C) and pH conditions (3–6) [8]. Under environmental stress conditions such as a shortage of nitrogen, its recognition in optical microscopy is easy thanks to the appearance of a fat globule inside the cell at the beginning of the sporulation process [8]. In its sporulated form, the asci of *Metschnikowia* are long and clavate, containing one to two acicular to filiform spores [1].

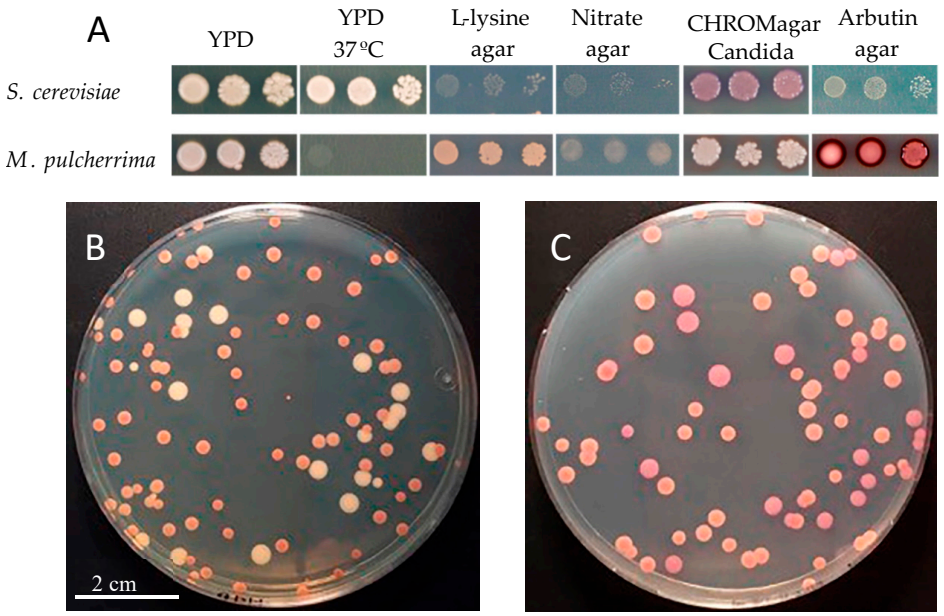

Figure 2. (**A**) Development and colony appearance in several growth media and different culture conditions (temperature). (**B**) *Metschnikowia pulcherrima* (*Mp*) orange colonies, some of them surrounded with white halos and *Saccharomyces cerevisiae* (*Sc*) white/creamy colonies in YPD media. (**C**) *Mp* and *Sc* in CHROMagar® media. Sc: bigger colonies with light pink color, *Mp*: smaller orange colonies, some of them with white halos.

The fermentative power of *Mp* is low, with many strains easily reaching 4% *v/v* in ethanol [3], although previous studies have observed the production of ethanol up to 6–7% *v/v* [9]. This feature, together with the fact that the presence of *Mp* in freshly pressed must is about 19–39% of the yeast ecology [9], makes it necessary to use *Mp* together with other yeast with a high fermentative power such as *Sc* or *Schizosaccharomyces pombe* to fully ferment grape sugars [10]. Its volatile acidity is also quite moderate, ranging from 0.3 to 0.4 g/L expressed as acetic acid [3]. Moreover, some strains are able to decrease the formation of H_2S during fermentation [11].

The fermentative performance of *Mp* is lower than that observed for other non-*Saccharomyces* species. The CO_2 production during fermentation yielded lower amounts for *Mp* than for *Sc* with 4.5 g per 100 mL vs. 12.9 g per 100 mL, respectively [12]. *Mp* has an intermediate acetoin production during alcoholic fermentation with respect to other species, such as *S. cerevisiae* and *B. bruxellensis* with low acetoin production and *C. stellata* and *K. apiculata* with the highest production of acetoin. The metabolic pathway for the production of this secondary metabolite from fermentation is shown in Figure 3. In addition, the amount of 2,3-butanediol produced by *Mp* is usually lower than that produced by *Sc*.

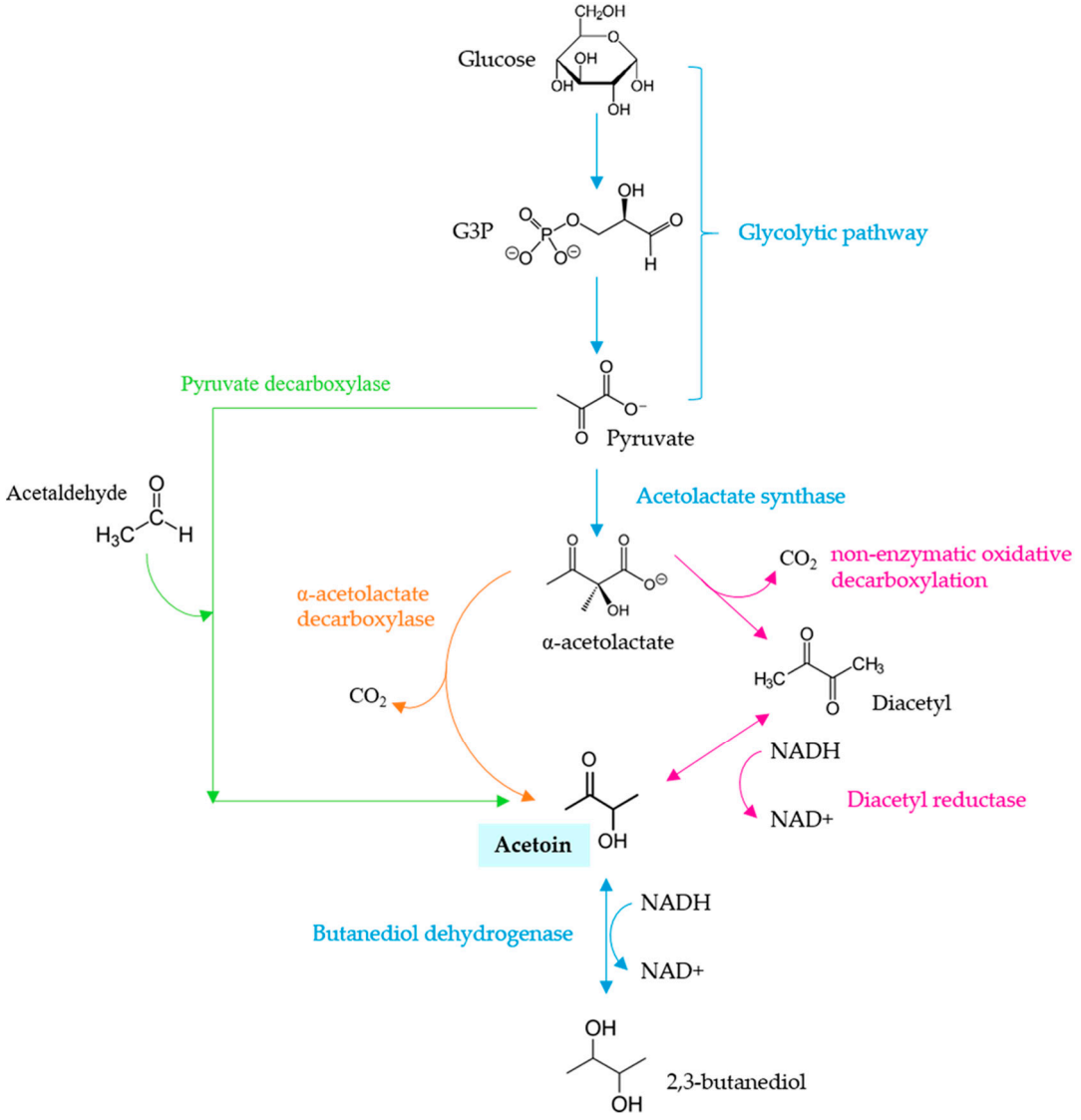

Figure 3. Metabolic route for the biosynthesis of acetoin by yeasts (adapted from Romano and Suzzi, [13]).

In mixed cultures with *S. cerevisiae*, viability was found to decrease rapidly after a few days of fermentation because of the low resistance to the ethanol produced by *S. cerevisiae* [14,15]. The use of emerging physical technologies that are able to strongly reduce the wild yeast content in grapes [16] can facilitate the prevalence of *Mp* during a longer period until the sequential inoculation of Sc, thus also increasing its effect on the sensory profile of the wines.

The sensibility of *Mp* to SO_2 is lower than that observed in Sc, *Saccharomycodes ludwigii* or *S. pombe*, but *Mp* shows a medium resistance compared with other non-*Saccharomyces* species [7]. A certain sensibility to some antimicrobials such as carvacrol and thymol has also been observed [17]. Regarding the use of dimethyl dicarbonate (DMDC), the growth of *Mp* strains during the fermentation of grape must is delayed, but not inhibited, after the addition of 400 mg/L DMDC [18]. The total inhibition of the microbial population can be achieved with 500 mg/L of DMDC. *Sc* can survive the addition of 200 mg/L DMDC, whereas the growth of other species of the genus *Saccharomyces* is inhibited with 150 mg/L DMDC.

2. Antimicrobial Bio-Tool

Mp can be used as a biological control agent thanks to its ability to produce natural antimicrobial compounds, namely pulcherrimin, an insoluble red pigment with antifungal activity. This peculiar antimicrobial activity is produced by the depletion of iron in the medium through the precipitation of iron(III) ions caused by the interaction with pulcherriminic acid, a precursor of pulcherrimin secreted by *Mp*. In this way, the environment becomes inhospitable to other microorganisms that require iron for their development. Pulcherrimin has shown effective inhibitory activity against several yeasts: *Candida tropicalis* and *Candida albicans*, as well as the *Brettanomyces/Dekkera*, *Hanseniaspora* and *Pichia* genera; and fungi: *Botrytis cinerea*, as well as *Penicillium*, *Alternaria* and *Monilia* spp. [19–24]. However, *S. cerevisiae* seems not to be affected by this antimicrobial activity [21,22]. Therefore, the use of *Mp* as a selected starter in sequential or mixed biotechnologies with *Sc* could be of great interest in modern enology.

Mp, as well as other yeast species such as *Wickerhamomyces anomala* (formerly *Pichia anomala*) and *Torulaspora delbrueckii* (*Td*), has a broad killer spectrum against some spoilage yeasts [25,26], of which *C. glabrata* had the highest sensitivity against the toxins from this species [27]. *Mp* has also been described as biofungicide capable of effectively reducing the incidence of *Botrytis* development in postharvest fruits [28]. Its antagonistic mechanism is mainly based on its competition for nutrients [29].

3. Aroma Compounds

The single use of *Mp* has led to excessive production of ethyl acetate with negative sensory repercussions [30]. However, the mixed use of *Mp* with *Saccharomyces uvarum* reduces the production of ethyl acetate, simultaneously favoring the formation of 2-phenyl ethanol and 2-phenylethyl acetate [30]. The use of co-inoculations of this type (mixed fermentations with *Mp*/*Sc*) has produced high contents of acetate esters and β-damascenone with lower levels of C_6 alcohols in ice wines made from the Vidal blanc grape variety [31]. An improvement in the aromatic complexity of the wines can be obtained by the use of *Mp* as a co-starter with *Sc* [3,32], mainly due to its high production of esters derived from its intense extracellular enzymatic activity [10,33]. Similarly, sequential fermentations with *Mp* showed a higher production of higher alcohols, with particularly high concentrations of isobutanol and phenylethanol [4].

4. Enzymatic Activities

Activities of the following enzymes have been described in *Mp*: pectinase, protease, glucanase, lichenase, β-glucosidase, cellulase, xylanase, amylase, sulphite reductase, lipase and β-lyase [11,33–35]. This is because *Mp* one of the non-*Saccharomyces* yeast species able to express more extracellular hydrolytic enzymes. Its high proteolytic activity makes it a very interesting fermentation partner for *Sc*, since the amino acids released (including those from autolysis) can serve as a source of nutrients for

Sc [36]. In addition, its intense glucosidase activity [2], higher under aerobic conditions [37], promotes the release of varietal aromas from the grape by hydrolyzing bound monoterpenes. However, it is important to always remember that the intensity of the enzymatic activity depends not only on the species, but also on the strain [32].

Concerning aroma enhancement, the expression of β-D-glucosidase favors the release of free terpenes and this activity has been evaluated using the substrates 4-methylumbelliferyl-β-D-glucoside (MUG) and *p*-nitrophenyl-β-D-glucoside (*p*NPG), showing a good intensity with medium-to-low degradation of color by the effect on anthocyanin glucosides [38]. The commercial *Mp* L1781 (Flavia™ MP346, Lallemand) expresses α-arabinofuranosidase; this activity helps to release precursors of volatile terpenes [39,40] (Figure 4) and thiols [32,41], which help to enhance fruity smells in some varieties. This strain has shown an enzymatic specific activity of 0.22 U/mg when used as a dry yeast or fresh culture [41]. This has been measured by the hydrolysis of 11 µmol de *p*-nitrophenyl-α-L-arabinofuranosidase (*p*NPA) per minute [42].

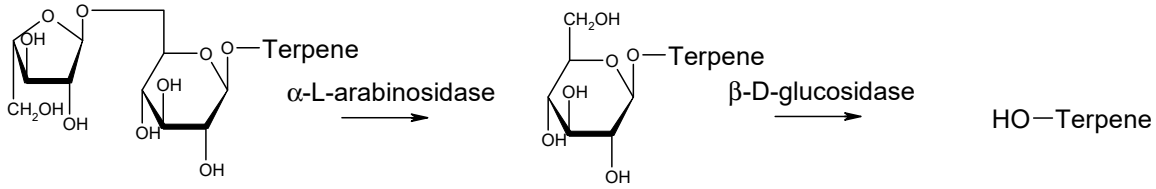

Figure 4. Effect of sequential α-arabinofuranosidase and β-D-glucosidase activities on the transformation of bonded terpenes into free forms, enhancing the aromatic profile.

Intracellular β-glucosidase of *Mp* has been purified by ion-exchange chromatography on amino agarose gel [6] and subsequently characterized. The optimum catalytic activity was observed at 50 °C and pH 4.5. The enzyme shows hydrolytic activity on β-(1→4) and β-(1→2) glycosidic bonds. The stability in alcoholic media (12% *v*/*v*) is good but it is affected by low pH.

5. Aerobic Metabolism/Alcohol Degree Reduction

The sequential use of *Mp* and *Sc* has proved to be somewhat effective in lowering the ethanol content of wine [11,43–46]. This is connected with the aerobic respiratory metabolisms of *Mp* that, in suitable aeration conditions, can aerobically metabolize more than 40% of sugars, thus significantly reducing the ethanol yield. An example of this application can be seen in the study developed by Contreras et al. (2014), where an average reduction in the alcoholic strength of 1.6% *v*/*v* was achieved when *Mp* was used in sequential fermentation with *Sc* (inoculated on the fourth day) in the production of red wine of the Syrah variety from a must with 240 g/L of sugars (potential alcoholic strength of 14% *v*/*v*). Therefore, the use of certain non-*Saccharomyces* yeast species, such as *Mp*, has been suggested as a biotechnological strategy aimed at producing wines with lower levels of ethanol [47]. In this last study, a kind of "collaboration" was seen between populations of *Mp* and *S. uvarum*, that is, a synergistic effect, achieving a lower ethanol production than in pure fermentations with each yeast. Recently, Mestre Furlani et al. (2017) evaluated the metabolic behavior of different non-*Saccharomyces* native yeasts to reduce the ethanol content during winemaking. They report that two out of the three strains of *Mp* isolated from grapes have a sugar to ethanol conversion ratio greater than >19 g/L/% *v*/*v* [48]. This confirms the usefulness of *Mp* to obtain wines with lower ethanol content.

6. Improvement of Wine Color Stability

Some non-*Saccharomyces* adsorb lower contents of anthocyanins during fermentation than *Sc* [49]. In *Sc*, the adsorption can range between 1 and 6% in total content of anthocyanins [50], but can reach up to 30% for some specific anthocyanins [51]. Adsorption is influenced by the composition and structure of the yeast cell wall. *Mp* shows a low adsorption of anthocyanins in cell walls when compared with

other yeasts such as *Sc*, *Td* or *Lachancea thermotolerans* (*Lt*) in grape skin agar (Figure 5), according to the methodology described by Caridi et al. [52].

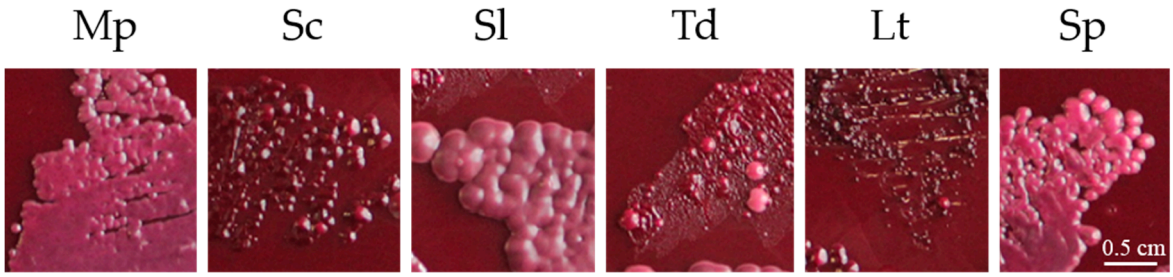

Figure 5. Adsorption of grape anthocyanins in yeast cell walls (*Saccharomyces* and non-*Saccharomyces*) during growth in a specific plating medium containing pigments. *Metschnikowia pulcherrima* (*Mp*), *Saccharomyces cerevisiae* (*Sc*), *Saccharomycodes ludwigii* (*Sl*), *Torulaspora delbrueckii* (*Td*), *Lachancea thermotolerans* (*Lt*), *Schizosaccharomyces pombe* (*Sp*).

The effect of *Mp* in the formation of stable pigments (pyranoanthocyanins and polymers) during fermentation has been studied in sequential fermentations with *Sc* and *S. pombe* [10].

7. Conclusions

The versatility of *Metschnikowia pulcherrima* lies in its ability to ferment in combination with other yeast species as well as modulate the synthesis of secondary metabolites of fermentation to improve the sensory profile of the wine. It is characterized by a medium fermentation power and a high enzymatic capacity to release aromatic precursors from the grape. In addition, this yeast has potential as a biocontrol agent in order to limit competition with other yeasts in the fermentation medium.

The abovementioned applications and features of *Metschnikowia pulcherrima* may be of great interest in order to address one of the major concerns in today's winemaking industry, such as excessive alcoholic strengths and the increasing prevalence in the market of flat wines from a sensory point of view. *Mp* could help solve these issues. The only important thing is to select the proper combination, as well as the right time and ratio of inoculation, between *Mp* and another yeast species capable of completing the alcoholic fermentation.

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ORIGINAL RESEARCH ARTICLE

Agaricus bisporus chitosan influences the concentrations of caftaric acid and furan-derived compounds in Pinot noir juice and base wine

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ABSTRACT

Chitosan is a fining agent used in winemaking, although its use in juice and wine beyond fining has been limited until now. Therefore, this study's first aim was to determine if chitosan derived from *Agaricus bisporus* (button mushrooms) could reduce caffeic and caftaric acid concentrations in Pinot noir grape juice (Study A). The second aim was to determine if chitosan, when added to base wine, could influence the synthesis of furan-derived compounds during storage (Study B). In Study A, Pinot noir grape juice was stored at 10 °C for 18 hours after the following treatments: control (no addition), bentonite/activated charcoal (BAC), low molecular weight (< 3 kDa; LMW) chitosan, med. MW (250 kDa; MMW) chitosan, and high MW (422 kDa; HMW) chitosan (all 1 g/L additions). Caftaric acid was decreased, and total amino acid concentration was increased in the LMW chitosan-treated juice, while the estimated total hydroxycinnamic acid content, turbidity, and browning were decreased in the MMW chitosan-treated juice compared to the control. In Study B, Pinot noir base wine destined for sparkling wine was stored at 15 and 30 °C for 90 days with the following treatments: control (no addition), LMW chitosan, MMW chitosan, and HMW chitosan (all 1 g/L additions). The three chitosan treatments stored at 30 °C had increased furfural, homofuraneol, and 5-methylfurfural formation in the base wine compared to the control. At 15 °C, furfural and homofuraneol had greater concentrations in all chitosan-treated wines after 90 days of storage. Our results demonstrate the potential of mushroom-derived chitosan to remove caftaric acid from grape juice and suggest that chitosan can influence the synthesis of furan-derived compounds in wine after short-term storage.

KEYWORDS: Base wine, caftaric acid, chitosan, grape juice, furan-derived aroma compounds

INTRODUCTION

Wine grapes destined for the traditional method of sparkling wine production are typically hand-harvested and whole bunch pressed to minimise the extraction of phenolic compounds into the juice (Charnock *et al.*, 2022). The excessive accumulation of these compounds may be unavoidable in some years to attain appropriate ripeness levels for sparkling wine production (Chamkha *et al.*, 2003). In these years, the removal of phenolic compounds from grape juice is possible through fining agents such as polyvinyl-polypyrrolidone (PVPP) or activated carbon (Spagna *et al.*, 2000). More recently, crustacean- and fungi-derived chitosan have been proposed as sustainable alternatives to these fining agents (Vendramin *et al.*, 2021).

Chitosan is a naturally synthesised biopolymer derived from crustacean exoskeletons and the cell walls/root structures (mycelium) of fungi (such as *Aspergillus niger* and *Agaricus bisporus*) (Silva *et al.*, 2020). Formed via the deacetylation of chitin, it is one of the most abundant biopolymers on Earth (Vendramin *et al.*, 2021). Chitosan molecules are chemically defined by their molecular weight (MW) and degree of deacetylation (DD), which refers to the proportion of the monomer units that are *N*-deacetylated (Abd El-Hack *et al.*, 2020). In 2009, the International Organization of Vine and Wine (OIV) authorised the use of fungal chitosan (derived from *A. niger*) in the winemaking process to improve flocculation of suspended solids and proteins, thereby decreasing protein haze and turbidity (Castro Marín *et al.*, 2020a). In addition to the treatment of protein haze, Spagna *et al.* (1996) revealed that chitosan could remove phenolic compounds (flavanols, proanthocyanidins, and hydroxycinnamic acids (HCAs)) from white wines. Nevertheless, the mechanism/s involved, specifically which compounds interact with chitosan, have not, to our knowledge, been elucidated. Therefore, it is necessary to determine the effect of chitosan on specific phenolic compounds in grape juice and wine.

In sparkling wine, caffeic acid and its tartaric acid ester, caftaric acid, are the HCAs typically found at the highest concentrations (Bosch-Fusté *et al.*, 2009; Chamkha *et al.*, 2003; Pozo-Bayo *et al.*, 2003; Serra-Cayuela *et al.*, 2013). The removal of these phenolic compounds from wine (Spagna *et al.*, 2000) and model wine (Chinnici *et al.*, 2014) by crustacean-derived chitosan has previously been investigated, but not its ability to reduce specific phenolic compounds in grape juice. At the juice stage, polyphenol oxidase (PPO) is yet to be inactivated by ethanol, thereby exposing the juice to the risk of developing browning via enzymatic oxidation (Li *et al.*, 2008).

The impact of chitosan on wine aroma and flavour, positive and/or negative, is important to establish with respect to its overall contribution to wine quality (Castro Marín *et al.*, 2020a). For instance, Castro Marín *et al.* (2020b); Castro Marín *et al.*, 2021) reported that the addition of chitosan (*A. niger*) to sparkling wine prior to the second alcoholic fermentation elicited a higher concentration of furfuryl alcohol, decanoic acid, and acetovanillone (aroma-active compounds) compared to the control wine. Nunes *et al.* (2016) reported that chitosan films present in Encruzado red wine enhanced the aroma contributions of furfural and benzaldehyde, known products of the Maillard Reaction (MR). The MR is a non-enzymatic set of chemical reactions categorised by the initial reaction of a reducing sugar with an amino acid, protein and/or peptide, generating many flavour compounds (Medeiros *et al.*, 2022). Some of these compounds, such as 2-furfurylthiol and furfural, have been found in aged wines, as well as those that have been heated (Medeiros *et al.*, 2022; Tominaga *et al.*, 2000). The ability of mushroom-derived chitosan to influence the formation of furan-derived aroma compounds in wine, such as furfural, is currently unknown.

The presence of Maillard reaction (MR)-associated products has been reported in a variety of wine styles (Charnock *et al.*, 2022; Le Menn *et al.*, 2017; Medeiros *et al.*, 2022; Pereira *et al.*, 2014; Sawyer *et al.*, 2022). Of particular interest are their contributions to aged sparkling wine aroma and flavour (Tominaga *et al.*, 2003). Studies that targeted aged sparkling wines elucidated many of the aroma compounds responsible for these empyreumatic compounds, such as 5-methylfurfural and 2-furanmethanethiol (Le Menn *et al.*, 2017; Tominaga *et al.*, 2000). Sawyer *et al.* (2022) recently studied the influence of base wine composition on the oxidative character of sparkling wine after 6, 12, and 24 months of storage on or off yeast lees. They reported that the length of time the wines aged, and not yeast lees contact, was the main factor that affected aroma synthesis, particularly for the furanone compound homofuraneol (Sawyer *et al.*, 2022).

The main challenge in assessing the contributions of age-related compounds in older wines is the length of time required for their synthesis (Escudero *et al.*, 2000; Le Menn *et al.*, 2017). Studies that have relied on analysing aged wines have often included only limited information on the winemaking and storage conditions that inform their conclusions. To address this and determine concentrations of aroma compounds that change during ageing, researchers have used heating techniques to accelerate wine ageing (Bosch-Fusté *et al.*, 2009; Elcoroaristizabal *et al.*, 2016; Pereira *et al.*, 2014). This accelerated ageing allows for rapid analysis of compounds contributing to the aroma of aged wine, as well as browning and HCAs (Pickering *et al.*, 1999).

The aims of this study were two-fold: to determine (Study A) the ability of mushroom-derived chitosan to reduce the concentration of caffeic and caftaric acid in Pinot noir grape juice and (Study B) to establish if mushroom-derived chitosan added to sparkling base wine influences the synthesis of furan-derived compounds during storage.

MATERIALS AND METHODS

1. Chemicals and Standards

Potassium metabisulphite (KMS) and potassium bitartrate (cream of tartar) were purchased from Vines to Vintages (Jordan, Ontario, Canada). IOC-2007 yeast, GoFerm®

yeast metabolite, Most-Rein bentonite/ activated-charcoal (product ID 31-15040, 20 kg), and Scottzyme® KS enzyme were purchased from Scott Laboratories Ltd (Niagara-on-the-Lake, Ontario, Canada). < 3 kDa (LMW), > 98 % DD chitosan mushroom oligosaccharide (CAS 9012-76-4, ≥ 99 %), 250 kDa (MMW), 98 % DD mushroom chitosan (CAS 9012-76-4, ≥ 99 %), and 422 kDa (HMW), > 98 % DD mushroom chitosan (CAS 9012-76-4, ≥ 99 %) were purchased from ChitoLytic Inc., (St. John's, Newfoundland, Canada). Milli-Q water was obtained from Millipore (Saint-Quentin-en-Yvelines, France).

2. Study A: Influence of chitosan on caffeic and caftaric acid concentrations in Pinot noir grape juice

2.1. Experimental Design

250 kg of Pinot noir grapes (clone 667) were hand-harvested from a vineyard in Niagara-on-the-Lake, Ontario. Healthy whole bunches were stored overnight at 10 °C in a temperature-controlled room without sulfur dioxide (SO_2) additions. Berries were whole bunch pressed (20 L hydraulic bladder press) 22 hours later to 1.5 bar (1.48 ATM) pressure into a 200 L stainless steel (Criveller Company, Niagara Falls, Canada) to ensure homogeneity of the juice. The juice was then divided into 15 × 11.5 L clear glass carboys and gassed with carbon dioxide (CO_2). Juice samples were collected for laboratory analysis before treatment additions. SO_2 was not added to the juice to prevent potential interference with the chitosan (Castro Marin *et al.*, 2021). Treatments were added directly to carboys in triplicate according to the following treatments: No addition (control), 1 g/L bentonite/ activated-charcoal (BAC) slurry made to manufacturer recommendations, 1 g/L < 3 kDa (low MW – LMW) 98 % DD chitosan, 1 g/L 250 kDa (med. MW – MMW) 98 % DD chitosan, and 1 g/L 422 kDa (high MW – HMW) 98 % DD chitosan.

Juices were placed in a refrigerated room and cold-settled at 10 °C for 18 hours before racking. Juice samples were taken after treatment additions prior to inoculation for laboratory analyses. Each replicate was separately inoculated with IOC-2007 yeast (Scott Laboratories Ltd., Niagara-on-the-Lake, Ontario, Canada) after being chaptalised from 15 to 18 °Brix using granulated cane sugar (Redpath Sugar Ltd. Toronto, Ontario, Canada). Fermentation took six days, 50 mg/L of SO_2 was added to each replicate once they reached dryness (< 3 g/L RS), and wines were kept at 4 °C to settle for six days. After settling, the wines were racked off yeast lees. 4 mL/hL of Scottzyme® KS pectinase enzyme (Scott Laboratories Ltd., Niagara-on-the-Lake, Ontario, Canada) was added to each wine to clarify them due to excess pectin haze. Pectin was not treated prior to fermentation to prevent potential interference with chitosan. The wines settled for three days at 15 °C before cold stabilisation (–2 °C) with 4 g/L of potassium bitartrate (Vines to Vintages, Jordan, ON, Canada) and coarse filtration (6 µm, Buon Vino Super Jet, Buon Vino Manufacturing Inc., Cambridge, Ontario, Canada), and were then stored with CO_2- in the headspace until the use of the control wine in Study B six weeks later.

3. Study B: Influence of chitosan on furan-derived compound formation in base wine during storage

3.1. Experimental Design

The wine used in Study B was the Pinot noir base wine made in Study A (control) that did not undergo any juice treatments. The study B base wine treatments were made in triplicate in screwcap bottles (200 mL narrow mouth HDPE plastic screw-cap bottles (VWR® International, Radnor, Pennsylvania, USA)) by the addition of *A. bisporus*-derived chitosan (200 mg), which remained in the base wine during storage. Treatments were as follows: No addition (control), 1 g/L < 3 kDa (LMW) 98 % DD chitosan, 1 g/L 250 kDa (MMW) 98 % DD chitosan, and 1 g/L 422 kDa (HMW) 98 % DD chitosan. The concentration of chitosan (1 g/L) was chosen because it is the maximum recommended dose for chitosan in winemaking set by the OIV (International Organisation of Vine and Wine, 2015). The chemical analysis of the wine was carried out prior to chitosan additions (Table 1). Bottles were gassed with argon gas before cap closure to prevent oxidation and kept for 90 days at 30 °C (moderate accelerated ageing, per Medeiros *et al.,* (2022)) or 15 °C (approximate cellar temperature) in an insulated, dark temperature-controlled room. After the storage period, duplicate laboratory analysis of each replicate wine was conducted consisting of the following parameters: total HCA estimation (A.U. at λ_{320nm}), brown pigmentation (A.U. at λ_{420nm}), caffeic acid concentration (mg/L), and nine furan-derived compounds (µg/L).

4. Standard Juice and Wine Chemical Analyses

Titratable acidity (TA g/L tartaric acid eq.) and pH were determined by auto-titrator (Hanna Instruments® HI 84502 Woonsocket, Rhode Island, USA). Free and total SO_2 was determined by the aspiration method (Iland *et al.,* 2013) using a Sartorius Biotrate digital burette (Model LH-723082) and Isotemp condenser (Thermo-Fisher Scientific, Waltham, Massachusetts, USA). Ethanol (% v/v) was analysed according to Nurgel *et al.* (2004) by gas chromatography (Agilent 6890 model) coupled with a flame ionisation detector (GC-FID). Acetic acid (g/L), L-malic acid (g/L), yeast assimilable nitrogen (YAN mg N/L), and residual sugars (mg/L D-glucose & D-fructose) were determined by enzymatic kits: K-ACET 02/17; L-LMALL 06/07; K-PANOPA 08/14; K-AMIAR 12/12; and K-FRUGL 05/17, respectively (Megazyme International Ltd, Wicklow, Ireland). °Brix and temperature (°C) were determined by a glass hydrometer and mercury thermometer, respectively. Turbidity (NTU) was measured with a turbidity meter (Hanna Instruments® HI 98703 Woonsocket, Rhode Island, USA), while dissolved oxygen (DO mg/L) was determined by a DO meter (Hanna Instruments® HI 9146 Woonsocket, Rhode Island, USA). Total HCA estimation and degree of browning were analysed according to Iland *et al.* (2013) using a Cary 60 UV-Vis spectrophotometer (Agilent Technologies Canada Inc., Mississauga, ON).

TABLE 1. Chemical analysis of the base wine used in Study B.

Chemical Parameters	Pinot noir Base Wine
pH	2.75 ± 0.01
Titratable acidity (TA g/L)	9.1 ± 0.0
Fructose (g/L)	3.5 ± 0.1
Glucose (g/L)	0.3 ± 0.1
Malic acid (g/L)	3.3 ± 0.0
Acetic acid (g/L)	0.42 ± 0.00
YAN (mg N/L)	7.2 ± 0.2
Alcohol (% v/v)	9.7 ± 0.0
Free SO_2 (ppm)	2 ± 1
Total SO_2 (ppm)	60 ± 3
Total HCA Estimate (A.U)	0.13 ± 0.00
Brown Pigmentation (A.U)	0.08 ± 0.00

± indicates the standard deviation between sample means (n = 2).

TABLE 2. Retention times (min), quantifying ions (m/z), qualifying ions (m/z), regression coefficient (R^2), calibration range (µg/L), LODs, and LOQs of six furan-derived compounds analysed in Study B.

Compound	Retention Time (min)	Quantifying Ion (m/z)	Qualifying Ion(s) (m/z)	Regression Coefficient (R^2)	Calibration Range (µg/L)	LOD (µg/L)	LOQ (µg/L)
Furfural-d_4	24.0	100	70, 99	-	-	-	-
Furfural	24.0	96	67, 95	0.9999	2.5–300	0.76	2.5
Ethyl-2-furoate	41.2	95	112, 140	0.9978	1.76–75	0.53	1.76
Homofuraneol	24.0	97	101	0.9995	1.59–300	0.48	1.59
5-Methyl furfural	35.2	110	53	0.9988	4.9–300	1.47	4.9
Furfuryl ethyl ether	27.2	81	98, 126	0.9999	1.09–300	0.33	1.09
2-Acetylfuran	30.3	110	95	0.9995	1.03–300	0.31	1.03

5. Liquid Chromatography-UV Diode Array Detection (LC-UV/DAD) and Headspace Solid Phase Micro-Extraction-Gas-Chromatography/Mass Spectrometry (HS-SPME-GC/MS) Analyses

LC-UV/DAD and HS-SPME-GC/MS methods for quantifying caffeic/caftaric acid and furan-derived compounds, respectively, are detailed in Medeiros *et al.* (2022), including the chemicals and reagents used for both methods. Limits of detection (LOD) were calculated by adding the Limit of Blank (LoB) to the standard deviation of the 1 µg/L standard multiplied by 1.645 ($LOD = LoB + 1.645_{SD\ 1\ \mu g/L\ sample}$) (Table 2). Limits of quantification (LOQ) were calculated as the LOD multiplied by 3.3 (Armbruster and Pry, 2008).

6. Statistical Analyses

The statistical software used was XLSTAT (2021.1.1, Addinsoft, Paris, France), and all data were analysed using the Shapiro–Wilk test for normality to determine which model would be applied to analyse the variance between sample means. Standard chemical parameters were analysed pre- and post-treatment by two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test to separate sample means for normally distributed data at $\alpha = 0.05$. For non-normally distributed data, the Kruskal–Wallis (KW) test followed by the Conover–Iman procedure was used at $\alpha = 0.05$. Post-treatment data, as well as total HCA estimation, degree of browning, LC-UV/DAD, and HS-SPME-GC/MS data, were analysed by one-way ANOVA with Tukey's $HSD_{0.05}$ test for normally distributed data, while the KW test followed by the Conover–Iman procedure was used for non-normally distributed data, at $\alpha = 0.05$.

RESULTS

1. Study A: Influence of chitosan on caffeic and caftaric acid concentrations in Pinot noir grape juice

Standard chemical analysis of the Pinot noir grape juice pre- and post-treatment addition can be found in Table 3. Post-fermentation standard wine chemical analyses, total

TABLE 3. Chemical composition of Pinot noir juice analysed pre- and post-treatment addition.

Parameter	Control		BAC		LMW Chitosan		MMW Chitosan		HMW Chitosan		Significance (Treatment* Timepoint)
Time of Analysis	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	-
Brix (°)	15.1 ± 0.0	15.1 ± 0.1	15.1 ± 0.2	15.1 ± 0.1	15.1 ± 0.0	15.0 ± 0.1	15.1 ± 0.1	15.0 ± 0.0	15.2 ± 0.1	15.1 ± 0.1	NS
pH	3.14 ± 0.02a	3.07 ± 0.00cd	3.11 ± 0.02b	3.05 ± 0.02d	3.09 ± 0.00bc	3.09 ± 0.01bc	3.09 ± 0.01bc	3.07 ± 0.00cd	3.10 ± 0.01bc	3.07 ± 0.01cd	***
Titratable Acidity (TA g/L)	9.7 ± 0.7a	9.2 ± 0.3ab	9.6 ± 0.1a	8.6 ± 0.2b	9.2 ± 0.2ab	9.2 ± 0.4ab	9.2 ± 0.2ab	8.5 ± 0.2b	9.3 ± 0.3ab	8.9 ± 0.3ab	*
Malic Acid (g/L)	4.7 ± 0.1a	4.7 ± 0.3ab	4.6 ± 0.1ab	4.5 ± 0.0ab	4.6 ± 0.0ab	4.5 ± 0.1ab	4.6 ± 0.1ab	4.4 ± 0.1b	4.6 ± 0.1ab	4.5 ± 0.2ab	*
Acetic Acid (g/L)	0.01 ± 0.00cd	0.02 ± 0.01bc	0.02 ± 0.01bc	0.02 ± 0.00bcd	0.02 ± 0.00bc	0.05 ± 0.01a	0.02 ± 0.00b	0.01 ± 0.00d	0.02 ± 0.01bcd	0.02 ± 0.01bcd	***
Ammonia (mg N/L)	83.5 ± 10.0	76.8 ± 1.7	77.9 ± 4.6	77.6 ± 3.0	80.9 ± 2.0	80.1 ± 0.3	77.9 ± 2.5	78.6 ± 0.6	76.8 ± 3.7	78.4 ± 1.1	NS
Amino Acids (mg N/L)	83.0 ± 3.3b	79.7 ± 2.9b	78.9 ± 3.8b	58.3 ± 2.4c	81.7 ± 4.0b	115.5 ± 3.7a	76.8 ± 3.4b	79.2 ± 1.7b	79.2 ± 4.5b	78.1 ± 1.2b	***
Total YAN (mg N/L)	166.5 ± 11.9b	156.6 ± 1.8b	156.8 ± 2.6b	135.9 ± 2.9c	162.6 ± 4.8b	195.6 ± 4.0a	154.7 ± 2.7b	157.8 ± 1.5b	155.9 ± 5.5b	156.1 ± 1.2b	***

± represents the standard deviation of the means (n = 6), and multiple comparisons of treatment means were carried out using two-way ANOVA with interactions, followed by Tukey's HSD (honestly significant difference) test, using treatment and the time points of analysis as independent variables. Different letters represent means that were separated based on the model applied. Significance: NS = $p > 0.05$, * = $p < 0.05$, *** = $p < 0.001$.

TABLE 4. Chemical composition of the Pinot noir base wine in Study A.

Parameter	Control	BAC	LMW Chitosan	MMW Chitosan	HMW Chitosan	Significance
pH	2.96 ± 0.00ab	2.93 ± 0.02b	2.96 ± 0.03a	2.94 ± 0.01ab	2.94 ± 0.01ab	*
Titratable Acidity (TA g/L)	8.4 ± 0.1b	8.4 ± 0.1b	8.6 ± 0.1ab	8.4 ± 0.3b	8.8 ± 0.3a	*
Glucose (g/L)	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
Fructose (g/L)	3.2 ± 0.6a	1.3 ± 0.4c	1.5 ± 0.4c	2.0 ± 0.2bc	2.4 ± 0.3b	***
Total Residual Sugar (g/L)	3.3 ± 0.6a	1.4 ± 0.4c	1.6 ± 0.3c	2.1 ± 0.2bc	2.5 ± 0.3b	***
Malic Acid (g/L)	4.0 ± 0.1	3.8 ± 0.0	3.8 ± 0.0	3.9 ± 0.0	3.9 ± 0.0	NS
Acetic Acid (g/L)	0.33 ± 0.00b	0.29 ± 0.01c	0.37 ± 0.01a	0.32 ± 0.01bc	0.31 ± 0.02bc	**
Ammonia (mg N/L)	ND	ND	ND	ND	ND	NS
Amino Acids (mg N/L)	7.0 ± 0.5b	7.8 ± 0.5b	41.5 ± 3.3a	9.1 ± 0.7b	8.0 ± 1.5b	***
Total YAN (mg N/L)	7.0 ± 0.5b	7.8 ± 0.5b	41.5 ± 3.3a	9.1 ± 0.7b	8.0 ± 1.5b	***
Alcohol (% v/v)	9.8 ± 0.0	10.0 ± 0.0	9.9 ± 0.2	9.9 ± 0.1	9.8 ± 0.1	NS
Free SO_2 (mg/L)	9 ± 1	9 ± 0	9 ± 0	9 ± 0	10 ± 1	NS
Total SO_2 (mg/L)	62 ± 1	66 ± 2	63 ± 2	63 ± 1	63 ± 1	NS
Dissolved Oxygen (mg/L)	4.9 ± 0.5a	4.8 ± 0.2ab	4.5 ± 0.1bc	4.3 ± 0.1c	4.4 ± 0.0c	*
Turbidity (NTU)	11.9 ± 1.3a	11.9 ± 0.3a	12.7 ± 0.6a	9.2 ± 0.3b	12.0 ± 0.9a	***
Total HCA Estimate (A.U)	0.4 ± 0.1a	0.4 ± 0.0a	–	0.3 ± 0.0b	0.4 ± 0.0a	**
Brown Pigmentation (A.U)	0.21 ± 0.03a	0.23 ± 0.02a	–	0.17 ± 0.01b	0.20 ± 0.00a	**

± represents the standard deviation of the means (n = 6), and multiple comparisons of treatment means were carried out via one-way ANOVA followed by Tukey's HSD test at $p < 0.05$. Different letters represent means that were separated based on the model applied. Significance: NS = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. – signifies no data collected for this treatment due to colour interference with the spectroscopic method.

HCA estimation, brown pigmentation, and caffeic and caftaric acid concentrations data are presented in Table 4.

1.1. Juice Chemical Composition

Juice pH in the control and BAC samples decreased after treatment ($p < 0.001$) but not in any chitosan treatments ($p > 0.05$). TA decreased by 1.0 g/L after the BAC treatment was applied to the juice ($p < 0.05$), but no difference in the chitosan-treated juices nor the control was observed. The acetic acid concentration was decreased in the MMW chitosan treatment by 0.01 g/L compared to the pre-treatment juice ($p < 0.05$) but was increased in the LMW chitosan treatment by 0.03 g/L ($p < 0.001$), and the higher acetic acid concentration persisted post-fermentation compared to the other treatments ($p < 0.001$).

In the LMW chitosan-treated juice, YAN increased by 35 mg N/L post-treatment ($p < 0.001$). This increase was due to a higher concentration of amino acids in the juice and was higher than the other treatments ($p < 0.001$). However, the BAC treatment had decreased amino acid levels by 20 mg N/L ($p < 0.01$) compared to the control.

1.2. Base Wine Chemical Composition

Wine pH and TA differences were observed between treatments, though the differences were small (Table 4). The control retained the highest concentration of fructose relative to the other treatments, followed by the HMW and

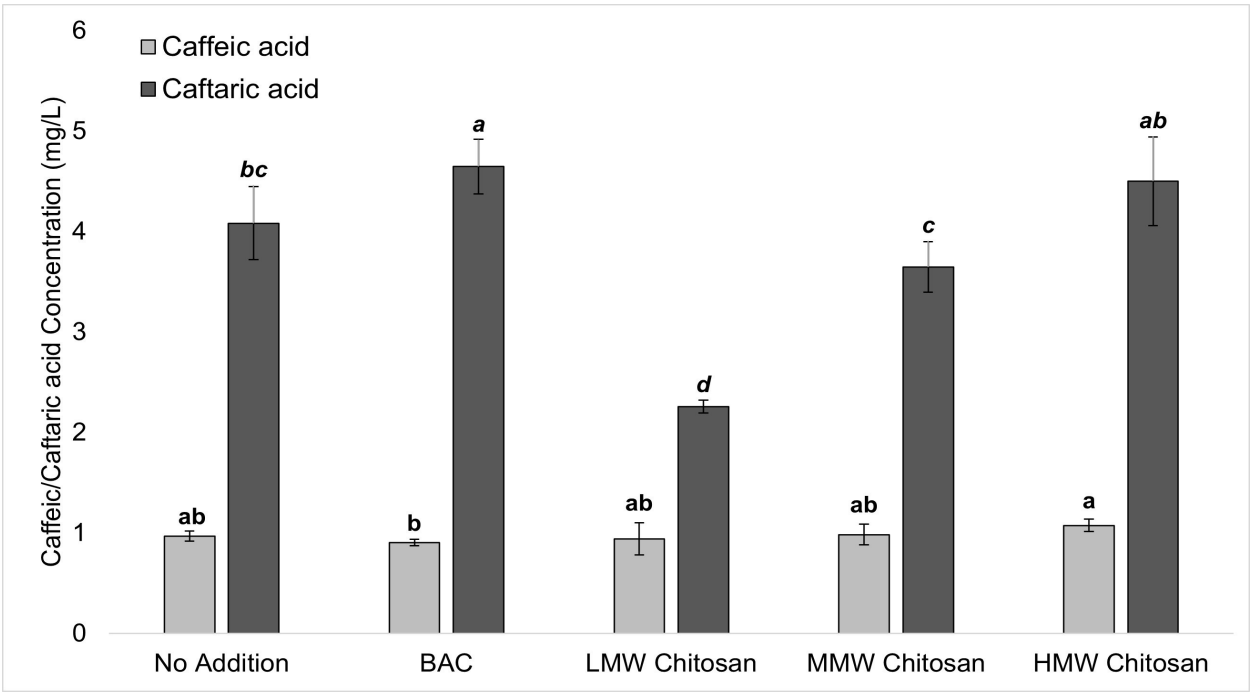

FIGURE 1. Caffeic and caftaric acid concentrations (mg/L) in base wine after juice treatments and fermentation.

Error bars represent the standard deviation of sample means (n = 6). Multiple comparison of treatment means was carried out via the KW test followed by the Conover–Iman procedure for caffeic acid data (bold), and a one-way ANOVA was performed followed by Tukey's HSD test for caftaric acid data (bold/italics). Different letters represent means that were separated based on the model applied at $p < 0.05$.

MMW chitosan wines, while the LMW chitosan and BAC wines had the lowest fructose. The LMW chitosan wine contained a higher concentration of acetic acid ($p < 0.01$) and amino acids ($p < 0.001$) compared to the other treatments. Turbidity and DO measurements differed between treatments– the control had higher DO compared to the three chitosan treatments. The MMW chitosan wine had the lowest turbidity compared to the other treatments.

1.3. Caffeic and Caftaric Acids in Base Wine

The LMW chitosan wine contained 2.3 mg/L caftaric acid, lower than the other treatments ($p < 0.0001$) (Figure 1). Caftaric acid concentration in the MW chitosan-treated wine was 3.7 mg/L, which did not differ from the control but was lower than for the BAC wine ($p < 0.0001$).

2. Study B: Influence of chitosan on furan-derived compound formation in base wine during storage

2.1. Total HCA Estimation, Degree of Browning, and Caffeic Acid Following 90 Days of Storage

MMW and HMW chitosan-treated wines had a lower estimated HCA content compared to the control (Table 5). Under the 15 °C storage condition of these two chitosan treatments, the HCA content was estimated at zero, while some HCAs were estimated to be present in the MMW (0.08 A.U) and HMW (0.09 A.U) chitosan-treated wines at 30 °C. The LMW chitosan imparted a visible orange hue to the wine, which prevented spectroscopic analysis. For brown colouration, the MMW and HMW chitosan wines were again found to have reduced absorbance values compared to the control. Under the 15 °C storage condition, only the MMW chitosan wine was lower than the control. Overall, a higher degree of browning was seen in the wines stored at 30 °C compared to 15 °C, though no visible browning was discernable to the naked eye. The caffeic acid variability due to treatments was minimal under both 30 °C and 15 °C storage, and only the HMW chitosan treatment was higher than the control ($p < 0.05$).

2.2. Furan-Derived Compounds

Six of the nine compounds analysed were determined at concentrations greater than their LOQ in wines stored at 30 °C (furfural, homofuraneol, ethyl-2-furoate, 5-methylfurfural, furfuryl ethyl ether, and 2-acetylfuran) but at 15 °C, only furfural, homofuraneol, and ethyl-2-furoate were quantified (Table 6).

Furfural, homofuraneol, and ethyl-2-furoate were found at higher concentrations in wines stored at 30 °C than at 15 °C. Furfural and homofuraneol concentrations increased in the chitosan-treated wine, with the greatest increase observed in the HMW chitosan wines for both compounds. Furfural concentrations in the MMW chitosan wine at 15 °C were indistinguishable from the LMW chitosan but 2 µg/L lower than the HMW chitosan ($p < 0.01$). However, at 30 °C, the MMW chitosan wine's furfural concentration was 209 µg/L, higher than the LMW chitosan ($p < 0.0001$) but like HMW chitosan.

TABLE 5. Total HCA estimation, brown pigmentation, and caffeic acid concentrations of base wines stored at 15 and 30 °C for 90 days.

Treatment	Total HCA Estimation (A.U)		Brown Pigmentation (A.U)		Caffeic Acid Concentration (mg/L)	
	15 °C	30 °C	15 °C	30 °C	15 °C	30 °C
Control	0.13 ± 0.01a	0.28 ± 0.01a	0.08 ± 0.01a	0.15 ± 0.00a	1.6 ± 0.1b	1.6 ± 0.1b
LMW Chitosan	–	–	–	–	1.7 ± 0.1ab	1.6 ± 0.1ab
MMW Chitosan	0.00 ± 0.00b	0.08 ± 0.01b	0.06 ± 0.01b	0.11 ± 0.00b	1.7 ± 0.2ab	1.6 ± 0.1ab
HMW Chitosan	0.00 ± 0.00b	0.09 ± 0.01b	0.07 ± 0.01ab	0.12 ± 0.00b	1.7 ± 0.1a	1.7 ± 0.1a
Significance	***	***	*	***	*	*

± represents the standard deviation of the means (n = 6). Multiple comparison of treatment means was carried out via one-way ANOVA followed by Tukey's HSD test for normal data, and the KW test followed by the Conover–Iman procedure for non-normal data. Different letters represent means that were separated based on the model applied. Significance: * = $p < 0.05$, *** = $p < 0.001$. – signifies no data collected for this treatment due to colour interference with the spectroscopic method.

TABLE 6. Concentrations (µg/L) of furan-derived compounds in Pinot noir base wine after 90 days of storage at 15 °C and 30 °C.

Treatment	Furfural		Homofuraneol		Ethyl-2-furoate		5-Methyl furfural	Furfuryl ethyl ether	2-Acetyl furan
	15 °C	30 °C	15 °C	30 °C	15 °C	30 °C	30 °C	30 °C	30 °C
Control	6 ± 0c	91 ± 3c	4 ± 1c	89 ± 3c	2.9 ± 0a	10 ± 1a	9 ± 0c	1.6 ± 0b	2.8 ± 0c
LMW Chitosan	26 ± 0b	691 ± 9b	23 ± 1b	701 ± 15b	2.9 ± 0a	11 ± 0a	23 ± 1b	2.1 ± 0a	4.9 ± 0a
MMW Chitosan	26 ± 1b	899 ± 48a	23 ± 1ab	889 ± 48a	2.6 ± 0b	9 ± 0b	34 ± 2a	1.6 ± 0b	3.2 ± 0b
HMW Chitosan	28 ± 3a	955 ± 60a	24 ± 1a	930 ± 68a	2.5 ± 0b	9 ± 1b	37 ± 5a	1.6 ± 0b	3.0 ± 0b
Significance	**	***	*	**	*	**	***	**	*

± represents the standard deviation of the means (n = 6). Multiple comparison of treatment means was carried out via one-way ANOVA followed by Tukey›s HSD test for normally distributed data, while the Kruskal–Wallis test followed by the Conover–Iman procedure was performed for non-normally distributed data, at $p < 0.05$. Different letters represent means that were separated based on the model applied. Significance: NS = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. 5-Methylfurfural, furfuryl ethyl ether, and 2-acetlyfuran concentrations could not be quantified at 15 °C due to being below the LOQ of the method.

For homofuraneol, a similar pattern was noted between treatments: LMW and HMW chitosan wines were different at both 15 °C ($p < 0.05$) and 30 °C ($p < 0.0001$) temperatures. At 15 °C, the MMW chitosan wine contained a similar concentration of homofuraneol compared to both the LMW and HMW chitosan wines. At 30 °C, the MMW chitosan wine was 188 µg/L greater than the LMW chitosan treatment ($p < 0.001$) but similar to the HMW chitosan treatment.

The control and LMW chitosan wines contained higher concentrations of ethyl-2-furoate compared to the MMW and HMW chitosan treatments, regardless of the temperature condition. At 15 °C, the MMW chitosan wine had lower concentrations of ethyl-2-furoate compared to the control ($p < 0.01$) and LMW chitosan ($p < 0.01$). At 30 °C, the MMW chitosan wine had lower ethyl-2-furoate concentrations again compared to the control ($p < 0.001$) and LMW chitosan treatment ($p < 0.0001$). These results were similar for the HMW and the MMW chitosan wines.

5-Methylfurfural concentrations were impacted by the chitosan treatments, with the highest amounts found in the MMW and HMW chitosan wines. The LMW chitosan wine contained lower concentrations compared to the MMW chitosan ($p < 0.0001$) and the HMW chitosan ($p < 0.0001$) but was still higher than the control ($p < 0.0001$).

The concentrations of furfuryl ethyl ether in the control, MMW, and HMW chitosan wines did not differ, while the LMW wine contained a higher amount compared to the control, MMW and HMW chitosan ($p < 0.01$). 2-acetylfuran concentrations followed a similar trend; LMW chitosan wine

contained the highest concentration compared to the MMW, HMW chitosan, and control ($p < 0.0001$). The MMW and HMW chitosan wines were indistinguishable, though they were both higher in concentration than the control ($p < 0.05$).

DISCUSSION

1. Study A: Influence of chitosan on caffeic and caftaric acid concentrations on Pinot noir grape juice

1.1. Impact of Chitosan on Grape Juice

In contrast to the study by Castro Marín *et al.* (2020b), acetic acid concentrations in our study were higher in the LMW chitosan wines compared to the control but only by 0.1 g/L. However, the increased amino acid content in the LMW chitosan-treated wine is in agreement with Castro Marín *et al.* (2020b), who found elevated protein content (38 mg/L) compared to the control (26 mg/L) after 12 months ageing the wine on lees with 250 mg/L of *A. niger*-derived chitosan (80–90 % DD, 10–30 kDa). Those differences are smaller than in our study, which is notable given the shorter contact time of chitosan with juice (18 hours vs. 12 months). Castro Marín *et al.* (2020b) attributed the increase in total protein content to the greater speed of yeast autolysis caused by polar interactions of the chitosan with the yeast cell walls, favouring lysis and amino acid release. The chitosan treatments in our study were carried out on un-inoculated juice, so the sole source of proteins available for release via cell lysis was grape proteins, yeast present on skins/stems, and bacteria. These could have been responsible for the increased amino acid content observed in the LMW chitosan treatment. The assessment of chitosan as a clarification agent by Spagna *et al.* (1996) included measurements of wine turbidity and reported a correlation between the reduction in brown pigmentation formed and the turbidity of the wine. The MMW chitosan lowered the turbidity in the wine compared to the control, in agreement with Spagna *et al.* (1996).

Total estimated HCA levels in the wines decreased in the MMW chitosan treatment compared to the control, as previously reported by Spagna *et al.* (1996), who found a 40 % reduction in HCAs in Italian wines treated with 0.4–4.0 g/L of chitosan (60–78 % DD, 190–250 kDa). However, only one of the treatments in our study (HMW) reduced the total estimated HCA content. This is in contrast to caftaric acid concentration determined via LC-UV/DAD, in which only LMW chitosan-treated juice reduced caftaric acid. Adsorption of other HCAs or spectroscopically relevant compounds in the λ_{320nm} range could potentially account for this discrepancy.

1.2. Caffeic and Caftaric Acids in Base Wine

As reported in previous studies by Castro Marín *et al.* (2020b) and Spagna *et al.* (1996), our chitosan-treated wines had lower concentrations of caftaric acid compared to the control and BAC wines, especially the LMW chitosan, which had the lowest concentration of caftaric acid compared to the control. Chien *et al.* (2007) also determined that in apple juice, the lower MW of the chitosan (12 kDa), the greater its ability to scavenge hydrogen peroxide and exhibit adsorptive properties compared to higher MW (318 kDa) chitosan when DD was the same (98.5 %). These results indicate that lower MW chitosan, which at $pH < 6.5$ is water-soluble (Tian *et al.*, 2015), is likely to interact with phenolic constituents in wine better than higher MW chitosan. This may be due to decreased cation repulsion of adjacent chitosan polymer subunits, resulting in a better ionic attraction between the chitosan oligomers and negatively charged small particles (Chien *et al.*, 2007).

In contrast, Castro Marín *et al.* (2020b) determined that a low MW *A. niger*-derived chitosan (10-30 kDa, 80–90 % DD) did not influence the concentration of caftaric acid during a second alcoholic fermentation in a bottle, nor during 12-months ageing on lees. Caftaric acid concentrations in Pinot gris/Pignoletto sparkling wine in their study were within 1–2 mg/L of those determined in our study, though no variation in HCA concentration from chitosan addition was reported. Given the similar chemical parameters and *A. niger* source of the chitosan products used in both studies, this discrepancy could be explained by the rate of chitosan addition; 1 g/L, compared to 250 mg/L in Castro Marín *et al.* (2020b). Our finding agrees with Spagna *et al.* (2000), who tested 0.4 g/L and 0.8 g/L concentrations of shellfish-derived chitosan on Trebbiano and Albana wines. Under a short (< 2 hr) contact time, the authors found that the 0.4 g/L chitosan decreased total HCAs by 20 %, while the 0.8 g/L chitosan treatment decreased HCAs by 40 %, which is comparable to the 54 % decrease in caftaric acid observed from the LMW chitosan treatment in our study. This improved adsorption at higher concentrations requires further exploration to establish the appropriate chitosan dosage for wine for optimal HCA removal.

Concerning caffeic acid, our results align with those of Castro Marín *et al.* (2020b), who reported that 250 mg/L of *A. niger*-derived chitosan did not affect caffeic acid concentrations during 12-month ageing on lees. In fact, an increase in caffeic acid concentration was recorded for the HMW chitosan-treated wine compared to the BAC-treated wine and control. However, these differences in caffeic acid concentration were extremely small (< 0.1 mg/L), and, therefore, these results should be taken with caution.

2. Study B: Influence of chitosan on furan-derived compound formation in base wine during storage

2.1. Total HCA Estimation, Degree of Browning, and Caffeic Acid Following 90 Days of Storage

Compared to the control wine, both MMW and HMW chitosan-treated wines had lower HCA content after 90 days of storage under both temperature conditions, with the concentration in 15 °C chitosan wines below the 1.4 A.U limit for HCA estimation (Iland *et al.*, 2013). This result was expected based on results in Study A, where the MMW chitosan treatment reduced total HCAs compared to the

control wine. Additionally, an increase in HCAs was found in the wines stored at 30 °C compared to those kept at 15 °C, which agrees with the findings of Medeiros *et al.* (2022), where the control wine stored at the lower temperature had lower estimated HCA content compared to the wines kept at 30 °C.

Caffeic acid content from the control wine in Study A was used to make treatments in Study B, which had increased slightly over the time period, likely due to the hydrolysis of caftaric acid into caffeic acid (Vallverdú-Queralt *et al.*, 2015). These concentrations align with previous results by Ferreira-Lima *et al.* (2013), who reported caffeic acid concentrations in the range of 2.25–2.75 mg/L in Goethe white wines over the course of four months of ageing at 25 °C.

2.2. Furan-derived Compounds

2.2.1. Furfural

Furfural is a volatile furan-derived compound that contributes a sweet, bready, and fruity aroma in wine (Nunes *et al.*, 2016). It accumulates over time in aged sparkling wines (Jeandet *et al.*, 2015; Tominaga *et al.*, 2003) and wines subjected to heating (Pereira *et al.*, 2010). Furfural was found at higher concentrations in chitosan-treated wines compared to the control. In wines stored at 30 °C, the furfural concentration amongst treated wines was 849 µg/L, almost 10× higher than the concentration in the control wine. In comparison, Tominaga *et al.* (2003) found that the furfural concentration of bottle-aged Champagne in a 10-year-old Louis Roederer Champagne was just below 1000 µg/L**,** compared to the 955 µg/L achieved by the addition of 1 g/L HMW chitosan in just 90 days at 30 °C in our study. The influence of chitosan on furfural formation was less at 15 °C. Despite the lower temperature, furfural concentration was higher in all of the chitosan-treated wines.

The odour detection threshold (ODT) is the lowest concentration that an odour can be reliably detected (Czerny *et al.*, 2008)**.** The ODT of furfural is 14 mg/L (in 11 % (*v/v)* aqueous ethanol, with 7 g/L glycerol, and 5 g/L tartaric acid at pH 3.4) according to Ferreira *et al.* (2000). This is higher than the concentrations in our study; thus, it is unlikely that furfural would directly affect the sensory characteristics of the wines at this stage of production.

These results are in accordance with two separate experiments performed on model wine solutions containing arabinose (a pentose sugar), in which chitosan increased furfural concentrations (Nunes *et al.*, 2016). Rocha *et al.* (2021) also treated a model wine with chitosan, generating almost 3000 µg/L of furfural in just over 100 days at room temperature under magnetic stirring. These studies demonstrate additional factors of surface area–contact and agitation on chitosan reactivity, aspects that were not covered in our study.

The formation of furfural under wine-simulated conditions indicates that arabinose, and possibly other pentose sugars, contribute preferentially to furfural formation rather than fructose or glucose (Rocha *et al.*, 2021). Chitosan-based reactivity with pentose sugars is also supported by a measured decrease in galactose, mannose, and arabinose determined in Albariño white wines treated with 1 g/L of *A. niger*-derived chitosan (No Brett Inside from Lallemand) (Arenas *et al.*, 2021). Similar to chitosan adsorbing HCAs and other phenolic compounds via electrostatic interactions caused by $-NH_3^+$ residues on the polymer backbone, Arenas *et al.* (2021) postulated that this chemical behaviour could be responsible for the reduction in pentose sugars observed in their experiment. It is possible that via these electrostatic interactions, the dehydration of hexose and pentose sugars may be enhanced. This is supported by the established understanding that acid-hydrolysis of sugars is the most likely contributing pathway to furfural formation in wine due to the low pH environment (Charnock *et al.*, 2022; Pereira *et al.*, 2010). However, the mechanism behind furfural synthesis in wine treated with chitosan or stored with chitosan immersed in it has not been elucidated in our study.

2.1.2. Homofuraneol

Homofuraneol (2-ethyl-4-hydroxy-5-methyl-3(2H)-furanone), also referred to as ethyl-furoate, has been reported in several studies as a compound with a relatively low ODT (10 µg/L) (Kotseridis *et al.*, 2000; Czerny *et al.*, 2008; López *et al.*, 2003; Roscher *et al.*, 1997). Homofuraneol has been associated with aged Champagne and bestows a caramel aroma on wine (Escudero *et al.*, 2000). In our study, it was the only furan-derived compound found at concentrations higher than its ODT, and the treatments increased its concentration and exceeded the ODT value. At 15 °C, the chitosan-treated wines increased their concentrations above 10 µg/L, while the control (4 µg/L) remained below this value. Therefore, the addition of chitosan may have had an actual impact on the aroma of the Pinot noir base wine after 90 days of ageing at 15 °C, though this is based solely on the quantification of a single compound exceeding its determined ODT and is not representative of a true sensory evaluation. No studies could be found that reported the influence of chitosan on homofuraneol synthesis, although the influence of the chitosan treatments on homofuraneol concentrations was large in our study. The mechanism by which homofuraneol is synthesised in wine has not yet been elucidated. However, Escudero *et al.* (2000) reported that homofuraneol was previously identified as a component of the MR via a pathway involving acetaldehyde. Given the similar concentrations obtained between furfural and homofuraneol in our study, we suggest a potential link in their formation is possible. If homofuraneol is the reaction product of acetaldehyde and a sugar (arabinose or xylose), it is possible that the aldol condensation of these compounds might yield a chemical similar in structure. The tautomerisation and cyclisation could then take place in a similar manner to furfural or 5-hydroxymethylfurfural (another furan-derivative found in aged wines), yielding the product homofuraneol (Wang *et al.*, 2019). As this would require the loss of $-CH_2O$ in the same way as the furfural mechanism**, t**his could explain the increase in homofuraneol synthesis in the chitosan-treated

wines. The exact mechanism of homofuraneol formation in wine is outside the scope of this study, and it requires further investigation.

2.1.3. Ethyl-2-furoate

Associated with vanilla and burnt aromas, ethyl-2-furoate is potentially a relevant aroma compound in aged wines (Mariscal *et al.*, 2016; Pereira *et al.*, 2014). Ethyl-2-furoate concentrations did not increase much at 30 °C compared to 15 °C throughout the 90 days of storage, in contrast to a previous study that used Chardonnay base wines (Medeiros *et al.*, 2022). However, results from this study align with those of Pereira *et al.* (2014), who subjected a dry Tinta Negra wine to 45 °C storage conditions for 3 months and found the wine to contain 12 µg/L of ethyl-2-furoate, a concentration comparable to the 10 µg/L observed in the control wine in our study. Whether ethyl-2-furoate at this concentration contributes to aged wine odour cannot be established, given the lack of information on an ODT in existent literature.

2.1.4. 5-Methylfurfural

5-Methylfurfural correlates with wine ageing, contributing to almond and caramel aromas (Burin *et al.*, 2013; Le Menn *et al.*, 2017; Pereira *et al.*, 2014). The ODT of 5-methylfurfural has been reported as 16 mg/L (in 14 % EtOH (v/v) solution (Moreno *et al.*, 2005), which is greater than the concentrations found in our base wines. A similar trend to that of furfural and homofuraneol formation was observed for 5-methylfurfural at 30 °C. The MMW and HMW chitosan-treated wines had greater concentrations of 5-methylfurfural compared to the LMW chitosan and control wines. No relationship between chitosan and 5-methylfurfural formation could be established from previous studies, although Castro Marín *et al.* (2020b) mentioned that by treating their Pinot gris/Pignoletto wine with 250 mg/L of *A. niger*-derived chitosan (80–90 % DD, 10–30 kDa), furfural, HMF, and furfuryl alcohol were found at higher concentrations. Given the chemical similarities between these three compounds and 5-methylfurfural, one could anticipate that it would have been affected alongside them; however, 5-methylfurfural was not among the compounds determined. Therefore, it is likely this is the first time an association between chitosan and 5-methylfurfural formation in wine has been reported. Additionally, although the concentrations of 5-methylfurfural and furfural were below their ODT, they could still increase during the cellar ageing of the final wine.

2.1.5. Furfuryl ethyl ether

Furfuryl ethyl ether has been previously associated with ageing in wine and beer (Spillman *et al.*, 1998; Vanderhaegen *et al.*, 2004). It was quantified at 2 µg/L for all wine samples, and an increase was observed in wines treated with LMW chitosan compared to the other treatments and control. Spillman *et al.* (1998) determined the ODT of furfuryl ethyl ether in white wine as 430 µg/L, higher than the concentrations in our study. However, the concentrations of furfuryl ethyl ether were in accordance with those found by Vanderhaegen *et al.* (2004) in beer kept at 20 °C for 100 days, during which time < 10 µg/L of furfuryl ethyl ether was detected. Further research into the mechanism and factors surrounding the formation of furfuryl ethyl ether is necessary to determine its potential contribution to sparkling wine aroma.

2.1.6. 2-Acetylfuran

2-Acetylfuran (also known as 2-furyl methyl ketone) has previously been identified in wine, although its contributions to wine aroma and flavour have yet to be established (Burin *et al.*, 2013; Le Menn *et al.*, 2017). 2-Acetylfuran concentrations were higher in wine treated with LMW chitosan compared to the control and the other wines. Similar to 5-methylfurfural, no association between 2-acetylfuran and chitosan could be established from previous studies, and so this is likely the first time its influence on 2-acetylfuran in wine has been described. 2-Acetylfuran has previously been quantified at concentrations similar to those in our study. Burin *et al.* (2013) found 2-acetylfuran concentrations in Alsatian and Bordeaux white wines to be 1–6 and 2–21 µg/L, respectively, while Le Menn *et al.* (2017) reported concentrations ranging from 3–15 µg/L in Champagne wines. The aroma contribution of 2-acetylfuran at these concentrations has not been established since its ODT in wine is yet to be determined.

3. Limitations of the study and further recommendations

Only Pinot noir grape juice was tested, which limits the scope of our findings on the applications of chitosan to how it impacts solely this grape variety. Fully elucidating the efficacy of mushroom-derived chitosan in winemaking requires data across a range of grape varieties from multiple stages of winemaking and should include organoleptic characterisation of the treated wine. Only two of the six HCAs present in grape juice were evaluated in determining chitosan's adsorbent capabilities, but this was justified since caffeic and caftaric acid are found at the highest concentrations in grape juice. The adsorption of the other HCAs and various phenolic compounds by chitosan may have also occurred. Additionally, this study only used a single chitosan source (*A. bisporus*), although many other sources, such as shellfish, fungi, and insects, also exist (Vendramin *et al.*, 2021).

Concerning Study B, alternative and multiple concentrations of chitosan addition would help establish the bounds of efficacy in winemaking and help with making recommendations on its application. Here, we only used the maximum OIV recommended dose. Testing chitosan sourced from crustacean or *A. niger* in addition to the *A. bisporus*-derived chitosan under the same conditions would help determine the relative effects of chitosan source on the chemical interactions in wine. Finally, we encourage further research to elucidate the impact of chitosan treatment of juice and wine on other aroma compound groups. Further research into chitosan's ability to adsorb undesirable compounds and increase the generation of desirable ones will further inform its application in winemaking. Further consideration of the impact of chitosan on wine aroma and flavour development,

such as during extended lees ageing, should be given in future research.

CONCLUSION

Study A showed that the addition of mushroom-derived chitosan to Pinot noir juice as a pre-fermentation fining agent could be a viable strategy for reducing the concentration of caftaric acid, which can contribute to non-enzymatic browning of wine. Additionally, turbidity and browning of the wine were reduced by the addition of chitosan when compared to the control and BAC treatment. Study B determined the positive effect of chitosan on furfural, homofuraneol, and 5-methylfurfural formation in Pinot noir base wine for the first time. This effect was higher in wines that were kept at 30 °C for the 90-day storage period to simulate accelerated ageing. The addition of 1 g/L HMW chitosan to base wine stored for this duration resulted in furfural formation comparable to that observed in a 10-year-old Champagne (Tominaga *et al.*, 2003). Additionally, it is possible that while the concentrations of furfural and 5-methylfurfural are currently too low to influence wine aroma and flavour based on their ODTs, they could continue to increase during cellar ageing, and, therefore, the chitosan treatments of base wine could accelerate "ageing" of the sparkling wines in the cellar. The implementation of chitosan as a winemaking tool requires further understanding of factors such as contact time, temperature, chitosan source, and chemical parameters (MW) and how these factors impact wine quality.

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Review

Chitosan: Sources, Processing and Modification Techniques

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Abstract: Chitosan, a copolymer of glucosamine and *N*-acetyl glucosamine, is derived from chitin. Chitin is found in cell walls of crustaceans, fungi, insects and in some algae, microorganisms, and some invertebrate animals. Chitosan is emerging as a very important raw material for the synthesis of a wide range of products used for food, medical, pharmaceutical, health care, agriculture, industry, and environmental pollution protection. This review, in line with the focus of this special issue, provides the reader with (1) an overview on different sources of chitin, (2) advances in techniques used to extract chitin and converting it into chitosan, (3) the importance of the inherent characteristics of the chitosan from different sources that makes them suitable for specific applications and, finally, (4) briefly summarizes ways of tailoring chitosan for specific applications. The review also presents the influence of the degree of acetylation (DA) and degree of deacetylation (DDA), molecular weight (M_w) on the physicochemical and biological properties of chitosan, acid-base behavior, biodegradability, solubility, reactivity, among many other properties that determine processability and suitability for specific applications. This is intended to help guide researchers select the right chitosan raw material for their specific applications.

Keywords: chitin; chitosan; deacetylation; chitosan modification; chitosan processing

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1. Introduction

Chitosan is a copolymer composed of glucosamine and *N*-acetyl glucosamine derived from chitin. As summarized in many elegant reviews compiled in Table 1, chitosan is emerging as a versatile raw material for the synthesis and manufacturing of a wide range of products with application ranging from food, medical, pharmaceutical, health care, agriculture, industry, and environmental pollution protection. This is due to the reactive amino and hydroxyl groups which confer chitosan with many functional properties including polyelectrolyte, antimicrobial, antioxidant, gel-forming, biocompatibility, metal chelating and easy processability [1]. This impressive list of characteristics of chitosan is continuously and rapidly expanding its applications in many areas never imagined before. Chitin, the parent compound of chitosan, is a biopolymer present in many organisms including exoskeletons of the crustaceans (for example, lobster, shrimps, krill, barnacles, crayfish etc.), mollusks (for example, octopus, cuttlefish, clams, oysters, squids, snails), algae (for example, diatoms, brown algae, green algae), insects (housefly, silkworms, ants, cockroaches, spiders, beetles, brachiopods, scorpions) and cell wall of fungi (*Ascomycetes*, *Basidiomycetes*, and *Phycomycetes* for example, *Aspergillus niger*, *Mucor rouxii*, *Penicillium notatum*, *Trichoderma reesi* cell walls) [2,3]. Generally, the amount of chitin in these organisms ranges from 15–30% in crab cuticles, 20–30% crustacean exoskeletons [4], 30–40% shrimp cuticles, 5–25% insect cuticles [5] and 2–44% fungi cell walls [6,7]. Currently, the chitosan used for industrial application is mainly derived from crustaceans, especially crab, prawns

and shrimp shells, whose exoskeletons are readily available as waste derived from the food processing industry. However, it is increasingly becoming available as a side-stream product from the breeding of cocoons from silk industry, a by-product of proteins extraction from insects for food/animal feed industries and fungal fermentation. Although fish scales are made of chitin, it is often discarded because its yield is very low constituting only 1 wt% of its whole weight [8]. According to some estimates, 10^{12}–10^{14} tons of chitosan from crustaceans are produced every year [9] and the global market for chitin and its derivates was valued at US$2900 million in 2017, growing at a Compounded Annual Growth Rate (CAGR) of 14.8%) [2]. It is expected to reach US$63 billion by 2024 [2]. A reasonable number of companies including Chinova Bioworks, Heppe Medical Chitosan GmbH, Golden-Shell Biochemical and G.T.C. Bio-corporation are leading players in the market producing a wide range of products for food, drug, medical, textile and waste treatment with chitosan sourced from shrimp shell that occupies almost 80% of the total market [10].

Table 1. Summary of major applications of chitin and chitosan in the different fields.

Field of Application	Applications	References
Biomedical and Pharmaceutical applications	Antioxidant: free radical scavenger/quencher Antimicrobial agent: positively charged chitosan-NH_2 groups interact with negatively charged microbial cell membrane creating pores Drug delivery: mucoadhesive properties increase drug permeation of intestinal, nasal, and buccal epithelial cells, Gene therapy: Delivering various genes and siRNA Chitosan based drugs. For example, lowering effect of cholesterol for obesity treatment Regenerative technology/tissue engineering: bone, neural, cornea, cardiac and skin regenerative technology. Provides a three-dimensional tissue growth matrix, activate macrophage activity and stimulate cell proliferation Wound management: homeostatic agent, participate in repair, replacement, activation of humor immunity, complement system, and CD4+ cells, enhances granulation as well as the organization of the repaired tissues. It slowly degrades into *N*-acetyl-β-d-glucosamine that stimulates fibroblast proliferation, regular collagen deposition in addition to stimulating hyaluronic acid synthesis at the wound site.	[11–33]
Health care products	Cosmetics formulations: Antimicrobial, antifungal, UV absorbing abilities exploited in various cosmetics formulations including in shampoos, rinses, colorants, hair lotions, spray, toothpaste formulations and tonics. Sunscreens, moisturizer foundation, eyeshadow, lipstick, cleansing materials, and bath agent, toothpaste, mouthwashes, and chewing gum as a dental filler.	[34–37]
Food Industry	Packaging, edible coatings, body filling, emulsifying agent, natural flavor extender, texture controlling, thickening and stabilizing agent, food preservation (antimicrobial agent), antioxidant agent. Flocculation/Clarification and deacilification of fruits and beverages	[38–45]
Agriculture	Antimicrobial activities against various plant pathogens. Fruit preservative. controlled delivery of fertilizers, pesticides, and insecticides. Increase in the auxin concentration and urea release in the soil, germination capacity, root length and activity, and seedling height	[46–48]
Industrial application	Functional materials: Graphitic carbon nanocapsules/composites, tungsten carbide chitin whiskers, etc. are used in the production of micro-electrochemical systems and 3D networks	[49–51]
	Electrolyte: Sulfuric acid and chitosan combination has the ability to discharge high voltage Chitosan provides ionic conductivity and can be used in the production of solid-state batteries Photography: fixing agent for color prints	[52–59]
	Paper manufacture: Production of filter papers, water-resistant papers, biodegrading packages, water-resistant papers	[59–63]
	Enzyme carrier: immobilizing enzymes on solid materials	[64–66]
Construction industry	wood adhesive, fungicide, wood quality enhancer, and preservative	[67–69]
Waste treatment	Flocculating, and negative charge (chelating agent), for dye, heavy metal ions removal and decontamination. Used for various processing plants such as whey, dairy, poultry, and seafood processing plants	[70–75]

One of the most prominent and well-established biomedical application of chitosan is producing homeostatic agents for wound management and wound healing promoting commercial products already approved by the United States Food and Drug Administration (USFDA) [10]. A number of companies are also emerging specializing in the production of fungi-based chitosan products including the Belgium-based company, KitoZyme whose products have already been recognized as safe by the Food and Drug Administration (FDA) and, the European Food Safety Authority (EFSA) for use in medical, food and beverage for example, in winemaking process (clarification, fining etc.) and dietary fiber, microbeads hydrogel for treating osteoarthritis [10]. Similarly, in Canada, Mycodev is producing chitosan for biomedical and pharmaceutical applications through fermentation while in China, Chibio is producing chitosan for food and pharmaceutical applications.

Given chitosan's rapidly increasing industrial importance and ongoing intensive research, this review summarizes ongoing research in exploiting different sources of chitosan, extraction techniques and highlights some of the exploited functional properties based on source, extraction and processing techniques. This is intended to help guide researchers choose the right chitosan raw material for desired applications.

2. Biosynthesis of Chitin

To understand the basis of exploitation of chitosan, it is important to briefly discuss various origins, chemical composition, similarities, and differences from different sources which influence not only the choice of extraction techniques but also the final properties of the chitosan. In Chitin, although similar to cellulose, the C_2 hydroxyl (OH) groups of the glucose units are substituted with acetyl amine groups, hence termed poly-β-[1,4]-*N*-acetyl-d-glucosamine (Figure 1).

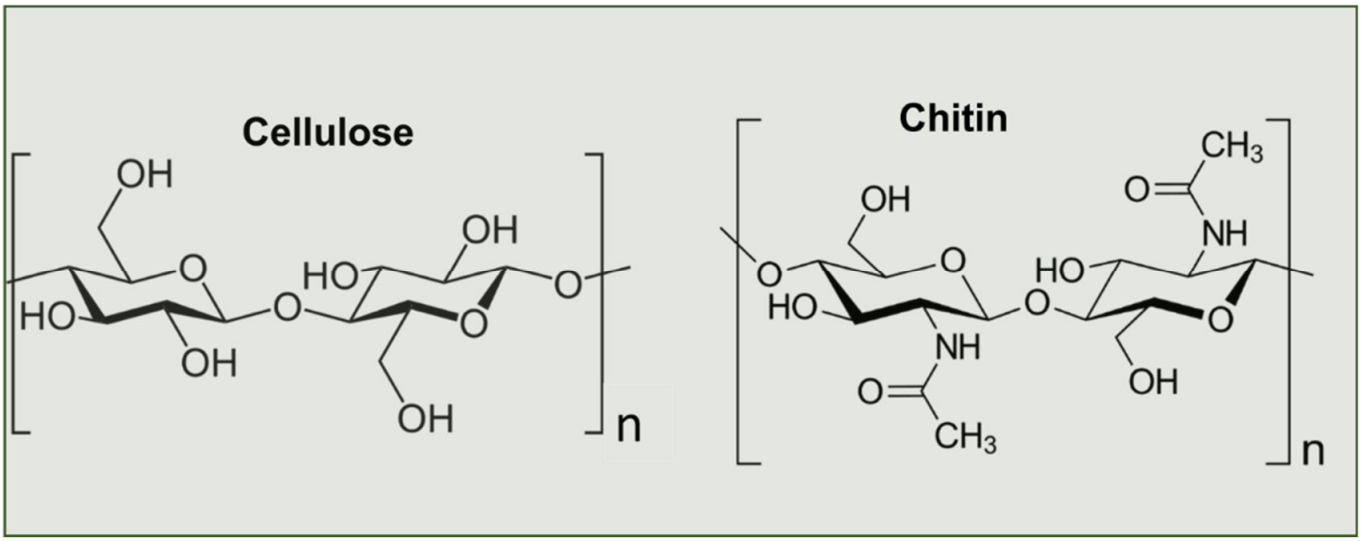

Figure 1. Structures of cellulose and chitin.

The chitin biosynthesis pathway is highly conserved in all organisms, from algae to crustaceans and from fungi to insects. As summarized in Figure 2, chitin biosynthesis pathway can be generally divided into five distinct phases namely; (1) synthesis of *N*-acetylglucosamine-6-phosphate from sugars such as glucose, glycogen or trehalose via the hexosamine pathway [75], (2) synthesis of amino sugar uridine diphosphate *N*-acetylglucosamine-(UDP-*N*-acetylglucosamine), (3) polymerization UDP-*N*-acetylglucosamine by the action of chitin synthase into chitin and (4) deposition of chitin along the cell membrane and release into the extra-cellular space and finally, (5) assembly into chitin nanofibrils [76–78]. As elegantly summarized in previous reports [76–78] and shown in Figure 2, trehalose is first converted into glucose by trehalase, and the glucose is further converted into glucose-1-phosphate by phosphorylase. The formed glucose-1-phosphate is then converted into glucose-6-phosphate by

phosphomutase, which is further converted into glucose-6-phosphate by a hexokinase. The fructose-6-phosphate is then converted into glucosamine-6-phosphate by an aminotransferase using L-glutamine. The glucosamine-6-phosphate is converted into *N*-acetylglucosamine-6-phosphate by *N*-acetyltransferase using acetyl co-A as a substrate. Further phosphate group is moved from position 6- to 1-phosphate position by phosphoacetylglucosamine mutase. A pyrophosphorylase using triphosphate as the co-substrate converts *N*-acetylglucosamine-1-phosphate into UDP-*N*-acetylglucosamine. In the last step, chitin synthase uses UDP-*N*-acetylglucosamine to produce chitin.

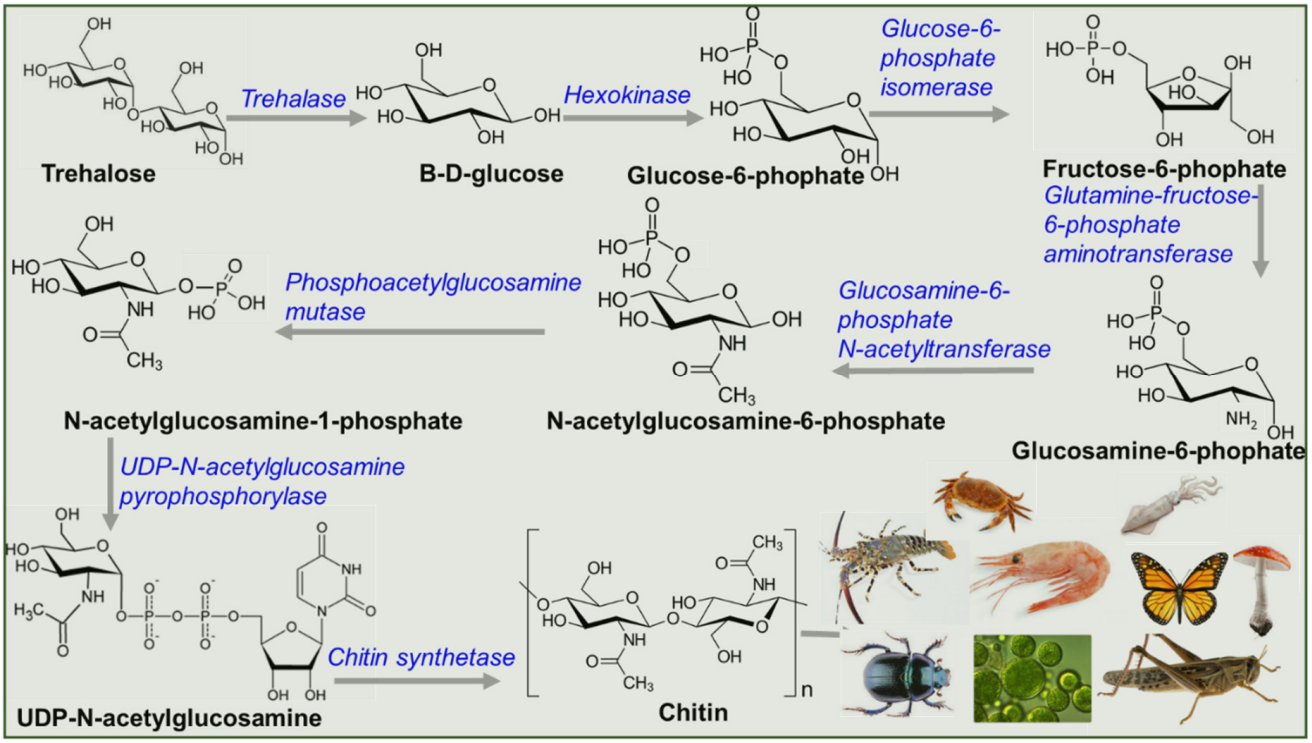

Figure 2. Chitin biosynthesis pathway.

The synthesized linear chitin chains are then assembled into microfibrils and organized in the extracellular matrix (cell walls, cuticles, peritrophic matrices) [79]. The composition of chitin varies between organisms, season, gender, age, habitat and other environmental conditions [80]. Based on X-ray diffraction studies, chitin microfibrils seem to orient themselves in three crystalline allomorphic forms namely; α-, β- and γ-chitin (Figure 3). These microfibrils also differ in orientation, number of chains, degree of hydration and unit size. The α-chitin crystalline structure is the most abundant form present in arthropod exoskeletons such as krill, lobster, crab, as well as in the insect cuticle [77].

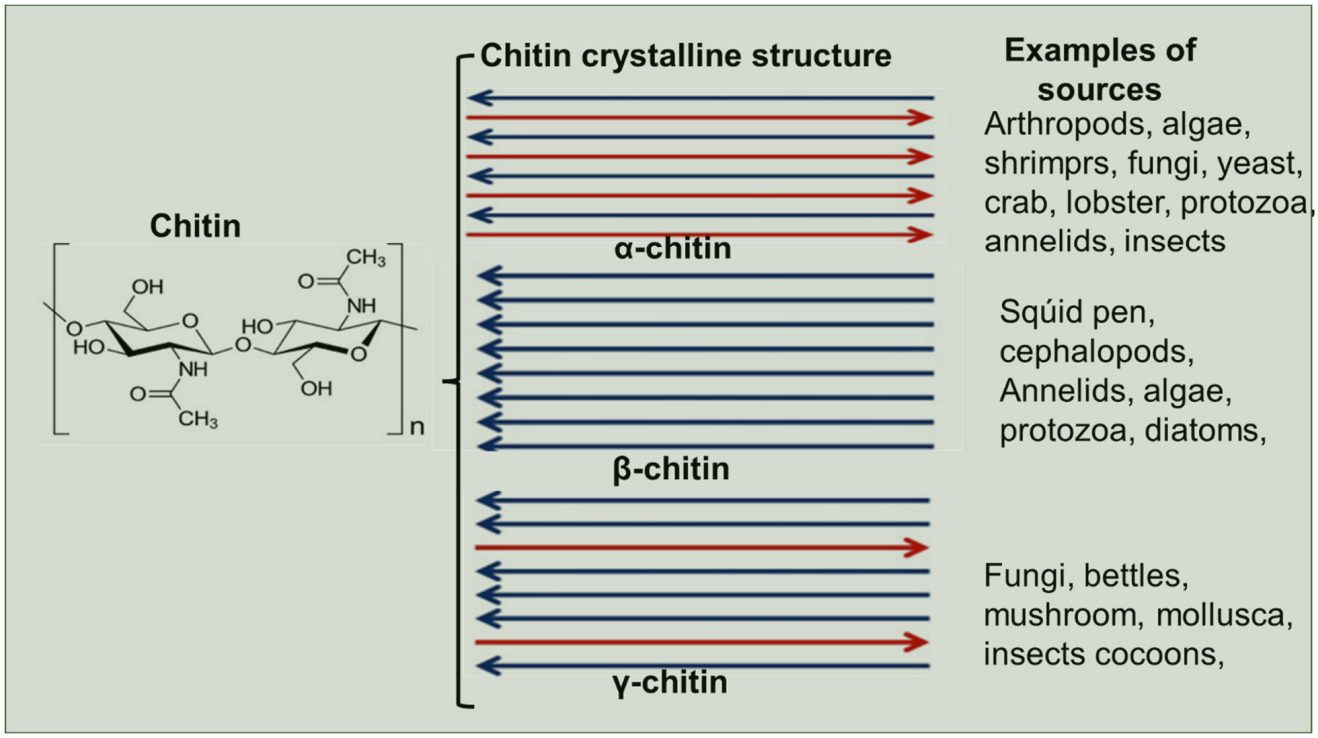

Figure 3. Orientation and arrangement of chitin microfibrils in α-, β- and γ-chitin [81].

The α-chitin is the most stable crystalline form of chitin. Each microfibril consist of approximately twenty single chitin chains arranged in an antiparallel to each other and resulting in a densely packed polymer with increased intra- and intermolecular hydrogen bonds that confers it a remarkable thermodynamical stability [2,78,82,83]. Unlike α-chitin, found mainly in arthropod exoskeletons, β-chitin is found in diatoms and squid pens microfibrils are organized in parallel [84]. This confers them with flexibility. The γ-chitin microfibrils found in fungi, yeasts, and insect cocoons [84], containing a mixture of parallel and antiparallel chains which confers them with both properties of α-form and β-form microfibrils [1]. The γ-chitin microfibrils show random chains as shown in Figure 3 [2,85]. Generally, chitin is also closely associated with other biological components, such as proteins, minerals, carbohydrates, lipids and pigments present in the shells. For example, fungal chitin contains α-chitin found in cell walls associated with glucans, and in insects they are embedded in a proteinaceous matrix.

3. Chitin Extraction Techniques

Chitin is found in association with other biopolymers in different organisms. For example, in fungi, chitin is covalently bonded directly or indirectly via peptide bridges to glucans in cell walls while in insects and other invertebrates, it is either covalently or none covalently associated with certain proteins. This variation implies that different extraction techniques maybe necessary. For example, insect and crustacean chitin forms part of the exoskeleton while in fungi chitin makes a complex flexible compound in cell walls covalently linked to glucans [86]. Further, chitin of marine organisms such as crustaceans are found associated with minerals mainly inorganic carbonate salts, chitin–protein complexes and also contains carotenoids (mainly astaxanthin) and lipids [87]. Although both arthropod and insect chitin are associated with proteins, lipids, minerals, pigments, their abundances vary. Generally, the shells of crustaceans contain 20–30% of chitin [88], 30–40% of proteins, 0–14% of lipids [89], 30–50% of minerals [90]. These percentages vary depending on the source, or even the species, from which chitin is isolated [91]. Distinct from crustaceans, insects generally contain 30–60% protein, 10–25% lipid, 5–25% chitin, 5–15% pigments and 2–10% minerals [84,92]. Fungal cell wall are flexible complex structures composed mainly

of 2–44% [6] chitin chemically linked through α- and β-linkages to glucans (80–90%), 3–20% glycoproteins [2] and minor proportions of lipids, pigments, and inorganic salts [10].

Processes for industrial production of chitin from crustacean shell waste are well established exploiting the abundance of shells of crab, shrimp, and prawn from food processing industries. Crustaceans contribute 69–70% of chitin production [93]. The traditional extraction process involves various steps, namely, demineralization, deproteination, bleaching/discoloration and finally deacetylation to form chitosan as summarized in Table 2. Dissimilar to fungi and insect, the presence of minerals in crustaceans makes demineralization a crucial step. Demineralization is achieved through acid treatment using sulfuric, hydrochloric, nitric, acetic, oxalic and formic acids [94]. Mohan et al. [95], demonstrated that the use of hydrochloric acid during the extraction of chitin from insects produced chitin with superior quality as compared to other acids [96]. Acid treatment breaks down calcium carbonate into calcium chloride and carbon dioxide. While hydrochloric acid is the most preferred reagent for the demineralization of both insect and crustacean shells, attempts are being made to replace with more environmentally friendly organic acids [10]. Fungal extraction does not require the demineralization step but requires deproteinization using bases and strong acids at high temperatures and further neutralization [97]. A typical extraction process of chitin from fungi requires first treatment with alkali, usually 1 M NaOH at 60–120 °C for 0.5–12 h), to remove proteins, lipids, and other alkali-soluble carbohydrates [98]. The remaining alkali insoluble material containing mainly chitin is further treated with acids such as 2–10% acetic acid at 50–95 °C [99] in order to remove acid soluble material. The obtained acid soluble material, rich in chitosan, is then treated with alkali up to 2 N NaOH followed by centrifugation and washing with acetone and ethanol [100,101], followed by centrifugation and washing with acetone and ethanol [98]. Alkaline conditions degrade cell wall material resulting in insoluble proteins and chitin which is then further treated with an acid such as hydrochloric, lactic or acetic acid. Acetic acid is preferred for effectively removing phosphates and insoluble materials. It should be noted that high alkali concentration can cause chitosan oxidation, extensive chain degradation especially at high temperatures and long deproteinization incubation time. Similarly, acid treatment can also affect the final yield of chitosan during the extraction process. Lactic acid produced higher yield of chitosan than hot sulfuric acid even under lower temperature [98], formic acid (6% v/v) gave a higher yield of chitin compared with acetic acid [99]. Although hydrochloric acid causes a greater extent of hydrolysis of the acetyl moieties, it produces chitosan with higher DDA compared with acetic and formic acid [9]. Generally increasing the concentration of acids results in increased DDA and darker colored chitosan [9]. This procedure does not include the deproteinization nor the demineralization processes required in the extraction of chitosan from crustacean sources. The fungal chitin extraction process has been shown to result in chitin free of proteins that could cause allergic reaction, making them suitable for biomedical applications [102]. Extraction of both marine and fungal sources may require discoloration or bleaching to remove pigments that are naturally present in the organism [6]. Discoloration is easily achieved using organic solvents for example, acetone while bleaching is achieved using sodium hypochlorite or hydrogen peroxide [103]. For example, decolorization of crustacean chitin is achieved using bleaching agents such sodium hypochlorite potassium permanganate and oxalic acid or hydrogen peroxide, while a mixture of methanol–chloroform or alcohol–chloroform has been found effective for decolorizing insect's chitin [84].

Table 2. Summary of different chitin extraction techniques.

Extraction Techniques	Process Conditions	Advantages	Disadvantages	References
Chemical methods	Deproteinization conditions: NaOH, KOH, Na_2SO_3, Na_2CO_3 Temp: 25–100 °C, 30 min–72 h Demineralization: HCL, HNO_3, CH_3COOH, HCOOH Temp: 25–100 °C, 30 min–48 h Decolorization: organic solvents such as acetone, ethyl alcohol, diethyl ether Bleaching: $KMnO_4$, $NaClO/H_2O_2$; Temp: 20–60 °C, 25 min–12 h Recovery: precipitation with 5–10%NaOH Deacetylation: NaOH/KOH 30–50% *w*/*v*, Temp: 80–150 °C, Time 1–8 h	Short processing time Produces chitin with high DA% Accompanied by deacetylation Process used at industrial scale	Multistep process Deacetylation unavoidable Environmentally unfriendly generate large quantities of waste that cannot be used as human and animal nutrients. Calcium carbonate lost to waste stream	[88,104,105]
Biological and enzyme based methods	Demineralization: fermentation using lactic acid producing bacteria or lactic acid Deproteinization using enzymes (cellulases, pectinases, chitinases, lipases, papain, hemicellulases, pepsin and lysozyme produces chitooligosaccharides, lysozyme Protease deproteinization and demineralization: in (10% HCl solution at 20 °C for 30 min) at 55 °C and pH of 8.5 Combined deproteinization and demineralization: microorganisms producing proteases or proteases Protease demineralization at 25 °C for 20 min in the presence of lactic acid ratio of 1:1.1 *w*/*w* and acetic acid ratio of 1:1.2 *w*/*w*) Deproteinized with chitinase at 45 °C and a pH of 6.0 with shaking at 150 rpm Alcalase, esperase and neutrase in deproteinization, followed by deacetylation by alkaline treatment, reached the highest degrees of deacetylation with 61.0–63.7% NaOH for 14.9–16.4 h Combination of species, including Serratia marcescens and L. plantarum, increased deproteinization and demineralization activity Decoloration: acetone or organic solvent, Deacetylation: chitin deacetylase producing by bacteria Lactic acid ratio of 1:1.1 *w*/*w* and shells: acetic acid ratio of 1:1.2 *w*/*w*) had a maximum demineralization	High quality of final product Sustainable process Environmentally safe; specific, fast in action, reduces the use of energy, chemicals and/or water compared to conventional processes Regular deacetylation and MW	Long processing time (days) Process still under development enzymatic method had a higher degree of acetylation (19.4%) and viscosity than that prepared by chemical method (17.2%).	[106–120]
Ionic liquids	Complete dissolution followed by the selective precipitation of chitin. Treatment with [C2C1im] [CH3COO] [121]. causes swelling swell Ionic liquids 1-ethyl-3-methylimidazolium acetate [C2mim] [OAc], 1-butyl-3-methylimidazolium chloride [C4mim]Cl, [C2mim]Cl, [C2mim] [OAc], and 1-allyl-3-methylimidazolium acetate [Amim] [OAc], are effective against chitin from shrimp shells, crab shell waste, and squid pens. Combination of steam explosion and ionic liquid pretreatments for efficient utilization of fungal chitin	Scaling-up the process were successful leading to the establishment of a company 525 Solutions at industrial scale [122]. Dissolution and coagulation of the polymer combined with enzymatic hydrolysis, reduces its crystallinity, making the polymer more accessible to the enzyme	Harsh totally dissolves chitin Toxicity and nonbiodegradability DESs are the ability to perform a three-step process in single step, including demineralization, deproteinization and chitin dissolution	[121–128]

Table 2. *Cont.*

Extraction Techniques	Process Conditions	Advantages	Disadvantages	References
Deep eutectic solvents	Demineralization, deproteinization and chitin dissolution perform a three-step process in single step Mixture of hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), choline chloride (ChCl) is commonly used as an HBA, while HBDs include lactic acid, malonic acid, and citric acid 150 °C Incubating different ratio mixtures of DESs (ChCl/citric acid, ChCl/L-lactic acid, and ChCl/malic acid) with chitin sources at temperatures between 50–150 °C for 2–6 h DES plus Microwave: DES ratios of 1:5, 1:10, and 1:20. Next, the mixture was heated under 700 W microwave irradiation (Haier MZC-2070M1) for different durations of time (1, 3, 7, and 9 min) Demineralization was carried out by the malic acids. When choline chloride–malic acid was applied to the shrimp shells, minerals, which are mostly in the form of crystalline $CaCO_3$, were removed by the malic acid, leaving the proteins and chitin. The spacing between the chitin–protein fibers was filled with proteins and minerals; thus, the removal of minerals resulted in a weakening of the linkages within the inner structural organization of the shrimp shells. Since the minerals are removed by the malic acids, in order to conduct demineralization, one component of the DESs used in the chitin extraction should be an acid.	Single step for simultaneous removal of protein and minerals Demineralization, deproteinization and chitin dissolution perform a three-step process in single step Low melting temperature, non-flammability, highly chemical and thermal stability and superior biodegradability. No deacetylation Solvent recycling possible	High solvent viscosity causes difficulty at large scale DESs are a new class of ionic liquid analogues derived from inexpensive commercially available raw materials with a melting point lower than that of each individual component. DESs are biodegradable, cheap and easy to produce	[129–134]
Ultrasound extraction	Ultrasound's cavitation effect solubilizes protein associated with chitin, dissociates covalent bonds in polymer chains and disperses aggregates Uses high-intensity Ultrasound signals at 750 W power and 20 kHz ± 50 Hz operating frequency to enhance the efficiency of extraction of chitin,	Reduces the extraction time and avoids the requirement of high temperatures.		[135–137]
Microwave-assisted extraction	Microwave heating involves two main mechanisms: (i) dipolar polarization and (ii) ionic conduction Increasing the microwave irradiation to 130 watts of power for 15 min resulted in high deproteinization (11.46%) and a low ash content (5.4%) at 700 °C for 2 h using 50% of NaOH solution in a power range of 500–650 W resulted in a low DDA, and the deacetylation reaction was more than 80% completed after 10 min. MAE allowed the production of chitosan with medium and high MW (300–360 kDa).	Fast deacetylation of chitosan in 24 min, compared to conventional heating method that requires 6–7 h Upscaling possibility		[138–142]

Besides chemical-based chitin extraction techniques, several other methods including biobased methods, the use of ionic solvents, deep eutectic solvents and ultrasound-assisted techniques summarized in Table 2 are emerging suitable methods too. Microbial fermentation technology that employs lactic acid producing microorganisms or the use of biologically produced organic acids are proving efficient systems to obtain high quality

chitin. Biological extraction processes either use fermentation processes exploiting the ability of such microorganisms like *Lactobacillus* for example, *L. paracasei*, *L. plantarum*, and *L. helveticus* to produce organic acids.

These acids are efficient in demineralizing chitin. Microbial fermentation using Aspergillus sp., Pseudomonas sp., and Bacillus sp. has also been shown to be effective [143]. Biological deproteinization process uses proteases produced by microorganisms. Approximately 95.3% deproteinization and 99.6% demineralization is achieved without comprising the quality of chitin [111]. Among the enzymes, microbial proteinases, fish entrails proteases for example, intestines of sardinella (Sardinella aurita) and grey triggerfish (Balistes capriscus) [144] have proved useful deproteinization agents [105]. Enzyme based methods share the same demineralization mechanism with chemical methods. Nevertheless, despite promising, biological extraction approaches suffer from commercial scalability. However, intensive research is ongoing to make this approach industrially feasible. Other emerging extraction techniques include the use of ionic liquids, deep eutectic solvents (DES), microwave, ultrasound, and pulsed electric field technologies for the deproteinization [142]. Of these approaches, microwave-assisted extraction, ionic liquids, deep eutectic solvents and ultrasound-assisted extraction [133,145] offers enhanced process control, energy-efficiency, and cost-effectiveness [130,141] in comparison with the conventional chemical approaches. Especially the use of emerging green solvents (ionic liquids and deep eutectic solvents (DESs)) have been applied to many fields, such as biomass for separation and purification, pretreatment and synthesis of polymers including chitin extraction. For example, the use of ionic liquid 1-ethyl-3-methylimidazolium acetate ([C_2mim] [OAc]) resulted in successful extraction of chitin from shrimp, fly larva, crab and lobster with different properties, reconfirming specie dependence [146]. Ionic liquids (ILs) are salts generally composed of a large organic cation and a smaller organic or inorganic anion, possessing a melting temperature below 100 °C. Chitin dissolution is complex and depends not only on the strong hydrogen bond acceptor ability of the IL anion and its interaction with the cation, but also on the chitin type and degrees of acetylation and crystallinity [122]. Regarding chitin, the highest solubility was reported for [C_2C_1im] [CH_3COO], being approximately 20 wt% with microwave irradiation [147]. The extraction of chitin from crustaceans using ILs focused mainly on its complete dissolution followed by the selective precipitation of chitin to obtain clean achieving a maximum of 94 wt% chitin yield from crustacean shells with [C2C1im] [CH3COO] [121]. Scaling-up the process was also successful, leading to the establishment of a company 525 Solutions at industrial scale [122]. Nevertheless, due to the toxicity and non-biodegradability of ionic liquids, deep eutectic solvents (DESs) are emerging as alternative with similar properties to those of ionic liquids. DESs are a mixture of an acid and a base formed through complexing a hydrogen bond acceptor (HBA), usually a quaternary ammonium salt, with a hydrogen bond donor (HBD) or a metal salt. They are emerging as a new class of ionic liquid analogues derived from inexpensive commercially available raw materials with a melting point lower than that of each individual component. DESs are biodegradable, cheap and easy to produce [123]. Compared with traditional ionic liquids, they are cheap, environmentally friendlier and easy to prepare [148]. DESs contain hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) that posses a strong hydrogen bond interaction and electrostatic interaction. Normally, DESs are two-component or three-component systems. They are mixtures of quaternary ammonium salts, metal salts (for example, choline chloride, betaine) while HBD such as polyols, polyacids, and polyamines (for example, ethylene glycol, lactic acid, oxalic acid, urea) [133]. The DESs are able to preform both demineralization and deproteinization. For example, demineralization of chitin by choline chloride–malic acid is attributed to malic acid leaving the proteins and chitin weakening of the linkages within the inner structural organization of the shrimp shells [123]. The demineralization is realized through releasing hydrogen ions from DESs which react with the calcium carbonate in crustaceans resulting in solubilization and formation of calcium salts, water and carbon dioxide [129]. The removal of the calcium carbonate from crustaceans results in a less tight chitin-protein

polymer. The DESs strongly interact with protein hydroxyl, carboxylic and amine groups consequently resulting in interruption of intra- and intermolecular hydrogen bonds within the network of chitin-protein fibrils and subsequent separation of chitin-protein fibrils [129]. Although the DESs have been observed to dissolve up to 9 wt% of chitin for example, in ChCl/thiourea used in 1/2 molar ratio under 100 °C, the dissolved chitin could easily recovered by using water or ethanol [132]. The extraction with ionic liquids produced chitin from shrimp with the strongest fibers, while weaker fibers were obtained with crab and lobster chitin although the latter were twice as elastic and, fly larvae chitosan were the weakest and least elastic fibers [146]. Besides crustaceans and fungi, another potential emerging source of chitin is that from insects. Insects constitute over 900,000 species out of the total 1.3 million different species on the earth [5]. Increasing demand for insects as an excellent alternative source of protein will eventually lead to increased availability of their chitin. By 2016, more than 120 companies had been registered farming insects for animal and human nutrition. By 2019 more than 6000 tons insect black soldier fly and the yellow mealworm protein meal was produced in Europe alone [149]. Many species of insects including honey bees, silkworms, and synanthropic files can be artificially reared and used as a promising new chitin source for industrial purposes [150]. Insect's chitin extraction procedures are similar to those applied for crustacean sources except that insect chitin contains very low quantities of mineral when compared to crustacean shells [151]. This simplifies processing of insect chitin for application in the biomedical and pharmaceutical industries. A comparative study of chitin in exoskeleton of seven orthopteran species (Aiolopus simulatrix, Aiolopus strepens, Duroniella fracta, Duroniella laticornis, Oedipoda miniata, Oedipoda caerulescens, Pyrgomorpha cognate) showed that chitin content varied between 5.3 to 8.9% [152]. In summary, it is important to note the harsher the chemical extraction techniques employed during demineralization, deproteination and discoloration treatments with regards to chemicals used, pH, temperature and incubation time, the higher the degree of hydrolysis and may affect the quality of obtained chitin. Despite the advances in developing new environmentally friendly, efficient chitin extraction techniques, chemical extraction techniques remain, to date, the preferred routes due to the availability of chemicals and the possibilities of scalability.

4. Chitin Deacetylation Techniques

Deacetylation converts chitin into chitosan. The process involves the removal of the acetyl groups attached to amino group to expose the -NH_2 groups. The degree of acetylation (DA) of chitin is a significant parameter influencing the biological, physicochemical, and mechanical properties and an important parameter that determines its classification whether it is chitin or chitosan. The deacetylation process results in a polymer containing both *N*-acetyl-glucosamines and glucosamines units. If the deacetylation produces a polymer with >50% *N*-acetyl-glucosamine units, it is still referred to as chitin if it is lower, than it is termed chitosan. Thus, deacetylation does not only affect acid-base behavior, electrostatic characteristics, biodegradability, self-aggregation, solubility, sorption properties, ability to chelate metal ions among many other properties but also determine its classification and affect its suitability for specific applications [95]. The percentage of *N*-acetyl-glucosamine units is termed the degree of acetylation (DA) and can vary from 50% to 100%.

During the deacetylation process, random depolymerization also occurs due to the extreme process conditions (for example, strong base, high temperatures and pressures) leading to the production of chitosan with varying chain length and water-solubility properties. Although chitin can be deacetylated using either acids or alkalis, glycosidic bonds are very susceptible to acid hydrolysis therefore alkali-deacetylation using NaOH at high temperature is increasingly being used more frequently to avoid unwanted chain termination [96]. Satisfactory deacetylation is achieved with concentrated NaOH or KOH (40–50%) at temperatures above 100 °C [59,153]. This industrial approach hydrolyzes the amide bonds makes it possible to produce several chitosan products in the form of flakes, fine powder, beads, or fibers. Generally, the extent of deacetylation is the function of

concentration of NaOH, reaction time, temperature, density, and molecular weight of the chitin initial polymer [59]. Approximately 82% deacetylation is achieved during treatment of chitin with 50 wt% NaOH for 1 h at 100 °C [59,95]. This process can lead to chitin with DDA as low as <10% and the molecular weight as high as 1–2.5 × 10^6 Da corresponding to a degree of polymerization of ca. 5000–10,000 and chitosan with DDA ranging from 40% to 98% and the molecular weight ranges between 5 × 10^4 Da and 2 × 10^6 Da [59]. However, chitosan generally has a DDA between 13 and 40% and molecular weight (M_w) between 2 × 10^5 to 1 × 10^6 Da [153]. It is important to note that due to the higher reactivity of β-chitin it is much easier to destroy its crystalline structure compared to α-chitin during deacetylation because of the loose arrangement of chitin molecules completely converting it into amorphous unlike highly crystalline chitosan from α-chitin [154]. Generally, alkaline deacetylation consumes high quantities of energy; large amounts of alkali solution produce chitosan with varied DDA and broad M_w distribution [155]. As noted by Jug and Zhao, several studies have found that the chemical treatments alter the structural properties of chitin, due to swelling, dissociation of hydrogen bonds, and rearrangements of polymeric chains, and the different forms of chitin responded differently such as weakening inter-sheet hydrogen bonds and decreasing crystallinity index [156]. Alkali- or acid treatment of β-chitin converts them into α-chitin, that affects its original functional properties, its high reactivity and susceptibility toward solvents [157]. Strategies to minimize chain degradation include avoiding the use of acids which easily hydrolyze glycosidic bonds, reducing the amount of alkali added by using water miscible solvents like 2-propanol or acetone [155], or reducing deacetylation reaction time.

Alternatively, other gentler extraction techniques such as microwave-assisted extraction, combined steam explosion and deep eutectic solvents integrated with microwaves and enzymatic deacetylation techniques are emerging as highly promising also environmentally friendly processes to produce chitosan. Microwave-assisted chitin deacetylation using sodium hydroxide increased deacetylation efficiency beyond >90% in 3 h as compared 21 h during conventional alkali treatment. Steam explosion has also been shown to facilitate deacetylation of chitosan [158]. During steam explosion, chitin is treated in a puffing gun with saturating steam at increased pressure and temperature for several minutes followed by explosive decomposition. The conversion of the steam energy into thermomechanical force breaks the intermolecular interactions of molecules and frees chitin. Chitin with 75% moisture content exhibited maximum DDA (43.7%) when compared to chitin containing 50% and 35% of moisture which resulted in only 40% and 32% DDA [158]. Chitin extracted by deep eutectic solvents had high purity (74–91.345) and yield (12.71–26%) compared to the conventional acid/alkali method (purity 91% and yield 6.5%) [158]. Further combining microwave with deep eutectic solvents resulted in effective deproteinization efficiency (88–93% rate of removal) in shrimp chitin [159]. Further, enzymatic deacetylation using chitin deacetylases obtained from different biological sources such as fungi and insects [90,160] present an efficient alternative strategy [160].

Various proteinases and deacetylases are emerging as competent technologies for deproteinating and deacetylating chitin [105]. Chitosan deacetylases are mainly derived from bacteria, fungi and a few insects. Among the prominent fungal chitin deacetylases are those produced by Mucor rouxii, Absidia coerulea, Aspergillus nidulans and Colletotrichum lindemuthianum [161]. It is important to note that the different deacetylases show different catalytic efficiency. Chitinolytic hydrolyzing enzymes are classified according to their mode of action into endo- and exochitinases can completely hydrolyze chitin. The endo-chitinases hydrolyze internal glycosidic bonds producing fragment ranging from dimers to polymers while the exo-chitinases act on the non-reducing end of chitin releasing monomeric and dimeric *N*-acetyl glucosamine units. For example, a deacetylase from *M. rouxii* performs sequential exo-type deacetylation at the non-reducing end of the oligomer, while deacetylase from C. lindemuthianum hydrolyzes a single acetyl group before dissociating and forming a new active complex [162]. Generally, most of the bacterial chitosan deacetylases preferably act on low-molecular-weight chitosan. Of all the chitin deacetylases, those ob-

tained from from *Rhizobium* spp. and Vibrio cholerae [162] are known to efficiently produce chitosans [163,164]. Due to chitosan´s similarity with carbohydrates such cellulose, it is also important to note that enzymes such as cellulases and lysozymes are also able to hydrolyze chitosan [162]. Indeed, chitosan oligomers with 5–30 kDa have been produced using cellulases, pepsin and lysozyme [165]. The enzymatic deacetylation process tends to produce homogenous chitosan, although this approach is currently not industrially feasible due to the use of high cost of enzymes [90]. However, it should be noted that chitin deacetylases are not efficient in deacetylating insoluble chitin. It is therefore important to pretreat chitin.

5. Structure-Function Properties of Chitosan

5.1. Influence of DDA and Molecular Weight (M_w) on Chitosan Properties and Applications

The DDA, polydispersity and M_w of chitosan are the most significant parameters influencing its biological, physicochemical, mechanical properties and hence its application. For example, DDA and M_w influences solubility, reactivity, acid-base behavior, electrostatic behavior, flexibility, polymer conformation, viscosity, crystallinity, porosity, tensile strength, conductivity, ability to chelate metals and photoluminescence. Moreover, the same two parameters listed above (DDA and M_w) also affect many biological properties such as biodegradability, biocompatibility, mucoadhesion, hemostatic, analgesic, adsorption enhancer, antimicrobial, anticholesterolemic, antioxidant among many other properties that determine the material's suitability for specific applications [10]. When protonated, the -NH_2 group enables chitosan to make complexes with negatively charged derivatives for example, proteins, dyes, enzymes, tumor cells, bacteria cell wall proteins, DNA, RNA as well as various metal ions its neutral or negatively charged hydroxyl groups of D-glucosamine [10]. Under certain conditions, its insolubility at neutral and solubility under alkali conditions makes it a versatile polymer for application in polymer synthesis, solution or as solid polymer [10]. Generally, a high number of acetyl groups prevents chitosan's enzymatic degradation (by enzymes such as lysozyme), making it suitable for producing drug delivery systems [166]. Although chitin/chitosan is readily soluble in many organic solvents and dilute organic acid solutions such as acetic acid and formic acid, its poor solubility in water has been one of the hindering challenges towards its full exploitation. To this effect many studies have been developing techniques to enhance the solubility of chitosan in water [9]. Acid hydrolysis with concentrated hydrochloric acid conducted at 80 °C produces chitosan oligomers with a degree of polymerization between 1 and 40 [9]. Nitrous acid treatment is also effective resulting in selective, rapid, and easily controlled, stoichiometry products. Nitrosating agents instead attack the glucosamine but not the *N*-acetylglucosamine moieties and cleave the glycosidic linkage. Chemicals such as hydrogen peroxide and hot phosphoric acid are also used. The use of acids produced in the human body such as acetic acid, HCl, lactic acid, citric acid, and pyruvic acid can potentially be also used to solubilize chitosan in water 9 except phosphoric acid) [167]. A Second strategy involves the deacetylation combined with hydrolysis of long chain chitosan polymer into lower M_w oligomers. Increasing the amino groups during deacetylation and whilst, respectively, decreasing the acetyl groups lead to enhanced solubility. This is because, under acidic pHs below 6, the amino groups are fully protonated. However, increasing pH beyond 6 gradually decreases its solubility due to deprotonation of the amino groups [168]. Increasing the DDA leads to oligomers with higher protonated amino groups which facilitate its solubility.

Thirdly, chitosan M_w's affect its water-solubility properties due to the presence of free amine without need of acidification [169]. Acid hydrolysis also leads to chitosan with decreasing M_w while concomitantly increasing its solubility [105]. Chitosan with M_w < 30 kDa is easily water-soluble without adding any acid. Solubility of chitosan with M_w between 22 and 30 kDa can be enhanced by adding acid [105]. This also applies to chitosan with M_w > 30 kDa. When the M_w of chitosan is above 30 kDa, protonation of its amino groups becomes a prerequisite to dissolve it in water. It is known that chitosan M_w lower than

2×10^6 Da and containing 7% w/w nitrogen is suitable for textile, food, photography, medical and environmental applications [170]. However, although deacetylation is crucial in converting chitosan into water soluble oligomers, higher M_w tend to increase inter- and intra-molecular hydrogen bonds between chitosan chains leading to poor solubility [171]. High Mw chitosan ranges from 310 to 375 kDa [172], medium Mw ranges from 190 to 310 kDa (O'Callaghan & Kerry, 2016), and low Mw is below 90 kDa [173]. For example low MW chitosan is mainly preferred for drug delivery applications. Chitosan hydrolysis can easily be achieved using several hydrolytic enzymes including lysozyme, chitinases, some cellulases and lipases [59] and chemically using HCl, HNO_2, H_2O_2 and potassium persulfate while sonication, electromagnetic irradiation, gamma irradiation, microwave irradiation and thermal treatment constitute the commonly used physical processes. The DDA and solubility properties of chitosan influence their functional properties and application. For example, chitosan with higher DDA is suitable for making films with higher tensile strength, water transmission while membranes with DDA between 65 and 80 were effective in inducing inflammation reactions [174]. Chitosan with a DDA of 45–55% is highly soluble in water and weak acid [175] making it suitable for making flexible and transparent materials [176]. As summarized in altering the DDA changes the biological functions of chitosan into antibacterial anti-tumor, anti-inflammatory, wound healing properties, immune activation [106]. Similarly, chitosan DDA also found to influence biocompatibility, biodegradability, hydrophilicity, muco-adhesion, hemostatic, analgesic, anticholesterolemic, antioxidant, and adsorption-enhancing properties of chitosan-based biomaterials [174]. For example, chitosan sponges produced from chitosan with different DDA and Mw shows that cell spreading was much higher on sponges made with higher DDA which led to increased activities of alkaline phosphatase, osteopontin, vascular endothelial growth factor-A (VEGF), interleukin-6 (IL-6), and reduction in monocyte chemoattractant protein-1 (MCP-1), sclerostin (SOST) and dickkopf related protein-1 [177]. Sponges made from chitosan with lower DDA increased secretion of osteoprotegerin and SOST as compared to higher DDA while a combination of high DDA and Mw increased secretion of VEGF and IL-6, reduced the secretion of osteopontin as compared to chitosan with similar DDA but with lower Mw [177]. These observed variations clearly indicated the possibility to introduce desired tailored conditions in tisuue engineering or wound management. Materials produced with DDA value > 70% have been shown to be suitable for making material suitable for drug delivery applications [178].

The DDA and Mw of chitosan has also been shown to influences its antibacterial properties. This is because the presence of positively charged amino group that interact with the negatively charged bacteria membrane depends on concentration of -NH_2 reactive free groups. Low molecular chitosan inhibited *Escherichia coli* and *Pseudomonas aeruginosa* [179] and *phytopathogens* [179–181]. The -NH_2 groups alter the bacterial surface morphology which result in increased membrane permeability and loss of intracellular substances [182]. Commercially available chitosan antimicrobial compounds include HidroKi®, Axiostat®, Chitopack®, Tegasorb®, and KytoCel® CWD [11]. Chitosan having a lower M_w of approximately 2×10^6 Da and 7% *w/w* nitrogen is suitable for textile, food, photography, medical and environmental applications while because of their stiffness and higher mechanical properties, chitosan with high crystallinity higher are good for making tissue engineering platforms [183]. Higher DA makes chitosan less senstive to enzymatic biodegradation making them useful as delivery systems [166]. In contrast, low M_w chitosan is also suitable for producing efficient protein-based delivery systems for transport and release of intestinal drugs and bioactive compounds. Low M_w chitosan (<300 kDa) is suitable for the synthesis of wound dressings, food preservation materials, wastewater treatment, molecular imprinting and chelating materials. Chitosans with DDA (70–80%) and high M_w (>300 kDa) is recommended for the synthesis of drug delivery systems, scaffold materials for tissue engineering, cell and enzyme immobilization platforms, encapsulation, food packaging while chitosan with DDA (70–90%). Chitosan with low DDA (55–70%) and high molecular weight (>300 kDa) is suitable as emulsifying agent and for application in various pharmaceutical

applications, synthesis of nanoparticles and application in food formulations. In contrast, chitosan with low DDA (55–70%) and low M_w (<300 kDa) is suitable for gene and drug delivery, plant protection and plant growth stimulator [2]. Chitosan with moderate M_w has higher anti-cholesterol activity [184]. Generally it has been observed that increasing DDA enhances stronger biological effects, decreasing the M_w generally increases the bioactivities [96], especially when the M_w is lower (for example, <20 kDa) than higher (for example, >120 kDa) [185]. The DDA, DA and M_w are therefore very important characteristics to consider when using chitosan for specific applications.

5.2. Influence of Origin of Chitosan

In addition to DA, DDA and M_w, the source (origin) of chitosan also influences its application. For example, comparing the three allomorphic forms of chitosan namely α-, β-, and γ-chitosans, shows that β-chitosan has higher solubility than the α-chitosan. This is attributed to the weaker binding forces among the chains of the β-chitosan. Due to their higher crystallinity, α- chitosan is not only less soluble but also stiffer. This stiffness confers it with higher mechanical strength, which makes it suitable for producing tissue engineering platforms. The higher reactivity of β-chitosan derived from squid pen than α-chitosan due to its hydrated structure and weaker intermolecular hydrogen bonds makes it more suitable for the synthesis of thin films, medical, food applications and biosensors products [186]. The higher solubility of β-chitosan compared to that of α-chitosan is attributed to weaker binding force that enhance its biological activities. Furthermore, squid pen chitosan with 31–49% DDA is free from calcium carbonate, carotenoids, and minerals which makes it suitable for biomedical applications [186]. γ-chitin contains both α- and β forms and hence properties from both forms [1]. However, low availability remains a barrier for its mass production and commercial application. The occurrence of chitosan of high purity and concentration in fish makes it more attractive for biomedical and pharmaceutical applications than chitosan of microbial origin [187]. The α-chitosan derived from marine crustaceans was the first most abundant chitosan readily available in larger amounts from food processing industries. It was broadly used for the production of biomedical products, although there are increasing concerns due to the extra effort needed to ensure that they are free from heavy metals [85]. They are extensively used in medicine (drugs, wood management, artificial organs, membranes, anti-coagulant, anti-microbial agent, in tissue generation of artificial bones and skins) [11], pharmacology (fungicides and drug carriers) [188], food systems (preservatives, coatings, antimicrobial and antioxidant agents), and cosmetology (body creams, hair additives, and lotions) [189]. The higher M_w of crustaceans (approximately1.5 $\times$ 10^6 Da) makes it poorly soluble at neutral pH values, resulting in highly viscous solutions than that of fungi (1–12 $\times$ 10^4 Da) and makes it attractive as thickening agent and for making tissue engineering platform and film [190]. Contradictory as it might seem, the presence of protein, lipid and chitosan in marine crustaceans has also been seen as a source of nutrients that can be used in formulating functional foods for therapeutic applications [191]. Although crab chitosan (15–30%) is composed of high mineral (30–50%) and protein (15–50%), it exhibits excellent antioxidant properties, thereby generating interest for developing, products that combat oxidative damages caused by free radicals [192]. Another important crustacean, Krill, contains protein (72.9–75.8%), lipid (12–50%), and chitin (20–30%), is suitable for incorporation into food formulations and application in health [191]. Krill-based chitosan also have higher porosity which makes them suitable for sulfate modification [10]. The presence of protein, lipids, pigments, and $CaCO_3$ associated with shrimp chitosan [190] is viewed as potential source of high value-complex added products. Shrimp has been demonstrated to possess potent antimicrobial activities against pathogenic microorganisms (*Staphylococcus aureus, Enterococcus faecalis, Enterobacter aerogenes*, and *E. coli*). Further, shrimps chitooligomers have been found effective as replacement for antibiotics in animal feed products [193]. Furthermore, chitooligomers (12.3%) from shrimps can be used in animal feed, replacing antibiotics, particularly when the focus is on developing antibiotic residue-free animal

products [193]. Chitosan from fish is emerging a good source of high-quality chitosan for biomedical applications [187] and agrochemical industry [103]. However, the main problem has been its availability since it constitutes <1% of fish´s body weight.

However, increasing interest in insect as a new alternative source of protein is making its chitosan more attractive due to foreseen increasing availability. Chitosan obtained from the larvae of Chrysomya megacephala shows excellent antioxidant activity with an IC_{50} value of 1.2 mg/m [194] while chitosan from the larvae of Lucilia cuprina was shown to have superior anti-bacterial activity against Bacillus subtilis and Klebsiella pneumoniae [194]. The mealworm beetle chitosan was effective against Staphyloccocus aureus, Escherichia coli, Listeria monocytogenes and Bacillus cereus [195]. The ability of the insect chitosan to cause cell deformation and leakage of cell contents, which leads to the breaking of the cell. It has already been shown that honeybee chitosan is suitable as food additive for preserving food [196]. Beetles have also been shown to be a good source of chitosan (~36.6%) [196]. The superior elasticity of insect chitosan is advantageous in polymer production [197]. Chitosan derived from insects has been found suitable for agriculture application (seed coating, plant protection, gene transferring), and biomedical applications for example, drug delivery, and as biomedical platforms.

Another even more prominent and fast emerging source of chitosan are fungi. In fact, their abundance is ranked second, next only to insects. Fungal chitosan is increasingly becoming attractive due to easy production under controlled conditions through fermentation. Although generally insect-derived chitosan has similar properties to those obtained from crustaceans, the superior particle size uniformity, even distribution of acetyl groups. lower Mw and unique Mw homogeneity, viscosity distribution and the absence of heavy metals makes fungal chitosan suitable for wide applications [191,198]. Their unique uniformity of particle size and antimicrobial effect has found them preferred for application in water cleaning, beer-brewing, wound management and textile production [198]. Their viscosity which is 3–5 times lower and M_w (1–2 $\times$ 10^5 Da) but higher DDA (70–90%) [190]), make them suitable for application in food, healthcare, and pharmaceutical industries [199]. For example, chitosan marketed by Sigma "Kitozyme" is isolated from Agaricus bisporus and is an ingredient for wound healing, biosurgery, cell therapy, drug delivery, and vaccines [200]. Chitosan-based edible food coatings have also been used extensively for extending the storability and quality of fresh and processed foods, owing to their antifungal and antibacterial activities [201]. Although yeast contain far less chitin, it has been found suitable for the development of stabilizers and emulsifiers for food and nutraceutical applications [202]. Yeast derived chitosan was successfully used as food stabilizer, emulsifier and for nutraceutical applications [202]. Mucor indicus and Rhizopus oryzae chitosans were demonstrated to be effective in controlling field infestation by the entomopathogenic fungus (*M. anisopliae*). Interestingly, shiitake mushroom (*L. edodes*) chitosan has been shown to have a complex immune stimulant property [203]. "Kitozyme" isolated from Agaricus bisporus is used for making wound healing promoting agents, in biosurgery, cell therapy, drug delivery, and vaccines [201]. Aspergillus niger chitosan with residual glucans content lower than 2%, viscosity in 1% acetic acid higher than 15 Cps and settled density < 0.7 g/cm^3 is the only chitosan allowed in winemaking since 2009 [204], aimed at reducing protein as an alternative to the commonly used bentonite as well as an antimicrobial agent. In summary, the lower viscosity, M_w and higher DDA of fungal chitosan, makes it appropriate for application in food, beverage, healthcare, and pharmaceutical industries [199].

Although algal chitosan from marine sources for example, coralline algae Clathromorphum compactum matrix is less studied, the presence of collagen makes them attractive for cell immobilization applications and as food additives [205]. Further, the α-chitosan nanofibrils from microalgae (Phaeocytis globosa) showed tensile strength comparable to β-chitosan nanofibrils obtained from squid (Loligo bleekeri) and tubeworm (Lamellibrachia satsuma), making them good candidates for making tissue engineering scaffold materials [206].

6. Tailoring Chitosan for Specific Applications

The presence of reactive amino groups on C_2 position, primary hydroxyl group on position C_3 and the secondary hydroxyl group on position 6, offers a myriad of possibilities for modifying and exploiting chitosan for various applications through reactions summarized in Figure 4. These reactions have been extensively summarized in [204,207–211] to which the reader is referred to for detailed reactions. However, here we present in brief possible reactions targeting each functional group highlighted in Figure 4. For example, amino group targeted modification is achieved through such reactions as alkylation, acylation, quaternization, phosphorylation, acylation, nitration, sulfonation, xanthation, *N*-succinylation, thiolation, and graft copolymerization etc. while the hydroxyl groups are mainly modified through o-acetylation, sulfonation, methylation, hydroxylation, cross-linking/grafting [88]. Of these reactions, acylation, alkylation, carboxymethylation, *N*-phosphomethylation and Michael addition, quaternisation, carboxyalkylation, hydroxylation, phosphorylation, sulfation, and copolymerization are the mostly commonly used approaches. Amino group targeted substitutions reactions for example, with quaternary ammonium produces hydrophilic chitosan derivatives extensively studied for their antimicrobial, hemostatic. anticoagulant, hydrogel, film forming properties [212]. This approach is achieved firstly by turning the -NH_2 into quaternary ammonium salt, introducing quaternary ammonium compounds or quaternary phosphonium compounds resulting in products with improved water solubility [213]. One of the most important quaternary chitosan derivative achieved through this process is *N*,*N*,*N*-trimethyl chitosan chloride with excellent solubility in aqueous solution which has found applications as a fluid absorption enhancer, antibacterial agent and gene vector, improving bioadhesion, biocompatibility, solubility, viscosity and swelling index properties of obtained polymers [213,214]. Amino group targeted phosphomethylation produces products with improved solubility, bactericidal, heavy metal chelating and tissue engineering properties) while *N*-targeted modification with *N*-methylene phosphonic, *N*,*N*-dicarboxymethyl, *N*-[(2-hydroxy-3trimethylammonium) propyl] produces soluble chitosan [211]. Selective amino group substitutions reactions while protecting –OH groups in the C_3 and C_6 of chitosan is an effective strategy for synthesize homogeneous *N*-quaternarised chitosan derivatives without *O*-methyl substitutions and *O*-silytation [211].

Dual functionalized chitosan hemostatic wound dressings were formulated using varying ratio of quaternized chitosan and phosphorylated chitosan along with tannic acid which acted as adjuvant hemostat and a crosslinker and poly-ε-lysine to impart the elastic and adhesive properties [215]. In contrast, *N*-acylation produces hydrophobic for example, by grafting fatty acids through amidation of –COOH groups of fatty acids with –NH_2 of chitosan. This chemical process uses such chemicals as acyl halide or acid anhydride in pyridine, chloroform/pyridine, or methanol/water/acetic acid. This reaction also leads to the O-alkyl chitosan the C_2 and C_6–OH groups. To avoid this, protection with trityl groups is necessary [204]. Two alkynoyl-chitosan derivatives that could serve as a useful tool for linking other molecules through click chemistry, one containing alkyl spacers which are soluble in organic medium and another spacer soluble in water were synthesized by introducing alkyne functionalities on the amino group of chitosan without the use of protection groups [216].

Hydroxyl group (–OH) targeted reactions usually require initially protecting the -NH_2 group. This is usually achieved using phthalic anhydride that allows for regioselective processes for example introducing sugar branches that improve water solubility of chitosan [217]. The phthaloyl group is then easily removed by introducing an electron-withdrawing group (for example, –NO_2, –Cl) into the phthaloyl aromatic ring that deprotect the amino group [218]. Products with antifungal activity, flexible films have been produced through this strategy [219]. It should also be noted that while the C_2–NH_2 or C_6–OH groups are easily accessible and modifiable, steric hindrance of the C_3-OH group often makes it is tricky to modify, although it is easily chemically modified through methylation, acylation, sulfation) [220]. Sulfonation produces chitosan with many bioactive activities

such as antioxidant and anticoagulant properties. For example, chlorosulfonic chitosan has improved antioxidant activity [221] while sulfonation of chitosan for metallic implants increased hydrophilicity of the implants while decreasing calcium deposits [211]. Chitosan thiolation adding different compounds such cysteine, thiolactic acid, thioglycolic acid, homocysteine, thiobutylamidine, glutathione, etc. is used to produce films, hydrogels, and nanoparticles for biomedical and food applications while phosphorylation performed using phosphorous pentaoxide in methane sulfonic acid as a solvent result in chitosans with high water solubility and metal chelating important for application in tissue engineering, drug delivery intermediates and in food industries [222]. Carboxyalkylation of chitosan produces water soluble and amphoteric chitosan excellent water soluble, nontoxic, biocompatible and biodegradable polymers suitable for biomedical applications as antimicrobial agents, in biosensor, wound healing, food industry and bio-imaging [222]. O-alkylating chitosan makes it soluble in chloroform, ethanol, water and acetic acid, etc. [88] while phosphorylation, quaternization and carboxymethylation of chitosan significantly improve the solubility of this polymer in different solvents at ambient conditions.

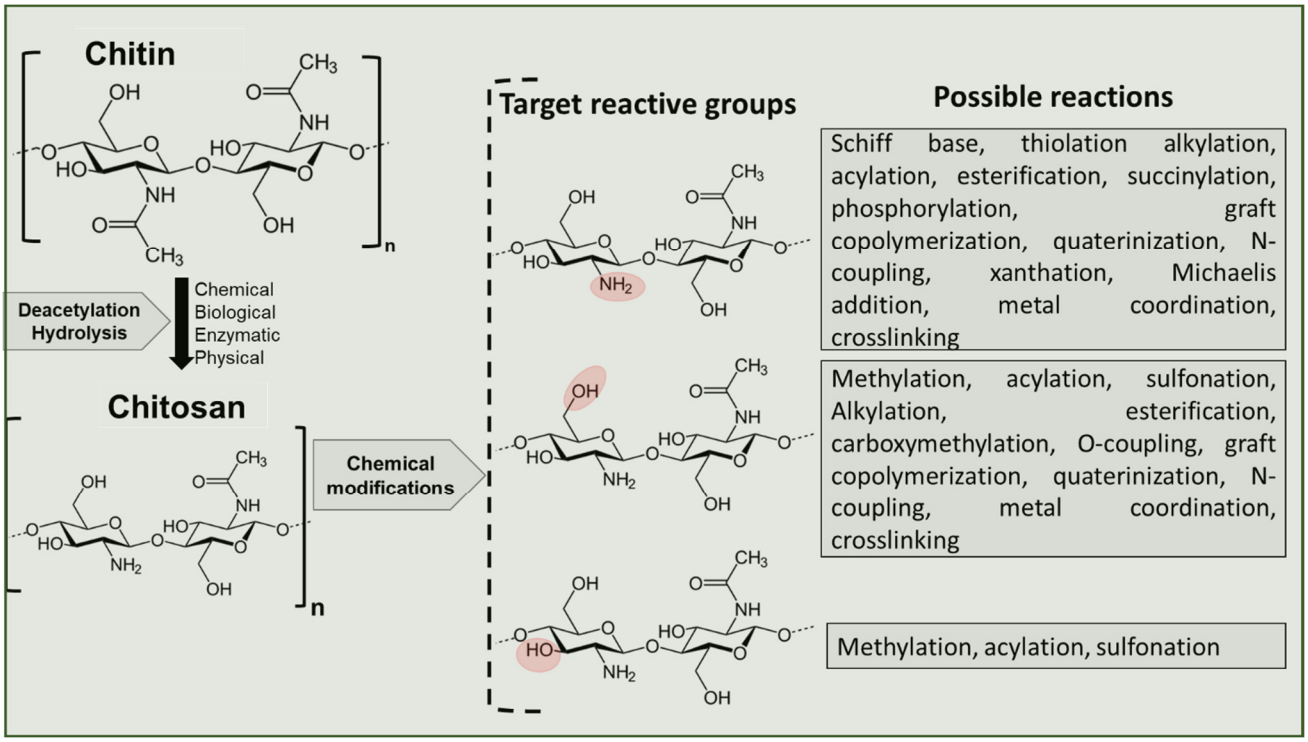

Figure 4. Target functional chitosan molecules (–OH, –NH_2) groups for chemical modification.

Graft copolymerization/cross-linking are also very important strategies used to tailor and broaden chitosan applications. To achieve this, various redox initiator molecules such as Fenton's reagent, ceric ammonium nitrate, ammonium and potassium persulfate, potassium diperiodatocuprate and ferrous ammonium sulfate, enzymes, and microwave irradiation and γ-irradiation are widely used [96,222]. Free radical initiated grafting is one of the mostly commonly used approaches. For example chitosan-graft-poly (*N*-hydroxy ethyl acrylamide) using potassium persulphate initiator [223], polyacrylonitrile-g-chitosan (PA*N*-g-CS) in the presence of an initiator ceric ammonium nitrate [224], binary grafted chitosan with two monomers [acrylamide and (2-methacryloyloxyethyl) trimethyl ammonium chloride] via γ-radiation [225] graft copolymer of chitosan with poly [2-(acryloyloxy)ethyl trimethylammonium chloride] in the presence of potassium persulphate initiator [226], chitosan-g-polyaniline in the presence of APS [227]. Similarly, various crosslinking molecules such as simple phenolic compounds, glutaraldehyde, epichlorohydrin, ethylene glycol, diglycidyl ether and sodium tripolyphosphate etc. are used [11,88,227,228]. Glutaraldehyde mediated

cross-linking of chitosan by forming a Schiff base (imine) is the most studied technique which leads to condensation reaction between the aldehyde function and a primary amine group from the chitosan chain in the presence of labile hydrogen. Cross-linking using glutaraldehyde produces polymers with high adsorption tendency for several metal ions as follows: Cd > Cu > Ni > Ag > Pb > Zn [222]. Benzoyl chitosan biopolymers that play a significant role during drug delivery, cosmetics, wound healing management derived from the synthesis of o-benzoyl chitosan derivatives of benzoic acid and *p*-methoxybenzoic acid were produced in trifluoroacetic acid anhydride/phosphoric acid mediated acylation [222]. Recently chitosan-based hydrogels with fast gelling, tunable elasticity and mechanical properties were obtained through Schiff-base crosslinking of dialdehyde debranched starch with chitosan amino groups [229]. On the other hand, epoxidation reactions are used to obtained chitosan hardened polymers [206,230].

Radiation-induced modification of chitosan is an emerging toxic free alternative process to the use of chemical crosslinking molecules. Several studies have demonstrated the possibility of introducing a variety of functional molecules into chitosan backbone through radiation including synthesis of chitosan-g-maleic acid copolymers [231], grafting of acrylic acid, acrylamide and acrylonitrile on to chitosan via microwave radiation [232]. Similarly to radiation, enzymes are also emerging as the safest strategy to modify or synthesize chitosan-based polymers. Many chitosan derivatives with unique properties such as increased water-solubility, thermal stability, pH-sensitive, adhesive have successfully been synthesized. TEMPO/laccase redox system has been used to selectively oxidize the chitosan C_6 group in order to generate water soluble chitosan [233], synthesizing chitosan-based hydrogels using lignin and simple phenolics as crosslinker [169,230,234]. For example, phosphorylase was used to produce amylose-grafted chitosan copolymers by reacting chitosan and α-d-glucose 1-phosphate [235]. Summarizing, there is no shortage developing strategies for tailoring chitosan for many industrial applications.

7. Conclusions and Future Perspectives

Significant progress is being made in developing technologies for producing chitosan from other novel and attractive emerging sources such as insects and fungi, thanks to advances in insect farming (insect biotechnology) and fungal fermentation processes. These new sources not only providing a new source of chitosan but rather chitosan with superior properties that can easily and safely be used in food and medical, pharmaceutical applications and overcome the challenges often encountered with marine based chitosans obtained as by-product of food processing industry. The observation that chitosans with different DDA and Mw greatly influence their inherent properties and, hence, their function increases the scope of tailoring it for specific applications especially in tissue engineering and wound healing process. The fast-increasing demand for chitosan associated with increasing understanding its properties, extraction techniques and increasing numerous ways of chemically modifying and tailoring its properties is significantly expanding field of applications of chitosan. Although marine crustacean chitosan has predominantly been used in industry due to huge availability generated from the food industry, fungi and insect chitosan will in future become more mainstream raw materials due to increasing availability driven by advances in biotechnological processes for their mass production. For example, the recent increase in exploiting insects and the fast-expanding insect biotechnology field, as a new source of protein for both human and animal feed and increasing fermentation technology knowledge in fungal biomass and excellent properties, are once again propelling chitosan into an important strategic raw material for the future. The real utilization potential of chitosan for industrial applications is only just starting.

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Effectiveness of chitosan as an alternative to sulfites in red wine production

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Abstract and figures

Part of sulfur dioxide used to preserve wine reacts with several wine compounds and it is not anymore active and capable of performing its antioxidant and antiseptic functions. In this work, with the aim to decrease the amount of SO2-bound to acetaldehyde, the use of chitosan in pre and post fermentative phases during the production of Aglianico red wine was investigated. Two winemaking batches, one made of 100% of healthy grapes and one composed of 50% healthy grapes and 50% berries damaged by acid rot, were considered. Results highlight that the use of chitosan just after the end of the fermentation allowed a lower production of SO2-bound to acetaldehyde in finished wines. Furthermore, when chitosan was used in post-fermentative phases, a higher content of polymeric pigments and a lower content of tannins reactive towards proteins was observed. All these effects were less evident when partially acid rot contaminated grapes were used.

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ORIGINAL PAPER

Effectiveness of chitosan as an alternative to sulfites in red wine production

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Abstract
Part of sulfur dioxide used to preserve wine reacts with several wine compounds and it is not anymore active and capable of performing its antioxidant and antiseptic functions. In this work, with the aim to decrease the amount of SO_2-bound to acetaldehyde, the use of chitosan in pre and post fermentative phases during the production of Aglianico red wine was investigated. Two winemaking batches, one made of 100% of healthy grapes and one composed of 50% healthy grapes and 50% berries damaged by acid rot, were considered. Results highlight that the use of chitosan just after the end of the fermentation allowed a lower production of SO_2-bound to acetaldehyde in finished wines. Furthermore, when chitosan was used in post-fermentative phases, a higher content of polymeric pigments and a lower content of tannins reactive towards proteins was observed. All these effects were less evident when partially acid rot contaminated grapes were used.

Keywords Red wine · Chitosan · Pre- and post-fermentation · Acetaldehyde · Free and bound SO_2

Introduction

Sulfur dioxide (SO_2) is widely used as a preservative in winemaking due to its antimicrobial and antioxidant properties. Unfortunately, the addition of SO_2 to wines raises health-related objections due to serious allergic reactions incurred by sulphite-sensitive individuals [1], and concerns over sulfites have resulted in regulatory restrictions set by World Health Organization (WHO) and International Organization of Vine and Wine (OIV). Furthermore, excessive quantities of SO_2 in wine can give an unpleasing aroma to wines [2]. For all these reasons the market of wines with low SO_2 levels have been increased in last decades.

However, the use of SO_2 is necessary in several phases of winemaking to produce wines with positive sensory quality. Especially prior of alcoholic fermentation the addition of SO_2 to the must is very important to shorten the fermentation time by repressing non-*Saccharomyces* yeasts and promoting the growth of sulfite-tolerant *Saccharomyces* yeasts (microbial selection) [3]. This protection is especially important to limit the microbial spoilage of must obtained from not completely healthy grapes as them affected by the presence of rotten bunches which present a variegated and high microbial charge [3]. This microbiota significantly influences the flavour of must and wine and the production of secondary products that could invalidate the future protection by sulfur dioxide added at the end of fermentation [4]. Also after fermentation completion SO_2 addition is fundamental to prevent chemical oxidation and microbial spoilage.

It is important to consider that not all SO_2 added to must or wine has antioxidant and/or antimicrobial properties but it is split in several forms and the knowledge of specific activity of each form in wine is an important step to better manage SO_2 addition for preventing wine spoilage without altering healthiness and sensory quality of it. At wine pH from (3 to 4), the majority of SO_2 (>95%) exists in the form of bisulfite (HSO_3^-), with the remainder existing as "molecular" SO_2 and a negligible portion existing as sulfite (SO_3^{2-}). The sum of these forms (molecular SO_2, HSO_3^-, and SO_3^{2-}) is referred to us as "free SO_2". Additionally, HSO_3^- may form covalent

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Similar research

Effect of Different Enological Tannins on Oxygen Consumption, Phenolic Compounds, Color and Astringency Evolution of Aglianico Wine

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Molecules

Luigi Picariello · Alessandra Rinaldi · Martino Forino · [...] · Angelita Gambuti

Background: In the wine industry, in addition to condensed tannins of grape origin, other commercial tannins are commonly used. However, the influence of oxygen uptake related to different tannin additions during the post fermentative phase in wine has not been completely investigated. In this study, we evaluated the influence of four different commercial tannins (namely, condensed tannins, gallotannins,...

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Impact of 5-year bottle aging under controlled oxygen exposure on sulfur dioxide and phenolic composition of tannin-rich red wines

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Aim: This study aims at understanding the impact of the initial phenolic composition on the evolution of red wines after long bottle aging. Materials and results: three different red wines rich in tannins, Aglianico, Casavecchia and Pallagrello, bottled with the same amount of total sulfur dioxide and different amounts of free sulfur dioxide, were analysed after 5 years of bottle aging under controlled exposure to...

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Due to climate change and the consequent rise in grape pH, there is often the necessity of acidifying musts or wines during winemaking. In this study, the effect of early (on musts, during fermentation) and late (on wines, after the end of the fermentation) acidification was evaluated. The experimental design consisted of the preparation of seven wines from the same batch of grapes fermented in a first tank ...

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In order to evaluate the effect of the application of chitosan to red wines with different initial composition, four wines showing tannins/anthocyanins ratio T/A ranging from 0.15 to 2.44 were treated with this amino polysaccharide. As one of the main factors involved in red wine ageing is the oxidation, even a forced oxidation test was applied on all the samples. The addition of chitosan determined a...

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Angelita Gambuti · Luigi Picariello · Alessandra Rinaldi · Luigi Moio

Changes in phenolic compounds, chromatic characteristics, acetaldehyde, and protein-reactive tannins associated with oxidative aging were studied in Sangiovese wines with varied tannin T/anthocyanin A ratios. For this purpose, three Sangiovese vineyards located in Tuscany were considered in the 2016 vintage. To obtain wines with different T/A ratios, two red wines were produced from each...

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Review

Emerging Non-Thermal Technologies as Alternative to SO_2 for the Production of Wine

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Abstract: SO_2 is an antioxidant and selective antimicrobial additive, inhibiting the growth of molds in the must during the early stages of wine production, as well as undesirable bacteria and yeasts during fermentation, thus avoiding microbial spoilage during wine production and storage. The addition of SO_2 is regulated to a maximum of 150–350 ppm, as this chemical preservative can cause adverse effects in consumers such as allergic reactions. Therefore, the wine industry is interested in finding alternative strategies to reduce SO_2 levels, while maintaining wine quality. The use of non-thermal or cold pasteurization technologies for wine preservation was reviewed. The effect of pulsed electric fields (PEF), high pressure processing (HPP), power ultrasound (US), ultraviolet irradiation (UV), high pressure homogenization (HPH), filtration and low electric current (LEC) on wine quality and microbial inactivation was explored and the technologies were compared. PEF and HPP proved to be effective wine pasteurization technologies as they inactivate key wine spoilage yeasts, including *Brettanomyces*, and bacteria in short periods of time, while retaining the characteristic flavor and aroma of the wine produced. PEF is a promising technology for the beverage industry as it is a continuous process, requiring only microseconds of processing time for the inactivation of undesirable microbes in wines, with commercial scale, higher throughput production potential.

Keywords: sulfur dioxide; pasteurization; pulsed electric field; high pressure processing; microbial inactivation; *Brettanomyces*

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1. Introduction

Oxidation and the undesirable activity of specific microorganisms have a negative effect on wine quality and shelf life. Sensory quality is the most important factor for wine consumers. Most sensory related attributes are largely dependent on wine's phenolic composition which determines color, bitterness and astringency [1]. The loss of wine quality during storage is often accelerated due to exposure to sunlight, high temperatures, oxygen, vibration, pH, contaminants from the storage environment surrounding the wine or cork, microbial spoilage and the failure of bottle closures. The storage of wine for ageing and maturation depends on chemical composition and equilibria, with specific flavors and characteristics, which increase wine quality, developing during this period. On the contrary, the quality of white wines typically does not improve during storage, so they can be sold and consumed straight after production within the first 1 to 2 years. On average, red wines have a longer shelf life than white wines due to their higher phenolic concentration, which reduces their susceptibility to oxidation.

In winemaking, sulfur dioxide (SO_2) is often used at different stages in the production process (e.g., after harvesting the fruit, after crushing, added to the must before fermentation, before maturation, before bottling). SO_2 has the ability to control oxidative processes including polyphenol oxidase and to inhibit Maillard reactions. If left untreated, oxidation can lead to a decrease in the sensorial and nutritional quality of wine [2,3]. In addition to antioxidant action, SO_2 also exhibits antimicrobial capacity against spoilage

microorganisms, inhibiting the growth of molds in the must during the early stages of wine production, as well as undesirable bacteria and yeasts during fermentation, preventing unwanted secondary fermentation and the formation of yeast haze [4], and thus avoiding microbial spoilage during wine production and storage. The addition of SO_2 to wine before bottling leads to an increased shelf life, with less likelihood of the formation of off-odors. SO_2 exists in a bound and free form, the latter being the active form of the compound. The amount of each form present depends on the pH of the wine [3,5]. As wine pH increases, antimicrobial capacity decreases. The addition of SO_2 can also increase the extraction of phenolic [2,3].

The excessive use of SO_2 can have a detrimental effect on wine quality including the neutralization of wine aroma, the formation of hydrogen sulfite, unwanted aromas and flavors and cloudiness after bottling [3,6]. Moreover, SO_2 can have adverse effects in humans including allergic reactions, headaches, asthma, dermatitis, abdominal pain, diarrhea and bronchoconstriction. As SO_2 is a commonly used preservative in the wine industry, it is also important to consider the cumulative effect it has on the consumer [4]. This led to the establishment of strict regulations and limits governing SO_2 application in wineries. The SO_2 regulatory limits for wine preservation are constantly being reviewed and reduced [2,3]. Currently, the International Organization of the Vine and Wine (OIV) recommends 150 mg/L total SO_2 for red wines, the European Union limits the total use of SO_2 to 160 mg/L for red wines and 210 mg/L for white and rosé wines and Australia permits 350 mg/L total SO_2 for all wines [3]. The use of fungal-source chitosan for the inactivation of *Brettanomyces* has also been authorized by the OIV and European Union (Regulation (EC) No 606/2009) [7]. Although SO_2 free wines are considered healthier, more natural and sustainable, it is a challenge to produce wines without the addition of SO_2. Consequently, the wine industry is interested in finding alternative strategies to reduce or eliminate SO_2 in wine production, while maintaining wine quality. To be successful, the alternative must provide the same level of microbial stability and antioxidant activity while also safeguarding the quality of the wine produced, and be less harmful to humans [3]. The use of thermal technologies is unacceptable for the wine industry because of their detrimental effects on the delicate organoleptic characteristics of wine (e.g., flavor, aroma and color) [8]. Thus, the application of non-thermal technologies to produce, age and preserve wine is an area of great interest. Ideally, these technologies will allow the reduction in the use of SO_2 additive in wine production, while keeping or improving the original characteristics of the produced wine [4,9–12]. Van Wyk et al. (2018) compared sensory, microbiology and other quality parameters in wine subjected to SO_2 addition, HPP and PEF treatments during one year storage [13]. No sensory differences were detected between HPP and PEF treated wines and the untreated wines after being stored for one year [13]. The inactivation of polyphenoloxidase enzyme by US, PEF and HPP has been demonstrated [14], and this could be another way to control the undesirable change in the polyphenol profile of wines.

In this investigation, a review of the application of the following non-thermal technologies for wine pasteurization was carried out: pulsed electric fields (PEF), high pressure processing (HPP), ultrasound (US), high pressure homogenization (HPH), low electric current (LEC), ultraviolet irradiation and filtration. The specific objectives were: (i) to present the fundaments of the non-thermal technologies mentioned and their benefits in terms of wine quality; (ii) to review and introduce the main microorganisms of concern that can potentially spoil wine; (iii) to investigate the effect of non-thermal technologies on microbial inactivation and compare the technologies in terms of the efficiency of key microbes' inactivation in wine; (iv) to discuss the commercial viability of using non-thermal technologies to reduce or eliminate the use of sulfur dioxide in the wine industry.

2. Non-Thermal Cold Pasteurization Technologies for Wine Preservation

2.1. Pulsed Electric Fields (PEF)

PEF, a relatively novel non-thermal technology, is a promising alternative for wine preservation because it operates in continuous mode [4] (Figure 1). Depending on the application, it can have a relatively low energy consumption compared to other food preservation technologies [15]. Since PEF is a continuous process, it is easier to integrate into existing industrial processes. In operation, short microsecond pulses of high electric field strength are applied to pumpable beverages flowing between two electrodes [16]. The shape of the applied pulses is either exponential, where the voltage rises quickly to its maximum before decaying slowly to zero, or square where it remains constant for a few microseconds [16–18].

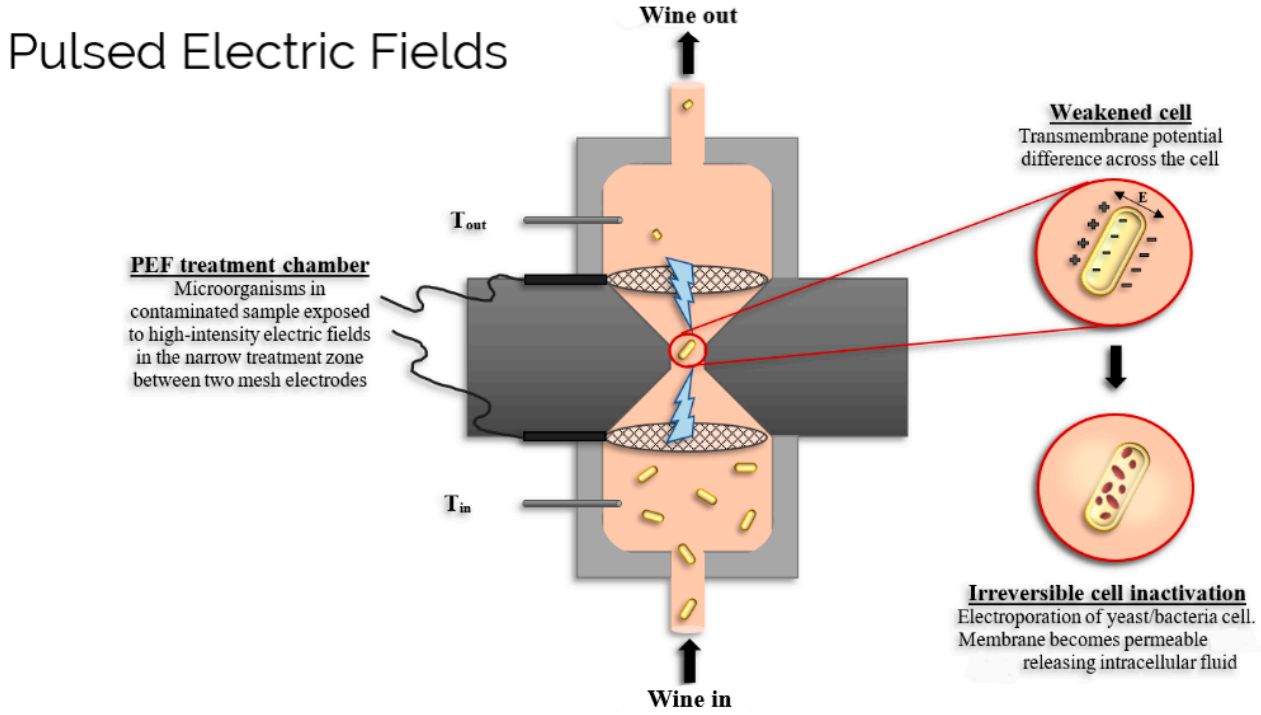

Figure 1. Diagram showing the pulsed electric field (PEF) inactivation of wine spoilage microorganisms (T refers to temperature which is below 40 °C for a non-thermal process).

A typical PEF system is based on a high voltage pulse generator along with a treatment chamber, a suitable fluid handling system, and monitoring and controlling devices [4,16]. PEF is able to inactivate spoilage microorganisms, often without any significant effect on beverage quality [16,17]. Figure 1 shows an example of a PEF unit including details of some components (e.g., PEF treatment chamber, electrodes, thermocouples) and also information on the mechanism of microbe inactivation in the beverage. Microbial inactivation is thought to occur due to the formation of a potential difference across the microbial cell membranes. This in turn causes the permeabilization or electroporation of the cell membrane, leading to the loss of intracellular fluids [19]. The electric field strength required for the inactivation depends on the size and shape of the cell as well as the composition of its membrane. The bigger the size of the cells, the more susceptible the cells are to the applied electric field pulses; for example, vegetative yeasts are more susceptible than vegetative bacteria cells [20]. Rod-shaped cells required five times stronger electric fields than spherical cells of similar dimensions [19].

Most authors have reported a temperature increase associated with increasing treatment electric field strength or energy [16]. This increase in temperature also increases the rate of inactivation by improving the cell membrane fluidity and mass transfer from the cells [19]. As this temperature increase is not desirable for wine, proper processing

conditions must be selected to ensure a non-thermal process, namely flow rate, initial wine temperature and electric field intensity.

Examples of PEF processed beverages include grape-, strawberry-, orange- and apple juices, milk, soup, liquid egg and beer [15,16,18,20–22]. PEF can also be operated or designed as a batch process, thus cured fish and meat products have also been processed [23]. One of the main reasons why PEF has not been commercially implemented is the need for high-voltage electric pulses, which pose a significant safety risk in commercial scale operations.

Impact on Wine Quality

Abca and Evrendilek (2014) [20] found that subjecting red wine to three μs bipolar PEF pulses at 31 kV/cm ($T \leq 40$ °C), resulted in no changes to sensory quality and the treatment had no effect on total phenolic content, antioxidant activity or color. Using 100 pulses of the same electric field strength (31 kV/cm), Puértolas et al. (2009) [17] concluded that the color and odor of PEF treated red wine remained unchanged. Red wine processed using 20 kV/cm ($T \leq 42$ °C) for 10,000 μs, had no effect on the anthocyanins present in the wine, but led to a slight increase in tannin concentration and slight changes in the total phenolic index and color intensity [19].

PEF also has other applications in wine production. The technology has been applied to grape pomace and grape skins during the early stages of wine production to improve final wine quality or reduce maceration time. Puértolas et al. (2010a) [24] concluded that a pilot-scale PEF unit was able to produce a high phenolic content wine that can subsequently be used to create a high-quality oak aged red wine. The crushed Cabernet Sauvignon grapes were treated using 50 pulses at 5 kV/cm (122 Hz, 3.67 kJ/kg, <30 °C), after which potassium metabisulfite was added before fermentation [24]. The treatment of Cabernet Sauvignon grape juice with skins using square pulses at 5 kV/cm for a residence time of 0.41 s (122 Hz, 50 pulses, 3.67 kJ/kg, ≤17 °C), produced a finished wine that required a shorter maceration time and had a higher Folin-Ciocalteu index, polyphenol concentration and color intensity [22]. López et al. (2008) [25] tested the effect of PEF treatment (10 kV/cm, 50 pulses, 1 Hz, 6.7 kJ/kg, <30 °C) of grape skins on wine quality factors. The prior PEF treatment reduced the duration of maceration during vinification and increased the quantity of phenolic compounds in the final wine. A low electric field strength of 5 kV/cm to treat Cabernet Sauvignon grape pomace led to an increase in the color intensity, total phenolic content, tannins and anthocyanins of the final wine produced [26]. López et al. (2009) also found that the PEF treatment of grape pomace was able to reduce the required maceration time from 268 to 72 h [26].

2.2. High Pressure Processing (HPP)

HPP is a modern commercial non-thermal technology that can be used to extend shelf life by inactivating spoilage and pathogenic yeasts, molds and bacteria in wine, beer, juices, solid foods and other products through the application of isostatic and uniform pressures usually between 100 and 600 MPa [4,27–35]. Microbial inactivation can generally be increased by increasing pressure or holding time [29,30,35]. According to previous research, HPP has a greater effect on larger molecules such as enzymes, proteins, and lipids, whereas smaller molecules including vitamins, flavor and several bioactive/health related compounds are generally unaffected. Thus, HPP can better maintain the quality of food, without any significant impact on flavor [32,36–38]. Microbial inactivation can occur due to the disruption of cell membranes, protein denaturation and solute loss (Figure 2). Research suggests that microbial inactivation by HPP involves the perforation of the cell membrane, the formation of dimples and swellings or the overall shrinkage of the volume of the cells [35,39,40]. The financial feasibility of HPP is also greatly increased by using the shortest possible processing time that still achieves the desired level of pasteurization [4].

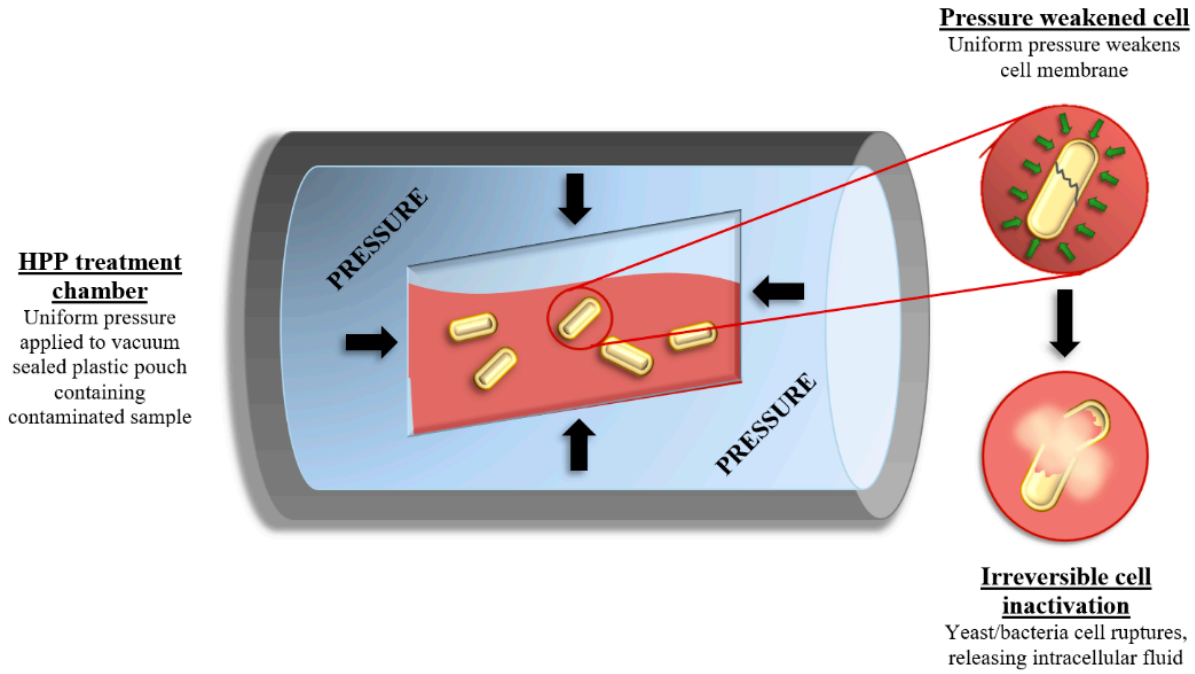

Figure 2. Diagram showing the high-pressure processing (HPP) inactivation of wine spoilage microorganisms.

A HPP unit consists of a vessel, a pressure generating pump and a yoke for safety. In the HPP chamber, pressure is applied uniformly throughout a pressure medium and the sealed product. Usually, vacuum-sealed flexible pouches are used for the "in pack" process, as they can withstand both a decrease in volume during compression and the associated expansion on decompression. More recently, Hiperbaric developed a technology named "in bulk" HPP for processing liquids before packaging, allowing the use of any type of rigid container (e.g., glass, can, etc.). Due to the adiabatic compression inside the chamber, a temperature increase of around 3 °C per 100 MPa occurs [11,28]. The increase in temperature is dependent on the properties of the food and can be reduced by decreasing the rate of pressurization [4]. The maximum temperature after compression can be reduced by starting the process with a low initial temperature of the beverage.

Most HPP units are operated in batch, but semi-continuous units for pumpable products have now also been developed by industry [41]. Due to HPP being independent of the shape and size of the product, the scale-up to commercial size is relatively simple. When HPP is unable to produce the required level of microbial inactivation, it can be combined with reduced quantities of preservatives or other preservation technologies [42].

Since 2000, the usage of HPP technology worldwide has increased exponentially and has been implemented commercially in Japan, the USA and across Europe. At the end of 2010, the annual world-wide HPP production capacity reached 250,000 tons. The total number of installed HPP units has been increasing quickly in the food and beverage industry: 315 in 2015, >400 by 2016, etc. [41]. The current industry suppliers of HPP units include Avure (Ohio, USA), Hiperbaric, S.A. (Burgos, Spain), Uhde High Pressure Technologies GmbH (Hagen, Germany) and BaoTou Kefa High Pressure Technologies (BaoTou, China). In 2013, Hiperbaric and Avure introduced the largest HPP unit with a capacity of 525 L and throughput of 3000 L or kg per hour [41]. HPP processed products currently available on the market include fruit and vegetable juices/smoothies, other beverages, pre-cooked meals and meat- and fish-based products [41,43,44]. The application of HPP is currently limited to high-value products due to the high initial capital investment required [41]. The cost of production must therefore be offset by the increase in the value of the product produced through an increase in quality, shelf life, consumer convenience, reduction in preservatives and lower transportation, labor or storage costs. In conclusion, the advantages of HPP include: (i) operation at or below room temperature; (ii) microbial

inactivation with no heat damage and fewer preservatives and other additives, improving sensorial and nutritional quality; (iii) instantaneous transfer of pressure, regardless of liquid size and geometry; and (iv) the creation of novel functional food properties, including new structures and textures [45]. This makes HPP an attractive alternative technology for use in the wine sector.

Impact on Wine Quality

In 1996 Lonvaud-Funel et al. [46] investigated the effect of high pressure on microbial inactivation in wine for its stabilization. HPP treatment of 650 MPa for 15 min caused no change in the overall sensory quality of red wine and 11 sensory attributes [47]. The same study found that total phenolic content, tartaric esters, flavonols and tannins decreased when wine was subjected to the same HPP treatment. Using milder conditions of 500 MPa for 5 min, Santos et al. (2013a) [48] found no change in the antioxidant activity and b* color parameter of red wine directly after processing. Storing the wine in bottles caused minor changes in the wine sensory properties after 9 months, and higher L^*, a^* and b^* color parameters and lower antioxidant activity after 12 months in-bottle storage [48]. Puig et al. (2003) [9] detected no effect on organoleptic quality when subjecting red wine to the same conditions (500 MPa, 5 min). Mok et al. (2006) [27] found that HPP treatment at 350 MPa for 10 min had no effect on the mouthfeel, aroma and taste of red wine.

Regarding white wine, Puig et al. (2003) [9] detected no organoleptic difference between untreated and 500 MPa, 5 min treated wine. Santos et al. (2013b) [49] found that the same HPP conditions had no effect on the antioxidant activity and total phenolic content of white wine. Processing liquor white Sauterne wine using 355 MPa for 10 min resulted in no significant difference in the sensory quality [50]. Red grape must subjected to 551 MPa for 10 min did not have any effect on the color and little or no effect on the sensory quality of the final wine produced [51].

Research has shown that HPP can lead to the acceleration of wine ageing [45,48]. This suggests that HPP has the potential to shorten the time required to produce a desirable, high quality 'aged' wine. HPP can also change wine color involving decreased color intensity and an increase in brownish color often associated with aged wines [48,49].

2.3. Power Ultrasound (US)

Power ultrasound (US) is a non-thermal technology with the potential to be used as a wine preservation technique. Sound intensity or acoustic intensity between 10 and 1000 W/cm^2 [52–54] producing waves with frequencies between 20 and 100 kHz are applied to liquid samples causing the formation of small bubbles. When these bubbles collapse, a phenomenon known as cavitation occurs. Localized high pressure (50,000 kPa) and temperature (5000 °C) regions are formed as well as free radicals, shock waves and shear forces. High frequency small bubbles result in a less intense but more uniform acoustic field with a greater rate of cavitation collapse during sonication [55]. In ultrasound units, a generator converts electricity into high-frequency alternating current, followed by the conversion of current into mechanical vibrations [56]. Liquid foods are sonicated by the direct contact of a probe, also known as a sonotrode, horn, finger or ultrasonic tip [56]. US units have also been designed to operate in batch or continuous mode, with cooling water jackets to control temperature during operation [57], as a temperature increase is expected [55,58,59]. The microorganisms are inactivated due to the disruption of their cell membranes/walls. The technology has also been found to be good for food deteriorative enzyme inactivation (e.g., polyphenoloxidase) [14,56,58]. Cavitation may affect the chemical and physical properties of processed foods and can accelerate the wine aging process [15,60].

The efficiency of US is dependent on the viscosity, vapor pressure and surface tension of the liquid sample treated. Microbial inactivation by US also depends on the shape, size and species of microorganism treated, with smaller cells believed to be less sensitive than larger cells [53,55,61,62].

Potential uses of US in the wine industry include wine aging/maturation, improved fermentation, sanitation of barrels and equipment, and the extraction of bioactive aromatic and phenolic compounds from grapes and must. US can also be used to improve the penetration of the wine into the structure of oak barrels and chips, improving oak vanilla, caramel, cream, earthy and spice flavors [4]. US has been found to be more effective at sterilizing wine barrels compared to existing methods, while also providing a significant cost reduction [55]. As this technology is already used commercially for food applications (e.g., extraction of compounds, emulsification, and degassing), it should be easy to scale up for pasteurization purposes. According to Gracin et al. (2016) [54], the scale-up of continuous flow US is possible, which significantly improves its ability to be utilized in the wine or other beverage industry.

Impact on Wine Quality

Singleton and Draper (1963) [63] found that using ultrasound treatment at 90 kHz for 60 min in direct contact with wine, increased the tannin concentration and changed the overall flavor of red wine. Indirect water bath ultrasound treatment (30 kHz, 20 kPa) of red wine over a prolonged period of 10 days resulted in increased anthocyanin concentration, and a decrease in L^* color parameter [64]. Similar to Singleton and Draper (1963) [63], Luo et al. (2012) [53] found that treating red wine (24 kHz, 0.2 W/mL, 22 mm radius probe) led to significant changes in flavor and aroma. Zhang and Wang (2017) [65] investigated the effect of US on red wine directly after treatment and also after 70 days storage. Directly after US processing using 20 kHz, 950 W for 14 min, color density and visual characteristics increased. After storage, the trend in changing phenolic composition was similar in the US treated and untreated wine. Lastly, Singleton and Draper (1963) [6] studied the effect of US on white wine. Treatment conditions of 90 kHz and 35 W for 60 min resulted in an increase in tannin concentration as well as the generation of a 'scorched' off-flavor. One other potential application of US in the wine industry is the acceleration of wine maturation. Ultrasound treatment was found to accelerate changes in wine quality associated with wine aging, thereby shortening vinification time [63,65].

2.4. Ultraviolet (UV) Irradiation

UV light is an energy efficient technology that employs the use of UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm) radiation for microbial inactivation [5]. The UV microbial inactivation mechanism is thought to involve damage to microbial cell DNA, inhibiting reproductive and other cell functions [5]. UV-C penetrability and effectiveness depend on the absorbance, color, density, soluble solids and suspended materials in the beverages [5,66]. Industrial applications of UV include the disinfection of water, surfaces and food packages.

Impact on Wine Quality

Falguera et al. (2013) [6] used UV-visible irradiation (250–740 nm, 0–210 min, 25 °C) to process 800 mL of white wine must. The same level of preservation (polyphenoloxidase inactivation) was achieved as with the use of SO_2 without a significant change in quality parameters including pH, alcohol and tartaric acid content. The ability of UV-C (254 nm) to inactivate yeasts (*Brettanomyces bruxellensis* and *Saccharomyces cerevisiae*) and bacteria (*Lactobacillus plantarum, Pediococcus acidilactici, Oenococcus oeni* and *Acetobacter aceti*) in grape juice and wines was investigated [66]. Dosages of up to 3672 J/L (flow rate of 4000 L/h) were able to cause microbial log reductions within a range of 4.3 to 5.0 in Chardonnay and Pinotage wines, and Chenin Blanc and Shiraz juices. UV-C was able to stabilize the grape juice and wine, reducing the required level of SO_2 [66]. However, wine color deteriorated as polyphenol-oxidase activity was not prevented, so a residual use of SO_2 was still necessary [6]. As expected, ultraviolet was more effective in white wine than red wine [5].

2.5. High Pressure Homogenization (HPH)

HPH is mainly known for preventing milk creaming in homogenized milk. The main differences between HPP and HPH is that the latter can be used in a continuous operation mode and employs much lower pressures than HPP.

Impact on Wine Quality

Comuzzo et al. (2015) [67], found that HPH treatments between 50 to 150 MPa produced wine with stronger fruity aromas and less off-flavors. Additionally, multiple HPH passes at 200 MPa resulted in no significant impact on the sensory attributes of red and white grape musts. HPH technology can also be used to disrupt and break microbial cell membranes [68].

2.6. Filtration

Filtration has the ability to decrease microbial growth, reduce browning and prevent haziness through the removal of colloids [69]. The suitable pore size for wine filtration depends on a number of factors including the effectiveness of removal of targeted microorganism, throughput and economic viability. To remove yeasts and bacteria, it is generally recommended to use a pore size of 0.45 µm [69]. However, it has been reported that the cell size of yeasts such as *B. bruxellensis* decreases as a result of SO_2 treatment [70]. As a result, filtration using 0.45 µm pore size after SO_2 treatment could be insufficient for the removal of damaging spoilage microorganisms such as *B. bruxellensis* [71]. Due to membrane fouling, wine quality and permeation rates are often reduced. This in turn decreases the economic viability of filtration [19,69].

Impact on Wine Quality

Filtration can often have a detrimental effect on wine characteristics, most significantly sensorial properties [43,69]. The smaller the filter/membrane pore size, the greater the of negative impact on wine quality. Due to its effect on the colloidal structures of wine, filtration reduces the color intensity, viscosity and body of wine [17,72,73]. Arriagada-Carrazana et al. (2005) [74] conducted a study investigating the effect of membrane filtration on the phenolic quality and aromatic profile of a Cabernet Sauvignon wine. A 1.2 µm pre-filter followed by a 0.65 µm filter led to a reduction in color intensity and polyphenolic profile. A sensory panel, conducting a triangle test, confirmed the reduction in color and polyphenolic quality. Membrane adsorption was thought to have caused the observed difference in wine quality [74].

2.7. Low Electric Current (LEC)

As opposed to PEF, LEC uses a constant low-power electric charge ($\leq$200 mA) over prolonged treatment times up to several months to reduce the integrity of the membranes of spoilage microorganisms [69].

Impact on Wine Quality

LEC treatment of red wine using 200 mA for 60 days had no effect on wine color or odor [73]. The ability of LEC (200 mA, 90 days, <15 °C) to prevent the formation of undesirable flavors by *D. bruxellensis* during storage in oak barrels was compared to SO_2 treatment [75]. A sensory panel of 18 analyzed the wines after four months in the barrels and found that there was no difference between the two wines in terms of phenolic content or sensory properties. Low-voltage treatment was shown to have a positive effect on grape juice fermentation by subjecting SO_2-free grape must to 200 mA for 16 days [76]. LEC had no effect on the growth of *S. cerevisiae* while increasing the death rate of apiculate (lemon-shaped cell morphology) yeasts.

3. Microbial Wine Spoilage

The microbes are the main targets of the preservation technologies presented in the following Section (Section 4—Effect of PEF, HPP and other non-thermal technologies on microbial inactivation in wine). This section is based on a previous publication by Van Wyk and Silva (2019) [4]. Yeasts and bacteria are common types of wine spoilage microorganisms which can have negative effects on wine quality and shelf life, leading to detrimental economic losses. As the yeast *Saccharomyces cerevisiae* is generally more tolerant to high ethanol concentrations compared to other microorganisms [77], it is widely employed for several industrial fermentation processes, including the production of alcoholic beverages. With respect to wine, *S. cerevisiae* is the most abundant microorganism found in the final wine at the end of fermentation, converting the must sugars into alcohol and generating important compounds (e.g., aroma), which are vital for the final wine properties. However, it is important to control the activity of this oenological yeast after fermentation to keep the wine desirable properties and stability during storage. This fermenting yeast can be controlled in wine by SO_2 additions or inactivation with non-thermal processes, as investigated by a number of authors.

Contaminant yeasts detected in wines belong to the genus *Brettanomyces/Dekkera*, *Saccharomyces*, *Schizosaccharomyces* and *Zygosaccharomyces*, while spoilage bacteria include *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Pediococcus* and *Acetobacter* [2]. Off-odors, haziness and precipitation are indicators of microbial spoilage. The source of contamination can be the grape skins, insects, winery walls and other equipment in contact with the grapes, grape juice, must or wine. As red wines' grape skins and stems are in contact for longer periods of time with the juice/wine during fermentation, these wines are more frequently contaminated than any other wine type [2,78,79]. The wine industry prevents microbial contamination by ensuring grape quality, proper sanitation of winery equipment including oak barrels, and controlling oxygen and sulfite levels [79].

3.1. Brettanomyces Yeast

Brettanomyces or *Dekkera* (name given to the spore forming microorganism) poses a great threat to the wine industry, leading to detrimental economic losses worldwide [2,79,80]. This yeast has been detected in wines and wineries across the world, including all major wine producing countries [79,80]. The genera *Brettanomyces/Dekkera* consists of five species: *B. custersianus*, *B. naardenensis*, *B. nanus*, *B. anomalus* and *B. bruxellensis* [80]. *B. bruxellensis* is mainly found in barrel-aged red wines with low SO_2 content and high pH. The presence of less than 10^4 cfu/mL can have a detrimental effect on the sensory quality of wine, resulting in unpalatable off-odors and -flavors [2,78,79]. *Brettanomyces* yeasts are infamous for causing mousy off-flavors, also known as '*Brett*. character'. The off-odors produced are characterized as being 'barnyard-like', 'medicinal', 'Band-aid®' or 'horsey'. The chemical compounds responsible for the off-flavors and -odors are 4-ethylguaiacol (4-EG) and 4-ethylphenol (4-EP). *Brettanomyces* is the only known microorganism to cause the formation of these compounds in wines [78–81]. Depending on the species and strain, *Brettanomyces* can also cause the formation of acetic acid known as vinegar taint. When volatile fatty acids are produced, they can cause rancid and/or cheesy flavors and odors [80,81]. Survival studies conducted by Barata et al. (2008) [82] found that most *D. bruxellensis* strains are able to grow in environments with ethanol concentrations as high as 15% (*v*/*v*). Temperatures exceeding 36 °C over a period of 12 h resulted in complete loss of viability [82]. Additionally, these yeasts are able to survive and grow in environments with low pH and nutrients [69,78,81], although these environmental stresses can cause the yeasts to change into a viable but non-culturable (VBNC) state. While in this dormant state, the cells cannot be cultured without resuscitation even though they are still alive. Some researchers have found that this can sometimes occur directly after the addition of sulfur dioxide [69].

3.2. Other Spoilage Yeasts

Zygosaccharomyces bailii is another yeast that can cause cloudiness in bottled wine through the formation of flocculants and granular deposits. It can also produce acetic acid and metabolize malic acid resulting in off-odors and pH increase. Due to *Z. bailii*'s high resistance to yeast inhibitors including sulfur dioxide and tolerance to high ethanol (18%) environments, it can be difficult to control this yeast in wine [2,83]. Film-like growths can form on the surface of wines due to the presence of *Saccharomyces cerevisiae, Saccharomyces bayanus*, *Zygosaccharomyces fermentati* and species of *Candida, Pichia* and *Hansenula* [83].

3.3. Bacteria

The number of bacteria found on grapes varies depending on their condition, with healthy fruit typically having considerably less than damaged grapes. Spoilage caused by lactic acid bacteria typically occurs in warm environments with a pH higher than 3.5 and insufficient sulfur dioxide. *Lactobacillus brevis* and *Oenococcus oeni* cause the transformation of tartaric acid to lactic acid, which leads to a rise in pH, a dull red-brown color in red wines, an increase in carbon dioxide, cloudiness, and the formation of viscous deposits and mousy off-odors. *L. brevis* and *Lactobacillus buchneri* can also cause bitterness. Furthermore, *O. oeni* and *Pediococcus* can cause ropiness, characterized by the flotation of silky threads in spoiled wines [2,83]. Since the 19th century, acetic acid bacteria, including strains of *Gluconobactera* and *Acetobacter*, have been known to cause the oxidation of ethanol to undesirable acetic acid and the oxidation of polyols to ketones. Due to the ability of these acetic acid bacteria to survive anaerobic conditions, they are able to grow in barreled and bottled wines. Bacteria of the genus *Bacillus* cause the formation of sediment and earthy, musty off-odors [83]. Bacteria can be kept under control in wine by maintaining a low pH and temperature environment, minimizing the concentration of oxygen and adding sulfur dioxide [83].

3.4. Molds

Molds including *Aspergillus, Penicillium, Alternaria, Botrytis, Cladosporium, Mucor, Oidium, Plasmopara, Rhizopus* and *Uncinula* are known to infect grapes. These can enter in the process in the crushing stage, decreasing the juice yield and increasing the grape pressing time. Molds deteriorate the wine quality by altering its composition, producing off-flavors, and encouraging the undesirable growth of spoilage yeasts and bacteria. The resistance of molds to HPP is very variable, depending on the species [84]. Molds can easily be controlled in wine as they are unable to survive due to their susceptibility to alcohol concentration of $\geq$3% and SO_2 [2].

4. Effect of PEF, HPP and Other Non-Thermal Technologies on Microbial Inactivation in Wine

This literature review showed limited studies of microbial inactivation in wine by some of the seven non-thermal technologies described previously in Section 2. Therefore, this section presents results of microbial inactivation in wine by PEF, HPP and US, and allows a comparison of the efficiency of each technology. The microbial inactivation for different non-thermal PEF, HPP and US conditions is expressed in terms of log reductions and shown in Tables 1–3. The wine composition, the microorganism species and strain are factors that affect the level of inactivation. For example, the wines' electrical conductivity is directed related to its composition, and is crucial for PEF efficiency. The microbial reduction is expected to increase with process intensity and duration (time). Depending on the process, the intensity is given by the electric field strength for PEF (kV/cm), the pressure for HPP (MPa) and the acoustic power density for US (W/mL). With respect to continuous PEF processing, the real treatment time is calculated from the residence time (dependent on the flow rate), the frequency of the pulses, and the pulse width, with low flow rate conditions maximizing the treatment time. Being a batch process, the HPP

conditions are straightforward, i.e., the pressure and time set for the constant pressure phase of the HPP cycle. The US can be set in batch or continuous mode.

Table 1. Inactivation of *Brettanomyces bruxellensis* yeast in wine by non-thermal PEF, HPP and US technologies *.

Pasteurization Technology	Wine	Alcohol Content (% *v*/*v*)	Processing Conditions	Treatment Time	Log Reduction	Reference
PEF	Red	13.0	31 kV/cm, 1 Hz, 100 pulses, batch, T < 30 °C	–	5.2	[17]
PEF	Red	nr	20 kV/cm, 0.5 Hz, 10 µs pulse width, T ≤ 37 °C	6000 µs	>4.8	[19]
PEF	Red	13.5	50 kV/cm, 100 Hz, 1.7 µs pulse width, T < 40 °C	39 µs	3.0	[12]
HPP	Red Cabernet Sauvignon	13.4	400 MPa	5 s	>7.0	[11]
HPP	White Chardonnay	13.0	200 MPa	15 s	>7.0	[10]
HPP	Rosé	12.5	200 MPa	120 s	>6.0	[10]
HPP	Red Pinot Noir	13.0	200 MPa	180 s	6.0	[10]
HPP	Red & white	nr	500 MPa	300 s	6.0	[9]
HPP	Red Cabernet Sauvignon	13.5	200 MPa	180 s	5.8	[10]
HPP	Red Syrah	12.5	200 MPa	180 s	5.0	[10]
HPP	Red SO_2-free Cabernet Merlot	13.7	200 MPa	180 s	3.8	[10]
HPP	Red Dolcetto Syrah	10.5 14.0	200 MPa	180 s	3.0 4.2	[10]
US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.24	[53]

* HPP was carried out at room temperature, maintaining nonthermal conditions; PEF—pulsed electric fields; HPP—high pressure processing; US—power ultrasound, nr—not reported.

4.1. Brettanomyces Bruxellensis

Table 1 shows a summary of *Brett* inactivation expressed in terms of log reductions for different non-thermal PEF, HPP and US conditions. An electric field strength of 20 kV/cm applied to red wine for 6000 µs, led to more than 4.8 log reductions of *Brettanomyces bruxellensis* [19]. Puértolas et al. (2009) [17] achieved 5.2 log reductions of *Dekkera bruxellensis* and 5.8 log reductions of *Dekkera anomala* in red wine using 100 pulses at 31 kV/cm. These results suggest that *D. bruxellensis* in more resistant to PEF inactivation than *D. anomala*. Van Wyk et al. (2019) [12] could reduce the treatment time to as low as 39 µs by increasing the electric field intensity to 50 kV/cm, to obtain 3.0 log reductions in *B. bruxellensis*.

Non-thermal HPP treatment at 400 MPa for only 5 s resulted in the complete inactivation (>7.0 log reductions) of *Brettanomyces bruxellensis* in Cabernet Sauvignon wine [11]. The same study concluded that the strain of *B. bruxellensis* had a significant effect on HPP inactivation. Strain AWRI 1499 proved to be the most resistant, with 3.0 log reductions in red wine after processing at 150 MPa for 10 min. Puig et al. (2003) [3] achieved at least 6.0 log reductions of *B. bruxellensis* using 500 MPa for 5 min (=300 s). Treatment at 100 MPa resulted in no significant *B. bruxellensis* inactivation [11,85]. This suggests a minimum threshold pressure below which no inactivation occurs. The results confirm the microbial inactivation dependence on HPP pressure and time [11,85,86]. Van Wyk & Silva (2017a) [10] investigated the effect of wine intrinsic properties on the inactivation of *B. bruxellensis*, by performing HPP studies in seven different wines, including red, white and rosé wines. HPP treatments at 200 MPa for 3 min resulted in 3.0, 3.8, 5.0, 5.8 and 6.0 log reductions in Dolcetto Syrah, SO_2-free Cabernet Merlot, Syrah, Cabernet Sauvignon and Pinot Noir, respectively. Complete inactivation (>6.0 log reductions) was achieved in rosé wine using 200 MPa for 2 min, while only 15 s was required to achieve complete inactivation (>7.0 log reductions) in the Chardonnay wine [10], showing the effect of wine composition on *Brett* inactivation. Additionally, results showed that alcohol concentrations above 12.0% *v*/*v*

had a significant effect on *Brett* inactivation with an increase of log reduction from 3.0 for 10.5–12% to 4.2 for 14% red Dolcetto Syrah wines, while wine pH from 3 to 4 in Cabernet Sauvignon wine was found to have no effect on *B. bruxellensis* inactivation [10].

Ultrasound (US) set at a low acoustic power density of 0.2 W/mL was not efficient for *Brett* inactivation, even after a long processing time of 20 min, which only reduced the yeast in 0.24 log in red wine [53]. When using thermo-sonication, the combination of thermal conditions of 50 °C with US treatment for 1 min, Gracin et al. (2016) [54] achieved 3.0 log reductions of *Brettanomyces bruxellensis* yeast in red wine and 2.0 log reductions of lactic acid bacteria. However, high temperature has a negative impact on wine sensory properties and is not recommended.

Research has shown that LEC is an effective technology for the inactivation of wine spoilage microorganisms. Lustrato et al. (2010) [73] investigated the inactivation of *D. bruxellensis* by LEC in red wine (13.5% alcohol) using 200 mA for 60 days. After 1 and 30 days, 4.0 and 5.2 log reductions were achieved, respectively [73]. Lustrato et al. (2015) [75] found that, after 30 days storage in oak barrels, there was no significant difference in the viable cell count of *D. bruxellensis* between the LEC treated (200 mA, <15 °C) wine and wine treated using SO_2. At the end of the experiment, SEM (scanned electron microscope) images showed the rupturing of the yeast cells which caused its irreversible inactivation [75].

4.2. Yeasts Important for Wine

Table 2 shows a summary of inactivation of different yeasts in wine submitted to different technologies and processing conditions. Abca and Evrendilek (2014) [20] found that 31 kV/cm bipolar pulses resulted in 4.5 log reductions of *Saccharomyces cerevisiae* in red wine. The same electric field strength applied to *Saccharomyces bayanus* in red wine led to significantly higher inactivation of 5.4 log reductions [17]. Abca and Evrendilek (2014) [20] also looked at the inactivation of *Candida lipolytica* and *Hansenula anomala* in red wine and found that 31 kV/cm caused 4.4 and 3.2 log reductions, respectively. Thus *H. anomala* was more resistant to PEF than *C. lipolyitica* and *S. cerevisiae*.

Similar to the *B. bruxellensis* results, Puig et al. (2003) [9] achieved 6.0 log reduction of *Saccharomyces cerevisiae* in red and white wine using 500 MPa for 5 min. With a reduced pressure of 300 MPa for 6 min, Tonello et al. (1998) [86] achieved more than 7.0 log reductions of *S. cerevisiae* in wine. Tonello et al. (1996) [87] found that HPP treatment at 321 MPa for 180 s resulted in more than 7.0 log reductions. Using 400 MPa for 20 s, Tonello et al. (1996) [87] attained more than 3.5 log reductions of *S. cerevisiae* in white wine and >3.7 log reductions of *S. ludwigii* in rosé wine, respectively. It was also concluded that HPP inactivation depends on the type and size of microorganism targeted [87]. Environmental scanning electron microscopy analysis images of *S. cerevisiae* spores demonstrated its death after HPP treatment of 600 MPa for 5 min, showing the release of intracellular content and change in the shape and size of the cell [40]. Lastly, alcohol concentration proved to be an important factor for microbial inactivation, with higher log reductions achieved in wines with alcohol concentrations above 13% v/v [10,86,87].

Residual yeast inactivation ($\leq$0.6 log reductions) was registered in red wine, even after a very long and unrealistic US treatment time of 20 min at 0.2 W/mL [53]. The highest yeast inactivation was a 0.6 log reduction of *Pichia membranefaciens* and the lowest a 0.13 log reduction of *Schizosaccharomyces pombe*.

HPH treatments of 150 MPa was able to induce a 2.2 log reduction of *Saccharomyces bayanus* [67]. Subsequently, further studies of Comuzzo et al. [88,89] revealed 3.5 log reductions using multiple HPH passes (10 L/h) at <40 °C. Puig et al. (2008) [68] found that subjecting red and white grape musts to 200 MPa using a flow rate of 120 L/h, caused complete inactivation of wild yeasts and lactic acid bacteria.

Table 2. Inactivation of different yeasts in wine by non-thermal PEF, HPP and US technologies *.

Yeast Species	Pasteurization Process	Wine	Alcohol Content (% *v*/*v*)	Processing Conditions	Treatment Time	Log Reduction	Reference
Saccharomyces cerevisiae	PEF	Red	12.0	31 kV/cm, 3 µs square bipolar pulse, 40 mL/min, T ≤ 40 °C	–	4.5	[20]
Saccharomyces bayanus	PEF	Red	13.0	31 kV/cm, 1 Hz, 100 pulses, batch, T < 30 °C	–	5.4	[17]
Candida lipolytica	PEF	Red	12.0	31 kV/cm, 3 µs square bipolar pulse, 40 mL/min, T ≤ 40 °C	–	4.4	[20]
Hansenula anomala	PEF	Red	12.0	31 kV/cm, 3 µs square bipolar pulse, 40 mL/min, T ≤ 40 °C	–	3.2	[20]
Saccharomyces cerevisiae	HPP	nr	15.0	300 MPa	360 s	>7.0	[86]
Saccharomyces cerevisiae	HPP	Red & white	nr	500 MPa	300 s	6.0	[9]
Saccharomyces cerevisiae	HPP	White	nr	400 MPa	20 s	>3.5	[87]
Saccharomyces ludwigii	HPP	Rosé	nr	400 MPa	20 s	>3.7	[87]
Saccharomyces cerevisiae	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.30	[53]
Schizosaccharomyces pombe	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.13	[53]
Zygosaccharomyces bailii	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	No inactivation	[53]
Pichia membranefaciens	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.60	[53]

* HPP was carried out at room temperature, maintaining nonthermal conditions; PEF—pulsed electric fields; HPP—high pressure processing; US—power ultrasound, nr—not reported.

4.3. Bacteria

Table 3 shows the results of bacteria inactivation in wine by non-thermal technologies. Only 2.7 log reductions of *Lactobacillus delbrueckii* ssp. *bulgaricus* was achieved in red wine using 31 kV/cm bipolar pulses [20]. Puértolas et al. (2009) [17] treated red wine containing *Lactobacillus plantarum* and *Lactobacillus hilgardii* using 100 pulses at 31 kV/cm, resulting in 4.8 and 5.2 log reductions, respectively. The magnitude of bacteria inactivation was similar or slightly lower than with yeasts (5.2 to 5.8 log reductions), using the same process. Lastly, 20 kV/cm applied for 6000 µs led to >1.0 and >5.3 log reductions of *Pediococcus parvulus* and *Oenococcus oeni* in red wine, respectively [19]. Therefore, research has shown that the size of the microorganisms has a significant effect on PEF inactivation, with larger yeast cells being less resistant to inactivation than smaller bacteria cells [17,20].

A number of HPP inactivation studies have been conducted on spoilage bacteria in red and white wines (Table 3). Puig et al. (2003) [9] used 500 MPa for 5 min to investigate the inactivation of *Lactobacillus plantarum, Acetobacter aceti*, *Acetobacter pasteurianus* and *Oenococcus oeni* in red and white wine, resulting in 8.0 log reductions for all four bacteria. Tonello et al. (1996) [87] found that 400 MPa applied for only 20 s resulted in >4.2 log reductions of *A. aceti* in wine. Tonello et al. (1996) [87] also looked at the inactivation of *Pediococcus damnosus* in wine using 400 MPa for 20 s, which led to more than 3.4 log reductions.

Regarding US, similarly to yeast, the inactivation of bacteria was almost none, ranging from log reductions of 0.13 with *Lactobacillus plantarum* to 0.35 with *Pediococcus* sp. bacteria [53] after a 20 min treatment (0.2 W/mL).

Table 3. Inactivation of bacteria in wine by non-thermal PEF, HPP and US technologies *.

Bacterium Species	Pasteurization Process	Wine	Alcohol Content (% *v*/*v*)	Processing Conditions	Treatment Time	Log Reduction	Reference
Lactobacillus plantarum	PEF	Red	13.0	31 kV/cm, 1 Hz, 100 pulses, batch, T < 30 °C	–	4.8	[17]
Lactobacillus hilgardii	PEF	Red	13.0	31 kV/cm, 1 Hz, 100 pulses, batch, T < 30 °C	–	5.2	[17]
Lactobacillus delbrueckii ssp. bulgaricus	PEF	Red	12.0	31 kV/cm, 3 µs square bipolar pulse, 40 mL/min, T ≤ 40 °C	–	2.7	[20]
Pediococcus parvulus	PEF	Red	nr	20 kV/cm, 0.5 Hz, 10 µs pulse width, T ≤ 42 °C	6000 µs	>1.0	[19]
Oenococcus oeni	PEF	nr	nr	20 kV/cm, 0.5 Hz, 10 µs pulse width, T ≤ 38 °C	6000 µs	>5.3	[19]
Lactobacillus plantarum	HPP	Red & white	nr	500 MPa	300 s	8.0	[9]
Pediococcus damnosus	HPP	Red	nr	400 MPa	20 s	>3.4	[87]
Oenococcus oeni	HPP	Red & white	nr	500 MPa	300 s	8.0	[9]
Acetobacter aceti	HPP	Red & white	nr	500 MPa	300 s	8.0	[9]
Acetobacter aceti	HPP	Red	nr	400 MPa	20 s	>4.2	[87]
Acetobacter pasteurianus	HPP	Red & white	nr	500 MPa	300 s	8.0	[9]
Lactobacillus plantarum	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.13	[53]
Pediococcus sp.	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.35	[53]
Oenococcus oeni	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.22	[53]
Acetobacter pasteurianus	US	Red	14.0	24 kHz, 0.2 W/mL, T ≤ 25 °C	20 min	0.60	[53]

* HPP was carried out at room temperature, maintaining nonthermal conditions; PEF—pulsed electric fields; HPP—high pressure processing; US—power ultrasound, nr—not reported.

5. Comparison of Technologies and Final Remarks

PEF and HPP proved to be effective wine pasteurization technologies, as they inactivate key wine spoilage yeasts and bacteria in short periods of time, feasible for application in the wine industry. Both technologies have the potential to complement or be used as alternatives to SO_2 addition to must, grape juice and finished wine at different stages of wine production, to control undesirable microbial growth or stop fermentation, and stabilize and preserve the quality of the finished wine until consumption. In fact, PEF is a promising technology for the wine industry as it is a continuous technology, requiring short processing times, in the magnitude of microseconds, for the inactivation of microbes of concern in the wineries. This enables commercial scale production with higher throughput. In addition, the same PEF unit also has the potential to decrease wine maceration time during the early stages of production. HPP and US have been investigated for the acceleration of wine ageing, reducing the required vinification time. US produced insufficient inactivation even after application of unrealistically long processing times.

Despite the encouraging results demonstrating less or no SO_2 addition to wine by using non-thermal technologies such as HPP [90] and PEF [91], more research is needed to determine the extent to which the use of SO_2 can be reduced or eliminated in the production/stabilization of different types of wine. The role of SO_2 in wine is complex and more research is required involving simultaneous assessment of microbial inactivation and wine quality after processing and during storage. Further wine stability studies with SO_2 free wines are needed to compare the quality of the wine produced using non-thermal methods vs the conventional addition of SO_2. More wine stability/quality studies should focus on the combination of a non-thermal method with a reduced amount of added SO_2 preservative.

Another important aspect is the investigation and comparison of costs of non-thermal technologies in terms of capital investment, energy requirement and environmental impact. In addition, although some technologies such as HPP are already used at commercial scale for other beverages and packed foods, others are not. Fortunately, the modern wine consumer's increasing demand for healthier and preservative free novel wines serves to promote research as well as applications and improvements of non-thermal technologies to the wine industry.

This review shows the potential of both HPP and PEF for wine preservation, as these technologies have minimal effect on overall wine sensory quality (flavor and aroma) and biochemical quality factors such as antioxidant activity, phenolic content and anthocyanins.

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Review

Protective, Biostimulating, and Eliciting Effects of Chitosan and Its Derivatives on Crop Plants

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Abstract: Chitosan is a biodegradable and biocompatible polysaccharide obtained by partial deacetylation of chitin. This polymer has been gaining increasing popularity due to its natural origin, favorable physicochemical properties, and multidirectional bioactivity. In agriculture, the greatest hopes are raised by the possibility of using chitosan as a biostimulant, a plant protection product, an elicitor, or an agent to increase the storage stability of plant raw materials. The most important properties of chitosan include induction of plant defense mechanisms and regulation of metabolic processes. Additionally, it has antifungal, antibacterial, antiviral, and antioxidant activity. The effectiveness of chitosan interactions is determined by its origin, deacetylation degree and acetylation pattern, molecular weight, type of chemical modifications, pH, concentration, and solubility. There is a need to conduct research on alternative sources of chitosan, extraction methods, optimization of physicochemical properties, and commercial implementation of scientific progress outcomes in this field. Moreover, studies are necessary to assess the bioactivity and toxicity of chitosan nanoparticles and chitosan conjugates with other substances and to evaluate the consequences of the large-scale use thereof. This review presents the unique properties of chitosan and its derivatives that have the greatest importance for plant production and yield quality as well as the benefits and limitations of their application.

Keywords: chitosan; biostimulants; biotic elicitor; polycationic polymers; secondary metabolites

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1. Introduction

Chitosan is a biopolymer obtained from chitin, which is the second most common natural polysaccharide after cellulose [1,2]. The discovery and first research on this compound date back to the 19th century, when the links between chemistry, botany, and medicine were discerned. Chitin was probably discovered in 1799 by English scientist A. Hachett, who extracted the compound from shells of marine invertebrates and described it as "a material with particular resistance to ordinary chemicals". However, he did not conduct further research on this compound. For this reason, it is believed that chitin, originally called fungine, was first isolated from fungi by French researcher H. Braconnot and subsequently described by Swiss chemist A. Hoffman in his doctoral thesis. Almost 20 years later, the same compound was isolated from insect cuticle by A. Odier and named chitin (Greek: chitōn—tunic, coat). In turn, chitosan was discovered accidentally in 1859 by French physiologist C. Rouget during the production of natural soap. The process of boiling water-dissolved chitin with the addition of concentrated potassium hydroxide resulted in the deacetylation of chitin in the alkaline solution yielding chitosan. Intensive research on the structure and properties of chitin and chitosan was carried out in the following years. The modified form of chitin was eventually named "chitosan" by German chemist and physiologist F. Hoppe-Seiler in 1894; however, the chemical structure of this compound was determined only in the mid-20th century [3–7].

Currently, the term chitosan denotes a group of biopolymer substances obtained in the process of chemical or enzymatic deacetylation of chitin with different (but not lower

than 50%) deacetylation degrees. Both chitosan and chitin are linear copolymers consisting of D-glucosamine and N-acetyl-D-glucosamine linked together by β-1,4-glycosidic bonds. The main difference between these polymers is their deacetylation degree (DD) expressed as a percentage and defined as the ratio of the number of amino groups ($–NH_2$) to the total number of acetylamino groups ($–NHCOCH_3$) present in chitin [1,4,8]. In addition, the solubility in dilute acids is a practical criterion for discriminating chitosan from chitin. The extraction of chitin begins with demineralization aimed at dissolving the calcium carbonate by acid treatment followed by deproteinization which uses, depending on the method (biological or chemical), enzymes or alkaline solutions. In order to obtain pure and colorless chitin, the last step is decolorization (Figure 1). The deacetylation process results in the removal of the acetyl groups ($–CH_3CO$) from chitin and substitution of reactive $–NH_2$ groups, thus resulting in the formation of chitosan, whose deacetylation degree depends primarily on the duration of the reaction, the temperature (90–120 °C), and the concentration of the aqueous NaOH solution (40–50%) used in the process. Although it occurs naturally in the cell walls of some fungi, bacteria, and algae as well as insect cuticles, chitosan for industrial and laboratory use is obtained from chitin derived mainly from the shells of marine invertebrates (shrimps, crabs, lobsters, and krill), which are wastes of marine food processing industry [1,8–10]. Until recently, the use of marine resources was considered one of the most effective methods for the extraction of chitin and the production of chitosan. An additional advantage was the possibility of disposal of a huge amount of waste generated by the marine food sector. However, some disadvantages and threats posed by this practice have been noticed in recent years, e.g., dependence on the fish industry, environmental hazards, and allergenicity of the final product, which depends on its purity. According to Ravindranathan et al. [11], chitosan thoroughly purified from impurity proteins and endotoxins is low-allergenic. However, the global chitin and chitosan market is developing very dynamically, and it has become important to look for alternative sources of these polymers for commercial and sustainable production. Currently, an increase in the number of studies on the production and applications of chitosan from various insect species can be observed. The primary cause of the interest is the speed of insect reproduction and the possibility of year-round breeding. Additionally, the results of many studies show that insect chitosan has even more favorable properties than that derived from shellfish. Recently, there have been attempts to produce chitosan from chitin derived from cell walls of fungi, which are the second-largest source of chitin after marine invertebrates [10,12–15], and biological methods are increasingly being used to obtain these polymers [2].

Chitosan is a compound with a number of physicochemical properties determining its suitability to be used in medicine, pharmacy, environmental protection, and agriculture as well as food, cosmetic, textile, and paper industries. These properties include nontoxicity; biodegradability; biocompatibility; hydrophilicity; film-forming properties; high sorption capacity; and high affinity for metals, proteins, and dyes. A summary of the extraction and applications of chitosan is presented in Figure 1. However, chitosan also exhibits many physicochemical properties that impede its wider use in some areas. The main limitation is its solubility, which largely depends on the isolation conditions, degree of deacetylation, type of acetyl group distribution, and molecular weight. There are many types of chitosan which are well soluble in both alkaline and neutral media, but in most cases, the best solvents for this group of polysaccharides are some acids. Additionally, chitosan swells strongly in an acidic environment and is characterized by poor mechanical and chemical strength. Therefore, chitosan is subjected to various types of physical and chemical modifications primarily aimed at improvement of its solubility and other physicochemical properties, e.g., mechanical strength, thus expanding the spectrum of its potential applications [8,13,16–19]. Three basic groups of chitosan modification techniques are used, i.e., methods leading to an increase in the molecular weight or extension of the chitosan chain; generation of chitosan derivatives through substitution reactions; and methods of physical, chemical, or enzymatic depolymerization. Chitosan derivatives

obtained through modifications are an important object in research on their application in biotechnology, agro-technology, and medicine as well as food, pharmaceutical, and textile industries [20,21].

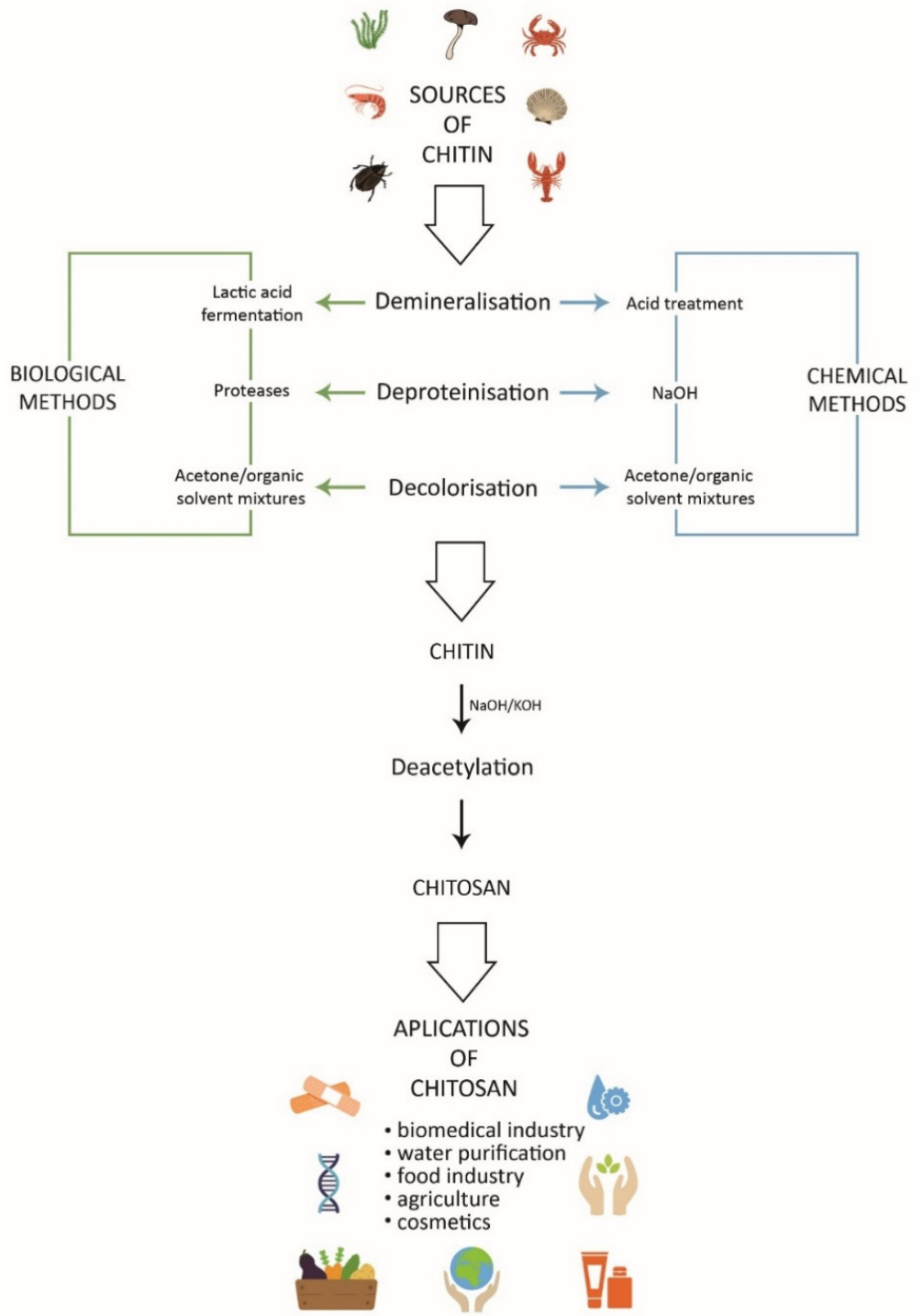

Figure 1. Extraction, preparation, and applications of chitosan and chitosan derivatives.

2. Biological Activity of Chitosan

Chitosan is a biopolymer with a wide potential for application in plant production mainly due to its biocompatibility and biodegradability as well as high biological activity. Although it is not part of the structure of plant tissues, chitosan exerts a considerable impact on plant growth and development. It initiates and modulates various types of physiological reactions, e.g., defense and immune responses in plants, and regulates metabolic

processes [22]. Chitosan also exhibits strong antibacterial, antifungal, and antiviral properties. The first investigations of its antimicrobial activity were reported in 1979 by Allan and Hadwiger, whose publication contributed to the growing interest in the potential application of chitosan in the agricultural industry. However, the practical use of this compound in plant production became possible with the wider availability of industrial amounts of chitosan. The compound has been used for protecting plants against pathogens and fighting diseases affecting plants during the vegetation and postharvest period only since the 1990s [23].

The mechanism of the antimicrobial activity of chitosan has not been fully elucidated yet, but many literature reports indicate that it is most probably related to its polycationic nature. The interactions of chitosan molecules with negatively charged molecules on the surface of microbial cells lead to agglutination of their cell walls, loss of intracellular components, and cell death. Another hypothesis assumes that chitosan limits the entry of pathogens into plant cells by reduction in stomatal opening. In turn, the antifungal activity of chitosan results from its ability to bind and inhibit the synthesis of toxins and stimulate induced systemic resistance and production of many secondary fungistatic metabolites, e.g., phytoalexins, abscisic acid, methyl jasmonate, and phenolic compounds, and various enzymes such as hypha-degrading chitinase and β-glucanase. Additionally, microscopic studies have shown that chitosan induces clear morphological changes in fungal cells at various stages of development. This polymer also reinforces plant cell walls through lignification and constitutes a mechanical barrier limiting plant contact with adverse external factors [4,24–29]. Consequently, the effectiveness of chitosan action against microorganisms is associated with its bidirectional action: control of the presence of pathogens and induction of plant defense reactions [30].

The high biological activity of chitosan in the induction of plant immune response may be related to the processes taking place in plant cell walls. Acid pectins present in the cell walls bind calcium and form chain dimers at higher concentrations of the element. Cationic chitosan can interact with negatively charged pectin and pectin dimers, thus influencing their supramolecular conformation, which induces a specific alarm signal informing plant cells about the degradation of cell walls and the presence of pathogens. The plant response to chitosan–pectin dimer complexes is considerably stronger than that in the case of separately interacting components [31].

Chitosan has been shown to have antioxidant activity consisting in the neutralization of such reactive oxygen species (ROS) as superoxide anion radicals, free hydroxyl radicals, and hydrogen peroxide (H_2O_2). It is also involved in interactions of hydroxyl and amine groups with metal ions resulting in metal chelation, adsorption, and ion exchange [27,32]. However, research results have indicated that chitosan can also induce the synthesis of H_2O_2 in plant cells as a signal molecule in defense reactions to stress and increase the activity of superoxide dismutase (SOD), peroxidases (POX), and catalase (CAT), i.e., enzymes involved in the direct neutralization of ROS [33]. As reported by Hawrylak-Nowak et al. [34], spraying plants with a chitosan lactate solution increased CAT and guaiacol peroxidase (GPOX) activity in *Ocimum basilicum* and ascorbate peroxidase (APX) in *Melissa officinalis*. Similarly, foliar application of chitosan nanoparticles increased the activity of CAT, APX, and glutathione reductase (GR) in *Catharanthus roseus* exposed to salt stress [35]. In turn, Quitadamo et al. [36] reported an important role of chitosan as a regulator of antioxidant responses to salinity in *Triticum durum*. It was observed that the use of this polymer neutralized the harmful effects of stress through a reduction in the content of superoxide radicals, H_2O_2, and malondialdehyde and via enhancement of CAT activity. In turn, the addition of chitosan as an elicitor in *Psammosilene tunicoides* hair root cultures was found to induce the production of nitric oxide and increase the activity of ROS-scavenging enzymes [37]. A similar effect was observed in *Curcuma longa* [38], where chitosan contributed to an increase in the activity of POX and polyphenol oxidase (PPO). Additionally, the results of research on the induction of oxidative responses by salicylic acid, chitosan, and exogenous H_2O_2 in *Capsicum annuum* plants demonstrated

that, in comparison with other substances, the foliar application of chitosan induced the lowest H_2O_2 accumulation in the leaves of this species [39]. The antioxidant activity of chitosan is also one of the criteria of its suitability to be used for enhancement of the storage stability of raw materials, which is described in detail in the next section. For example, in a study conducted by Wang and Gao [40], treatment of strawberries with chitosan induced a number of defense reactions; i.e., it increased the activity of CAT, GPOX, glutathione peroxidase (GSH-POX), dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR).

The biological activity of chitosan and the viscosity of its solutions, and thus the effectiveness of its use, are determined by a number of different factors, e.g., the origin, type of modifications, polymerization and deacetylation degrees, pH, positive charge, concentration, solubility, and chelation capacity [26,41]. Recent studies also indicate the importance of the pattern of chitosan acetylation, which may be an important molecular carrier of information [42].

The effect of chitosan depends on the plant species, developmental stage, and physiological condition. In turn, the antimicrobial activity of this polymer depends on the type of microorganisms. However, molecular weight is the most important parameter with a large impact on the level of biological activity of chitosan [26,33]. For example, it was observed in an experiment carried out by Kulikov et al. [43] that a decrease in the molecular weight of chitosan was accompanied by an increase in its inhibitory activity against mosaic virus infection in *Phaseolus vulgaris*. Animal-derived chitosan is a high-molecular-weight polymer. Moreover, it exhibits antimicrobial activity only in an acidic environment, in which it is soluble. Therefore, it is important to develop technologies for modification and adaptation of chitosan in order to elicit adequately targeted biological activity of this compound [9,44].

3. Application of Chitosan as a Biostimulant in Cultivation of Plants

Plant production is one of the most important elements of agriculture and the economy, which require continuous progress. The introduction of various novel technologies has contributed to the rapid increase in agricultural performance, but the search for ecological solutions increasing the efficiency of plant production has currently become essential [33]. Pro-ecological activities in this area are also enforced by the latest changes in agricultural policies, such as the European Green Deal, with one of the strategies aimed at a reduction in the use of plant protection products and the promotion of organic farming [45]. Hence, the interest in the use of natural-origin substances as stimulants of plant growth and development, the so-called biostimulants, has increased in recent years. The concept of biostimulants was proposed at the end of the 20th century but has not been clearly defined to date. Moreover, there are no adequate legal regulations for the systematization of available preparations or registration of new agents. However, the term "plant biostimulant" is assumed to denote any substance or formulation that is not a plant constituent, fertilizer, or pesticide but contains natural compounds (single or mixtures) or microorganisms. It is intended to be applied onto the whole plant, a part of a plant, or the rhizosphere in order to intensify natural physiological processes, increase plant resistance to stress, enhance the utilization of minerals, and improve the size and quality of crop yields [46–48].

Given its biological activity and film-forming properties, chitosan is widely used in treatments improving the propagation material, such as coating or encapsulation. It is most often used as an independent bioactive compound or a binding substance in combination with other agents stimulating plant growth and development and ensuring protection against pathogens. It is believed that coating the propagation material with a solution of this polymer mainly ensures adequate water and gas permeability, thus stimulating germination and development of seedlings [25,49]. It is also suggested that chitosan can activate hydrolytic enzymes required for the degradation and mobilization of storage substances, such as starch and protein [50]. A study conducted by Ruan and Xue [51] showed that coating *Oryza sativa* seeds with chitosan accelerated their germination and increased the

tolerance of these plants to salt stress. In turn, experiments carried out on tubers of freesia [52] showed that the application of a 0.2% chitosan solution increased the biomass and the number of progeny tubers in this species. Subsequent studies [53] demonstrated a positive effect of coating *Ornithogalum saundersiae* bulbs with a 0.5% aqueous solution of oligochitosan with 5000 or 100,000 g $\times$ mol^{-1} molecular weight on plant yields and most of the analyzed biometric and physiological indicators. It was also found that different doses of chitosan stimulated seed germination and had a positive effect on the fresh weight of cucumber roots and shoots [54]. Similar results were reported by Zeng et al. [55]; i.e., treatment with a chitosan solution stimulated germination and increased yields in soybean.

Current literature reports indicate a high effectiveness of foliar or soil application of chitosan in the stimulation of plant growth. This may be related to its stimulating effect on the uptake of water and essential minerals and its impact on the osmotic pressure in cells [41]. It was reported that a solution of this polymer sprayed on strawberry plants exerted a beneficial effect on plant growth and fruit yield [56]. This finding was confirmed in a study conducted by Rahman et al. [57], where foliar treatment of strawberry with chitosan had a positive effect not only on the growth and yield of fruits but also on their chemical composition. Furthermore, the results reported by Poterańska et al. [58] indicated that foliar application of chitosan-containing agents had a positive effect on the weight of haskap berries. In turn, *Eustoma grandiflorum* plants grown in soil with the addition of chitosan flowered much earlier and produced a larger number and greater weight of flowers [59]. Equally positive results were reported by Chookhongkha et al. [60], who additionally indicated a variable effectiveness of chitosan depending on its molecular weight. It was found that 1% high-molecular-weight chitosan applied to soil had a beneficial effect on fruit and seed yields in *Capsicum annuum*.

The literature provides information on the potential use of chitosan in the fertilizer industry as an ingredient for the production of sustained (SRF) or controlled release (CRF) fertilizers. The interest in this polymer has mainly been aroused by its beneficial effect on soil and plants and some physicochemical features. Given its film-forming properties as well as high biocompatibility and biodegradability, chitosan can be used as a coating material regulating the rate of release of minerals into the soil solution; additionally, it can be used to solve the problem with the disposal of residues of coatings produced from non-biodegradable polymers. In the agrochemical industry, chitosan nanoparticles are used both to optimize the activity and efficiency of various types of formulations and to reduce their toxicity to the environment [23,61,62]. An example of such an application was shown in an experiment carried out by Abdel-Aziz et al. [63], in which the foliar application of a nanochitosan-NPK fertilizer contributed to an increase in wheat growth and yields compared to plants treated with traditional forms of nitrogen, phosphorus, and potassium.

4. Chitosan as a Plant Protection Agent

The presence of harmful organisms poses a serious threat to agriculture and many industries worldwide. According to data published in 2019 by the Food and Agriculture Organisation of the United Nations (FAO), agrophages cause approximately 20–40% of losses of the world's crops annually [64]. The use of chemical plant protection products raises increasing concerns related to their negative impact on the environment despite their effectiveness. Given the growing ecological and consumer awareness and changes in the agricultural sector, the search for natural and safe methods for the protection of crops against pathogens and pests has become relevant. Chitosan is commonly described in the literature as a stimulant of plant resistance inducing natural defense mechanisms, which may reduce the amounts of synthetic plant protection products used in cultivation [33]. With its film-forming properties, this polymer can also act as a physical barrier limiting the contact between plants and pathogenic microorganisms [65]. Moreover, due to their potent antimicrobial activity, chitosan-based products can be used separately or in combination with other agents as biocides applied as hydrogels for coating tubers, fruits, and seeds; as a solution for soil or foliar application during or after vegetation; or as medium supplements

in hydroponic or tissue cultures [33]. Numerous studies have been carried out to assess the potential use of chitosan as a plant health-promoting agent. It was shown that soaking freesia tubers in a 0.2% solution of chitosan with different molecular weights (in the range of 2000–970,000 g × mol^{-1}) exerted a positive effect on their health [52]. In turn, the treatment of cucumber seeds with chitosan increased the resistance of seedlings to *Phytophthora capsici* in a concentration-dependent manner (0, 125, 250, and 500 ppm). The highest concentration ensured complete resistance of young cucumber plants to blight caused by the pathogen [54]. Treatment of soybeans with a chitosan solution was reported to reduce the presence of herbivorous insects [55]. Moreover, chitosan was found to induce an effective reduction in the viability of *Phomopsis viticola* spores [66]. Given its properties, it is also possible to use this polymer for the protection of herbal plants against pathogens. Szczeponek et al. [67] demonstrated the efficacy of a chitosan-based formulation against fungal species that infest lemon balm and peppermint most frequently. Chitosan can also be used to control pathogenic soil nematodes and increase plant resistance to these pests [41].

Chitosan is also one of the few active substances contained in formulations and fungicides approved for use in forestry for the protection of forest nurseries against pathogenic diseases [68]. As shown in an experiment carried out by Aleksandrowicz-Trzcińska et al. [69], foliar application of a chitosan-containing formulation increased the resistance of Scots pine to fungal infections. Other results of laboratory studies indicated that chitosan exerted a negative effect on growth and caused changes in the morphological and structural structure of *Cylindrocladium floridanum*, *Cylindrocarpon destructans*, *Fusarium acuminatum*, and *Fusarium oxysporum* fungi responsible for root rot in forest nurseries [70]. Moreover, there are a number of chitosan-based agents on the market for coating injuries and wounds in trees and shrubs.

5. Application of Chitosan in Storage

The main goal of storage is to reduce food losses by ensuring the longest possible shelf life, good quality of stored products, and the best possible protection of product stability before further distribution. An important role in this process is mainly played by the selection of optimal conditions and storage methods as well as the monitoring of such product quality parameters as the color, weight, firmness, content of bioactive compounds, and rate of ethylene and carbon dioxide production. However, in the case of low-processed raw materials, it is often necessary to employ various types of preservation methods. One of the options to preserve the quality of stored raw materials consists in a reduction in respiration and transpiration through the use of natural edible coatings. The potential of using chitosan in storage is associated with its physicochemical properties and biological activity. Given its natural origin, ability to form semipermeable coatings, and high antioxidant and antimicrobial activity, this polymer facilitates the maintenance and optimization of the postharvest stability of raw materials and food products and influences their chemical composition. Additionally, chitosan delays fruit ripening through the limitation of ethylene and carbon dioxide release. Therefore, it can be widely used in the production of biodegradable films, food packaging, fibers, gels, and films with high strength and flexibility. It can also be used as nanoparticles. Moreover, this polymer can be applied directly to fruits and vegetables as a single ingredient or in combination with other substances in order to create edible protective coatings with bacteriostatic and fungistatic properties [28,44,71]. Modifications achieved by combining chitosan with various types of substances offer many possibilities for the production of edible coatings and food packaging with required properties. For instance, conjugates of chitosan with phenolic (gallic and caffeic) acids produced durable films with adequate properties to serve as a barrier against water vapor and oxygen. They exhibited stronger antioxidant and antimicrobial activity than films based on traditional chitosan [72].

Numerous reports have demonstrated the suitability of chitosan for storage purposes, as its properties help to enhance the stability and quality of stored products. One of the examples is the research on the effect of chitosan coatings on the shelf life and quality of plum

fruit. The results of the experiment showed that the use of coatings containing 2% chitosan had a significant effect on the maintenance of the color, firmness, and weight of fruit stored at low temperature [73]. Similar results were obtained in a study of guava fruit stored at low temperature; i.e., the use of 2% chitosan coatings exerted a positive effect on the quality, firmness, and weight of fruit [74]. It was also observed that chitosan coatings increased the antioxidant properties of apricot fruits and contributed to the longer maintenance of a high total content of phenolic compounds [75]. Moreover, another experiment showed that chitosan coatings increased the storage stability of longan fruits and had a positive effect on their quality, color, and weight during storage [76]. A similar effect was reported in a study of *Actinidia melanandra* fruits, whose storage stability increased after the application of gel coatings containing this polymer [77]. In an experiment conducted by Zhu et al. [78], the application of chitosan coatings delayed the maturation and degradation of mangoes.

A study conducted by Tayel et al. [79] demonstrated that coating lemon fruit with chitosan inhibited the occurrence of *Penicillium* fungi. Moreover, the application of a chitosan-based formulation during the potato vegetation period was found to exert a limiting effect on the presence of *Fusarium* spp. and *P. carotovorum* subsp. *carotovorum* causing dry and wet tuber rot during storage [80]. A similar protective effect against the presence of the bacterial pathogen *Acidovorax citrulli*, which causes fruit blotch, was achieved in the case of watermelon seedlings [81]. In turn, as reported by He et al. [82], the use of a chitosan spray before harvesting strawberries had a beneficial effect on the quality and shelf life of the fruit through maintenance of the content of sugars; vitamin C; and numerous secondary metabolites, e.g., total phenolic compounds, flavonoids, and anthocyanins. Furthermore, the use of different concentrations of chitosan with different molecular weights was shown to increase the resistance of tomato fruit to grey mold caused by *Botrytis cinerea*. This was associated with both the direct antifungal activity of chitosan and the induction of a biochemical defense response in the fruit, which resulted in increased accumulation of phenolic compounds and enhanced activity of PPO [83].

6. Chitosan and its Derivatives as Biotic Elicitors

The potential for application of chitosan and its derivatives in the elicitation process is related to the biological activity of this compound, which primarily involves the ability to stimulate natural plant defense mechanisms and increase plant resistance to stress. This is associated with various types of physiological and biochemical changes, such as oxidative stress; accumulation of H_2O_2; synthesis of secondary metabolites (polyphenolic compounds, phytoalexins, flavonoids, alkaloids), enzymes (chitinase, glucanase, protease), and growth inhibitors (abscisic acid, jasmonic acid, salicylic acid); and accumulation of lignin and callose. The effect of chitosan on plants is reflected in changes in the chromatin structure, the inhibition of H^+-ATPase activity in the cell membrane, the activation of MAP kinases, and an increase in the cytosolic Ca^{2+} concentration [23,44,84]. Elicitation is aimed at stimulation of the biosynthesis of secondary metabolites present in plants or induction of the formation of new substances. It can solve problems related to the insufficient amount of bioactive compounds produced by plants, which does not cover the current needs, and bring benefits by providing high-quality raw materials with increased content of health-enhancing compounds [85]. The effectiveness of chitosan in elicitation largely depends on its solubility; therefore, chitosan salts, such as lactate or acetate, are the most commonly used derivatives of this polymer [21,34].

Numerous research publications report the effectiveness of chitosan and its derivatives in the elicitation process. Studies on *Curcuma longa* [86] indicated that the foliar application of a 0.1% chitosan solution triggered defense responses in the plants and had a positive effect on both plant growth and accumulation of curcumin in their rhizomes. A stimulating effect of 0.1% and 0.2% chitosan solutions was also reported in a study of *Stevia rebaudiana*. The application of chitosan increased the biomass and concentration of phenolic compounds and rebaudioside A [87]. A field experiment conducted on *Origanum vulgare* ssp. *hirtum* demonstrated that the use of different concentrations of chitosan oligosaccharides (50,

200, 500, or 1000 ppm) exerted a beneficial effect on plant growth and the content of polyphenolic compounds [88].

The study conducted by Gerami et al. [89] suggested the possibility of the use of chitosan as an elicitor increasing *S. rebaudiana* tolerance to salinity and reducing its phytotoxic effects on these plants. Similar results were obtained in an experiment conducted by Safikhan et al. [90], in which chitosan mitigated the harmful effects of salt stress, stimulated growth, and had a positive effect on the physiological parameters of *Silybum marianum*. Furthermore, a study conducted in water deficit conditions showed that chitosan treatment of *Salvia officinalis* reduced the negative impact of drought stress. It also had a positive effect on the quantity and quality of essential oil, the total content of phenolic compounds and flavonoids, and the antioxidant properties of sage extracts [91]. A stimulating effect of a chitosan suspension and a chitosan solution in 1% acetic acid on the production of flavonoids in *Ononis arvensis* in in vitro conditions was demonstrated as well [92]. A chitosan solution in acetic acid used as an elicitor in *Mentha piperita* suspension cultures produced a significant increase in the accumulation of menthol [93]. In turn, the treatment of *M. piperita* with chitosan in greenhouse conditions increased the total content of phenolic compounds and flavonoids and enhanced the antioxidant activity of the extracts [94]. Chitosan also stimulated the production of triterpenoid saponins in *Psammosilene tunicoides* hair root cultures [37]. Moreover, the polymer was found to have a beneficial effect on biomass accumulation in cell cultures of three basil species: *Ocimum basilicum*, *O. sanctum*, and *O. gratissimum* [95]. Other studies carried out on *O. basilicum* indicated the effectiveness of chitosan elicitation in the stimulation of biomass growth, intensified accumulation of total phenolic and terpene compounds, elevation of rosmarinic acid and eugenol concentrations, and enhancement of antioxidant activity [96]. The foliar application of chitosan lactate stimulated the biosynthesis of rosmarinic acid, anthocyanins, and phenolic compounds in *O. basilicum* and *M. officinalis* raw materials [34].

The effects of the application of chitosan and its derivatives on the accumulation of secondary metabolites in selected plant species are shown in Table 1.

Table 1. Effects of application of chitosan and its derivatives on the level of secondary metabolites in selected plant species.

Plant Species	Plant Growth Conditions	Chitosan Form	Dose, Method, and Number of Chitosan Applications	Effect of Chitosan on the Level of Secondary Metabolites	Reference
Artemisia annua	laboratory conditions; hairy root cultures	chitosan	50, 100, or 150 mg L^{-1} of chitosan added to hairy root cultures	increased artemisinin production	Putalun et al. [97]
Curcuma longa	field conditions	chitosan	0.1% chitosan; foliar application; seven treatments	increased curcumin content	Sathiyabama et al. [38]
Dracocephalum kotschyi	glasshouse; mixture of peat, sandy soil, and perlite substrate	chitosan	100 or 400 mg L^{-1} of chitosan; triple foliar application	enhanced biosynthesis of total phenolic and flavonoid compounds, including rosmarinic acid and apigenin	Kahromi and Khara [98]
Catharanthus roseus	greenhouse; sandy soil	chitosan nanoparticles	1% chitosan nanoparticles; single foliar application in salinity stress conditions	increased alkaloid accumulation	Hassan et al. [35]

Table 1. *Cont.*

Plant Species	Plant Growth Conditions	Chitosan Form	Dose, Method, and Number of Chitosan Applications	Effect of Chitosan on the Level of Secondary Metabolites	Reference
Fragaria × *annanasa*	field conditions	chitosan	125, 250, 500, or 1000 ppm chitosan; foliar application; six treatments	increased amount of phenolic compounds, carotenoids, flavonoids, and anthocyanins in strawberry fruits	Rahman et al. [57]
Ginkgo biloba	laboratory conditions; callus cultures	chitosan	25, 50, 100, or 200 mg L^{-1} of chitosan added to MS medium	enhanced production of total flavonoids and total phenolic compounds	Elateeq et al. [99]
Iberis amara	laboratory conditions; cell suspension cultures	chitosan	50, 100, or 200 mg L^{-1} of chitosan	enhanced total phenolic compounds, flavonoid, flavonol, and anthocyanin contents	Taghizadeh et al. [100]
Isatis tinctoria	laboratory conditions; hairy root cultures	chitosan	50, 100, 150, 200, or 400 mg L^{-1} of chitosan; hairy root cultures elicited for 6–96 h	increased total flavonoid accumulation	Jiao et al. [101]
Melissa officinalis	phytotron room; soil substrate	chitosan lactate	100 or 500 mg L^{-1} of chitosan lactate; single foliar application	increased accumulation of rosmarinic acid, anthocyanins, and total phenolic compounds	Hawrylak-Nowak et al. [34]
Mentha piperita	greenhouse; soil phosphate	chitosan	50 or 100 µM chitosan; single foliar application	increased content of phenolic and flavonoid compounds	Salimgandomi and Shabrangi [94]
Ocimum basilicum	greenhouse; potting substrate irrigated with a fertilizer solution	chitosan	0.01%, 0.05%, 0.1%, 0.5% or 1% chitosan; seed soaking (30 min.)	increased content of total phenolic and terpenic compounds (rosmarinic acid, eugenol)	Kim et al. [96]
Origanum vulgare ssp. hirtum	field conditions	chitosan oligosaccha-rides	50, 200, 500, or 1000 ppm chitosan oligosaccharides; single foliar application	increased accumulation of total flavonoids and total polyphenolic compounds	Yin et al. [88]
Psammosilene tunicoides	laboratory conditions; hairy root cultures	chitosan	200 mg L^{-1} of chitosan; hairy roots elicited by chitosan for nine days	enhanced accumulation of total saponins, increased content of quillaic acid, gypsogenin, and gypsogenin-3-O-β-D-glucuronopyranoside	Qui et al. [37]
Salvia officinalis	field conditions	chitosan	0.25 or 0.50 g L^{-1} of chitosan; single foliar application in reduced irrigation conditions	increased amount of total phenolic and flavonoid content; enhanced production of α- and β-pinene, limonene, α- and β-thujone, camphor, and 1,8-cineole in the essential oil	Vosoughi et al. [91]

Table 1. *Cont.*

Plant Species	Plant Growth Conditions	Chitosan Form	Dose, Method, and Number of Chitosan Applications	Effect of Chitosan on the Level of Secondary Metabolites	Reference
Satureja isophylla	greenhouse; sandy soil	chitosan	0.2 or 0.4 g L^{-1} of chitosan; foliar application	increased amount of essential oil; increased concentrations of essential oil constituents (carvacrol, β-bisabolene)	Salehi et al. [102]
Stevia rebaudiana	greenhouse; perlite and peat substrate	chitosan	0.2, 0.4, or 0.6 g L^{-1} of chitosan; double foliar application in salinity stress conditions	increased content of steviol glycosides: stevioside and rebaudioside A	Gerami et al. [89]
Sylibum marianum	laboratory conditions; cell suspension cultures	chitosan	0.5, 1, 2.5, 5, 10, 25, or 50 mg L^{-1} of chitosan in MS medium	enhanced accumulation of total flavonoids, total phenolic compounds, and silymarin	Shah et al. [103]

7. Prospects for New Applications of Chitosan

Due to the wide availability and a number of its beneficial properties, an increase in the number of potential applications of chitosan and its derivatives has been observed in recent years. Nevertheless, despite its numerous advantages, the practical commercial-scale use of this polymer is still limited by some of its physicochemical features and is dependent on progress in the development of optimal methods for chemical modification and adaptation of the polymer for specific applications. To date, the greatest success in this regard has been achieved in nanotechnology, which is regarded as one of the five most groundbreaking fields of science and technology of the last century [6,104,105]. The achievements of this discipline facilitate a wide use of chitosan nanomaterials in various types of biomedical, cosmetic, food, ecological, and agrotechnical innovations [13,16]. The application of nanoparticles facilitates more effective utilization of the biological properties of chitosan.

One of the most important prospects for the use of chitosan in nanotechnology is the concept of sustainability of the agrochemical industry through the production of the so-called agronanochemicals. It mainly involves the preparation of chitosan nanoparticles and chitosan nanocomposites or encapsulation of active substances in chitosan-based nanocarriers. The preparations primarily exhibit high efficiency and bioavailability; hence, they can constitute a more economical environmentally friendly alternative limiting the amounts of currently available chemicals [17,104,106,107].

Chitosan may exert a positive effect on plant photosynthetic efficiency, ability to biosynthesize and accumulate chlorophyll, and nutrient uptake [27,106,108,109]. The available research results demonstrate that chitosan nanoparticles have a greater impact on the macronutrient uptake efficiency than chitosan oligomers. Experiments carried out in greenhouse conditions showed that chitosan nanoparticles contributed to an increase in photosynthetic efficiency; chlorophyll content; and nitrogen, phosphorus, and potassium absorption in *Coffea canephora* [110]. Similar results were reported by Sathiyabama and Manikandan [111], who found that the foliar application of nanochitosan stimulated growth and increased the mineral content in *Eleusine coracana*.

Various research results also indicate the possibility of using chitosan to increase plant tolerance to abiotic and biotic stress factors and mitigate their phytotoxic effects [35,107,109]. Many reports show the effectiveness of chitosan in increasing plant resistance to salinity, drought, or low-temperature stress [112,113]. It was shown that spraying with a solution containing 1% chitosan nanoparticles alleviated the effects of salt stress through the

activation of defense mechanisms in *Catharanthus roseus* [35,109]. The foliar application of a solution containing chitosan nanoparticles (400 mg L^{-1}) to bananas enhanced their tolerance to low temperature and improved biometric parameters via a reduction in the oxidative stress intensity [113]. In turn, Kocięcka and Liberacki [112] reported the effectiveness of chitosan, its derivatives, and nanoparticles in the stimulation of the resistance of cereals to such abiotic stress factors as low temperature, drought, and salinity and in the enhancement of the health of plants and improvement of their quality.

This polymer can also be used in combination with other compounds not only to extend the spectrum of its activity but also to produce materials with more favorable mechanical, chemical, thermal, and barrier properties [17,107,114]. The use of chitosan in the production of nanocomposites may also increase the effectiveness of other chemical compounds or elements, e.g., zinc, iron, copper, and silver [107,114]. In addition to the stimulation of the efficiency of agrochemicals, these nanocomposites enhance the biosynthesis of secondary metabolites and may accelerate plant regeneration. An example of this type of use is the application of zinc encapsulated chitosan nanoparticles, which had a beneficial effect on the growth, health, and quality of maize kernels [115]. In turn, studies of *Capsicum annuum* tissue cultures showed that a composite of zinc oxide and chitosan nanoparticles contributed to a significant increase in its elicitation efficiency [116].

Chitosan nanoparticles can reduce the amount and frequency of application of plant protection products, ameliorate their negative effects, and help to monitor and remove their excess from soils and waters [23,117]. Due to its cationic nature and high affinity to metals and dyes, chitosan is also one of the preferred natural and relatively cheap adsorbents replacing the currently used active carbon, with similar sorption properties in water engineering [2,10,105,118]. In water remediation, similar to other applications, chitosan can be used alone or in combination with other substances, e.g., activated carbon or silicon dioxide. Nanocomposites made of chitosan and activated carbon, whose combination ensures a significant increase in their adsorption capacity, are one example [118,119].

The main applications of chitosan and its derivatives in plant production are summarized in Figure 2.

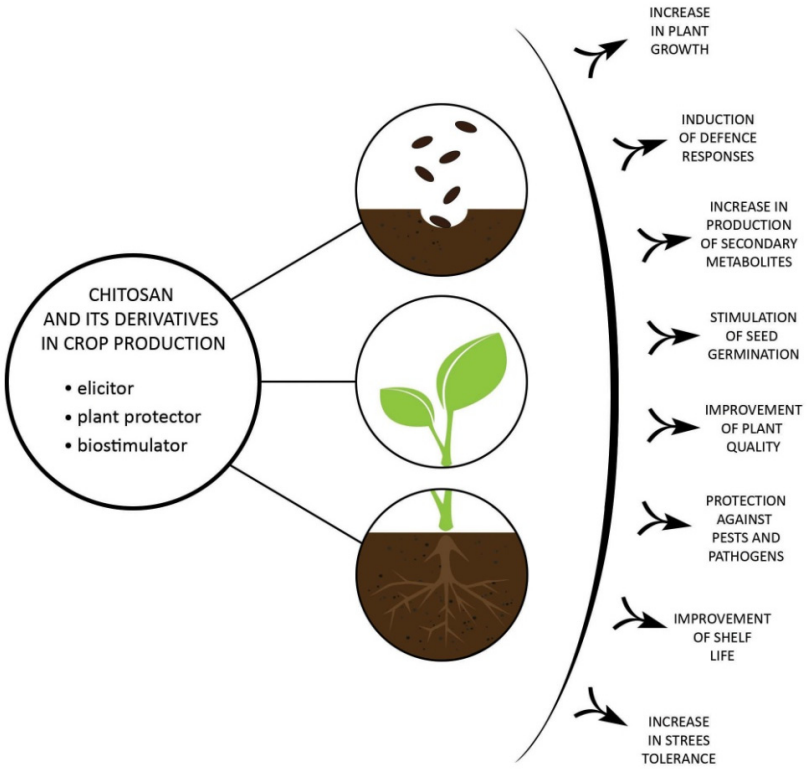

Figure 2. Application of chitosan and its derivatives in plant production.

8. Conclusions

Chitosan is an easily available environmentally friendly biopolymer with numerous favorable biological properties; hence, it has many applications in various fields. Additionally, it can be a potential solution to various ecological problems, especially in the production of plants by increasing their internal potential. Biodegradable and biocompatible chitosan and chitosan-based nanomaterials are becoming essential in agriculture due to their unique properties, such as biostimulating, eliciting, and antimicrobial activity and stimulation of plant growth and tolerance to environmental stresses. However, the use of other effective sources of chitosan, the methods for extraction and optimization of its physicochemical properties, and the practical implementation of laboratory results should be investigated comprehensively. Moreover, detailed studies on the potential toxicity of chitosan-based nanomaterials and the ecological consequences of their large-scale use are required. At present, these substances are not being widely used in agriculture, as the mechanisms of their biological activity in plants and action against pathogenic microorganisms have not been fully elucidated to date.

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Review

Chemical Methods for Microbiological Control of Winemaking: An Overview of Current and Future Applications

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Abstract: Preservation technologies for winemaking have relied mainly on the addition of sulfur dioxide (SO_2), in consequence of the large spectrum of action of this compound, linked to the control of undesirable microorganisms and the prevention of oxidative phenomena. However, its potential negative effects on consumer health have addressed the interest of the international research on alternative treatments to substitute or minimize the SO_2 content in grape must and wine. This review is aimed at analyzing chemical methods, both traditional and innovative, useful for the microbiological stabilization of wine. After a preliminary description of the antimicrobial and technological properties of SO_2, the additive traditionally used during wine production, the effects of the addition (in must and wine) of other compounds officially permitted in winemaking, such as sorbic acid, dimethyl dicarbonate (DMDC), lysozyme and chitosan, are discussed and evaluated. Furthermore, other substances showing antimicrobial properties, for which the use for wine microbiological stabilization is not yet permitted in EU, are investigated. Even if these treatments exhibit a good efficacy, a single compound able to completely replace SO_2 is not currently available, but a combination of different procedures might be useful to reduce the sulfite content in wine. Among the strategies proposed, particular interest is directed towards the use of insect-based chitosan as a reliable alternative to SO_2, mainly due to its low environmental impact. The production of wines containing low sulfite levels by using pro-environmental practices can meet both the consumers' expectations, who are even more interested in the healthy traits of foods, and wine-producers' needs, who are interested in the use of sustainable practices to promote the profile of their brand.

Keywords: sulfur dioxide (SO_2); chemical methods; antimicrobial activity; microbiological control; chitosan; sustainable approaches; winemaking

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1. Introduction

Wine fermentation is a microbiologically complex process which requires the monitoring of the microorganism load at different stages of the process. Winemakers manage the different steps of this process with the aim to obtain high-quality products according to their wishes and the expectations of their customers. However, the chemical characteristics and microbial composition of wine are in constant evolution throughout the process, and some parameters are difficult to control.

Microbial metabolism is one of the multiple factors affecting wine quality, by contributing to its complexity or, in some cases, leading to undesirable aromas. The control of wild microorganisms present in grape must is an advisable oenological practice to ensure the imposition of yeast starter cultures for adequate alcoholic fermentation, whereas the

control of microorganisms present in the wine is necessary both to assure the dominance of suitable bacteria strains for malolactic fermentation (MLF) [1], and to avoid wine spoilage due to the growth of undesirable yeasts, lactic acid bacteria (LAB), and acetic acid bacteria (AAB), with irreversible effects on wine quality and considerable economic losses. Some LAB and AAB species, such as *Lactobacillus* spp., *Pediococcus* spp., and *Acetobacter* spp., are primarily responsible for the loss of quality of musts and wines, in consequence of the formation of undesirable aroma and flavor compounds leading to defects such as a "vinegary," "nail polish-remover" taste. The major spoilage yeasts include species and strains of the genera, *Brettanomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Zygosaccharomyces*; in particular, yeasts belonging to the genus, *Dekkera* (anamorph *Brettanomyces*), are producers of 4-ethylphenol, 4-ethylguaiacol, and tetrahydropiridine, which are considered off-flavors in wine. To date, preservation technologies for winemaking have relied mainly on the addition of sulfur dioxide (SO_2), in consequence of the large spectrum of action of this compound, linked to the control of undesirable microorganisms and the prevention of oxidative phenomena [2].

However, in a context of societal concern regarding food and wine preservation, reducing the sulfite level in the wine now represents a major challenge for the wine industry. Different factors, such as increasing consumers' attention toward health concerns, the potential organoleptic alterations of the final product, and restrictive legislation on preservatives [3], along with the quest for environmentally friendly production, have driven the interest of scientific community and producers toward alternative methods to SO_2 [4]. Furthermore, the massive employment of SO_2 is not always compatible with the production of high-quality wines, as it can cause organoleptic alterations in the final product, neutralize the aroma, and even produce characteristic aroma defects, such as undesirable aromas of the sulfurous gas, when this compound is reduced to hydrosulfate and mercaptanes. In addition, the use of excessive SO_2 doses does not always avoid the risk of wine spoilage, in consequence of the emergence of tolerant/resistant spoilage microorganisms [5].

This paper is a review summarizing the main findings regarding chemical methods that have been so far studied to substitute or reduce the use of SO_2 in winemaking. After a summary on the main characteristics of SO_2 application in wine, the action mechanisms of the different methods will be discussed, including their efficacy, drawbacks, and effects on the final quality of the wine in order to claim that the new alternatives apply the same SO_2 properties in wine.

Special emphasis will be placed on very innovative methods, such as the use of chitosan extracted by unconventional sources, to develop an integrated perspective on how to produce a more natural, healthier, and sustainable wine.

2. Sulfur Dioxide

Sulfur dioxide (SO_2) is an additive commonly used for the microbiological control of foods, in particular for acidic foods, such as fruit juice and wine [6]. In the winemaking process, the wide use of this compound is correlated to its large spectrum of action, mainly linked to the control of undesirable microorganisms and the oxidative processes, as summarized in Table 1.

SO_2 is added to grape must or wine as a liquid or gaseous form; in Europe and Switzerland, the SO_2 forms which can be used as food additives are sulfur dioxide, sodium sulfite, sodium hydrogen sulfite, sodium metabisulfite, potassium metabisulfite, calcium sulfite, calcium hydrogen sulfite, and potassium hydrogen sulfite [6].

Table 1. Effects of SO_2 addition in grapes, must, and wine.

Process Phase	Times of SO_2 Addition	SO_2 Action
Grape and Must	Before the start of alcoholic fermentation	- Antimicrobial activity towards non-*Saccharomyces* yeasts, acetic and lactic acid bacteria (in white winemaking to avoid the malolactic fermentation) [7–9] - Antioxidant activity to avoid the must oxidation [2,10]
Wine	Filtration, decanting, and aging	- Antimicrobial activity towards *Brettanomyces* spp., *Candida* spp., *Pichia* spp., acetic acid bacteria [11–14] - Antioxidant activity to avoid the wine oxidation [11,15]
	Before bottling	- Antioxidant activity to avoid the wine oxidation [16–18] - In sweet wines to avoid the refermentation process by Saccharomyces cerevisiae [19,20]

Depending on the pH, different SO_2 forms can be found in the wine, which are sulfurous acid, or molecular SO_2, (H_2SO_3), bisulfite ion (HSO_3^-), and sulfite ion (SO_3^{2-}). At wine pH (ranging between 3 and 4), HSO_3^- is the predominant form, whereas the sulfite ion is negligible [21], as this form is found at pH > 7; the non-dissociated fraction, H_2SO_3, is commonly found at pH < 2. These three compounds form the "free SO_2", but the complex chemical equilibrium of the SO_2 in the wine results in different SO_2-combined compounds. Indeed, bisulfite ion can bind some wine compounds, such as acetaldehyde, glucose, quinones, anthocyanins, and ketoacids, forming compounds more or less active against microbiological spoilage. The stable combination with acetaldehyde makes SO_2 very poorly available for wine protection, conversely to the combination with other compounds species, which is reversible.

SO_2 is present in wine not only as a consequence of exogenous addition during the winemaking process, but it is also produced by yeast metabolism during alcoholic fermentation, the so-called "biological SO_2". In fact, yeasts use the sulfur present in the must for the synthesis of amino acids, and the production levels of sulfites by yeasts is highly strain-dependent [22].

As reported by Zara and Nardi [23], sulfate (SO_4^{2-}) in must is transported into the *Saccharomyces* yeast cell, where SO_4^{2-} is reduced to sulfite (SO_3^{2-}) and then into sulfide (S^{2-}). SO_3^{2-} is transported outside the cell, and S^{2-} is incorporated in methionine and cysteine, whereas the sulfide in excess diffuses outside the cell (Figure 1). At the end of alcoholic fermentation, yeasts produce sulfites and sulfides. An excessive quantity of these compounds can cause problems in finished wine because sulfides can lead to off-flavors (e.g., rotten eggs), and sulfites at a high concentration can delay the onset of MLF by inhibiting LAB [22].

In consequence of these metabolic activities, yeasts produce SO_2 both in must and finished wine, and, based on the production level of SO_2 quantity, the yeasts are divided into low (<10 mg/L), middle (10–30 mg/L), and high (>30 mg/L) SO_2-producers.

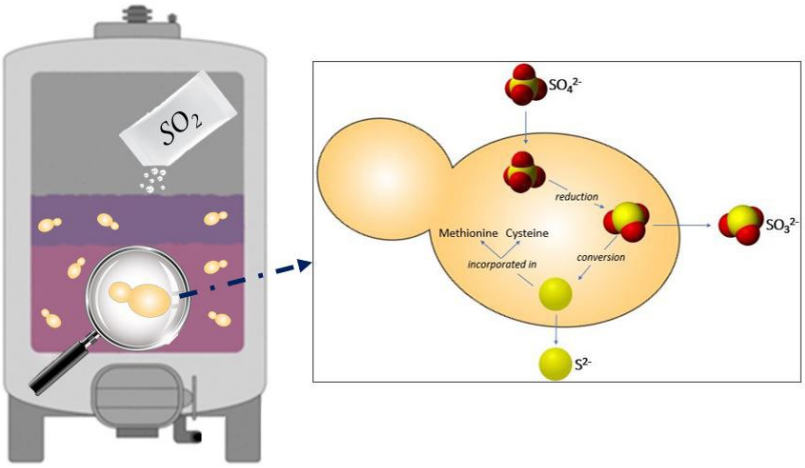


Figure 1. Sulfur metabolism in *Saccharomyces cerevisiae* yeast cell.

2.1. Antiseptic Activity of SO_2

As regards the antiseptic activity of SO_2, this compound inhibits the development of different microorganisms, with the highest antimicrobial activity against bacteria (LAB and AAB), followed by non-*Saccharomyces* yeasts, whereas usually *Saccharomyces* yeasts are highly resistant, although the sensitivity level is strain-specific [7].

The highest antimicrobial activity is shown by molecular SO_2, whereas bisulfite ions show weak antimicrobial activity, and the bound SO_2 exerts only a low antibacterial action.

2.1.1. Activity against Yeasts

As regards the antimicrobial activity against yeasts, the addition of SO_2 in the grape must is mainly to inhibit the growth of non-*Saccharomyces* yeast species prevalent at this stage. It was reported that, in spontaneous wine fermentation, concentrations of total SO_2 higher than 40 mg/L compromise the development of most of these yeasts, with the exception of *Hanseniaspora osmophila* and *Candida* spp. [24], whereas concentrations below 40 mg/L inhibit the development of *Metschnikowia* spp., and concentrations between 60 and 100 mg/L inhibit the development of *Torulaspora* spp. [25], *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe*, and *Saccharomycodes ludwigii* have been described as highly tolerant species to SO_2 [26,27], whereas *Kloeckera apiculata* and *Hansenula anomala* are highly sensitive to this compound [28].

The addition of SO_2 in the final wine is attributed mainly to the control of yeasts belonging to the genus, *Dekkera*/*Brettanomyces*. These are very harmful yeasts for the production of wine, as they are able to resist environmental conditions poor in nutrients, colonize and contaminate cellar equipment (especially wooden barrels), and produce high amounts of undesirable compounds, such as acetic acid, ethylphenols, and tetrahydropyridines [29,30].

Several studies have monitored the development of these contaminant yeasts in wine, showing the complexity in preventing the development of *Brettanomyces* spp. Survival models of *Brettanomyces bruxellensis* after the addition of sulfur dioxide [31] showed that the control of these yeasts can be obtained by adding relatively high doses of the antimicrobial compound (approximately 1 mg/L of the active molecular fraction). Furthermore, these authors confirmed previous findings [11], indicating that the effective control of *B. bruxellenis* should also be carried out by avoiding, as far as possible, the contact with oxygen, which favors the reactivation of yeast cells in the viable, but not culturable (VBNC), state. The maintenance of a free sulfur dioxide dose of 25–35 mg/L seems to be effective in eliminating viable *B. bruxellensis* cells [6].

The effect of sulfur dioxide on microbial cells has been studied for several decades, and mostly in the yeast, *Saccharomyces cerevisiae*. The molecular SO_2 enters the yeast cell through the plasma membrane with a simple diffusion mechanism [32]. Once inside the

cell, in consequence of the higher intracellular pH (about 6.5), molecular SO_2 is largely converted to HSO_3^- [33].

However, in the yeast cell, the dissociation of H_2SO_3 reduces the intracellular concentration of molecular SO_2, allowing further diffusion into the cells until the SO_2 concentration is balanced on both sides of the plasma membrane [34]. SO_2 is a highly reactive molecule, which it binds to many molecules in the cell (proteins, nucleic acids, coenzymes, cofactors, vitamins, etc.), interfering with intracellular processes [35]. This mechanism inhibits microbial growth.

The general tolerance to SO_2 exhibited by *S. cerevisiae* is correlated to detoxification mechanisms developed by this yeast. The main mechanisms developed by the yeast cell to resist sulfites are the following [36]:

- Metabolic pathways involved in the production of sulfur compounds, such as the amino acids methionine and cysteine [37];
- Sulfite detoxification by the membrane efflux [37];
- Production of SO_2-binding molecules, such as acetaldehyde [23];
- The cell entry into a VBNC state [38]. This physiological state is described as a protection strategy in which the cells can wait for more favorable conditions. In wine, the VBNC state allows spoilage yeasts and bacteria to survive throughout the wine fermentation process and into the wine bottle. In wine conditions, the presence of chemical stressors such as SO_2 has been shown to induce a VBNC state in *S. cerevisiae* and other yeast species, such as *Brettanomyces/Dekkera bruxellensis*, and reactivate later, when conditions become more favorable [39]. The removal of SO_2 from the wine environment can be obtained by increasing the pH in order to shift the chemical equilibrium of SO_2 with a decrease of the concentration in molecular SO_2, favoring the exit from the VBNC state [40].

2.1.2. Activity against Bacteria

The SO_2 provides, in addition to the control of undesirable yeasts, an antibacterial activity both in must and wine, although the action mechanism has not been clarified yet. Conversely to yeasts, the bacteria are weakly inhibited by bound SO_2, which exerts an antibacterial activity from 5 to 10 times lower than free SO_2, but it should be considered that bound SO_2 can be from 5 to 10 times more abundant in comparison to the free form.

The SO_2 antibacterial activity is mainly attributed to AAB and LAB.

As regards the AAB, the predominant species on the grapes, especially on berries with a non-optimal health state, are strains of the genera, *Gluconobacter*, *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* [41]. The proliferation of AAB, especially those of the *Acetobacter* genus, leads to oxidative phenomena, with undesirable consequences for wine quality. For this reason, the control of AAB is carried out by the use of SO_2 and by the control of oxygen, avoiding the contact of wine with oxygen [41].

As with *Brettanomyces* spp., AAB are also able to survive in the VBNC state when SO_2 is added and the oxygen is removed, maintaining their metabolic activity and resuming their development when the conditions permit [42]. Indeed, it was reported [11] that *Acetobacter pasteurianus* is able to survive in VBNC under anaerobic conditions and in the presence of SO_2, whereas it resumes the viable state with oxygen addition.

The most frequent LAB in wine are mainly species belonging to the genera, *Oenococcus*, *Pediococcus*, and *Lactobacillus* [6].

Oenococcus oeni, the LAB mainly responsible for MLF in wine, is able to overcome the stressful conditions of wine (low pH, high ethanol content), but it has a high sensitivity to SO_2 [43]. The sensitivity of *O. oeni* to SO_2 is a positive trait in the production process of white wines, where, usually, MLF is not desired, whereas for red wines, MLF is favored in part by the survival of the bacterium in VBNC, and in part by the inoculation of starter cultures that promote rapid bioconversion [8].

2.2. Technological Activities of SO_2

Other than the antimicrobial action, the addition of sulfur dioxide to wine has further activities. This compound is highly effective in preventing chemical and enzymatic oxidative processes in winemaking. Chemical, or non-enzymatic, oxidation processes may occur in grapes, must, and wine, and can cause changes in color and sensory characteristics. During this process, reactive oxygen species, produced by reduced transition metals ions (e.g., Fe (II), Cu (I)), react with many constituents of wine, especially phenolic compounds, causing oxidation [15]. These reactions lead to the formation of quinones, unstable compounds that can undergo further reactions, with the final formation of pigments, responsible for wine color alteration [44]. As an antioxidant, SO_2 is able to react with the oxygen-reduced form to inhibit aldehyde formation and to reduce quinones back to their phenol form [45].

The SO_2 addition, especially in the form of HSO_3^-, is fundamental to inhibit enzymatic oxidation processes [21], which occur in grapes and must as a consequence of the activity of polyphenol oxidase (PPO) enzymes, such as tyrosinase from grapes and laccase produced by *Botrytis cinerea*, and peroxidase (POD) from grapes [46]. This enzymatic process occurs rapidly during and after the crushing operation.

2.3. Negative Effects of Sulfur Dioxide

Despite the numerous advantages found in the use of SO_2 during winemaking, drawbacks in its use have to be considered, both on wine quality and health consumers.

As regards the influence on wine quality, excessive doses can cause the appearance of sensory defects, bad smells, and unpleasant aromas [44]. These off-flavors are related to the formation, starting from the added SO_2, of hydrogen sulfide and mercaptans, which is observed in fermentations performed in strict anaerobiosis conditions, and in cases of prolonged contact of the wine with the lees [6].

Furthermore, during alcoholic fermentation in the presence of excessive doses of SO_2 and of a must poor in nutrients, the yeasts degrade the sulfur dioxide, with formation of typical off-flavors related to sulfur compounds, such as rotten eggs.

As regards the health effects, the ingestion of sulfites can cause problems, especially in sensitive subjects. The observed reactions can affect the skin (urticaria, angioedema, hives, and pruritus), respiratory system (bronchospasm, bradycardia, etc.), and gastrointestinal apparatus (nausea, stomach cramps, and diarrhea) [47]. These symptoms are not common in all people; adverse reactions are caused in a very small population (about 1%) of "sulfite-sensitive" individuals, most being very mild [48].

In consequence of such evidence, the World Health Organization (WHO) estimated the allowable daily intake of SO_2 to be about 0.7 mg per kg of body weight, and the European Community imposed dose limits of SO_2 for the different foods. For winemaking, the European Regulation (EU Regulation No. 606/2009 and No. 479/2008) has established the maximum doses of total SO_2 contained in wine, which have to be 150 mg/L for red wine and 200 mg/L for white and rosé or pink wines, in cases of wines containing a maximum of 5 g/L of reducing sugars. These doses can be increased by 50 mg/L when the sugar content in the wine (glucose + fructose) exceeds 5 g/L, reaching, 200 mg/L for red wines and 250 mg/L for white wines. Furthermore, specific doses have been defined for some wines, for example, for special wines (Bordeaux superieur, Tokaji, Moscato of Pantelleria, etc.), where the maximum doses of SO_2 can reach up to 400 mg/L. The EU Regulation No. 203/2012 establishes the maximum doses for organic wine as 100 mg/L for red wine, and 150 mg/L for white and rosé wines. Moreover, the EU Regulation has obliged to label the wine bottle with the phrase, "contains sulfites", when the SO_2 concentration exceeds a quantity of 10 mg/L.

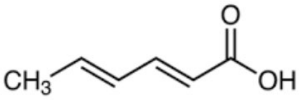
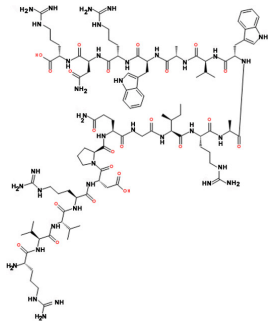
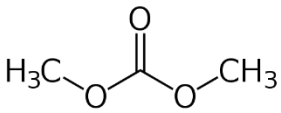
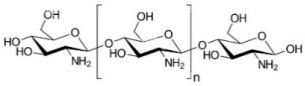
3. Alternative Methods to SO_2

Although sulfur dioxide is a compound with numerous advantages in winemaking, especially at a microbiological level, consumers' increasing attention towards "healthy"

products free of chemical additives is encouraging research on alternative preservation methods for reducing SO_2 use [6]. Furthermore, by considering the wide use of this compound in different food products, the risk is correlated to an excessive cumulative intake, and the World Health Organization (WHO) recommended the reduction of this preservative in sectors in which the use of SO_2 significantly contributes to daily intake, and this is the case for wine, particularly where it is regularly consumed.

For this purpose, several compounds have been authorized for the microbiological control of the winemaking process, in order to replace SO_2 or limit the used amounts (Table 2).

Table 2. Main applications, authorized doses, and antimicrobial activities of chemical compound alternatives to SO_2.

Compound	Chemical Structure	Admitted Amount	Winemaking Stage	Antimicrobial Activity
Sorbic acid		200 mg/L	Wine storage of sweet wines	Yeasts (*S. cerevisiae*, *Candida* spp.) in association with SO_2 [7,49]
Lysozyme *		500 mg/L (considered as cumulative, taking into account any additions to the must)	- Alcoholic fermentation in white wine - Maceration during alcoholic fermentation in red wine	Gram-positive bacteria (not active against Gram-negative bacteria and the yeast cell) [50]
Dimethyl dicarbonate (DMDC)		200 mg/L (with no residues in the marketed wine)	Prior to bottling in wine with sugar content ≥5 g/L	Yeasts (*Zygosaccharomyces bailii*, *Zygoascus hellenicus*, and *Lachancea thermotolerans*) [51]
Chitosan		10 g/hL	- Alcoholic fermentation in all the wines - Red wine aging in barrique	- Non-*Saccharomyces* yeasts, lactic acid bacteria [52,53], *Acetobacter* spp. [54] - *Brettanomyces* spp. [52]

* = part of the lysozyme chemical structure.

In this review, we will report the current state and recent updating of the main chemical methods actually approved or studied to reduce the sulfite content of the final wine, with special attention to environmentally friendly compounds to improve the sustainability aspects of winemaking.

3.1. Sorbic Acid

Sorbic acid is a short-chain unsaturated fatty acid not very soluble in water, but soluble in ethanol (112 g/L at 20 °C), used as an antimicrobial and antifungal in food preservation. In winemaking, it is used as potassium sorbate, which contains 75% sorbic acid and it is soluble in water [7].

The antimicrobial activity of sorbic acid is due to the combination of this molecule with the hydrosulfide group of the microbial enzymatic system, by destroying its activity [55].

The EU Regulation No. 606/2009 imposes a concentration limit of sorbic acid in wine on the market at 200 mg/L. These concentrations are highly tolerated by several contaminant yeasts present during winemaking, such as *Z. bailii* [56], *Brettanomyces* spp. [57], and *Saccharomycodes* spp. [58].

Sorbic acid is also inactive against acetic acid and malolactic bacteria, which are significantly affected by concentrations higher than 0.5–1 g/L [7].

In consequence of these considerations, sorbic acid plays its activity, in association with SO_2, against yeasts, such as *S. cerevisiae*, to avoid bottle refermentation during the conservation of sweet wines [49], and against flor yeasts (*Candida* spp.) able to develop on the wine surface [7]. Although the sorbic acid at the imposed concentrations does not modify the organoleptic characteristics of wine, in red wines, the LAB are able to react with sorbic acid, producing unwanted volatile compounds, responsible for a "geranium aroma" [7].

3.2. Lysozyme

Lysozyme is a muramidase enzyme isolated from egg white (EC 3.2.1.17) that can be proposed as a substitute of SO_2. It is a globular basic protein that consists of a single polypeptide chain of 129 amino acids, characterized by a molecular weight (MW) of about 14.4 kDa with an isoelectric point (pI) of 10.7, and the four disulfide bridges present in this molecule cause a high thermal stability of the enzyme. This enzyme is able to cause the lysis of the bacterial cell, leading to its death [59]; in particular, it breaks the glycosidic bonds between N-acetylmuramic acid and N-glucosamine in the bacterial wall of Gram-positive bacteria. For this reason, lysozyme is active against Gram-positive bacteria, and it is scarcely or not at all active against Gram-negative bacteria (AAB) and the yeast cell [50]. In winemaking, lysozyme is used for the partial replacement of sulfur dioxide in different stages of the technological process, and as a fining agent [60]. In grape must, lysozyme decreases the cell population of LAB [61] without affecting yeast activity and the progress of fermentation. Gram-positive bacteria responsible for must contamination consist of species such as *Pediococcus* spp., *Lactobacillus* spp., *Leuconostoc mesenteroides*, and *O. oeni* [62]. As reported by Delfini et al. [50], based on the composition of their cell wall, they show different degrees of sensitivity or resistance to lysozyme: *O. oeni* is more sensitive than *Lactobacillus* spp. and *Pediococcus* spp., which are resistant to higher concentrations of lysozyme.

The control of LAB is useful in white winemaking, where the addition of 125–250 mg/L of lysozyme allows the inhibition of MLF [62], and in red winemaking, to avoid the increase of volatile acidity and for the optimal regulation of MLF development. In this case, the addition of lysozyme inhibits the bacterial proliferation during prolonged maceration times, avoiding fermentation blocks and facilitating the subsequent inoculation of the selected malolactic starter [61].

As reported by Bartowsky et al. [63], in red wines, lysozyme is poorly effective, as the polyphenolic compounds can inhibit its effect, whereas the best effect is found in white wines. However, the lysozyme can induce heat instability (haze); consequently, clouding phenomena may occur due to protein haze, and a protein stabilization is necessary after the treatment of white wines with lysozyme.

As regards the limits of use, Regulation CE No. 606/2009 requires a maximum addition of 500 mg/L in the must, whereas in wine, this dose must be considered as cumulative, taking into account any additions to the must. Furthermore, by considering that lysozyme used in winemaking is isolated from egg white and it is designated as the egg allergen, Gal4, it might give rise to an allergic reaction in people sensitive to eggs, even in small amounts, and especially in wines not treated with bentonite, which leads to the absorption and precipitation of proteins [64]. In consequence of this, the current European regulation (directive 2007/68/CE) requires that for concentrations equal to or greater than 0.25 mg/L, the presence of lysozyme has to be reported on the label.

The addition of lysozyme, however, does not lead to negative changes in the alcohol content, pH, and organoleptic characteristics of the wine; in red wines, egg proteins containing lysozyme bind tannins by electrostatic interactions, preventing color loss [60]; furthermore, as reported by Sonni et al. [65], the use of lysozyme, together with the addition

of oenological tannins, improves the aroma of wine in consequence of the higher content of esters and acids.

Other studies [66] confirmed that wines obtained with the use of combinations of lysozyme and dimethyl dicarbonate (DMDC), another compound proposed as alternative to SO_2 (which will be discussed later), have better aromatic characteristics than wines treated with SO_2 alone.

All these considerations indicate that the use of lysozyme might lead to a reduction of SO_2, but it cannot be used for total replacement of sulfites alone, since it has only antibacterial activity, but it is not able to control the proliferation of contaminating yeasts and Gram-negative bacteria, nor oxidation phenomena in musts and wines.

3.3. Dimethyl Dicarbonate

Dimethyl dicarbonate (DMDC) is an organic compound possessing antimicrobial activity. The European Union, Australia, and the USA have authorized its use in winemaking, and the CE Regulation No. 606/2009 authorizes the use of DMDC up to a maximum dose of 200 mg/L, but without residues found in the wine on the market. Furthermore, the same regulation foresees the addition during the bottling only in wines with a sugar content equal or greater than 5 g/L, whereas in the USA, the DMDC can also be added during wine aging.

In wine, DMDC is quickly hydrolyzed to methanol and carbon dioxide within 12 to 24 h, though the concentration of these compounds does not cause toxicological effects in the wine; however, to ensure the efficacy of this compound, a rapid and adequate homogenization is necessary [51]. The most common commercial application is the addition to wines just prior to bottling [67]. Furthermore, Delfini et al. [68] have shown that it does not alter the organoleptic characteristics of wine.

The action mechanism has been known for some time; this compound reacts with the amino groups of some enzymes in the microbial cell, such as alcohol dehydrogenase and glyceraldehyde-3-phosphate [69], causing the methoxy carbonylation of nucleic residues [67], and leading to the death of the microbial cell.

DMDC is more efficient than SO_2 against yeasts, as it does not induce the yeast cell into a VBNC state, as it happens for SO_2, but it leads to cell death [70]. However, its use is recommended in addition to SO_2, because DMDC does not have antioxidant activity, and, at approved doses, it is ineffective against bacteria. In order to exert antibacterial activity, high and unapproved doses would be necessary. In fact, doses of 1000 mg/L are necessary to inhibit the growth of *Acetobacter aceti*, whereas doses of 500 mg/L are necessary for the inhibition of *Lactobacillus* spp. [68].

The antimicrobial efficiency, however, depends on the temperature, the pH, the ethanol content, the microbial species, and its initial concentration. Very early results [71] suggested that DMDC was more effective in wine rather than grape must due to the synergistic action with ethanol. Some authors [51] demonstrated that the species most sensitive to DMDC are *Z. bailii*, *Zygoascus hellenicus*, and *Lachancea thermotolerans*, whereas other species, such as *Sch. pombe*, *D. bruxellenisis*, *S. cerevisiae*, and *Pichia guilliermondii*, are also sensitive, but at slightly higher concentrations. More recent studies [72] evaluated the efficacies of the addition of the legal limit of DMDC against different yeasts associated with grapes and wines, confirming the high sensitivity for *Zygosaccharomyces* species, whereas for other species, such as *Candida*, *Metschnikowia*, *Meyerozyma*, or *Wickerhamomyces*, it was demonstrated that the additive may not provide long-term microbiological stability. The response of *Brettanomyces* to DMDC varied, indicating the need for additional research with a high number of strains in order to clarify the efficacy of this additive for long-term microbiological stability.

3.4. Chitosan

Chitosan is the main derivative of chitin, a biopolymer composed of N-acetylglucosamine units linked by β (1→4) linkages. This polysaccharide is the most abundant in nature after

cellulose, and it is found mainly in mollusks, crustaceans (the main commercial source), fungi, and insects [73]. Chitin is insoluble in common solvents, whereas chitosan, obtained by the removal of the acetyl group by deacetylation reaction, is a more soluble form [74,75]. Chitosan, indeed, is soluble in weak organic acid solutions at different concentrations, based on its degree of deacetylation (DD) and the MW [76]. These properties (DD and MW) influence the suitability of chitosan for different applications.

Actually, the biological activities of chitosan, including the antimicrobial activity against foodborne, filamentous fungi, yeast, and bacteria, have attracted notable interest for its use as a potential food preservative of natural origin [77].

Recently, in winemaking, chitosan has been accepted by the European Commission (Reg CE 53/2011) as a fining agent for the treatment of wines, for different purposes; in this sector, only chitosan derived from *Aspergillus niger* is admitted, in order to avoid any potential concerns of allergenicity correlated to the crustacean raw material.

The OIV (oiv-eno-338a-2009) has authorized the use of chitosan in different doses for the following applications:

- The addition of 100 g/hL is authorized to prevent hazing in wine, and to reduce the concentration of heavy metals (Fe, Pb, Cd, Cu). Different studies confirmed the clarifying action of chitosan and the prevention of protein haze phenomena, mainly in white wine, with the use at the permitted doses [78–80]. Similar results were found also in matrices different from the wine, such as beer [81] or fruit juices [82,83]. Chitosan also has an action of sorption of heavy metals, such as iron and copper, avoiding the formation of hazing phenomena in wine. A reduction of 70% of iron and 30% of copper is observed at a dose of 1000 mg/L [84], whereas other authors reported the ability to reduce the content of iron, lead, and cadmium in wine by adding doses of this polysaccharide ranging from 200 to 2000 mg/L [85].
- Doses of 500 g/hL are allowed to reduce any contamination by ochratoxin A (OTA), a mycotoxin which can be present in wine at a maximum dose of 2 µg/L (EC 2005), produced by fungi of the genera, *Aspergillus* and *Penicillium*, classified in group 2B as a "possible human carcinogen" by the International Agency for Research on Cancer [86]. Different studies demonstrated the ability of chitosan, at doses of about 4000–5000 mg/L, to remove a large percentage of OTA in wine [87,88].
- Amounts of 10 g/hL can be used to reduce the concentration of unwanted microorganisms, especially *Brettanomyces* spp. Details regarding the antimicrobial activity will be reported below (see Sections 3.4.1–3.4.3).

A further action of chitosan in the winemaking process is the antioxidant activity, although the use for this activity is not yet officially approved by the OIV. Indeed, Chinnici et al. [84] observed that the addition of 1000 mg/L of chitosan inhibits oxidation and, therefore, the browning of wine, with an action comparable to the addition of 80 mg/L of SO_2. Other authors [89,90] also showed the anti-radical action exerted by chitosan at doses ranging from 100 to 2000 mg/L. Furthermore, this compound has been the subject of a GRAS (*Generally Recognized as Safe*) notice to the United States Food and Drug Administration (US FDA) for its intended use in wine. However, due to its recent introduction in winemaking, the actual range of applications and the potential limitations of its use have not yet been fully clarified [91]; for example, Scansani et al. [92] reported that the addition of chitosan in fermentations with *S. cerevisiae* and *Sch. pombe* influences the final chemical composition of wine.

3.4.1. Antiseptic Activity

The antimicrobial mechanism of action of chitosan is not yet well elucidated; however, several mechanisms have been proposed (Figure 2).

In particular, the most accredited hypothesis is an electrostatic interaction between the positive charges of chitosan molecules and the negative charges of the teichoic acids in the cell wall of the Gram-positive bacteria (Figure 2A) [93–96]. These interactions cause a series of reactions, including a permeability increase, osmotic imbalance, interference with

the electron transport chain, and bacterial cell death. Furthermore, the different microbial cells show different degrees of sensitivity to chitosan, as a consequence of the different cell structure [94]. In fact, the microorganisms that contain chitin in their cell wall (as yeasts) or Gram-negative bacteria (their outer membrane essentially consists of lipopolysaccharides with negatively-charged phosphate and pyrophosphate groups) have been found to be less susceptible to chitosan [75,97].

Other authors [98] proposed that chitosan, once absorbed by the bacterial cell, interacts with its DNA, causing an inhibition of DNA transcription, mRNA synthesis, and, therefore, protein synthesis (Figure 2B).

A further mechanism was proposed by Ralston et al. [99], according to which, chitosan forms a layer on the yeast cell surface that hinders the entry of nutrients (as glucose) into cells, leading to their death (Figure 2C).

Finally, Guibal [100] and Kong et al. [101] observed that chitosan chelates micronutrients and metal ions important for the stability of the outer membrane in Gram-negative bacteria [102], leading to eventual cell death (Figure 2D).

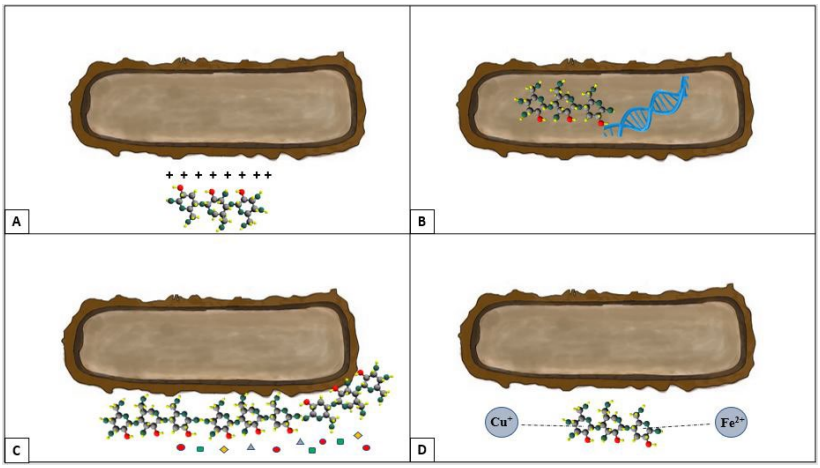


Figure 2. Chitosan antimicrobial mechanisms of action. (**A**) = Electrostatic interactions with the cell wall of Gram-positive bacteria [93–96]; (**B**) = Interaction with bacterial DNA [98]; (**C**) = Formation of chitosan layer on the yeast cell surface [99]; (**D**) = Chelation of micronutrients and metal ions with impact on the Gram-negative bacteria cell wall [100–102].

Generally, chitosan has a stronger antimicrobial activity against bacteria rather than against fungi [103]. Furthermore, the antibacterial effects of chitosan are dependent on its MW [104,105] and the DD [103].

3.4.2. Activity against Yeasts

In relation to wine yeasts, chitosan generally shows higher inhibitory effects towards non-*Saccharomyces* species than *S. cerevisiae* [52,106], and this aspect is very useful for the correct management of alcoholic fermentation. Indeed, it has been reported that chitosan has a biocidal action on *S. cerevisiae* only at doses higher than levels permitted in winemaking by the OIV [52,97]; only a 2–3 day increase in lag-phase has been observed, with differences correlated to the different *Saccharomyces* strains [107]. The observed differences among *S. cerevisiae* strains might be associated with the content of constitutive polyunsaturated fatty acids in the yeast cell membrane, which is correlated to the permeability and fluidity of the membrane. Strains with higher amounts of these compounds are more susceptible to chitosan, as this compound can enter more easily into their cytoplasm [108,109].

As regards the inhibition of non-*Saccharomyces* yeasts, the effectiveness of chitosan at a dosage of 400 mg/L on the most frequent species present in red grape must is lower than the effect of 50 mg/L SO_2 addition [110]. Other authors [111] analyzed the effect of chitosan in apple juice and elderflower against yeasts frequent in grape must. They reported that

a dose of 400 mg/L is able to inactivate *Hanseniaspora uvarum* and *Z. bailii*, and a dose of 300 mg/L is able to inactivate *Candida* spp. and *Rhodotorula* spp.

Among non-*Saccharomyces* species, particular attention should be paid to the effect on the contaminant *Brettanomyces/Dekkera* spp.; it was found that a 40 mg/L dose is able to reduce the *B. bruxellensis* contamination of red wine aged in barrique [112]. These authors reported that if the "batonnage", which favors the contact of the polysaccharide with the whole mass, is applied after the addition of the chitosan, this may favor the recovery of *Brettanomyces* cells, in consequence of the resuspension and oxygen incorporation. According to these authors, the activity of chitosan can be considered fungistatic rather than fungicidal at these concentrations. Petrova et al. [113] reported that the use of 80 mg/L reduced the microbial population by 3-log in 6–8 days after treatment, even if complete eradication was not observed, given the resumption of growth up to 10^5 CFU/mL at day 68.

Other studies performed on substrates different from wine showed the inactivation and inhibition of *Brettanomyces* spp., although different and higher doses than those authorized by the OIV were tested [52,106,114,115]. *B. bruxellensis* sensitivity to chitosan seems to be not affected by ethanol, whereas growth inhibition depends on the MW of the polysaccharide: chitosan, with low MW (107 KDa), is more effective than medium- and high- MW (310 and 624 KDa, respectively) chitosan [116].

3.4.3. Antibacterial Activity

The antibacterial action of chitosan has been investigated by several authors, with application in the food sector, although no direct studies have been carried out on wine or grape must. The addition of 0.3 g/L of chitosan to an apple/elderflower juice reduced the viable cells of LAB, with a positive effect compared to untreated juice. As the bacterial load was not knocked totally down by chitosan, the total bacterial count in treated juice tended to reach the same level of the untreated after storage for 8 days at 7 °C [111]. Furthermore, the different LAB species are differently sensitive to chitosan; indeed, *Lactobacillus* spp. showed a relatively higher resistance to chitosan than *Pediococcus* spp.; *Lactobacillus plantarum* was found to be more resistant than *Lactobacillus hilgardii*, *O. oeni*, and *Pediococcus* spp. [52,53].

Until now, very few data are available on chitosan activity against AAB; Valera et al. [54] reported that 200 mg/L of chitosan can reduce the population of *Acetobacter* spp. of 10^2 CFU in wine, showing similar results to the addition of 60 mg/L of SO_2, indicating that the efficacy of chitosan against AAB is similar to that of sulfites.

3.4.4. Sources of Chitosan

As previously reported, only the chitosan derived from *A. niger* is authorized for use in winemaking. Other sources from which the polysaccharide can be obtained are crustaceans and insects [73]. Currently, the main commercial source of chitin and chitosan comprises waste streams from the fishing industry, mainly the exoskeletons of crustaceans [117], since they contain from 15 to 40% of chitin in the exoskeleton [118], and are the major waste product from the marine food industry. However, the supply of crustacean waste is subject to seasonality and begins after spawning in spring [119], and the sustainability of crustacean farming is currently under debate [120]. Furthermore, the use of chitosan from crustaceans is not authorized in winemaking because of potential allergic reactions due to the release of fish protein into the product [79,121]. This supply chain is also characterized by a strong environmental impact, due to the pollution and waste generation during the processing phases. The global market for chitin and chitosan is expected to reach a volume of 282 thousand metric tons and $1.7 billion by 2027 [122,123], intensifying the need for a search of other sources to satisfy the growing market. A promising and sustainable alternative source is represented by insects, although they have not received much attention until now. Unlike crustaceans, insects are not subject to seasonality and can be easily reared, reproducing in laboratory-favorable conditions to their development, obtaining a large quantity due to their high fertility and reproductive rate [124].

In addition, comparing insect farms with traditional livestock and crustacean chains, it is possible to highlight the low environmental impact: they require much lower soil and water, and emit significantly lower levels of ammonia, carbon dioxide, methane, and nitrogen oxide [125–128].

Among insects, the most promising are the bioconverter species, such as *Hermetia illucens.* The larvae can feed on several different organic substrates of animal and vegetable origin [129–131], even decaying by-products and waste, reducing and converting them into larval biomass, rich in proteins, lipids, and chitin [132–134]. The bioconverter insect breedings, used for unconventional waste disposal methodologies and to produce animal feed or novel food production, generate huge amounts of side streams rich in chitin (pupal exoskeleton, called exuviae, and dead adults) that can be exploited to obtain chitosan with a view to a zero-waste circular economy.

Insect-mediated waste management is a growing trend; indeed, *H. illucens* is processed by around 80% of all EU insect-producing companies, and some industries utilize this insect also for high-quality chitin/chitosan production [135].

It is important to underline that the percentage of chitin varies among insect species and within the same species, in relation to different developmental stages. For example, in *Vespa crabro*, the chitin content for larvae, pupae, and adults is 2.2%, 6.2%, and 10.3%, respectively [136], whereas for *H. illucens* larvae, pupal exuviae, and adults, it is 13%, 31%, and 9%, respectively [137]. Moreover, the extraction method (chemical or biological) can affect the amount of chitin extracted from insects and the respective chitosan [137]. These variables also affect the chemical composition of the polymers, which showed a higher DD (around 90%) and a lower MW (lower than 100 KDa) compared to commercial chitosan. For this reason, it is always fundamental to take into consideration the source and the extraction process, in order to obtain chitosan with appropriate characteristics, according to the specific application.

Recent studies have also demonstrated the antimicrobial activity of *H. illucens* chitosan against Gram-positive and -negative bacteria [138]. These chemical and microbiological characteristics would make it optimal for the control of yeasts and bacteria during winemaking. Indeed, as previously reported, the highest antibacterial activity is given by low MW and high DD [116,139].

Moreover, some studies even reported potential insect allergies; to date, insects are not included as a major food allergen by the US FDA [140,141]. For this reason, it is possible to hypothesize that insect chitin and chitosan, which are polysaccharides, could not provoke an adverse reaction, and their usage in winemaking could be safe.

In conclusion, chitosan might be a promising tool for the reduction of sulfites in wine, as it combines antioxidant and antimicrobial properties, and it can be used in different steps of the winemaking process.

Further studies addressed to the use of this sustainable source of chitin and chitosan in order to support/substitute chitosan derived from *A. niger* (the only one authorized for use in winemaking) can add further value to this adjuvant in the winemaking process.

3.5. Other Substances

In this paragraph, other substances showing antimicrobial properties will be discussed. Some of them are admitted for applications other than microbiological stabilization, such as polyphenolic compounds, whereas others are innovative chemical additives, for which the use in winemaking is not yet permitted in EU, such as colloidal silver complex.

3.5.1. Phenolic Compounds

In recent years, the addition of phenolic compounds during winemaking as an alternative to SO_2 have been evaluated for their antioxidant and antimicrobial activity [13]. Phenolic compounds or polyphenols, naturally occurring in grapes and wines, are chemical additives permitted in enology by the OIV, following the EU legislation (Reg. EC No. 606/2009 and further modifications), when their amount in grapes and wines is too low.

Indeed, the natural concentration of wine phenolic compounds depends on various factors related to the grape (cultivar, time of the harvest, soil, climate, etc.) and enological practices (maceration time and temperature, fermentation with skins and seeds, enzyme addition, pressing, MLF, etc.) [142,143]. The major phenolic compounds present in wine are phenolic acids (hydroxycinnamic and hydroxybenzoic acids), flavonoids, condensed tannins, anthocyanins, and stilbenes (resveratrol). These compounds are responsible for many wines' organoleptic characteristics, such as color and astringency [144]. Moreover, the polyphenols are also associated with health benefits related mainly to cardiovascular and degenerative diseases [145], due to their well-known antioxidant, anticancer, or anti-inflammatory effects [146]. Several studies have demonstrated the role of phenolic compounds in wine for their antimicrobial activity against LAB and pathogenic bacteria [147,148], such as *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Salmonella enteritidis*, and *Pseudomonas aeruginosa* [149], suggesting their potential use as novel "natural antimicrobial agents" in winemaking. To date, the action mechanism of phenolic antimicrobial activity is not fully clarified. Several authors have reported that phenols increase cytoplasmic membrane permeability with a leak of bacterial intracellular constituents, and they can also alter the composition of fatty acids [150,151]. Some studies reported their inhibition of the synthesis of peptidoglycan, an essential component of the Gram-positive cell wall; furthermore, the inhibition of nucleic acid synthesis and interactions with cellular enzymes were observed [152–154].

In recent years, Garcia-Ruiz et al. [155] reported a comparative study of the potential inhibitory effects of 18 phenolic compounds (among them, hydroxybenzoic acids, hydroxycinnamic acids, phenolic alcohols, stilbenes, flavan-3-ols, and flavonols) on different LAB strains belonging to the species, *O. oeni*, *L. hilgardii*, and *Pediococcus pentosaceus*, isolated from wine. As expected, the results confirmed that the antimicrobial activity of the wine phenolic compounds is influenced by different phenolic chemical structures. In particular, flavonols and stilbenes showed the highest inhibitory effect on tested LAB growth; phenolic acids and their esters had a medium inhibitory effect, and the flavan-3-ols showed the lowest effect on the enological LAB strains studied. However, the LAB strains tested were more sensitive to the phenols in comparison with SO_2 and lysozyme. The antimicrobial activity of phenolics depends upon the microbial species and concentration added. For example, it was found that gallic acid and catechin used in concentrations normally present in wines (about 200 µg/mL) stimulate the growth and increase of the *L. hilgardii* population. On the contrary, at doses of 1000 µg/mL, these compounds had an inhibitory effect on bacterial development [156]. In addition, *O. oeni* seems to be more sensitive to phenolic compounds than *L. hilgardii* [152]. Syringaldehyde, one of the less-investigated polyphenols, represents a promising compound as a potential sulfite substitute because of the good inhibition results against spoilage bacteria (LAB and AAB) and non-*Saccharomyces* yeasts at doses of 250 µg/mL, a concentration that is higher than its sensory threshold in wine (50 µg/mL) [157].

In another study, Stivala et al. [153] showed that hydroxycinnamic acids (trans-p-coumaric and trans-caffeic acid) showed high inhibitory activity towards the growth of wine spoilage LAB strains (*L. hilgardii* and *P. pentosaceus*) in a synthetic wine-like medium (SWM), supplemented with 400 mg/L of each of the tested compounds, resulting in alterations of the bacterial cell integrity due to phenol adsorption.

Among these natural substances, resveratrol, belonging to the stilbene family, has been recently evaluated as an interesting and innovative substitute to SO_2 in red wines, for its antioxidant and antimicrobial activity against spoilage microorganisms, such as AAB, LAB, and the yeast of the genera, *Dekkera*, *Zygosaccharomyces*, and *Hanseniaspora* [158].

Phenolic compounds are also very abundant in winemaking by-products, such as grape pomace, seeds, and stems. The exploitation of wine production waste, in order to extract phenolic compounds that can be used as natural preservatives in wine, can constitute a sustainable approach based on the valorization of by-products, turning them into an interesting product with added value in the framework of a circular economy [149,154].

In this context, the olive mill waste extract, with a high hydroxytyrosol concentration, obtained from olive mill waste through a patented process [159], is a natural compound, recently tested as a potential alternative to SO_2 in winemaking. Hydroxytyrosol is a phenylethyl alcohol, naturally found in wine in a wide concentration range, with high antioxidant and antimicrobial capacity, and among other oil polyphenols, has been recently accepted as a protective compound against oxidative damage [160]. Although it was demonstrated that this olive hydroxytyrosol-enriched extract might be a suitable source of both antioxidants and antimicrobials, giving good results in model wine, the effectiveness of this compound in comparison to SO_2 depends on the microorganisms. Its antimicrobial activity was similar to that of SO_2 for *H. uvarum*, *Candida stellata*, *L. plantarum*, *Pediococcus damnosus*, and *A. aceti*; higher for *O. oeni*; and lower for *D. bruxellensis* and *Botryotinia fuckeliana*. However, this extract itself is not sufficient for the effective replacement of SO_2 in wines, but the well-known health properties and bioavailability of hydroxytyrosol would increase the added value of these wines with low SO_2 content. Although good results have been obtained with this compound in model wine, further experiments on real wines should be performed to confirm its usefulness.

3.5.2. Colloidal Silver Complex

The antimicrobial properties of silver have been known since ancient times. Silver nanomaterials are used in the food sector for water purification [161], and also in new packaging materials with antimicrobial properties [162]. Recent studies report the antimicrobial activity of these materials against a large scale of Gram-negative and Gram-positive bacteria, other than some antifungal and antiviral activities [163]. Although the exact action mechanism has not been fully elucidated yet, the existing experimental evidence supports different mechanisms correlated to the physicochemical properties of these materials, such as size and surface, which allow them to interact or cross cell walls or membranes, directly affecting intracellular components. The first action mechanism postulates that these particles cross the outer membrane, accumulating in the inner membrane, where the adhesion of the nanoparticles generates cell destabilization and damage, and, subsequently, its death. The second mechanism proposes that nanoparticles can also enter into the cell, where they interact with sulfur or phosphorus groups, present in DNA and proteins, altering their structure and functions. In the same manner, by interacting with thiol groups of the enzymes, they induce the formation of reactive oxygen species and free radicals, generating damage to the intracellular machinery. A third mechanism is the release of silver ions from the nanoparticles, which can interact with cellular components, altering metabolic pathways, membranes, and even genetic material. Colloidal silver complex was tested for its effectiveness as an antimicrobial agent instead of SO_2 in both white and red winemaking [164]. The authors found that doses of 1 g/kg of grapes are able to control AAB and LAB development, allowing the growth of *S cerevisiae* was at rates similar to those observed with SO_2. The silver concentration in finished white and red wines was 18.4 mg/L and 6.5 mg/L, respectively, which were below the legal limits of 100 mg/L, established by the OIV for silver content in the final wines. Other authors [165,166] reported the effects of two silver nanoparticles coated with biocompatible materials (polyethylene glycol and reduced glutathione) against relevant wine-related microorganisms. These new materials have exhibited great potential to be used as antimicrobials to control LAB and AAB after alcoholic fermentation, even more effectively than SO_2, and their action against yeast was greater than that of SO_2.

However, the precise effects on different wine-related microorganisms and the conditions for an effective application of these nanomaterials in wines needs further consideration. Furthermore, their toxicity and the impact of this application on the composition and sensory properties of wine have to be evaluated to ensure application at the winery level.

4. Conclusions

This review discussed the main chemical compounds with the potential to ensure the microbiological control of the winemaking process maintains the organoleptic properties of the wine as much as possible. Sulphur dioxide is still the main antimicrobial agent used in winemaking to decrease the risk of microbial spoilage, but due to the potential health problems that may arise by its usage, other treatments have been developed and applied to control the activity of undesirable microorganisms in wine.

Therefore, the other chemical compounds currently admitted by the official legislation, such as sorbic acid, lysozyme, DMDC, and chitosan, and other substances showing antimicrobial properties, for which the use for wine microbiological stabilization is not yet permitted in EU, were discussed. To date, a single compound able to completely replace SO_2 is not yet available, but a combination of different procedures might be useful to reduce the sulfite content in wine.

Among the proposed alternatives to SO_2, the insect-based chitosan might represent an innovation which can meet the consumers' expectations, who are even more interested in the healthy traits of food products and in the use of environmentally friendly practices in the production process. Furthermore, this approach can contribute to increase the competitiveness of wine producers, as the application of pro-environmental business practices may promote the profile of a brand and improve its image.

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Review of practices for the reduction of so2 doses used in winemaking

Status: In force

OIV Standards and technical documents Resolutions

Review of practices for the reduction of so2 doses used in winemaking

REFERENCE OIV-OENO 631-2020

REVIEW OF PRACTICES FOR THE REDUCTION OF SO_2 DOSES USED IN WINEMAKING

THE GENERAL ASSEMBLY,

IN VIEW OF THE ARTICLE 2, paragraph 2 b) ii of the Agreement of 3rd April 2001 establishing the International Organisation of Vine and Wine,

AT THE PROPOSAL of the “Microbiology” and the “Technology” Expert Groups,

CONSIDERING the interest of the wine sector in producing wines with low SO_2 levels,

DECIDES to adopt the following document as OIV guidelines for the reduction of SO_2 doses used in winemaking:

Background

The production of wines of high organoleptic quality is one of the priority goals for the world oenology industry. In parallel, in the last few years consumer demand for products free from chemical additives has grown. In this context, the OIV made the safety of consumers and their expectations one of the strategic axes of the Organisation's ve-year Strategic Plan 2015-2019 (Axis 4 "Contribute to the safety of the consumers and consider their expectations").

One of the issues of contemporary oenology is the use of sulphur dioxide (SO_2), the most frequently used chemical additive in winemaking. In the present document the term "SO_2" refers to the main forms of sulphur dioxide in equilibrium in wine (molecular SO_2), bisulphite ions- HSO_3^- and bound SO_2). SO_2 is a tool of choice to preserve wine quality, thanks to its antiseptic, antioxidant, and of enzymatic inhibition properties (Ribéreau-Gayon et al., 2006). During the phases preceding alcoholic fermentation, SO_2 makes it possible to limit oxidations and to reduce the total microbial load of must, favouring the selection of microorganisms most suitable for alcoholic fermentation such as Saccharomyces Cerevisiae, thus reducing the risk of fermentation defects. In nished wine, after malolactic fermentation if any, the use of SO_2 is aimed to eliminate spoilage microorganisms, such as lactic acid bacteria, acetic acid bacteria and yeasts of the Brettanomyces/Dekkera genera, which are responsible for the off-odour related to the production of volatile phenols. Oxygen in must and wine comes from the air, and it is dissolved during winemaking, ageing and storage operations. Depending on the wine composition and the level of oxygen exposure during winemaking, oxidation could have both a positive and negative impact on wine quality. SO_2 is effective in preventing the appearance of oxidative off- avours and degradation of numerous aromas and of the colour. An the form of HSO_3^- is able to inhibit oxidation enzymes in must and to prevent the oxidative browning (Du Toit et al., 2006; Waterhouse et al., 2016). Among oxidase enzymes, laccase from Botrytis spp. is less sensitive than grape tyrosinase, so musts produced from grapes affected by Botrytis cinerea require higher sulphiting levels (Du Toit et al., 2006). In wine, where chemical oxidation is predominant, sulphites react with hydrogen peroxide, and inhibit the Fenton reaction responsible for the oxidation of ethanol and other organic compounds by hydroxyl radical formation (Danilewicz, 2007). The reaction between sulphites and hydrogen peroxide also occurs in musts, although in these the chemical oxidation occurs to a much lesser extent. Another effect of sulphur dioxide is its ability to react with quinones formed during the initial stages of oxidative chain reactions, reducing them to phenols (Danilewicz et al., 2008; Waterhouse & Laurie, 2006). Moreover, it binds aldehydes (e.g., acetaldehyde), thus playing a role in reducing the perception of typical oxidative off- avours (Waterhouse & Laurie, 2006). Concerns have recently arisen regarding the safety of sulphites in food. Sulphur dioxide and sulphites have well-known toxic effects, both acute and chronic in sensitive subjects. They exacerbate respiratory, dermatological, cardiovascular and gastrointestinal symptoms, manifested mainly as asthmatic-like reactions in "sulphite sensitive" individuals, while severe allergic-like reactions (anaphylaxis) are uncommon

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on the market there is still a signi cant number of strains capable of producing signi cant levels of sulphites during alcoholic fermentation.

Today, following consumers demand, the tendency is to decrease the use of sulphite in winemaking. Different other additives and innovative physical methods, such as pulsed electric elds, have been proposed in order to achieve the various objective of the use of sulphur dioxide (Lisanti et al., 2019). Some alternative additives are already authorised in winemaking, while most of additives and innovative physical methods have been tested only on an experimental scale. However, based on current knowledge, none of these alternatives showed to be able to totally replace SO_2, which remains a useful, even indispensable additive in some cases.

These guidelines contain recommendations for the management of the entire winemaking process – from the vine to the bottle – to reduce the use of SO_2 without compromising wine quality in terms of organoleptic characteristics and microbiological stability.

General principles

A small and modulated oxygen intake could be necessary to enhance wine colour and aroma, especially in red wine production, always guaranteeing microbiological stability.

The factors to be considered are:

- the must and wine microbial load,
- the presence of oxidase enzymes derived from fungi in musts,
- the concentration of SO_2-binding substances in must and wine,
- pH,
- wine temperature,
- oxygen exposure,
- presence and concentration in wine of endogenous antioxidant compounds.

Some viti-vinicultural practices are able to in uence one or more of these factors, thus modifying the SO_2 requirement, by increasing or decreasing it.

The **phases** in which the addition of SO_2 is advised in order to prevent oxidation and microbial spoilage are, as a minimum:

- at harvesting, in the case of mechanical harvesting, in the production of white and rosé wines,
- pre-fermentative phases (grapes and must), especially for white and rosé wines,
- at the end of alcoholic fermentation (or malolactic fermentation, if applicable),

For the treatment of microbiological accidents, sulphitation through the addition of SO_2 in gaseous form (released from liquid SO_2) should be preferred for its greater effectiveness.

With the same total SO_2 concentration, the balance between its different forms (free, molecular and bound) depends on the chemical-physical characteristics of wine, in terms of pH, alcohol content, compounds capable of binding SO_2, as well as temperature. The fraction of SO_2 bound to acetaldehyde is very poorly available for wine protection, as this combination is very stable, therefore it should be minimised by reducing the formation of acetaldehyde in wine, both from chemical and from microbiological origin (Waterhouse et al., 2016; Capece et al., 2020).

In order to guarantee effective protection, reference values of 20-40 mg / L of free SO_2 can be considered for antioxidant protection, while to prevent microbial alterations in the nished wine 0.6 mg / L of molecular SO_2 in dry wines and at least 0.8 mg / L of molecular SO_2 in sweet wines are reported as reference values (Waterhouse, 2016). The reduction of the total SO_2 levels should be carried out by always guaranteeing adequate levels of protection, based on the characteristics of the wine, the storage conditions and the expected commercial life.

In the present document the entire winemaking process is analysed by identifying the Points of Intervention (PI) that are useful for the reduction of total SO_2 doses and giving indications for their proper management.

Classi cation of the Points of Intervention (PI)

In this document, PIs are coded as follows:

- PIa) point of intervention to limit the presence and the activity of oxidase enzymes;
- PIb) point of intervention to prevent must and wine microbial spoilage;
- PIc) point of intervention to limit dissolution and consumption of oxygen in musts (c1 enzymatic oxidation) and oxidation reactions (c2 chemical oxidation);
- PId) point of intervention to enhance SO_2 ef cacy (d1 increasing of molecular SO_2; d2 minimising the formation of compounds binding SO_2, including those derived from grapes affected by fungal diseases);
- PIe) point of intervention to avoid excessive or unwanted addition of SO_2;
- PIf) point of intervention to ensure the ef cacy of SO_2 protection.

All the oenological practices should be applied according to the OIV International Code of Oenological Practices. The present document should be updated following the admission of new oenological practices.

POINTS OF INTERVENTION:

- to ensure that the grape transport and winemaking equipment do not release metal ions (iron, copper and manganese) into must and wine (PIc2),
- to ensure that the grapes do not contain or contain low concentrations of metal ions from phytosanitary treatments such as copper (Bordeaux mixture) (PIc2),
- to ensure that pipes and the connections between pipes do not have any cracks or holes, and to periodically replace the tubes (PIc),
- to ensure that the internal surfaces of tanks and vats are perfectly intact and do not have any cracks (PIb),
- when choosing pumps to transfer must and wine, to consider that different types have different oxygen uptakes (e.g. centrifugal pumps are particularly disadvantageous if cavitation of the liquid which occurs during the transfer is not prevented) (PIc),
- to consider the oxygen porosity of the materials that make up the winemaking or storage vessels (PIc),
- to limit the oxygen uptake during the dynamic phases (turbulence due to the start / stop of pumps, Venturi effect due to poorly tight connections, transport in open compartments) and static phases (through holes and porous materials) (PIc),
- if SO_2 is supplemented in the form of potassium metabisulphite or solutions, to ensure that the product is not expired, that it has been properly stored and that the solutions have not crystallised (PIf),

Important note: It should be considered that SO_2 binding substances are generated by fungi from the vineyard as well as during alcoholic fermentation, and also in wine following contamination by aerobic yeasts and bacteria or following chemical oxidation reactions catalysed by metals such as iron and / or copper.

B. Vineyard management and harvest

- The selection of cultivation site and grape variety should be aimed at producing healthy grapes that have an adequate level of acidity at maturity, so that they have a suitable level of acidity upon reaching maturity. Any viticultural practice ensuring the sanitary state of grapes and/or low pH of the must should be applied in the vineyard (PIa, PIb, PId).
- As copper is a catalyst of chemical oxidation, its use in the vineyard should be limited as much as possible, but adapted to the protection of grapes from fungi development (PIc2).

- Especially for certain grape varieties and in hot climates, harvesting and processing a portion of the crop before maturity makes it possible to obtain a more acidic wine, which can be useful for increasing the acidity of the wine obtained from the remaining fraction harvested at full maturity ("double harvest"). As an alternative, it is possible to use a blend of different grape varieties at different stages of maturity. To do so, it is necessary to monitor the chemical parameters linked to technological maturity (soluble solids, total acidity, malic acid, tartaric acid, potassium and pH) (PId1).
- Harvesting should be selective in order to vinify separately bunches damaged by fungi in order to limit the presence of oxidase enzymes and spoilage microorganisms (PIa, PIb).
- In the case of mechanical harvesting, ensure that grape integrity is preserved as much as possible. Where possible, manual harvesting allows to limit the mechanical damage to the grapes (PIb).
- To avoid crushing of grapes and microbial proliferation, favoured by the release of the juice, whole healthy grapes should be transported in clean and well aerated containers, in order to avoid high humidity and mould growth. However, if grapes are damaged, it is preferable to vinify them separately, by using inerted containers to limit oxidation of the must and proliferation of aerobic microorganisms (Pia, PIb).

C. Grapes and must

- If musts from microbiologically altered grapes are obtained, these musts should be treated separately, from the reception of the grapes to the processing in the cellar, as they will need greater protection by the SO_2, therefore the use of higher doses (PIa-PIb).
- All pre-fermentative operations should be carried out as rapidly as possible, in order to limit oxygen exposure (PIc), except where hyperoxygenation of the must, followed by clari cation, is used as a practice to eliminate oxidisable compounds before vini cation.
- Pre-fermentative operations of destemming, crushing, pressing (or combinations of these) should be conducted as delicately as possible, in order to limit the extraction of potassium cations, thus preserving the must acidity (PId1), and reduce the extraction of oxidase enzymes (PIa).
- If necessary, acidi cation of musts should be performed. Acidi cation should be

- Early inoculation with selected microorganisms (yeasts and/or bacteria), even during the pre-fermentative phase, could be useful in preventing the development of undesirable indigenous microbial ora.

Speci cally for white or rosé winemaking:

- Antioxidant protection should be ensured during or soon after pressing. The addition of SO_2 to the mass should be perfectly homogeneous (PIc).
- To complement the action of SO_2, the addition of antioxidants (such as ascorbic acid, inactivated yeasts rich in glutathione, tannins) and antimicrobials (such as lysozyme, chitosan) may be considered (PIc, PIc).
- The addition of ascorbic acid, if any, must follow the addition of SO_2 (PIc2).
- Inert gas may be used in the more critical phases in oxidation terms, such as the transfer after destemming and / or crushing, pressing and must clari cation (i.e. static settling with or without enzymes or processing aids, otation, ltration or centrifugation) (PIc).
- The temperature of the musts in the pre-fermenting phase must be controlled so as to be compatible on the one hand with the chosen clari cation process and on the other with the limitation of the risk of development of a spontaneous microbial ora that would hinder the clari cation process. Although low temperatures slow down oxidative processes, the effect of low temperatures on the increase in oxygen solubility should be considered. As an indication, a temperature of about 15 °C in otation, less than 10 °C during static clari cation, of 20 °C in ltration or centrifugation can be recommended if the musts are treated continuously after their production and much lower the longer the time between mashing and treatment (PIc).

D. Alcoholic fermentation (AF)

- Inoculation with a starter yeast is advised; yeasts with good fermentative activity, high dominance, and low SO2, H2S, SO_2 binding compounds, and acetaldehyde production should be chosen. Yeasts should be properly stored. The preparation of yeast inoculums from active dry yeast or in other forms should be conducted according to manufacturer's instructions and should lead to an initial population suf cient to ensure a quick start to AF (approx. 1-2 x 106 CFU/mL), in order (PIb, PId, PIe).
- Co-inoculation with selected starter yeasts and lactic acid bacteria may favour the reduction of the lag period between the end of alcoholic fermentation and the start

SO_2 added in this phase would bind with carbonyl compounds, thus increasing the total SO_2 uselessly, at equal quantities to the free SO_2 present (PId2).

- Furthermore, the SO_2 added during AF is metabolised directly by fermentative yeasts with a proven risk of producing compounds responsible for reduction defects (H_2S).
- Oenological practices aimed at ensuring that AF runs normally and properly, and lowering the production of carbonyl compounds (among them thiamine addition, supplementation with nitrogen nutrient and growth factors, use of yeast hulls to detoxify the matrix, where necessary) and controlling the temperature should be implemented (PIb, PId2). Nitrogen can be added in mineral or organic form (sources such as yeast autolysate or inactivated yeasts).
- Fermentation kinetics should be monitored daily to verify that AF is running normally and properly. Stuck or sluggish fermentations should be detected as soon as possible. The use of automated temperature control systems is advised (PIb, PId2).
- In the case of stuck or sluggish fermentation, oenological practices to restart alcoholic fermentation must be applied as soon as possible. A proper addition of SO_2 is recommended in order to inhibit bacterial development prior to restarting AF. Detoxifying the matrix (elimination of fatty acids) using yeast hulls is also recommended. The volatile acidity should be monitored (PIb, PId2)
- The total transformation of fermenting sugars is advised for dry wines (reducing sugars < 2 g/L) (PIb).

E. Post-Alcoholic fermentation and malolactic fermentation

- In white and rosé winemaking, running off should be carried out while protecting the wine from the air. The receiving tank as well as the pipes and connections should be inerted with carbon dioxide, nitrogen or argon (or a mix of these) prior to wine input (PIc2).
- In red winemaking, soft pressing of pomace allows to reduce the extraction of potassium cations and then to limit pH increase (PId1).
- If malolactic fermentation is performed, it should be rapidly induced (PIb). The co-inoculation with yeasts and lactic acid bacteria or early inoculation with selected bacteria before or just after inoculation with yeasts could be considered useful to reduce the lag time between alcoholic fermentation and malolactic fermentation if conditions are suitable (PIb). Bacteria starter cultures should be prepared in

antimicrobial agent as chitosan complementing SO_2 action may be considered (PIb).

- Starting from the end of alcoholic fermentation, the concentration of molecular SO_2 should be monitored in order to ensure effective protection of the wine (PIf).

F. Stabilisation

- At the end of the alcoholic fermentation (or malolactic fermentation, if carried out), the wine should be sulphited. It is advisable to dose free acetaldehyde. The dose of SO_2 will therefore be chosen according to the pH, alcohol content and temperature, in order to guarantee antioxidant and antimicrobial protection (PIb-PIc2).
- In order to ensure effective protection of the wine, it is very useful to monitor, since the end of alcoholic fermentation, the quantity of total and free SO_2 and to calculate the molecular SO_2 concentration (PIf).

Physical treatments

- After alcoholic fermentation or malolactic fermentation, if any, the microbiological stabilisation of wine by physical (i.e. sterile membrane ltration and/or pasteurisation) and/or chemical methods (SO_2 possibly supported by other permitted antimicrobial chemical agents in wine, as chitosan) is recommended. Among these technical possibilities, the use of tangential micro ltration associated with permeate treatment seems to be particularly appropriate. Care should be taken to avoid subsequent recontamination (PIb).
- The initial and nal phases of different stabilisation practices such as ltration, micro- ltration, membrane processes and bottling are the most prone to introducing oxygen into wine, therefore the volume of the treated batch is critical and should be maximised. When possible, continuous processes are preferable (PIc2).
- The use of inert gas is advised for all of the processes mentioned in the previous point. If inert gas cannot be used for the whole duration of the process, consider that the most critical points are: the initial and nal phases of the process, the inerting of the empty apparatus (i.e. lters and pipes) and the inerting of feeding and receiving tanks. Consider that different types of lters may enrich wine with oxygen differently: careful attention should be paid to this characteristic when choosing between them (PIc2).
- The Venturi systems used for the addition of ning agents to wine and for their

electrodialysis) or products (carboxymethylcellulose, potassium polyaspartate, mannoproteins or metatartric acid) have to be considered, depending on the wine pro le and constraints (organic production for instance).

G. Conservation and ageing

- Tanks where wine will be transferred should be inerted and lled from the bottom to minimise the air that enters (PIc2).
- Implement a regular control plan for wines, based both on standard analytical parameters (pH, alcohol content, titratable acidity, volatile acidity, free and total SO_2), and on microbiological parameters (presence and level of contaminating microorganisms and products of their metabolism).
- Containers (tanks, barrels) should always be kept full (no headspace). Microbiologically stable wine should be used for topping up to avoid greater microbiological contamination. If the presence of headspace is unavoidable for a short period, use inert gas (PIb, PIc2).
- In case of yeast development at the surface of the wine, consider that these produce high quantities of acetaldehyde that binds with SO_2 in a stable way. For this reason, it is useless to try to eliminate these yeasts by adding SO_2 because the majority are extremely tolerant to it. Before re-establishing the free SO_2 content to protect the wine, remove the layer of yeasts by ltration and top up the container (PIb-PIc2-PId2-PIf).
- During wine conservation or ageing, maintain a constant temperature (indicatively, 13-18 °C). An overly low temperature increases oxygen solubility, while an overly high temperature is favourable to oxidation reactions and microbial proliferation (PIb, PIc2).
- Ageing on the lees of yeast and bacteria could be a useful practice in enhancing the antioxidant ef cacy of SO_2. This may be due to the consumption of oxygen for lipid oxidation, the bounding of carbonyl compounds and the release of reducing compounds. However, the volatile acidity and microbial load, as well as the free, bound and molecular SO_2, should be carefully monitored because products of yeast autolysis may favour the proliferation of spoilage microorganisms (acetic and lactic acid bacteria, Brettanomyces spp.). SO_2 protection should be ensured for its entire duration (PIc2).
- To complement the action of SO_2, the addition of antioxidant (such as tannins and inactivated yeasts rich in glutathione) and antimicrobial agents (such as lysozyme

- Consider that there are differences in oxygen permeability for wood from different botanical species, and for new barrels with respect to used ones, so these should be chosen carefully according to wine characteristics (i.e. polyphenol content, necessity for colour stabilisation, level of astringency) (PIc2)
- During wood ageing, efforts should be made to limit air penetration via the bunghole, or while topping up or opening for tasting. It should be ensured that there are no cracks in the wood (PIc2).
- Consider that the perfect sanitisation of old barrels is quite dif cult, especially with respect to contamination by Brettanomyces spp. Shaving and re- ring make it possible to remove the most contaminated wood layer, thus enhancing the ef cacy of sanitisation. Use effective techniques and materials in the deep layers of the wood. Potassium bitartrate crystals should be eliminated prior to sanitisation (PIb).
- Consider that the gaseous SO_2 used for wood container sanitisation may enrich the wine with SO_2 at the time of lling (PIe).

H. Packaging

- An effective hygienisation of the packaging line, including the bottles and the room, should be carried out, in order to avoid wine recontamination. When designing the process, prefer solutions that save energy and resources. If inert gas is used, this should be micro ltered, as well as the water for washing the bottles (PIb).
- Temperature at packaging should be maintained at around 15-20 °C (PIc2).
- Wine to be packed should be microbiologically stabilised. Microbiological control should be implemented prior to packaging, and carried out randomly on packed wine (PIb).
- Physical methods, such as sterile ltration and, in some cases, pasteurisation, ensure the reduction of wine microbial load and could allow for the use of lower doses of SO_2 (PIb).
- In wines that contain fermentable sugars, the use of products that complement the antimicrobial action of SO_2 (such as sorbic acid and dimethyl dicarbonate) may be considered (PIb).
- During packaging, if the wine contains a high quantity of dissolved oxygen (> 0.5 mg/L), it should rst be deoxygenated in a proper manner (PIc2-PIb).
- Bottle closures should be stored unopened in their original well-sealed packaging,

- The addition of SO_2 should take in account losses due to oxidation phenomena (quantity of dissolved O_2) and the expected shelf life of the wine (PIf).
- Ascorbic acid may enhance the antioxidant activity of SO_2, however its addition should be carefully evaluated considering the loss of SO_2during the expected wine shelf life. SO_2 antioxidant protection should be guaranteed until the expected time of wine consumption. Ascorbic acid should only be added after addition of SO_2 (PIb -PIc2).
- Inerting of the packaging line and of the containers (if possible and necessary) should be applied, paying particular attention to the beginning of the process of packaging and the last phases (PIb -PIc2).
- It should be ensured that the lling volume is properly regulated, also according to the temperature (PIc2).
- It should be ensured that the headspace is properly inerted after lling (PIb -PIc2).
- It would be good practice to measure the Total Package Oxygen (TPO) randomly during different phases of packaging, with a non-destructive optical method. The same method could be used to monitor the oxygen dissolved along the entire packaging line. Reference values should be taken into account to ensure these are not exceeded in wine after packaging (e.g. < 0.5 mg/L). It will be necessary to insert equivalent containers in the line that allow the measurement (transparent and equipped with sensor) (PIc2).
- The use of colourless glass bottles and transparent plastic containers should be avoided, with the latter material being permeable to oxygen. The use of green or brown glass bottles is advised (PIc2).
- It should be ensured that the homogeneity of the diameter of bottle necks is certi ed by the bottle manufacturer, as deviations from the nominal diameter may cause uncontrolled oxygen ingress (PIc2).
- Closures should be selected considering the oxygen permeability in relation to the wine compositional characteristics, in order to avoid oxidation. If using cork or plastic stoppers, choose products with short closure recovery times after compression (good elasticity). For wines particularly sensible to oxidation, the use of screw caps could be recommended (PIc2).

I. Shipping, storage and distribution

- High temperatures and direct light exposure should be avoided during shipping

and recording the temperature and humidity during transport should be provided for (PIc2).

- The nal wine distributor should be informed about the appropriate storage and distribution of the wine, in light of its low content of SO_2 (PIc2).
- Note: The OIV Good Practices Guide for Bulk Wine Transportation should also be taken into account with regard to this section.

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REVIEW ARTICLE

Chitosan and its applications in oenology

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ABSTRACT

This paper reviews the main applications of the biopolymer chitosan, the main derivative of chitin, a material usually obtained from natural sources accessible at low cost, i.e., industrial wastes from fisheries. Due to its natural origin, which confers biodegradability and biocompatibility properties, in addition to its low toxicity, chitosan has been gaining attention in numerous sectors, such as agriculture, food, medicine, pharmaceuticals, etc., including also important oenological applications due to its potential as a green alternative to the use of sulphite. Among the many applications that can be generated from these materials in the wine-making area, their use has been reported for the clarification of must; in the preparation of films for the removal of contaminants, whether organics such as ochratoxin A or inorganics such as some metal ions and their salts; the control of turbidity caused by protein precipitation; the encapsulation of yeasts of oenological interest and enzymes for the control of adverse microorganisms such as *Brettanomyces*; the manufacture of sensors and nanosensors for the quantification of contaminants, the quality control of starting materials and final products, the optimisation of fermentation processes, the monitoring of storage conditions, etc. As a result of this review, significant development of the applications of this material in the oenological area can be expected, especially due to the possibilities of preparing new derivatives, including the great variety of these that have been recently proposed through click reactions, as well as the growing incursion of chitosan in nanobiotechnology.

KEYWORDS: sulphite alternative, contaminant removal, nanosensors, ochratoxin A, clarification

INTRODUCTION

Wine is an old and very dear friend of man. It has also been considered since antiquity as one of the criteria marking the social evolution of humankind, as Herodotus states when referring to the advice given by the Lydian Sandamis to King Croesus in his struggles against the Persians. Throughout history, wine has also provided sublime examples of its use, such as the high regard given to it in Christianity as the blood of Christ, as well as biblical examples of the nefarious side of its abuse, as can be seen in the book of Genesis, chapter 9, verses 20–27, where it is narrated that one of Noah's first activities after the flood was to plant a vineyard, getting drunk afterwards with the wine produced and remaining naked in the sight of his youngest son Ham, who told his brothers Shem and Japheth, who covered him with their clothes but avoided seeing his nakedness. After waking up and realising what had happened, Noah cursed Canaan, son of Ham, condemning him to be a slave of Shem and Japheth.

Throughout history, many renowned scientists have made important contributions that have led to today's knowledge of winemaking processes. A brief summary to mention some of them includes the Dutchman Antoni van Leeuwenhoek, who developed high quality lenses for the time and was able to observe with his microscope, for the first time, some microorganisms he called "*animalcula*" (a word that has been translated as very small animals) in 1639 (van der Leeuwenhoek, 1939); the Frenchman Antoine Lavoisier, creator of the law of conservation of matter, who estimated in 1789 the proportions of sugars and water at the beginning of the fermentation reaction, adding yeast (calling it ferment) to continue the alcoholic reaction, and compared them with the proportions of alcohol and carbon dioxide obtained at the end, coming to the conclusion that sugars decompose into alcohol and carbon dioxide (Lavoisier, 1789), thus, providing a clear view of the basic principles of the chemical reactions necessary to produce alcohol; the French chemist Louis Joseph Gay-Lussac, in whose honour the degrees of alcohol in a wine are known as degrees GL, and who in 1810 carried out experiments with grape juice packed in closed bottles and heated for a time in boiling water, which were kept for a year without fermentation being observed, but after exposure to air were able to ferment, thus, concluding that heat inactivates yeast (Gay-Lussac, 1810); the German physiologist Wilhelm Friedrich Kuhne, who in 1878, to avoid the confusion caused by the double meaning of the word ferment, proposed to use the term "enzyme" for soluble substances that cause fermentation (even giving it a more general connotation and not restricted only to the fermentation process), and to leave the word ferment only to designate yeasts (Kuhne, 1878).

It is also important to mention that Gay-Lussac made calculations with the quantities of reactants and products in the fermentation processes, and his work in 1815 (Gay-Lussac, 1815) has been credited with the development of the chemical equation (1) describing the transformation of glucose into ethyl alcohol and carbon dioxide, although some researchers do not share this opinion (Barnett, 1998).

$$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2\text{-}OH + 2\ CO_2 \quad (1)$$

However, the real beginning of the systematic approach to the chemical and biological study of alcoholic fermentation can be dated back to 1857, in the work of the French chemist Louis Pasteur (Pasteur, 1857; Pasteur, 1860), who later became one of the most renowned bacteriologists of the modern world. Pasteur demonstrated, for the first time, that fermentation occurs only by the action of "live" yeasts, which transform glucose into ethanol, and that the process occurs in the absence of oxygen. From his experiments, he concluded that fermentation is a vital process that he called "airless respiration".

Another important point in the evolution of wine is related to the use of additives to prevent its decomposition, especially with the use of sulphur dioxide (SO_2), also known as sulphurous anhydride, sulphite, sulphur oxide, etc. Although the preservative and antiseptic effect of burning sulphur inside houses were known since ancient times, as mentioned by Homer in The Odyssey, the first recorded mention of SO_2 and its effects on wine seems to have also been made by Pasteur in 1866, demonstrating its antiseptic and antioxidant effects and recommended burning sulphur (Equation 2) inside wine barrels to make them more stable (Pasteur, 1866). At the time, the aim was to control the deterioration suffered by French wines during transport and storage for export (Nous les Vignerons de Buzet, 2017).

$$S_8 + 8\ O_2 \rightarrow 8\ SO_2 \quad (2)$$

Since then, SO_2 has remained an unrivalled additive in wine production, although, in more recent times, legal aspects have been regulated to control, and even try to eliminate in some wines, the use of sulphites. The main reasons that have led to these new practices include the need to improve the image of the naturalness of some wines and the prevention of some health damage associated with SO_2 consumption, such as allergies observed in sensitive consumers. In this regard, the acceptable daily intake established by the World Health Organization (WHO) for sulphites is 0.7 mg/kg body weight (World Health Organization, 2009), which means that the acceptable amount for an average person (~75 kg body weight) would be around 53 mg per day, a value that is reached with a daily intake of half a bottle (375 ml) of a red wine complying with the European Unión standard (up to 150 mg SO_2/l for red wines (European Union, 2019)) and even more easily in other countries such as the United States (up to 350 mg SO_2/L for red wines), where only one glass (150 ml) per day would suffice.

The search for alternatives to the use of SO_2 has led to the testing of various methods and substances for the protection and improvement of wines at different stages of their processing, whether these involve the application of physical processes (microfiltration, ultrasound, ultraviolet radiation, electrical pulses, microwaves, etc.), the use of chemical substances (sorbic acid, dimethyl carbonate, lysozyme,

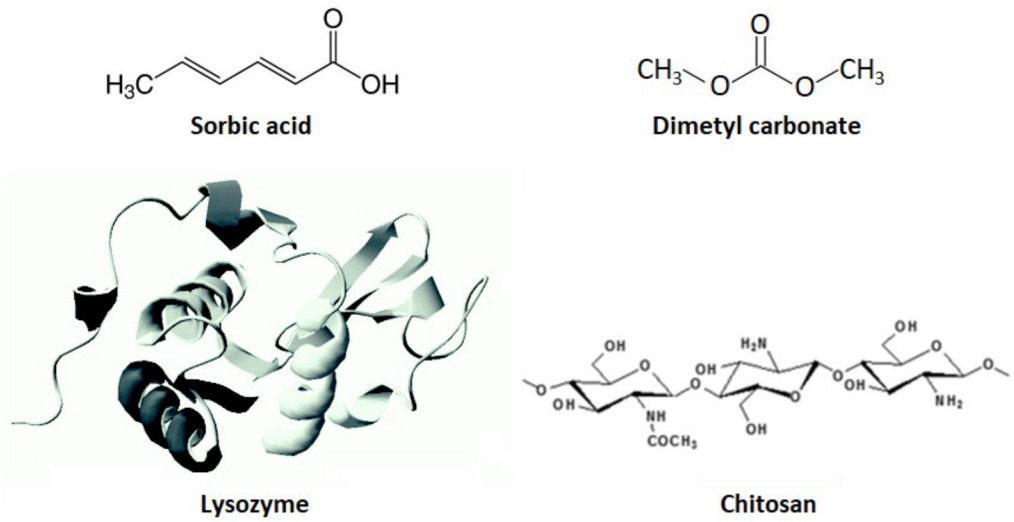

FIGURE 1. Structures of some additives currently used in oenological processes as green alternatives to SO_2.

chitosan, etc., see Figure 1) or the development of biological strategies such as the use of yeast strains with low capacity to produce SO_2 (Rauhut and Cottereau, 2009).

Despite the numerous studies carried out to date, with some of them showing satisfactory results, it has not yet been possible to completely replace the excellent duality of performance that this additive has for the preservation of wine properties, as an antimicrobial agent and as an antioxidant agent (Lisanti *et al*., 2019). Thus, over a long time, SO_2 has demonstrated a high efficacy in preventing the biological deterioration of wine and in the stabilisation of some of its most appreciated properties, such as fragrances and colour. Therefore, the current oenological task seems to be more focused on obtaining complementary methods to lower the sulphite content than on its total elimination.

Among the most prominent materials that have been investigated as an alternative to the use of SO_2 in oenological applications is chitosan. Its use has also been considered at various stages of the oenological process, including must clarification with approval of the Organization International of the Vine and Wine (OIV) (OIV, 2009a), during the fermentation process (Scansani *et al*., 2020), before packaging (Mármol *et al*., 2012), during storage (Nunes *et al*., 2016), etc. Additionally, chitosan could also be used as a matrix for the encapsulation of yeasts of oenological interest, like what has been tested in bioethanol production (Namthabad and Chinta, 2012), as well as for the preparation of membrane-based membranes for the nanofiltration of effluents in winemaking (Miao *et al*., 2008).

From the point of view of legal regulations for its use in oenology, chitosan obtained from safe and abundant food or biotechnological fungal sources, such as *Agaricus bisporus* or *Aspergillus niger*, was accepted in 2009 by the OIV for the improvement of some oenological processes (OIV, 2009a; OIV, 2009b; OIV, 2009c; OIV, 2009d). Similarly, the proposal of the Belgian company KitoZyme SA to consider chitosan, specifically that obtained from *Aspergillus niger*, as a "generally recognised as safe" (GRAS) material for use in the production of alcoholic beverages was accepted without objection by the US Food and Drug Administration (US FDA) in 2011 (US Food and Drug Administration, 2011), while in 2022, the same agency accepted without objection the proposal of the Canadian company Chinova Bioworks Inc. to consider chitosan, specifically that obtained from white mushrooms (*Agaricus bisporus*), as a GRAS material for use as an antimicrobial in food and alcoholic beverages (US Food and Drug Administration, 2022).

This paper presents an overview of the use of chitinous materials in oenological applications, with special emphasis on those works that promise possibilities to reduce the use of SO_2 without detriment to the preservation of the most appreciated properties of wines.

CHITOSAN APPLICATIONS IN OENOLOGICAL PROCESSES

1. Inhibition of chemical browning

Browning is one of the oldest known problems in winemaking. It derives from the set of chemical reactions that occur during winemaking, ageing, and storage. Due to these reactions, the colour of the wine can change, thus, affecting the quality of the final product. Many of the constituents of wine, such as phenolic compounds, certain metals, sugars, lipids, and amino acids such as tyrosine, aldehydes, etc., are susceptible to oxidation reactions during the manufacturing processes, which can also influence other sensory properties such as loss of flavour, aroma and nutritional value, increased astringency, etc., in addition to the colour changes. The main oxidation reactions can be caused by so-called Reactive Oxygen Species (ROS), which can originate due to some transition metal ions in their reduced form, which are usually present in them, e.g., divalent iron and copper ions (Oliveira *et al*., 2011).

For ferrous ions, the reaction would be initiated by its oxidation to give up an electron:

$Fe^{2+} \rightarrow Fe^{+3} + e^{-}$ (3)

which is transferred to the oxygen present in the system (triplet O_2, O≡O), generating the superoxide radical anion:

$O{\equiv}O + e^{-} \rightarrow {}^{-}O{=}O\bullet$ (4)

at the usual pH values in wines, it is in its protonated form (HO=O•, hydroperoxyl radical); the transfer of a second electron leads to the formation of the peroxide anion:

${}^{-}O{=}O\bullet + e^{-} \rightarrow {}^{-}O{=}O^{-}$ (5)

which exists in its protonated form (HO=OH, hydrogen peroxide). The subsequent transfer of one or two electrons generates a more reactive specie, the peroxide radical:

$HO{=}OH + e^{-} \rightarrow HO\bullet + OH^{-}$ (6a)

$HO{=}OH + 2e^{-} \rightarrow 2HO\bullet$ (6b)

which is capable of abstracting hydrogen atoms from various organic compounds present to form water, one of the end products of oxygen reduction, and an organic radical:

$R\text{-}H + HO\bullet \rightarrow H_2O + R\bullet$ (7)

The many different R-radicals that can form in wines lead to a variety of products that are ultimately responsible for the changes that occur in wines, including those that cause spoilage.

On the other hand, due to the antioxidant properties attributed to chitosan, some applications have been proposed in food preservation processes (Schreiber *et al*., 2013; Friedman and Juneja, 2010) and various oenological treatments (Castro-Marín *et al*., 2019; Chinnici *et al*., 2014). For the latter, only the use of chitosan of fungal origin, specifically those extracted from *Agaricus bisporus* or *Aspergillus niger*, has been considered, either in the treatment of musts, where it is used as a clarifying agent for the settling process (additionally preventing protein breakdown) (OIV, 2009a), or in wine, where it is used to reduce the content of heavy metals (iron, lead, cadmium and copper, preventing ferric or cupric cracking), and to reduce the presence of undesirable microorganisms such as *Brettanomyces* (OIV, 2009c). In this sense, it has been assumed that chitosan can act by complexing Fe^{2+} and Cu^{2+} ions, thus, decreasing Fenton reactions in the presence of tartaric acid (Rocha *et al*., 2020). Similarly, chitosan has been observed to reduce the content of some phenolic compounds, such as ellagic acid, which are actively involved in co-pigmentation with anthocyanins (Castro-Marín and Chinnici, 2020). In both cases, a decrease in wine colouring should be expected, although in the second case, the observed effect was marginal. Other mechanisms that have been considered for the inhibition of wine browning by chitosan include its reaction with HOO• and HO• radicals (Friedman and Juneja, 2010) which can abstract hydrogen from chitosan leading to its depolymerisation (Lárez-Velásquez and Zambrano Díaz, 2011), and the displacement of the tartrate anion from the tartrate/Fe(III) complex], thus, blocking Fe(II) regeneration (Castro-Marín *et al*., 2021).

2. Enzymatic browning

Wine browning can also occur through chemical reactions catalysed by enzymes generically known as phenol oxidases, which include catechol oxidase (also known by other names such as diphenol oxidase, phenol oxidase, polyphenol oxidase, phenolase, and tyrosinase, is also confused with other types of enzymes such as monophenol monooxygenase), laccase and o-aminophenol oxidase (Oliveira *et al*., 2011). These reactions occur mostly in the must because the activity of these enzymes is inhibited in wine by the alcohol present (Waliszewski *et al*., 2009). The oxidation of phenolic compounds involves (i) the hydroxylation at an ortho position to a hydroxyl group present in the phenolic substrate by an enzyme with cresolase activity, (ii) the subsequent oxidation of the product (ortho-dihydroxy-benzene) to ortho-benzoquinones by an enzyme with catecholase activity and (iii) the subsequent reactions of the generated quinones with other species present in the medium, such as other phenols, amino acids, proteins, etc., to generate condensation and polymerisation products (Oliveira *et al*., 2011), to generate condensation products and usually coloured polymers (see Figure 2).

Although studies on the effects of chitosan on enzymatic browning in wines are scarce, it has been reported that the use of chitin, added as an adsorbent that is removed by filtration before fermentation and packaging, resulting in a significant decrease in colour, catechins, and total polyphenols over storage time (Mármol *et al*., 2012; Mármol *et al*., 2009). Therefore, it can be assumed that these materials act by previously decreasing, by adsorption, the concentration of phenolic compounds in the must. Similar reasoning has been used to explain the decrease in browning of pear and apple

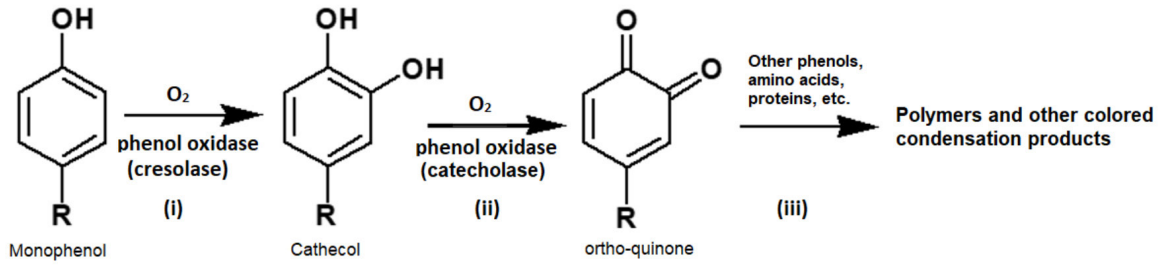

Figure 2. Summary of enzymatic oxidation processes leading to browning in the must.

juices when pre-treatments with chitosan solutions, followed by filtration, were applied before packaging and storage (Sapers, 1992); however, in the latter case, it was proposed that the decrease in browning is since the coagulation processes caused by chitosan allow a more efficient filtration of the smaller insoluble particles, to which the polyphenol oxidases are bound.

3. Control of harmful microorganisms

Due to their origin and taking advantage of the sugar content of fruits, oenological processes are affected by many harmful microorganisms. Among the most harmful microorganisms reported for wine are yeasts of the genus *Brettanomyces*, some of which can lead to the accumulation of volatile phenols in red wines, i.e., *Brettanomyces bruxellensis*, conferring them with strange fragrances that can even permanently damage them commercially (Paulin *et al*., 2020). For the control of these microorganisms, SO_2 has usually been used as a preservative agent, despite being considered a highly polluting and harmful compound for human health; additionally, in some cases, this compound can generate other complications in wines, such as unpleasant odours, allergic reactions, and headaches in consumers, etc., besides the difficulties arising from the emergence of resistant strains to this chemical may cause (Avramova *et al*., 2018).

Regarding the use of chitosan as an environmentally friendlier alternative to the use of SO_2 in the control of this type of harmful microorganisms, some studies have also been reported (Tika and Puspaningrat, 2022; Picariello *et al*., 2020). The antimicrobial properties of chitosan have been known for a long time, although the mechanisms of action have not yet been fully elucidated. Usually, its biocidal effect has been associated with its cationic nature (generated by the protonation of its amino groups), which can cause morphological changes, alteration of the cell membrane, and loss of intracellular material in several pathogenic microorganisms. In addition, other mechanisms have been proposed, such as the chelation of metals necessary for the development of pathogens (through their amino and hydroxyl groups), the alteration of their gene expression, the inhibition of protein synthesis, the blocking of sodium channels, etc. (Li and Zhuang, 2020; Lárez-Velásquez and Rojas-Avelizapa, 2020).

In this regard, it has been found that, under well-established conditions, chitosan does not impair the important properties of wine but, on the contrary, improves some of them, such as decreasing its browning (due to its antioxidant effects). However, it can delay the initial lag phase during the fermentation process with *Saccharomyces* strains (Castro-Marín *et al*., 2018), although it simultaneously acts as an antimicrobial agent, with variable effectiveness, against different strains of *Brettanomyces* (Paulin *et al*., 2020). Studies have also been reported where chitosan activity is highly selective, retarding the growth of *Brettanomyces* strains in the presence of *Saccharomyces* (Gómez-Rivas *et al*., 2004). Additionally, chitosan treatment is effective in reducing the activity of acetic acid bacteria during fermentation, with its remarkable effect being observed immediately after application, especially in the most active strains (Valera *et al*., 2017); similar effects have been observed against lactic acid bacteria (Elmacı *et al*., 2015).

In parallel to applications based on its recognised antimicrobial properties, chitosan can be used for the transport and release of bioactive substances, even at the nanoscale (Lárez-Velásquez, 2018). In applications related to oenological processes, for example, it has been reported that treatments with low molecular weight chitosan matrices, which act as an antimicrobial agent, loaded with antimicrobial fungal extracts (obtained by spray/drying), showed a notorious synergistic effect of the components for the control of *B. bruxellensis* (Choque *et al*., 2019).

4. Chitosan for the removal of contaminants from wine

The use of chitosan and chitin–glucan, both of fungal origin, in treatments for the removal of contaminants in wine has been approved in the European Union since 2011 (European Union, 2011). As mentioned above, chitosan has been tested in the removal of contaminants present in various stages of the oenological process, both for contaminants of an organic nature, such as ochratoxin A (Figure 3), perhaps the contaminant that has received the most attention for its removal due to its carcinogenic potential (Abbas *et al*., 2018), and inorganic ones, such as various metals and their salts. Thus, the treatment of different wine samples (red, white, and sweet) using chitosan as an adsorbent achieves notable reductions in the concentrations of iron, copper, and cadmium while simultaneously reducing the content of ochratoxin A (Bornet and Teissedre, 2008). Likewise, the use of nano adsorbents for the removal of ochratoxins, including those based on chitosan, seems to merit consideration by the oenological sector, as it has started to be tested in the food industry for the removal of mycotoxins (Song and Qin, 2022).

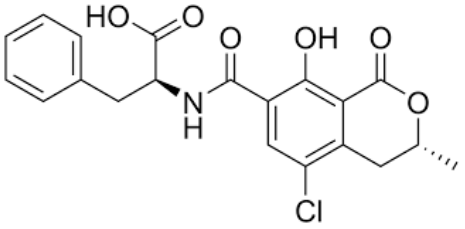

Figure 3. Chemical structure of the ochratoxin A.

On the other hand, Rizzo *et al*. (2010) reported a high efficiency of chitosan in the coagulation processes of wine wastewater, proposing its use as a sustainable alternative to the use of conventional metal-based coagulants, with the additional advantage of producing potentially reusable organic sludge; similarly, it has been reported that the use of chitosan-sepiolite nanocomposites allowed an efficient clarification of effluents from wine-making activities, associating its effect to a process of neutralisation of the charges in the colloids (Rytwo *et al*., 2013).

5. Chitosan as a support for enzymes and other additives in wine processing

The use of immobilised enzymes in different processes related to oenology (Ottone *et al*., 2020) is another area where the attractive properties of chitosan can be exploited. Many enzymes face difficulties due to their rapid inactivation, the lack of control over different reaction parameters, their low efficiency, etc. Therefore, their immobilisation, or co-immobilisation in enzyme aggregates, is an alternative whose development has become increasingly interesting. In this sense, some promising results that have been reported are the following: (a) the preparation of chitosan microspheres cross-linked with glutaraldehyde, loaded with β-D-glucosidase (βG) and α-L-arabinofuranosidase (ARA), with retention of enzymatic activity for 91 days of incubation under winemaking conditions, which makes them suitable for application in processes related to wine aroma enhancement (Tavernini *et al*., 2020), although it might be more advisable to test other less irritant crosslinking agents, such as genipin; b) the encapsulation of the proteolytic enzyme bromelain in clay/chitosan nanocomposites to control the opacification or turbidity caused by protein precipitation in white wines (Benucci *et al*., 2018), an instability phenomenon of non-microbial origin, leading to the search for mechanisms to control it (e.g., the use of proteolytic enzymes to hydrolyse proteins, including their encapsulation in appropriate and more environmentally friendly matrices); c) covalent immobilization of the enzyme lysozyme from hen's egg in chitosan spheres to control heterolactic fermentation in sherry wines, showing that the antimicrobial activity of the enzyme is not affected by SO_2 and phenols in white wine (Liburdi *et al*., 2016); d) immobilization of the urease enzyme on a chitosan-based support (*Chitopearl*) showed no appreciable difference in urea removal speed in white wines (with low tannin concentrations) with respect to soluble urease, however, since soluble commercial preparations usually have limitations for such removal due to their low protein contents (even when using the maximum legally allowed doses), the use of the immobilized enzyme could allow overcoming such limitations since the biocatalyst is insoluble in wine and can be easily removed (Andrich *et al*., 2010); e) it has been reported that the use of chitosan to encapsulate natural oxidizing agents, obtained as fungal extracts (*Aspergillus tubingensis*), allows to maintain their properties after the encapsulation process and that the retarding effect of chitosan on *Saccharomyces cerevisiae* seems to be diminished (Choque *et al*., 2019).

Another interesting point regarding the application of chitosan in the preparation of matrices for enzyme immobilisation is its chemical derivatisation, which can lead to controlled modifications of its chemical structure, allowing to obtain materials better adapted to the specific immobilisation of a particular enzyme, with the consequent improvement of the process to be controlled. In this sense, it has also been reported that, in general terms, enzymes immobilised on chitosan composites show better catalytic activity and operational stability than when they are in their free form (Nunes *et al*., 2021).

6. Chitosan as an antioxidant agent for thiols

Thiols are organic chemical compounds characterised by the presence in their structure of the functional group -S-H, known as thiol, sulfhydryl, sulfanyl, or mercaptan. These compounds have been identified as key aroma

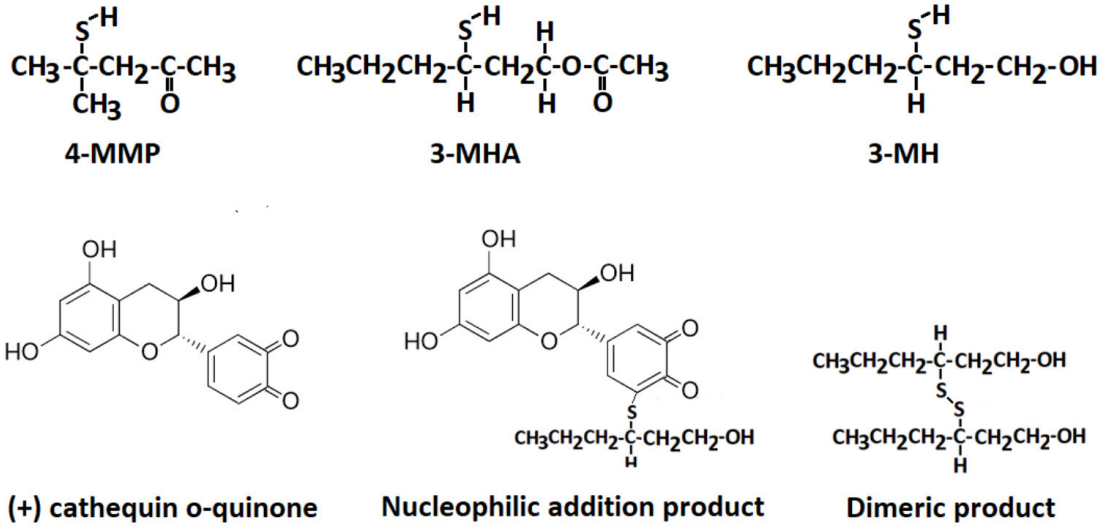

FIGURE 4. Chemical structures of some species of interest in the oxidation of thiols in wines.

components in young wines from different varieties, with 4-mercapto-4-methyl pentane-2-one (4-MMP), 3-mercaptohexyl acetate (3-MHA), and 3-mercaptohexan-1-ol (3-MH) (Figure 4) being among the most common in varietal wines (Roland *et al*., 2012).

Varietal wines are wines made from a single grape variety or at least 80 % of a single variety, as defined in the European Union (Salvador Insua, 2016), although in some countries such as Argentina, a minimum of 85 % is required (Murgo *et al*., 2019). In these wines, which constitute complex systems due to the presence of multiple chemical and enzymatic species, thiols are very sensitive to oxidation reactions that could generate a variety of products, among which those resulting from their nucleophilic addition to (+)-catechin-(o-quinone) and dimeric disulphides have been proposed (see Figure 4). The latter would be obtained through autooxidation catalysed by free radicals derived from the redox cycle of some of the metals present, especially the Fe^{2+}/Fe^{3+} couple. In both cases, such free radicals seem to play a major role (Nikolantonaki *et al*., 2010). On this point, chitosan can significantly lower the oxidation of thiols to maintain the varietal character of wines from aromatic grapes, especially when reduced amounts of sulphite are used in model wine solutions (Chinnici *et al*., 2014). However, contradictory results also have been reported for the early addition of chitosan in the grape processing stages, which results severely detrimental to the formation of varietal thiols although it was not possible to identify the exact mechanism by which chitosan affected thiol formation, suggesting some impact on enzyme systems (Dias Araujo, 2017).

SOME CONCERNS ABOUT THE USE OF CHITOSAN IN WINE TREATMENT

Although the preceding sections have highlighted many of the benefits of these materials for environmentally friendly applications in the main oenological processes, there are also some concerns regarding their use in some of them. Thus, chitosan for use in wine applications must be of high purity because the material containing proteins, i.e., tropomyosin, may cause intoxication problems in sensitive individuals (Amaral *et al*., 2016). Although this issue has been practically solved by obtaining chitosan from sources other than crustaceans, a few cases of allergy have also been reported using chitosan from zygomycetes (Kato *et al*., 2005). A further problem associated with the use of chitosan is the lack of reproducibility in the results usually obtained when using materials from different sources, or even from the same source but obtained by different methods, which implies additional work and costs to carry out tests each time chitosan of different origin is to be used. This is an issue that has generated a lot of attention for a considerable time but has not yet been resolved. Some observations related to the use of chitosan in oenological applications that that could be related to these facts are: (a) chitosan has shown a high capacity to reduce the ochratoxin A concentration in highly contaminated red wines, but also appreciably reduces colour intensity (contrary to chitin which reduces ochratoxin A without significantly affecting colour intensity) (Quintela *et al*., 2012), both reductions being dependent on the doses of chitosan applied (Kurtbay *et al*., 2008); b) it has been reported that chitosans with low deacetylation rates are not efficient for the removal of volatile phenols that affect the fragrance of red wines, and, therefore, do not help to ameliorate the negative impact of these, in contrast to chitosans with high deacetylation rates (Filipe-Ribeiro *et al*., 2018); c) it has been reported that chitosan can alter some organoleptic attributes related to astringency and bitterness, due to the removal of compounds such as cinnamic acid and phenols such as procyanidins (Spagna *et al*., 1996), which can decrease the concentration of vanillin-reactive flavanols (Picariello *et al.*, 2020), although the dose of chitosan added seems to be important to avoid obtaining contrasting results (Colangelo *et al*., 2018)

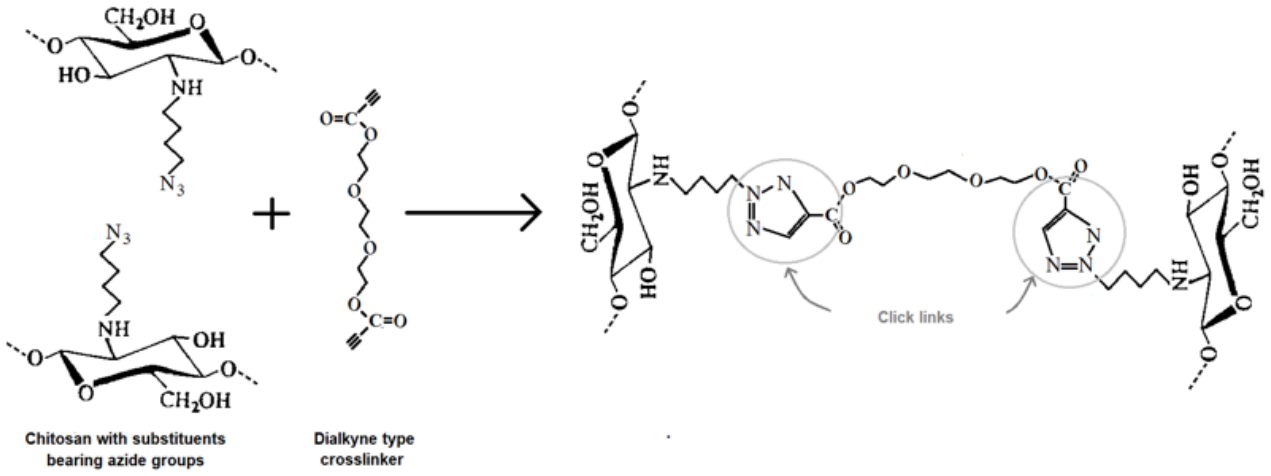

FIGURE 5. A hypothetical cross-linking, via click reaction, for chitosan with substituents bearing azide groups and a dialkyne-type cross-linker.

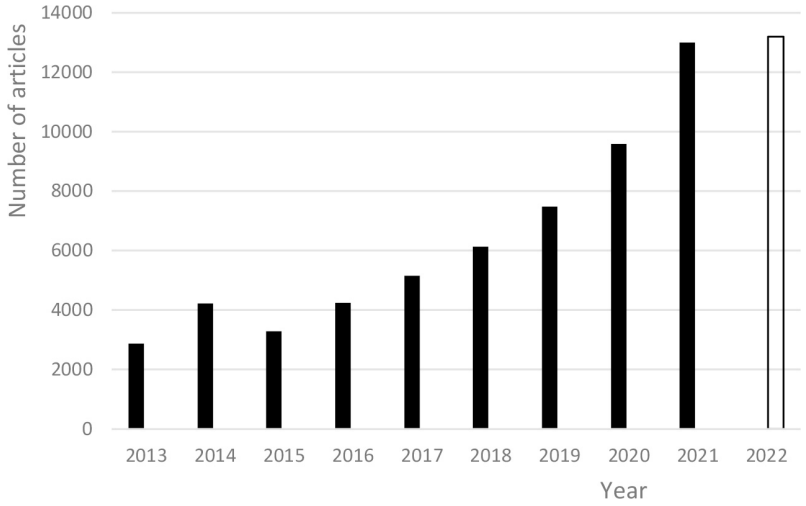

FIGURE 6. Evolution over the last few years of the number of articles found using the academic search engine Google Scholar for the keyword "chitosan-based nanomaterials". The white column represents the values for the year 2022 until 01/11/2022.

FUTURE TRENDS ON THE USE OF CHITOSAN IN OENOLOGICAL PROCESSES

Because chitosan is a material with multiple application possibilities in different fields, the intense research that has been carried out on its chemical modification has allowed obtaining increasingly novel derivatives with better control of its properties, including those obtained recently through the so-called click reactions (Truong *et al.*, 2014; Kritchenkov and Skorik, 2017; Rojas-Pirela *et al*., 2021) (see Figure 5), a new type of chemical reactions whose pioneers have received this year's Nobel Prize in Chemistry (Nobel Prize Organization, 2022).

In this sense, chitosan click reactions constitute a simple route to generate materials and systems that could quickly find applications related to the encapsulation of yeasts of oenological interest, such as those mentioned above for bioethanol production (Namthabad and Chinta, 2012); for the preparation of new methods for the immobilisation of proteolytic enzymes that help to control the opacification of wines derived from protein precipitation, similar to the systems reported (Benucci *et al*., 2018); the preparation of systems that promote the controlled release of substances enhancing the qualities of wine (Tavernini *et al.*, 2020); among others.

On the other hand, the development of different chitosan-based nanomaterials has been growing steadily over the last few years, as can be inferred from the number of publications in the last ten years for the Google Scholar search using the words "chitosan-based nanomaterials" (see Figure 6). Some systems based on these nanomaterials could easily be tested for the removal of contaminants in wine, like how some nano-adsorbents have been tested in other media for the removal of organic contaminants such as mycotoxins (Song and Qin, 2022), or inorganic contaminants such as metals and their salts (Haripriyan *et al.*, 2022). It is estimated that nano-adsorbents can generate better performance in the removal of various substances thanks to the unique properties derived from their nano-dimensions, such as high adsorption capacities, short-time adsorption equilibria, large surface area, and the presence of various active groups for ion binding, etc. (Ali *et al.*, 2020).

Similarly, chitosan offers wide possibilities for the manufacture of molecularly imprinted adsorbents (MIA) (Xu *et al.*, 2015; Karrat *et al*., 2020), whose preparation consists of using chitosan, or its composites, as a matrix for the prior moulding of an adsorbate of interest (usually molecules or ions but which can also be applied to viruses, bacteria, proteins, etc.) and whose subsequent extraction generates an adsorbent of very high specificity because its active sites have the shape of the adsorbate. Despite this, MIAs have been little exploited as adsorbents, especially in the oenological area, although some interesting studies have been reported in related applications, such as the removal of the toxin patulin in pear juices using a molecularly imprinted adsorbent that additionally possesses magnetic properties (Sun *et al.*, 2020).

The use of ever smaller and more specific sensors is also one of the areas of biotechnology, and nanobiotechnology, that has found a place in different stages of the oenological process, such as quality control of starting materials and final products, optimisation of fermentation processes, monitoring of storage conditions, etc. (Monge and Moreno-Arribas, 2016). Interesting results related to the use of chitosan-based nanomaterials have already been reported in many of these applications. Straightforward examples include the preparation of an amperometric nanosensor based on the immobilisation of the enzyme alcohol dehydrogenase on chitosan/carbon nanotube matrices for the quantification of ethanol in wine, beer, and spirits (Lee and Tsai, 2009) as well as the preparation of voltammetric sensors based on molecularly imprinted chitosan electrodeposited on a

boron-doped diamond electrode for the detection of catechol in wine (Salvo-Comino *et al.*, 2020). Other nanosensors that have been prepared for use in other areas should be of easy application in wine, such as the system for the detection of ochratoxin A prepared using a plasmon resonance biosensor based on chitosan and carboxymethyl-chitosan nanomatrices (Rehmat *et al.*, 2019).

CONCLUDING REMARKS

As can be seen from this brief overview of the multiple uses that chitosan has been experiencing in the oenological area, the possibilities of its utilisation seem to be widening more and more owing to the numerous investigations that are being developed with this material in different fields, in addition to being a material with proven sustainability benefits due to its natural origin and optimisations, in this sense, of the processes to obtain it in an environmentally friendlier way. An important point in favour of chitosan is its versatility to be easily integrated into the preparation of different types of nanomaterials, a previous step for the development of applications in nanotechnology and nanobiotechnology. In the case of oenology, for example, for the manufacture of nanosensors for the detection of contaminants such as ochratoxin A, the nanoencapsulation of strains of interest, the preparation of specific nano-adsorbents, etc.

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Review

Grapevine Responses to Heat Stress and Global Warming

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Abstract: The potential effects of the forthcoming climate change include the rising of the average annual temperature and the accumulation of extreme weather events, like frequent and severe heatwaves, a phenomenon known as global warming. Temperature is an important environmental factor affecting almost all aspects of growth and development in plants. The grapevine (*Vitis* spp.) is quite sensitive to extreme temperatures. Over the current century, temperatures are projected to continue rising with negative impacts on viticulture. These consequences range from short-term effects on wine quality to long-term issues such as the suitability of certain varieties and the sustainability of viticulture in traditional wine regions. Many viticultural zones, particularly in Mediterranean climate regions, may not be suitable for growing winegrapes in the near future unless we develop heat-stress-adapted genotypes or identify and exploit stress-tolerant germplasm. Grapevines, like other plants, have developed strategies to maintain homeostasis and cope with high-temperature stress. These mechanisms include physiological adaptations and activation of signaling pathways and gene regulatory networks governing heat stress response and acquisition of thermotolerance. Here, we review the major impacts of global warming on grape phenology and viticulture and focus on the physiological and molecular responses of the grapevine to heat stress.

Keywords: global warming; grapevine; heat stress; molecular responses; phenology; viticulture; *Vitis*; wine

1. Introduction

Climate change is one of the biggest environmental challenges that humanity will face over the next few decades according to reports from the Intergovernmental Panel of Climate Change (http://www.ipcc.ch). The release of greenhouse gases, especially CO_2, due to various anthropogenic activities is regarded as the main cause of climate change and in particular global warming [1,2]. During the 20th century, the concentration of CO_2 has escalated from 280 to 400 ppm resulting in an average temperature rise of 0.5–1 °C. It is expected that the CO_2 concentration will elevate further and the global average temperature will rise by 0.2–0.3 °C per decade, reaching values between 1.2 and 5.8 °C by the end of the twenty-first century [3]. Temperature records with emphasis on viticultural areas show that during the growing seasons from 1950 to 2000 the mean temperature has increased by about 1.6–1.8 °C in Europe and 1.2–1.4 °C worldwide [3,4]. Importantly, parallel to climate change, heatwaves are becoming more common and extreme high-temperature events more frequent.

Changes in grapevine phenology are regarded as one of the most unambiguous consequences of global warming. Recent studies show that temperature rise is highly correlated with an earlier onset of many growth stages in the grapevine [5,6]. Furthermore, the shortening of the duration of most

phenological stages due to increased global temperature may adversely affect the composition and quality of grapes and thus of wine. All these changes highlight the need to take adaptation measures such as the relocation of vineyard cultivation to northern zones or higher altitude areas with lower average temperatures in order to maintain the quality of the final products to the desired level [7].

Plant growth and many developmental processes are strongly influenced by ambient temperature fluctuations. Each species has a preferred temperature range, which is represented by optimum, maximum and minimum values. Extreme temperatures are among the most significant limiting factors for grapevine distribution [8]. During heat stress, the ambient temperature rises above the threshold level of plant tolerance and, when extreme or long-lasting, may cause irreversible damage. Grapevines often encounter heat stress during the growing season that perturbs cell homeostasis, may affect proper development and fruit metabolism and consequently exert constraints on grape yield and quality. Although the grapevine has a good ability to adapt to various environmental pressures, long-lasting extremely high temperatures or heatwaves may permanently affect yield attributes and vine physiology [9]. Due to the upcoming climate changes, the knowledge of grapevine responses to heat stress is of particular importance for the sustainability of viticulture and one of the most important topics in grapevine biology.

Grapevines (*Vitis* spp.), like other plants, have an internal adaptive mechanism to combat heat stress. Metabolic processes such as respiration, photosynthesis and transpiration are very sensitive even in short-term temperature fluctuations. Photosynthesis is the most critical process in plants that is directly or indirectly affected by temperature. Heat stress, which is often accompanied by drought, may significantly reduce stomatal conductance and water-use efficiency. Recent progress in molecular biology has uncovered the major stress response pathways in plants and has broadened our view of abiotic stress responses and plant tolerance [10]. The availability of the complete grapevine genome sequence has provided an opportunity for the identification and characterization of various genes, cis-regulatory elements and trans-factors implicated in stress response [11]. After employed "omics" technologies, many abiotic heat-stress-inducible genes and proteins have been identified, although our current knowledge on the particular mechanisms and complex regulatory networks governing heat stress response and acquisition of tolerance in the grapevine is still far from complete. In this review, we summarize the current knowledge and recent progress in heat stress studies on the grapevine. Particular emphasis was given to the impact of heat stress on altering major phenological stages and implications in viticulture and on the physiological and molecular responses.

2. Global Warming Impacts on Grapevine Phenology and Viticulture

Climate change is expected to affect many aspects of the natural world, while its impact on agricultural production may be of particular importance. Viticulture is one of the sectors of agriculture to be affected by climate change and specifically global warming. Climate is the main factor limiting the geographical distribution of grapevines over the world and also a basic element of the so-called "terroir" concept, which is of great significance for the wine industry [12]. The primary impact of global warming in viticulture is on phenology, i.e., the timing of annually recurrent biological events, as grapevine sensitivity to heat stress is directly related to the phenological stages [13,14]. Grapevine phenology is thought to be one of the main natural indicators of heat stress and may be utilized to measure the effect of environmental changes on most sensitive developmental stages like flowering, veraison and grape ripening [13,15]. Many models have been created based on the tight link between temperature and phenology to predict the onset and duration of phenological stages in the near future [14]. This prediction is of extreme significance in arranging appropriate viticultural activities and winemaking choices [16].

Among the most evident biological effects of global warming are the phenological shifts [5,17]. An analysis of data for four cultivars in south-west Germany, obtained from a previously published study [18], revealed an average 10–24-day shifts in the onset of most important grape phenological events from 1975 until 2015 (Figure 1). A significant advance is expected in most phenological stages,

although the duration of each stage is depended on the sort of soil and the grape variety [3,15]. A direct interaction between the average temperature during the growing season and the duration of the annual vegetation cycle has been documented [3]. The effect of heat stress on the phenology of several grape varieties has been well investigated [19]. Shortening of the growth intervals and a prior onset of most phenological stages is likely to occur. Research based on these models anticipates the advancement of two to three weeks until 2050, while the phenomenon in the northern hemisphere vineyards will become more apparent [20,21]. According to Caffarra and Eccel [22], the most prominent phenological shifts refer to flowering and veraison rather than to budburst. Projection models indicate that higher temperatures may not shorten the period length from bud break to anthesis, but rather the length from anthesis until maturation will be shortened significantly [23].

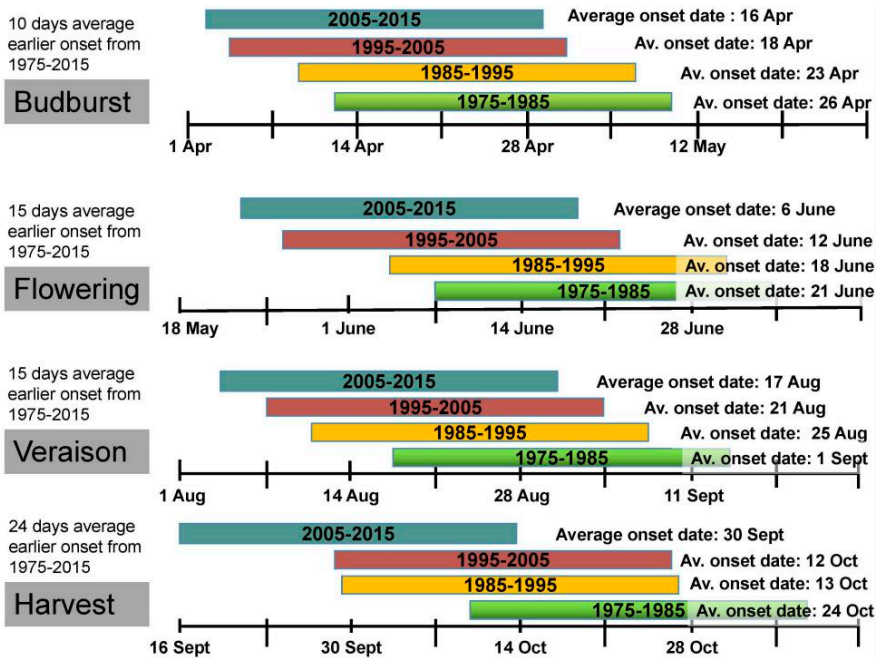

Figure 1. Phenological shifts based on average onset dates for bud break, flowering, veraison and harvest of four grapevine varieties ("Pinot Gris", "Pinot Noir", "Riesling" and "Muller Thurgau") grown in Hainfeld (Southwest Germany) from 1975 to 2015. Based on data from a previously published study [18].

Grape berry metabolism and juice quality are affected by both the onset and the duration of phenological stages [3]. In temperate zones of the northern hemisphere, the most suitable grape ripening period is usually in September, when the days are still warm and the nights are cool [20]. In such climates, early veraison dates would cause the berries to mature earlier under high-temperature conditions, which may have a negative impact on berry quality [14]. Early ripening results in the loss of wine typicity, changes in the aromatic character and loss of balance between sugar and acidity of the grape juice [21]. Grapes harvested earlier than expected thus result in wines with less organic acids, higher pH values, higher ethanol levels and altered sensory characteristics. In a future warmer climate, higher temperatures in wine-growing areas may also lead to the reduction of grape color due to the inhibition of anthocyanin biosynthesis [17,24].

Several wine-growing regions in southern Europe have already reached or even exceeded optimum thermal conditions for the currently cultivated varieties [25]. Fraga et al. [26] referred to recent elevated temperatures during grape berry maturation in the Iberian Peninsula, indicating a possible diminishing of wine quality in the near future. Likewise, higher temperatures during the growing season in Slovenia have resulted in a significant decrease in the total acidity content of early-ripening varieties [27]. The same changes have been also observed in many viticultural regions

in Europe, like Germany and France [5,17], and in some viticultural regions in Australia, where recent studies have shown an earlier onset of most phenological stages and an overall shortening of the whole growing season period [28]. Global warming, however, will probably have the most serious impacts on the Mediterranean region, which is located in the middle of the tropical climate of North Africa and the temperate rainy climate of central Europe [26]. The Mediterranean basin is one of the largest wine-growing regions in the world, characterized by long growing seasons with moderate to warm temperatures. Throughout the year there is little seasonal change in temperatures, and winters are generally warmer than those of continental climates. As the suitability of several southern European wine-making regions will decline, due to global warming effects, the projected warming in central and northern European regions will result in prolonged frost-free periods and growing seasons that will favor wine quality [3]. Many studies have projected the possible extension of viticultural zones to new vine-growing regions in Europe showing that an increment of 4 °C in the mean annual temperature will bring a general shortening of the annual growth cycle [29]. An eastbound and northward move of viticultural territories is thus expected to incorporate England, Poland, Romania, Belarus and Ukraine. Another reasonable result of the temperature increase is expanding the areas suitable for viticulture to areas with higher altitudes, where the temperatures for vines are currently too low [22]. Besides the rise of the mean annual temperature, heatwaves during the crucial development stages of grape berry have increased in the last decades, and under enhanced global warming they are expected to worsen, becoming more frequent and more intense [30,31]. These prolonged periods of excessive heat events may have dramatic impacts on both the quality and the yield of grape production, despite the overall suitable weather conditions [13,31].

3. High-Temperature Effects on Grapevine Physiology and Berry Composition

3.1. Effects on Photosynthesis

Among the main physiological functions, photosynthesis is the first process to be directly affected by temperature variations [32,33]. It is reduced before other symptoms appear when the temperature rises above an optimum limit, which differs among species [34,35]. The optimum photosynthetic temperature for the grapevine is between 25 and 35 °C [36]. When the temperature is below 10 °C, most of the physiological processes decline, and at temperatures over 35 °C, heat acclimation mechanisms are activated [15,37]. Extremely high temperatures, i.e., above 40 °C, have drastic effects on photosynthesis mainly due to the disruption of the photosynthetic apparatus.

Field measurements conducted on the photosynthesis of grapevine leaves at a temperature range between 20 and 40 °C showed that compared with 25 °C the average photosynthetic rate (Pn) decreased with increasing temperature and was inhibited by 60% at 45 °C [38]. Several studies on grape leaves clearly show that Pn does not decrease significantly at 35 °C, but it is limited at a temperature of over 40 °C [34]. Greer and Weedon [38] suggested that the Pn reduction may be attributed to a 15%–30% reduction of stomatal conductance. This is probably because heat and drought stresses are tightly linked and reduced stomatal conductance may consequently increase the symptoms of heat stress as leaf temperature rises [39]. However, as is the case for other abiotic stresses, the effect of heat stress on stomatal conductance differs among grapevine varieties. For instance, "Touriga Nacional", a Portuguese wine cultivar, keeps stomata open under mild heat stress, which is beneficial for the cooling of leaves via evaporation and thus may help keep photosynthesis unaffected [40].

The decreased photosynthetic rate could also be attributed to the disturbances of biochemical processes, such as decreases in ribulose-1,5-bisphosphate (RuBP) regeneration capacity and ribulose bisphosphate carboxylase/oxygenase (Rubisco) activation [41], as is shown in Figure 2. Photosystem II (PSII) is considered to be the most sensitive physiological system of the grapevine to heat stress, usually suspended or destroyed before other cellular functions are disrupted [36,42]. It is formed by a complex of essential proteins, including D1 and D2, and is vital for the electron transfer during the photochemical stage of the photosynthetic pathway. Under high-temperature regimes, these core

proteins are denatured and PSII impairment is observed after a few minutes to a few hours of heat exposure (Figure 3) [32,43]. High thermal stress even when applied at relatively short time periods, like 40 °C for 15 minutes, may cause serious and perhaps irreversible injury to the PSII of grapevine leaves [36]. As opposed to PSII, photosystem I (PSI) is relatively heat-stable.

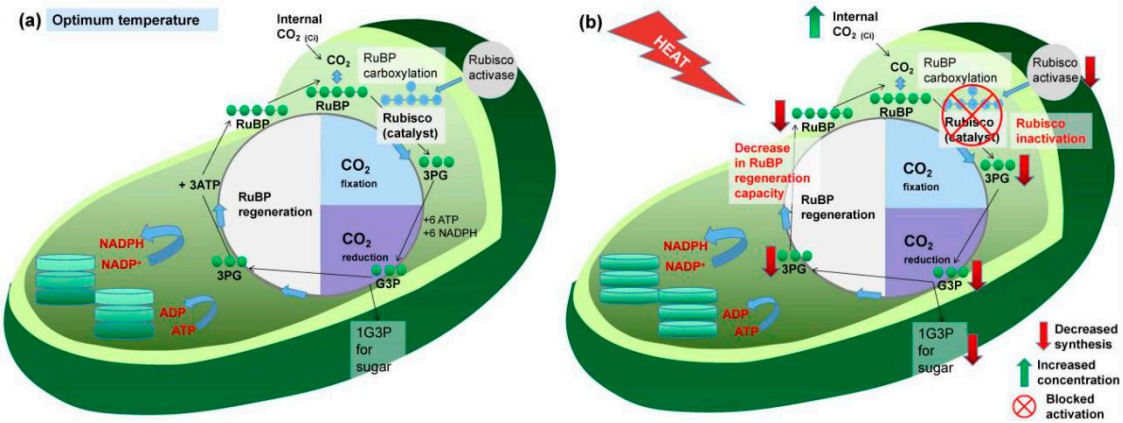

Figure 2. (**a**) Chloroplast function under optimum temperature; (**b**) disturbances of major biochemical processes of chloroplasts in grapevine leaves under heat stress. The activity of Rubisco activase is extremely heat-sensitive, and its inhibition blocks the activation of Rubisco and downstream reactions.

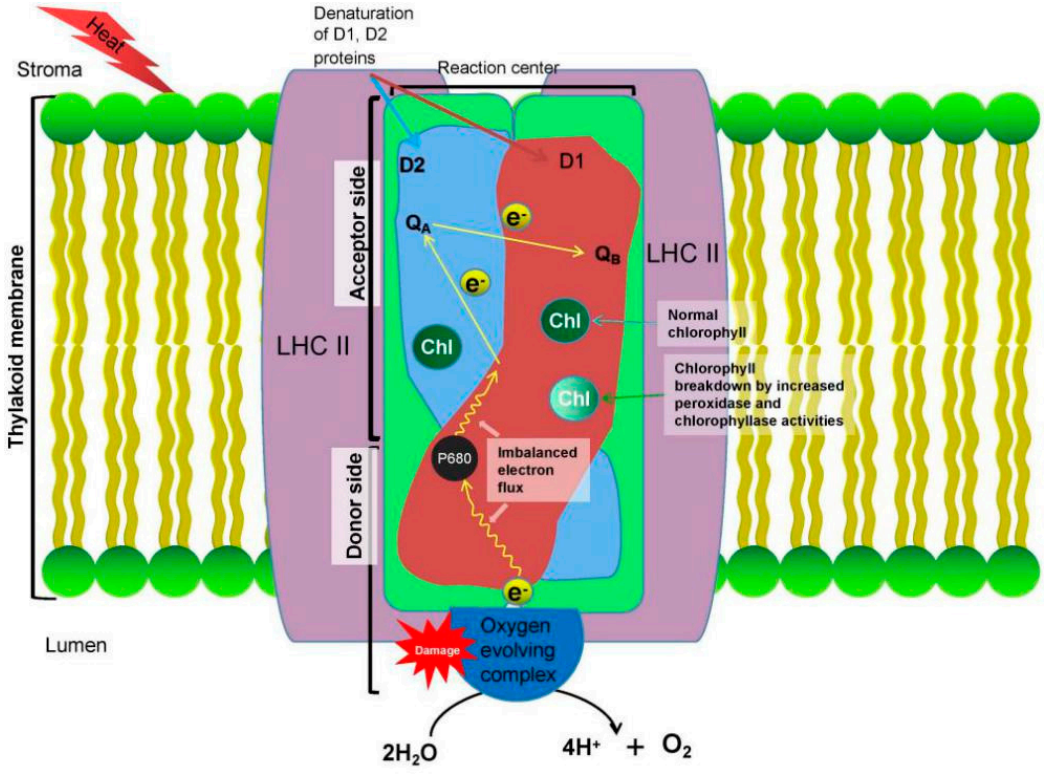

Figure 3. Photosystem II (PSII) is considered the most sensitive physiological system of the grapevine to heat stress. Extreme high temperatures cause dissociation to the oxygen-evolving complex (OEC), which results in the inhibition of the electron transportation from the OEC to the acceptor side of PSII. D1 and D2 are susceptible to heat inactivation, and under high-temperature regimes, chlorophyll degradation occurs due to the increased activities of peroxidase and chlorophyllase.

There are generally two main factors that influence PSII sensitivity to high temperatures. The first is the increased fluidity of the thylakoid membrane, resulting in the disconnection of the PSII

light-harvesting complex, and the second is the dependence of PSII integrity on electron dynamics [44]. In the grapevine, thylakoid membrane permeability is quite sensitive to heat stress [45]. A recent study showed that after heat treatments at 35 or 40 °C thylakoid membrane leakage, total chlorophyll content and chlorophyll fluorescence of "Cabernet Sauvignon" and *Vitis davidii* Foex. cv. Junzi vines did not change significantly, but the net photosynthetic rate was reduced. After heat treatment at 45 °C stress symptoms appeared with the fluidity of the thylakoid membrane increasing and total chlorophyll content decreasing [46]. It is noteworthy that prolonged high-temperature stress (e.g., a three-month period) mainly induced structural disorders of thylakoids. Chloroplasts in the mesophyll cells became round in shape, with smaller starch granules and more numerous plastoglobules than the control vines, indicating the beginning of senescence [45].

Damage to the thylakoid membranes is also associated with a decline in chlorophyll content [43]. Under high-temperature regimes, the chlorophyll-degrading peroxidase and chlorophyllase activities increase, resulting in a severe decline in chlorophyll content [47] (Figure 3). Decreased total chlorophyll content indicates inhibition of PSII. Thus, chlorophyll fluorescence measurements can be used to detect shifts in the photosynthetic machinery and as a good indicator for heat resistance in grape cultivars [48]. Strasser et al. [49] developed a method based on fluorescence transient analysis, namely the OJIP test, which explores changes in PSII photochemical performance and has been used as a measure of plant susceptibility to stress. This test can be applied to estimate many phenological and physiological expressions of PSII and is a unique method for in vivo examination of PSII behavior, including electron transportation and energy absorption [34,50]. The OJIP test may be used as a fast and simple method for measuring heat damage in grapes and can reveal information about the PSII electron transport chain. Xu et al. [51] conducted the OJIP test using different grapevine genotypes ("Riesling", spine grape and "Jingxiu") to investigate the response of the PSII electron transport chain to extreme heat stress (i.e., 47 °C). Results show that during the first 10 min of heat treatment the electron transport chain of PSII was highly sensitive to stress. High-temperature stress in grapevine may also cause serious damage to the oxygen-evolving complex (OEC) of PSII [52]. OEC participates in the splitting of water and the release of oxygen, resulting in an imbalance of the electron flux from the OEC toward the acceptor side of PSII [41] (Figure 3). The deactivation of the OEC is considered to be the cause of the reduced electron transport capacity caused by heat, especially at high temperatures. However, at moderate-high temperatures (e.g., 35°C) the damage in grapevine leaves is rather not significant, as leaves can easily alter the PSII properties to reduce OEC heat sensitivity [34].

The basic fluorescence (F0) and chlorophyll fluorescence, which is the ratio of variable fluorescence to maximum fluorescence (Fv/Fm), are parameters related to the tolerance of grapevine to heat stress [16]. However, it is not clear yet whether the inhibition of grape photosynthesis by high-temperature stress is due to a failure of electron transfer or to a reduction of Rubisco activity. Inactivation of Rubisco increases exponentially as temperature increases, and its activity drastically declines over 35 °C. Chlorophyll degradation due to heat stress can also reduce soluble protein content and change the speed of the Rubisco synthesis [16].

3.2. *Effects on Transpiration*

In the vineyard, heat stress is often accompanied by seasonal drought stress, which is a serious constraint in grapevine growth. Stomatal closure serves as the first-line defense from potential desiccation. However, transpiration is irreplaceable as part of the radiation energy is converted into latent heat through shifts in the opening of stomata [44]. Transpiration due to stomatal conductance is defined as the difference in intercellular and atmospheric water-vapor pressure divided by the total atmospheric pressure, which is often presented as a vapor pressure deficit (VPD) [53]. It is the main component of the energy balance of the leaves providing evaporative cooling to plants, necessary to keep leaf temperature below a maximum allowable limit [54]. Even a low transpiration rate can cause the leaf temperature to drop by a few degrees, which in some cases is the difference between growth and wilting. Average transpiration rates in grapevine leaves have been shown to increase five times almost

linearly at a temperature range between 15 and 40 °C, i.e., from 0.5 to about 2.5 mmol m^{-2} s^{-1} [55]. However, further temperature rise up to 45 °C had no additional effect on the transpiration rate. In "Semillon" vines, the transpiration rate increased substantially with the increase in leaf temperature, particularly at high heat stress conditions (above 35 °C), which is consistent with the need for enhanced evaporative cooling [56]. Similarly, transpiration rates of "Chardonnay" increased four-fold as the temperature rose from 15 and 30 °C, and the rate was even higher at 35–40 °C, while in "Cabernet Sauvignon" the transpiration rates increased almost linearly with increasing temperature from 20 to 40 °C [57]. It has been suggested that "Semillon" vines exhibit relatively higher transpiration rates as compared to other international cultivars [58], thus its cooling capacity may keep the canopy temperature a few degrees lower than the air temperature.

Although temperature may affect grapevine stomatal conductance across the whole temperature range, there is only a small overall shift and not a direct link between temperature and stomatal conductance [59]. Stomatal conductance in most plants declines under high VPD levels, up to a given threshold. Accordingly, stomata of "Chardonnay" leaves did not respond to temperatures below 30 °C, but stomatal conductance declined strongly at elevated temperatures and high VPD values [55]. As opposed, "Shirah" vines when heat-stressed at normal VPD conditions exhibited 62% higher stomatal conductance than the control plants [60]. Taken together, it is the interaction of temperature with VPD levels that regulate stomatal conductance rather than the temperature alone. The differences recorded in stomatal responses to temperature changes and succeeding transpiration rates have led to the classification of vines into isohydric and anisohydric varieties, according to the sensitivity of their stomatal conductance to VPD variations. Differences between isohydric and anisohydric behaviors in stomatal response to VPD have direct effects on the heat stress tolerance of grapevines [60]. Anisohydric behavior may contribute to heat dissipation, given that the soil available water is sufficient to maintain transpiration, whereas reduction in stomatal conductance in isohydric varieties may enhance the damaging effects of high-temperature stress [61].

3.3. Effects on Grape Berry Composition

The chemical composition of grape berries is quite complex comprising several hundreds of different compounds, mainly water, fermentable sugars, organic acids, nitrogen compounds, minerals, pectins, phenolic compounds and aromatic compounds. Global warming is expected to change the temperature range in major viticultural areas, leading to changes in the composition of berries. The rate of grape berry metabolism strongly depends on the ambient temperature. Elevated temperatures perturb several metabolic pathways resulting in alterations in the biosynthesis of basic compounds that are critical for the grape must quality [62]. More specifically, the rise in temperature is expected to lower the acidity and increase the sugar content of berries, resulting in unbalanced wines with higher alcohol content and deprived of freshness and aromatic complexity [15,63]. Anthocyanin content is also reduced by high temperatures (Figure 4).

Due to the elevated sugar content of grapes in the last decades, the ethanol content of wines has increased accordingly. Many of the wines that used to have 11%–12% vol. ethanol in the 1980s now have about 13%–14% vol. [64]. Titratable acidity decreases as the temperature rises, being lower at 30 than at 20 °C [65]. The role of acidity in winemaking is extremely important, and the taste of wine is directly related to acid concentration. The main organic acids are tartaric acid and malic acid, which together make up about 80% of the total organic acid content. The acid harmony, defined as the relative concentration of tartaric to malic acid, along with the potassium content characterizes the acidity of grape berry juice. Under heat stress conditions, especially at the stage of grape maturity, the potassium concentration of berries increases, thereby increasing the pH value and finally reducing the total acidity [15]. As the temperature increases, malic acid is also metabolized faster than tartaric acid. The optimum temperature for malate accumulation is 20–25 °C, and a dramatic decline is observed above 40 °C [66]. However, the biochemical and molecular mechanisms by which malate degradation enhances at high temperatures and the way downstream metabolic

pathways are affected are poorly explored [67]. When the temperature is relatively high during the day, low night temperature is essential to ensure a low pH. This is quite important for the sustainability of the grape/wine sector considering that global warming due to climate change is expected to be associated with elevated temperatures at night rather than during the day. High temperatures also affect the ratio of sugar-acid balance. Elevated temperatures can promote the accumulation of sugars and the concomitant degradation of organic acids, with the acidity being more drastically affected than the sugars. This results in lower acidity for the same sugar content in grapes grown under warmer conditions (Figure 4).

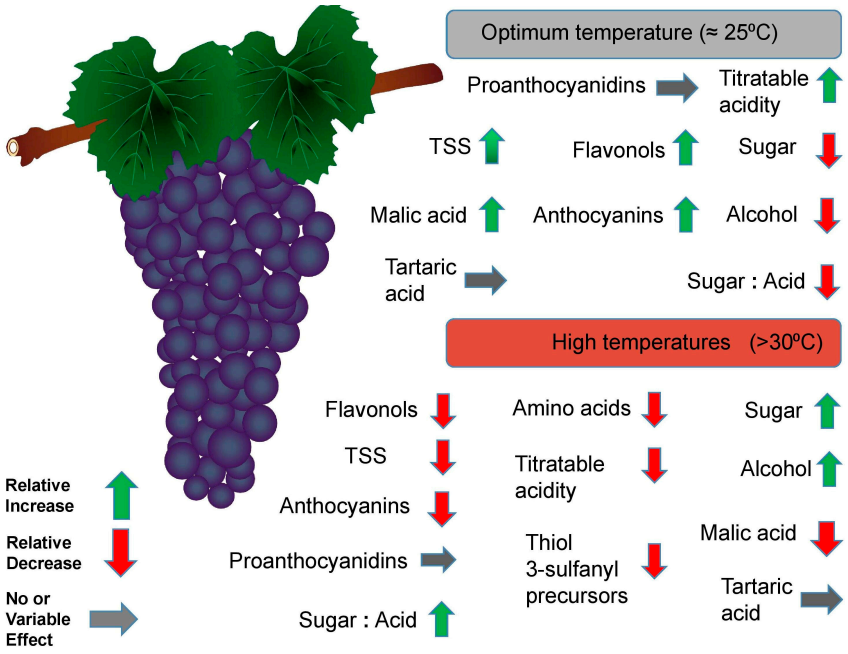

Figure 4. Summary of the effects of high-temperature stress on grape berry metabolism.

Heat stress restrains the formation of anthocyanins and flavor compounds in grapes grown in temperate regions [65,68]. For most common varieties, the ideal temperature during the grape maturation stage for the optimum formation of aroma compounds is within the range of 20 and 22 °C [62]. Reductions in color formation are observed when the temperature exceeds 30 °C, and at temperatures over 37 °C decreased grape color and increased volatilization of aroma compounds are observed [15,64]. The main components of the grape color are anthocyanins, mainly found in the grape skins of red grapes. Under high-temperature conditions, reductions of delphinidins, anthocyanins, petunidins and peonidin-based anthocyanins in grapes were observed but not in the biosynthesis of malvidin derivatives [15]. Anthocyanins, like other phenolic compounds, are also highly unstable and susceptible to thermal degradation. It is worth mentioning that the combination of heat and drought stress has less effect on the degradation of anthocyanins and sugars than heat stress alone, and this is because water deficiency may alleviate the deleterious effects of high temperature in the degradation of these compounds [69].

4. Molecular Responses to Heat Stress

Plants, like other living organisms, have the ability to perceive various abiotic stress signals from the environment through specialized sensor molecules and receptors and to consequently activate signaling pathways as a response to these stimuli. The identification of sensors for stress signals and the elucidation of the downstream signaling cascades are fundamental in plant science and big questions in grapevine biology. It has been shown that different receptors may perceive various types of stress signals [39]. However, there are both unique and overlapping abiotic stress signals and

there is much functional redundancy in genes encoding stress sensor proteins. High temperature may act as a sole stress factor in plants, but in the grapevine, as in other summer crops, heat is often combined with drought stress. Thus, plants have developed adaptive molecular mechanisms to counteract the detrimental effects of these combined constraints. Most of the known protein stress sensor molecules are located in the plasma membrane and include members of diverse gene groups or families, like receptor-like kinases (RLKs), G protein-coupled receptors (GPCRs), histidine kinases (HKs), ABA receptors and calcium sensors [70,71].

The perceived signals are then transmitted through secondary messengers, such as lipids like IP3, cyclic GMP, aquaporins and especially Ca^{2+}. During high-temperature stress, Ca^{2+}-binding proteins such as calmodulin (CaM), CaM-related proteins, Ca^{2+}-dependent protein kinases (CDPK) and calcineurin B-like protein (CBL) perceive the elevated Ca^{2+} concentration and initiate transcription networks and the activation of protein kinases (PKs), such as the mitogen-activated kinases (MAPKs). PKs in turn phosphorylate specific transcription factors (TFs) and induce stress-response genes that may act as molecular chaperones to provide heat and/or drought tolerance [71–73], as shown in Figure 5. Heat shock proteins (HSPs) and reactive oxygen species (ROS) scavenging enzymes are known targets of heat-stress-responsive TFs and play crucial roles in the adaptation of plants to heat stress [74].

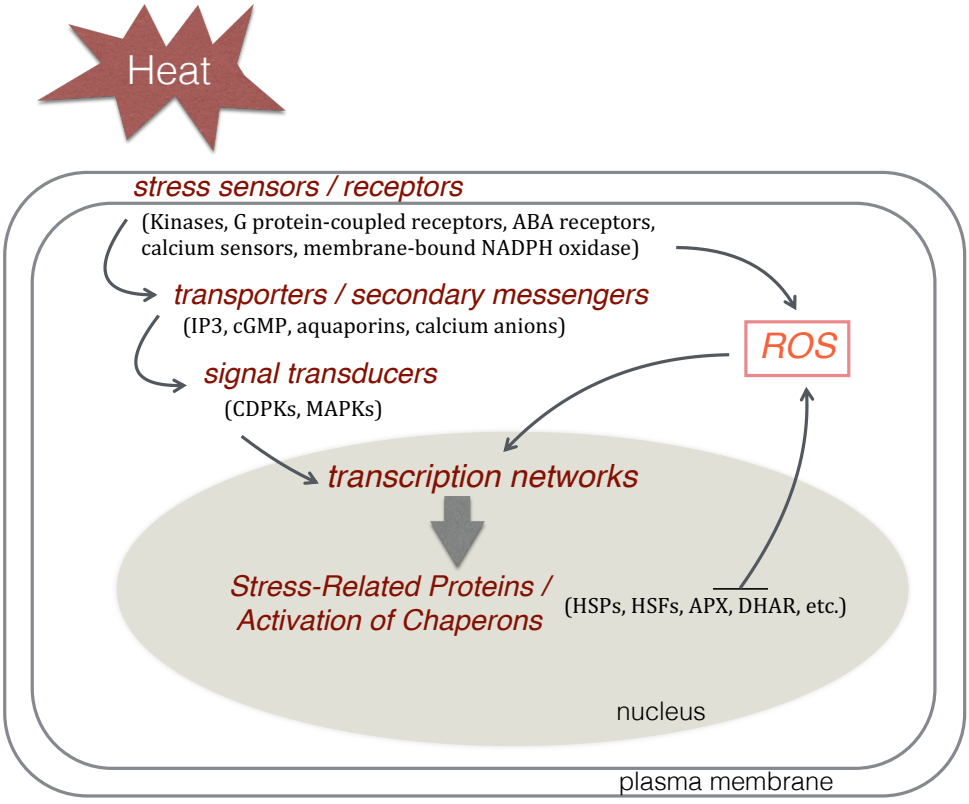

Figure 5. A simplified scheme for the heat-stress signal transduction pathway focusing on major regulatory components and stress-responsive genes identified so far in the grapevine. Protein stress sensor molecules perceive heat-stress signals and transmit them through secondary messengers initiating transcription networks to provide stress tolerance. Abbreviations: IP3, inositol 1,4,5-trisphosphate; cGMP, 3′,5′-cyclic guanosine monophosphate; CDPKs, calcium-dependent protein kinases; MAPKs, mitogen-activated protein kinases; HSPs, heat shock proteins; HSFs, heat stress transcription factors; APX, ascorbate peroxidase; DHAR, dehydroascorbate reductase; ROS, reactive oxygen species.

Following the characterization of the first *Arabidopsis* protein kinase receptor in the early 1990s, a number of different PKs have been identified in various plant species that regulate downstream signaling pathways [71,75]. Apart from their involvement in stress sensing and subsequent response,

PKs may also play critical roles in various developmental processes. Computational identification and classification of the entire collection of protein kinases in the grapevine genome (also referred to as kinome) have revealed a high number of genes (approximately 1200) grouped into 121 gene families. Among them, the calcium-dependent protein kinases (CDPKs) and the MAPKs are involved in a variety of developmental processes and also in grapevine responses to various types of stress [76]. Transcriptomics of heat-stressed grapevine leaves revealed upregulation of genes encoding components of calcium- or calmodulin-mediated signal pathways, including calmodulin and CDPKs, which induced the expression of heat stress transcription factors (HSFs) and heat shock proteins (HSPs), pointing to the implication of Ca^{2+}-mediated signals in grapevine heat stress response [77].

Both HSPs and HSFs are central players in the heat stress response and the acquisition of thermotolerance in plants [74,78]. HSPs are molecular chaperones that accumulate under heat stress to prevent misfolding and aggregation of proteins and to facilitate protein refolding under conditions of denaturing stress [79]. HSPs may be induced under various other abiotic stresses, including low temperature, oxidative stress, osmotic stress and desiccation, and also have roles in plant development and disease resistance [80–82]. There are five HSP families in plants, namely HSP100, HSP90, HSP70, the chaperonins HSP60 and the small HSP (sHSP) family [83]. Among them, the 90-kDa HSP90 family differs from most other molecular chaperones in that their known client proteins are signal transduction proteins, like transcription factors and kinases [84,85]. The *Vitis vinifera* HSP90 family has been identified and, in accordance with *Arabidopsis*, it comprises four cytoplasmic and three organelle-specific members [86]. Transcriptional analysis of VvHSP90 genes in various vegetative tissues and under different high-temperature stresses (30, 37, and 45 °C) uncovered the role of VvHSP90.1 as a bona fide heat-inducible gene. VvHSP90.1 was also differentially regulated with respect to the severity of the heat stress, suggesting that the HSP90-mediated grapevine heat stress response not only senses shifts in temperature but may also monitor the magnitude of the stress [86]. Various other members of the HSP family were shown to be upregulated during heat stress, and their transcripts declined dramatically after recovery, like the HSP101, HSP21 and HSP22, suggesting that some sHSPs may have important roles in the heat tolerance of grapevines [77]. The authors also showed the upregulation of ascorbate peroxidase (APX) and dehydroascorbate reductase (DHAR) genes under heat stress, suggesting an important role of these enzymes in grape leaves to scavenge ROS generated by heat stress. Besides toxic by-products, ROS may also act as signaling molecules, thus their concentration in the cell should be precisely controlled [87]. ROS in the cell respond early under heat stress conditions and induce the expression of HSFs and the accumulation of HSPs [88]. As expected, some members of the HSF family in the grapevine, like the HSF7 that promote the expression of HSPs, exhibited increased expression levels under heat stress and were downregulated after the subsequent recovery period. In addition, genes of the calcium- and calmodulin-mediated transduction pathways were also heat-induced or recovery-regulated, pointing to a prominent role of Ca^{2+}-mediated signals in the heat stress response of the grapevine. Interestingly, the upregulation of some RLK genes, which are also induced by wounding, pathogen attack and salicylic acid, points to overlapping pathways with other biotic, abiotic and ABA stress signaling pathways in grapevine leaves [77]. Extensive overlapping signaling pathways of heat stress with other stresses may explain the high percentage of grapevine genes (68% of the assembled genes) detected under high-temperature conditions (35–45 °C) through a high-throughput transcriptomic analysis [89]. Furthermore, after integrating transcriptomics and proteomics data, the authors verified previous results showing that HSPs are the main components of the heat tolerance machinery in grape, along with some important transcription factors such as Multiprotein Bridging Factor 1c and Heat Shock Transcription Factor A2.

5. Conclusions

Climate change has led to a significant advancement in the grapevine phenological stages over the last decades. If the annual temperature continues to rise and the global warming phenomenon to amplify, as climate models predict, worldwide viticulture is going to face a real threat in the

near future. Estimating the magnitude of future risk will help to develop rational and sustainable strategic approaches for grape growers [90]. Even though some local varieties may be adapted to such environmental conditions, most international cultivars that originate from cooler climates will probably not withstand such extreme heat-stress conditions [7,91]. The local grapevine germplasm from regions of warm and dry climate may serve as an alternative. Exploration and agronomic evaluation of the indigenous diversity for heat-stress-tolerant varieties or clones would be of particular importance for the sustainability of viticulture and the wine industry [25,92].

In addition, the ongoing research in grape physiology coupled with molecular biology data mainly acquired from "omics" approaches have uncovered a number of stress-responsive factors and molecular regulators with prominent implications to heat stress tolerance. Although the heat response mechanisms of the grapevine share many similarities with other crops and the model plant *Arabidopsis*, in particular, the grapevine as a perennial fruit crop species displays several distinct features, as it has been also shown in the case of the HSP90 family [86]. Therefore, it is important to identify the particular components of grape regulatory networks governing heat stress response and acquisition of tolerance. There is no doubt that the biggest challenge would be the application of this scientific knowledge in the vineyard, e.g., in breeding programs aiming to develop genotypes tolerant to environmental stresses. It is anticipated that the forthcoming advances in metabolomics and systems biology will further accelerate the development of stress-tolerant clones, so as to achieve sustainable viticulture.

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Perspective Article

Preparation methods and applications of chitosan nanoparticles; with an outlook toward reinforcement of biodegradable packaging

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ABSTRACT

Chitosan nanoparticles (NPs) are promising polymeric and bio-based NPs, which have received a lot of attention in the last decades. They have great potential as nanocarriers that encapsulate substances such as drugs or active compounds, deliver them to a specific place or site, and provide a controlled release. In the present review, we give a complete overview of the preparation methods, including novel and green procedures, and compile developed applications in medicine, agriculture, and wastewater treatment. Moreover, we highlight the future perspective of chitosan NPs use in PLA-based biodegradable polymers which can be one of the solutions for reducing plastic pollution in nature. Properties of chitosan NPs can be tuned in such a way that they can serve as reinforcement elements in biodegradable plastics leading to much-needed improvements, and also as functional elements that make the application in food packaging with e.g., enhanced antimicrobial activity possible.

1. Introduction

Nanosized materials may have improved or unexpected properties compared to the base material they stem from. Inorganic and organic NPs have been researched intensely, and numerous preparation routes (top-down, crosslinking, microbial) and possible fields of use (electronics, textile, and medicine) have been identified. Although it is good to point out that their effect on nature and the human body have been questioned. Polysaccharide-based NPs, are however known to be environmentally-benign, much less linked to concerns over toxicity, biodegradability, and physiological stability.

For example, chitosan a natural polysaccharide, is extensively used in medical formulations [1,2]. Chitosan, is derived from chitin and found as the primary component of cell walls of fungi, the exoskeletons of crustaceans and insects, and scales of fish. It is a cationic polymer composed of (1–4)-2-amino-2-deoxy-β-D-glucan that due its' pH sensitivity, biocompatibility, and bioactive functions has attracted more attention than its base polymer chitin [3,4].

From literature, it is clear that the properties of chitosan NPs can vary considerably depending on the preparation methods that are used, and the surface modification techniques that are applied, which can lead to applications in completely different fields. The field of medicine has been reviewed extensively, e.g. in the following reviews [5–9]. In addition to the great advances made in the field of medicine, researchers have concentrated on diverse applications over the last decades such as in agriculture, wastewater treatment, and cosmetics.

In addition, chitosan NPs may be used as filler material for biodegradable plastic matrixes which are in need of improvement in terms of mechanical and barrier properties [10–12]. In order to take steps in this field, more research is needed on the interaction strength between particle and based polymer, and that depends greatly on the size, and surface properties (which is a resultant of among others preparation method), that may be tailored by modification (chemical, physical, etc.).

This study aims to review recent developments in the preparation and application of chitosan NPs, and gives short overviews of the main fields of application, medicine/pharma, agriculture, water, and cosmetics. We bring this knowledge together for our main application field of interest: the preparation of nanoparticle reinforced packaging materials.

We first describe the preparation methods that are used for chitosan particle production and highlight the differences in the methods and particle properties that are obtained. The next section is dedicated to applications that are most recently described in different areas, and the modification techniques that lead to a. adjustment of surface hydrophobicity, b. surface charge, c. additional effects related to functionality. In the last section, we bring all insights together and give an outlook on how these particles can be best applied in packaging materials (for which aggregation and prevention thereof is an important point), and be

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instrumental in improving the properties of packaging materials in a more general sense.

2. Preparation of chitosan nanoparticles

Chitosan NPs were first characterized by Ohya et al., (1994) for the circulatory delivery of 5-fluorouracil, a chemotherapy medication. [13]. After this, researchers have studied chitosan NPs extensively and developed different methods considering diverse factors such as size, stability, drug loading capacity, and retention time. The basic approaches used to form chitosan NPs revolve around emulsification, precipitation, ionic or covalent crosslinking, or combinations thereof.

Emulsification and crosslinking was the first method described in the literature for the preparation of chitosan NPs using the amino group of chitosan and the aldehyde group of a crosslinking agent [13]. An emulsion consisting of an aqueous chitosan solution and an oil phase is made using Span 80 as a stabilizer, toluene, and glutaraldehyde as the crosslinker [13,14]. The phases are mixed intensely, and droplets form the basis of the NPs are formed after crosslinking. The separation of NPs from the emulsion can typically be done through centrifugation, multiple washing steps (with petroleum ether, acetone, sodium metabisulphite, and water) and vacuum- or freeze-drying. It is possible to obtain small size NPs with a narrow distribution. However, this method is no longer used since glutaraldehyde was noticed to cause overt toxicity and drug integrity issues.

Similar to the emulsification and crosslinking, the reversed micelles (microemulsion) method is another technique that based on covalent crosslinking; albeit that now water-in-oil reverse micelle structures assist the production of chitosan NPs. The aqueous phase including chitosan and glutaraldehyde is mixed with the organic phase that contains a lipophilic surfactant and an organic solvent. Usually, cetyltrimethylammonium (CTAB) bromide or sodium bis (2-ethylhexyl) sulfosuccinate (AOT) are used as surfactants, and *n*-hexane as an organic solvent is preferred [15,16]. The core of the micelle which contains chitosan works as a nanoreactor where chitosan NP are formed by crosslinking. Fig. 1 illustrates the preparation of chitosan NPs with the reversed micelles method. The isolation of NPs happens in three steps; the precipitation of surfactant with $CaCl_2$, dialysis for the elimination of unreacted materials, and freeze-drying [17]. It is possible to obtain ultrafine nanoparticles that have size below 100 nm, which is an important feature for many applications in which the specific surface area plays a role (loading capacity and sustained release). Lately, the preparation procedure has been adapted making use of non-harmful solvents and crosslinker which mitigates the issues with the classic method based on glutaraldehyde [16,18].

Chitosan NPs can also be produced by precipitation-based methods. The phase inversion precipitation method is based on emulsification combined with precipitation. The oil-in-water emulsion is prepared with an organic phase (dichloromethane and acetone) and an aqueous solution of chitosan, in the presence of a stabilizer (poloxamer). High-pressure homogenization is applied to obtain nanometer-sized well-dispersed emulsion droplets. Then methylene chloride is separated from the emulsion by evaporation at low pressure and room temperature, leading to acetone diffusing out of the droplets and concurrent precipitation of NPs [19]. Alternatively, the emulsion-droplet coalescence method, also called desolvation has been described, and which is based on the coalescence of two water-in-oil emulsions which induces precipitation of NPs because NaOH in one of the emulsions serves as a precipitation agent. Liquid paraffin and sorbitan sesquioleate are first mixed together, and serve as the continuous phase for the two emulsions, one with chitosan and one with NaOH. High-speed homogenization is applied to prepare emulsion containing chitosan. After two emulsions are combined, NaOH diffuses into the ultrafine droplets which decrease chitosan solubility, leading to nanoparticle formation and precipitation. NPs are obtained in three steps: centrifugation, washing with different solvents; toluene, ethanol, and water, respectively and freeze-drying [20,21]. In general, NPs obtained by precipitation methods are larger than 600–800 nm. Although both methods are not very preferred due to the use of organic solvents and high energy required homogenization applications and there are only a few studies in the literature, chitosan nanoparticles obtained by the phase inversion precipitation method have high encapsulation efficiency for hydrophobic drugs such as Cyclosporin A [18,19].

Chitosan nanoparticles have been investigated for more than two decades and ionic gelation is one of the most preferred preparation methods, which has first been described by Calvo et al., (1997) [22]. It is based on ionic crosslinking that happens in the presence of inversely charged groups; for example the protonated amino groups of chitosan and negatively charged groups of the polyanion such as sodium tripolyphosphate (TPP) [23]. Chitosan is added to an aqueous acidic solution (acetic acid solution in general), and then the aqueous solution of TPP is added under vigorous stirring. Anionic molecules diffuse into the mixture of positively-charged chitosan molecules and crosslinking occurs leading to nanoparticle formation. Fig. 2 shows the electrostatic interaction between chitosan and TPP, which ultimately forms spherical shaped nanoparticles. After a couple of centrifugation and washing processes with water, chitosan NPs can be obtained by oven-drying or freeze-drying. It is a straightforward technique without any harmful crosslinker or solvents. Besides, the process can be carried out at room temperature and the final nanoparticle size can be adjusted by changing the chitosan/TPP ratio, which is a crucial property that directly affects drug encapsulation efficiency and delivery [24,25].

Ionic gelation can also be used in combination with radical polymerization, which induces gelation of chitosan simultaneously with polymerization of acrylic or methacrylic acid [26]. Potassium persulfate is used as an initiator for the polymerization reaction which requires 6 h

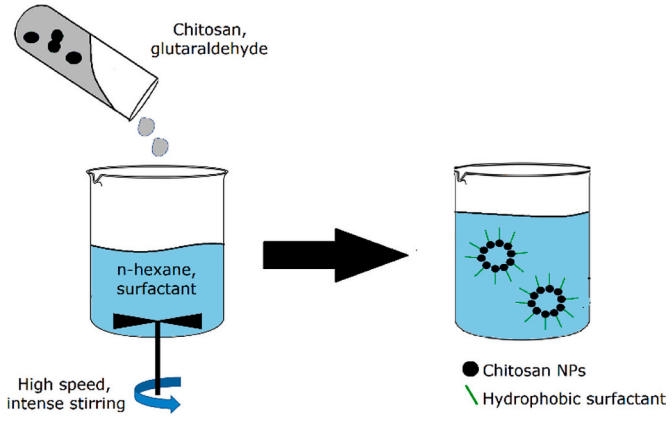

Fig. 1. Schematic illustration of chitosan nanoparticle forming via reversed micelles method.

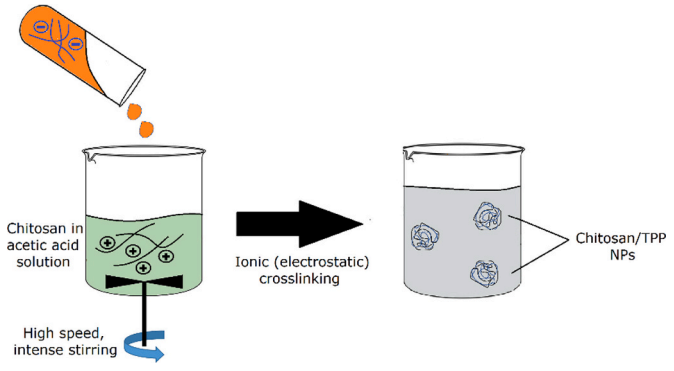

Fig. 2. Schematic illustration of chitosan nanoparticle forming via ionic gelation.

stirring at 60–70 °C [27,28]. As schematic representation of the interaction between chitosan and (meth)acrylic acid, and nanoparticle formation through radical polymerization can be seen in Fig. 3. The unreacted substances are removed by dialysis or multiple washing steps with water. This method has been used to successfully administrate insulin, silk peptide, and serum albumin through the oral route [18,28,29]. A limited number of applications are available today most probably due to long production process.

NP production can be occur by self-assembly which is a widely used method based on multiple simultaneous interactions that may be electrostatic, or hydrophobic in nature, or related to hydrogen bonding, and/or van der Waals forces between chitosan and other molecules [30,31]. The chitosan polyelectrolyte may form complexes with natural anionic materials such as hyaluronic acid or alginate, and this is mostly carried out by combining polymer solutions under stirring. Alternatively, the hydrophobicity of chitosan can be modified by grafting, which acyl-chitosan [32], stearic acid grafted chitosan [33], and PEGylated chitosan [34], that influences mostly the hydrophobic interactions during self-assembly. NPs obtained by self-assembly are especially favorable for encapsulating hydrophilic and lipophilic drugs [30] which allows the active to remain stable in the biocompatible matrix that can be adjusted readily with this mild process.

The top-down approach is also possible for the preparation of NPs, which includes two steps; acid hydrolysis of chitin to form chitin NPs and deacetylation (changing the acetyl group of chitin with an amino group). Basically, acid hydrolysis is applied with a strong acid such as hydrochloric acid for breaking glycosidic bonds and the amorphous part is removed by a couple of centrifugation and washing steps. Chitin nanocrystals are isolated after several centrifugation steps. Deacetylation is done by alkaline treatment with NaOH leading to chitin NPs that are called chitosan NPs above 60% degree of deacetylation [4,35]. Fig. 4 demonstrates chitosan NP production with top-down method. Unlike other methods where drug loading occurs simultaneously with NP formation, this method requires an extra loading step.

In recent years, green preparation routes and the use of 'mild' compounds have become popular among researchers. Spray drying is one these methods; chitosan is mostly dissolved in the aqueous acetic acid and NPs are formed by passing this solution through a nozzle using air temperatures from 120 °C-150 °C [27,36]. Magnetic chitosan NPs may also be produced by spray drying [37]. Generally, this method is used frequently in the production of chitosan microparticles and in the isolation of nanoparticles obtained by other methods. Supercritical-CO_2-assisted solubilization and atomization (SCASA) is one of the pioneering green methods as it is a process that is free of acid- or harmful solvent and uses only water and CO_2 during preparation. The dissolution of chitosan in water occurs through the acidifying effect of pressurized CO_2 under high pressure. After a relatively long dissolution step (48 h), the chitosan solution is fed to a fluidized bed by a spraying nozzle which

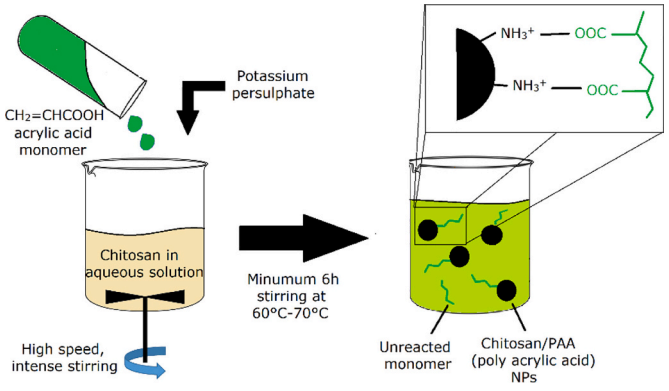

Fig. 3. Schematic illustration of chitosan nanoparticle forming via ionic gelation with radical polymerization.

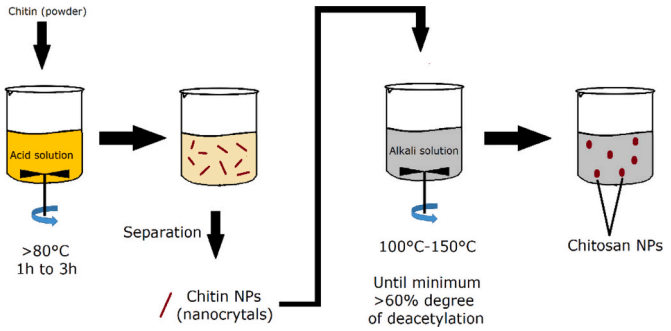

Fig. 4. Schematic illustration of chitosan nanoparticle forming via top-down method.

leads to atomization. NPs form due to the drying process, and are collected by a filter that is positioned on top of the fluidized bed [11,38]. The long processing time and the large particles and their distribution are seen as the disadvantages of this method.

Table 1 indicates the overall evaluation of preparation methods including advantages and drawbacks of each method in terms of ease of preparation, the use of harmful chemicals, and characteristics of NPs. Although it is not possible to nominate a particular method or principle as the best for all applications, methods that follow mild processes and produce NPs in a short time such as ionic gelation, self-assembly, and spray drying seem the most significant options from the points of view of human health and sustainable future.

3. Applications of chitosan nanoparticles

3.1. Medicine and pharmaceutics

Researchers have extensively studied chitosan NPs for various applications in medicine and pharmaceutics. The material is biocompatible and allows encapsulation and chain grafting of the drugs and active ingredients. Remarkable features such as preventing enzymatic degradation of drugs [40], and reducing the damage of non-targeted tissue or cells [41] make their use a great asset in drug delivery, cancer treatment and biological imaging and diagnosis [42]. Besides, the slow biodegradation of chitosan NPs has been reported to ensure controlled and continuous drug release [43]. The highly positive surface charge provides stable NPs that carry substances using various mechanisms in the human body [44,45].

The preparation of drug-loaded chitosan nanoparticle derivatives is generally done by two main techniques; nanoencapsulation and chemical modification. Nanoencapsulation relates to the formation of a nanostructure that contains absorbed drug at the surface or within the nanoparticle [46]. Table 2 shows chitosan NPs applications in medicine, NP properties and the performed modifications. Usually, the drug is encapsulated through a phase exchange process during which chitosan solidifies into NPs. This process helps to increase drug efficiency, specificity and accessibility to the target site while minimizing toxicity and side effects [47,48]. Studies on nanoencapsulation with chitosan NPs are mostly related to the delivery of therapeutic peptides such as insulin [49–51] and Cyclosporin A [46,47] and DNA [52–54] for gene therapy.

Chemical modification of chitosan NPs can be done starting from the base polymers or from the particles. The reactive amino and hydroxyl groups of chitosan are used for this purpose, and different alkaline conditions or temperatures have been reported. Examples are: thiolation [55,56], quaternization [57], carboxylation [52], PEGylation [53,58], and alkylation [59]. Even though chemical modifications are mostly performed as an auxiliary process for increasing encapsulation efficiency, solubility, enzymatic inhibition, and adhesive property, some applications also use direct grafting of the drug or active ingredient onto the polymer chain.

Table 1
The general overview of chitosan NPs preparation methods.

Method	Main Principle(s)	Advantage(s)	Drawback(s)	Ref.
Emulsification and crosslinking	Covalent crosslinking	• Simple process steps	• Use of harmful chemicals	[13,14,39]
Reversed micelles	Covalent crosslinking	• Ultrafine NPs below 100 nm	• Time-consuming process • Complex application • Use of harmful chemicals	[15–17]
Phase inversion precipitation	Precipitation	• High encapsulation capacity for specific compounds	• Requires high shear force • Use of harmful chemicals	[18,19]
Emulsion-droplet coalescence	Precipitation		• Requires high shear force • Use of harmful chemicals	[20,21]
Ionic gelation	Ionic crosslinking	• Use of mild chemicals • Simple process • Ease of adjusting NP size		[22,23]
Ionic gelation with radical polymerization	Polymerization and crosslinking		• Time-consuming process • Complex application	[26,28,29]
Self-assembly	Electrostatic and/or hydrophobic interaction	• Highly stable NPs • Use of mild chemicals • Adjustable procedure	• Hard to control when carried out a large scale	[30–32,34,40]
Top-down	Acid hydrolysis and deacetylation		• Time-consuming process • Complex application • Need an extra step for drug loading	[4]
Spray drying	Atomization	• Simple and fast process • Does not require another separation or drying steps	• Large particle size • Not suitable for use with temperature-sensitive substances	[27,36]
SCASA	Atomization	• Acid- or harmful solvent-free method • Does not require another separation or drying steps	• Time-consuming process • Requires a specially designed system • Large particle size	[11,38]

Table 2
Application of chitosan NPs in medicine and pharmaceutics.

Purpose of use	Compounds	NP production method	Particle size (nm)	Surface charge (mV)	Modification(s)	Ref
Insulin (INS) delivery	CsNPs, hydroxypropyl methylcellulose phthalate (HPMCP), INS	Ionic gelation	255	+30.1 ± 0.8	Spontaneous nanoencapsulation	[60]
	CsNPs, INS	Ionic gelation	289 ± 0.20–404 ± 18	+26 ± 2.4 +32 ± 0.6	Spontaneous nanoencapsulation	[49]
	CsNPs, INS (with different mass ratios)	Self-assembly	210.1–526.5	+2.83 +24.69	Spontaneous nanoencapsulation	[61]
	CsNPs, INS	Ionic gelation	346 ± 7	+36 ± 0.8	Spontaneous nanoencapsulation	[50]
	CsNPs, INS	Ionic gelation* (with termed flash nanocomplexation (FNC) system)	46.2 ± 2.7	+9.4 ± 1.2	Spontaneous nanoencapsulation	[51]
Cyclosporin A (CsA) delivery	CsNPs, CsA	Ionic gelation	293 ± 9	+37.5 ± 0.9	Spontaneous nanoencapsulation	[62]
	CsNPs, CsA	Spray drying	317.20 ± 78.69–681.30 ± 75.45	+22.0 ± 0.3 +30.5 ± 0.5	Spontaneous nanoencapsulation	[63]
DNA delivery (gene therapy)	Glycol chitosan NPs, 5β-cholanic acid, plasmid DNA (pDNA) (hydrophobized)	Self-assembly	277 ± 30–731 ± 51	1.1 ± 1.1 +3.3 ± 0.9	Direct grafting and spontaneous nanoencapsulation	[54]
	CsNPs, pDNA	Ionic gelation	190.51 ± 19.05–287.25 ± 14.12	+17.09 ± 0.71 +41.45 ± 0.43	Spontaneous nanoencapsulation	[53]
	Cs-N-2-hydroxypropyl trimethyl ammonium chloride NPs, pDNA	Phase inversion precipitation	91.8	~ 1	Quaternization and spontaneous nanoencapsulation	[57]
Cancer therapy	Thiolated CsNPs, curcumin (CRC)	Ionic gelation	150 ± 20	+48.2 ± 5	Thiolation and spontaneous nanoencapsulation	[55]
	Thiolated CsNPs, 5-fluorouracil (5-FU)		150 ± 40	+35.7 ± 3		
	Linoleic acid-modified carboxylated-Cs NPs, adriamycin	Self-assembly	417.8 ± 17.8	No information	Carboxylation and acid grafting	[64]
Circulatory system regulator	Thiolated CsNPs, heparin	Ionic gelation	323.1	+35.5	Thiolation and spontaneous nanoencapsulation	[56]
Gene silencing	Cs, methoxy PEG succinimidyl ester (mPEG-NHS), siRNA	Self-assembly	126.6 ± 2.18–175.6 ± 2.76	+19.2 ± 0.41 +26.7 ± 0.42	PEGylation and spontaneous nanoencapsulation	[34]

3.2. Agriculture

Studies on the use of chitosan NPs for agricultural applications have increased rapidly motivated by the need for sustainable and eco-friendly agrochemicals such as fertilizers and pesticides. Similar as in medicine, chitosan NPs are used mostly as nanocarriers that enhance the stability of active ingredients and as a means to create controlled release [65]. Through these effects, agrochemicals can be applied in lower doses and fewer treatments are needed, thus contamination risk of the environment and toxic effects to other non-targeted organisms are decreased

[64,66].

Table 3 shows a general overview of chitosan NPs applications in agriculture. In general, medium-size and highly positive charged NPs which provide better stability in aqueous environments are preferred to ensure the slow and continuous release of active ingredients in soil. Nanoencapsulation that occurs spontaneously during ionic gelation has been used to encapsulate agrochemical- or active ingredients e.g. essential oils in chitosan NPs [67–70]. Typical applications of chitosan NPs are for herbicide delivery for weed eradication [67,69,71], in insecticide [68,70,72], and fungicide treatment [72–74] and various deliveries such as plant growth regulator [75,76], and fertilizer for balanced nutrition of plants [77–81]. Besides, chitosan derivatives e.g. chitosan-poly(acrylic acid) (CS-PAA) [72], chitosan-poly(methacrylic acid) (CS-PMAA) [80] and alginate/chitosan (ALG/CS) NPs [75,76] have been applied.

3.3. Wastewater treatment

The lack of a cost-efficient, sustainable, and effective sorbent as alternative for the widely used activated carbon has motivated the study of bio-based alternatives [82]. Chitosan includes functional amino and hydroxyl groups, which makes these NPs interesting for the removal of a range of pollutants such as heavy metals, pesticides, and dyes [83]. Besides, NPs may exhibit higher capacity than conventionally used micro-sized sorbents due to their higher surface area [84].

The applications and particle properties of chitosan NPs in wastewater treatment are shown in Table 4. Unlike applications in other fields, ultrafine nanoparticles that are smaller than 100 nm are preferred for wastewater treatment, which leads to a higher surface area that can absorb more pollutants. Chitosan nanoparticle derivatives with increased electrostatic and magnetic properties have generally been suggested for wastewater treatment. Amination is carried out by grafting e.g. ethylenediamine, hexanediamine, or diethylenetriamine of which the NH_2 group(s) are responsible for the electrostatic interactions with the pollutants [85,86]. When used in conjunction with magnetic properties, chitosan NPs have been applied to improve heavy metal and hazardous dye removal [87–89].

3.4. Other applications

Studies have shown that chitosan NPs and their derivatives can bring innovative solutions and new approaches to various scientific fields. Although the progress and use are not as widespread and advanced as in medicine and agriculture, studies have also been carried out in cosmetics, food technology, and dentistry.

Chitosan NPs have been suggested as carriers of active ingredients that are used for skin and hair care. The use of chitosan NPs to deliver minoxidil sulfate (hair growth agent for which concerns consist of side effects) ensured a sustained release without dermal exposure [95,96]. Nanoencapsulation of retinol led to protection against degradation [97], and complexation of retinol in succinic-chitosan NPs increased the antioxidant activity compared to pure retinol [98].

A pioneering study was carried out by del Carpio-Perochena et al., [99] who used chitosan NPs in root canal treatment instead of EDTA. These researchers showed that the antibacterial and chelating ability of chitosan NPs makes them a proper nanomaterial for dental applications. Furthermore, Atta et al. [100], investigated amidated chitosan NPs that may be used for corrosion protection of steel.

Chitosan NPs have also been considered as a filler material in e.g. pectin based edible films to improve the mechanical strength and barrier properties [101–105]. Moreover, there are some studies on the antimicrobial activity of chitosan NPs and their potential use in (edible) food packaging [102,106,107].

4. Future perspective for the use of chitosan nanoparticles in biodegradable food packaging

Petrol-based plastics pose a societal challenge that concerns many. On the one hand, polymeric materials have excellent properties when considering e.g. strength in relation to the amount of material that is used, on the other hand, many of these plastics are not properly disposed of, thus creating a huge environmental issue, which also applied to biomaterials if they do not degrade within an acceptable period of time. For standard plastic degradation, time would need to be expressed in terms of centuries.

In 2020, one trillion food and drink packages are predicted to be thrown away only in Europe [108]. Disposable plastic packaging materials accounted for almost 40% of plastic production in 2018 [109].

Table 3
Application of chitosan NPs in agriculture.

Purpose of use	Compounds	NP production method	Particle size (nm)	Surface charge (mV)	Modification(s)	Ref.
Herbicide delivery	CsNPs, paraquat	Ionic gelation	~300	+45	Spontaneous nanoencapsulation	[67]
	ALG, CsNPs, imazapic (IMC) and imazapyr (IMR)	Ionic gelation	377.70 ± 9.70	30		[69]
	CsNPs, imazapic (IMC) and imazapyr (IMR)	Ionic gelation	478.60 ± 52.30	+26	Spontaneous nanoencapsulation	
Insecticide delivery	CsNPs, PAA	Polymerization and crosslinking	51.8	no info	–	[72]
	CsNPs, nicotine hydrochloride (NCT), sodium chloride (NaCl)	Ionic gelation	249.9	+35	Spontaneous nanoencapsulation	[70]
	CsNPs, fungal metabolites	Ionic gelation	~200	+24	Spontaneous nanoencapsulation	[68]
Fungicide delivery	CsNPs, *Cymbopogon martinii* essential oil (CMEO)	Self-assembly	455–480	+37.2 +39.3	Spontaneous nanoencapsulation	[74]
	CsNPs, clove essential oil (CEO)	Emulsification and crosslinking	268.47 ± 0.71	+22.45 ± 0.90	–	[73]
Plant growth agent delivery	ALG, CsNPs, gibberellic acid (GA_3)	Ionic (pre)gelation	~450	29.00 ± 0.3		[75,76]
	CsNPs, gibberellic acid (GA_3)	Ionic gelation	~195	+27.00 ± 3.0	Spontaneous nanoencapsulation	
Fertilizer delivery				+45.3 (with N)		[77–79]
	CsNPs, methacrylic acid, fertilizers (N, P and K)	Polymerization and crosslinking	~500–700	+33.6 (with P) +85.4 (with K)	Nanoencapsulation after NP production	
	CsNPs, fertilizers (N,P and K)	Ionic gelation	~500	+50	Nanoencapsulation after NP production	[81]

Table 4
Application of chitosan NPs in wastewater treatment.*

Purpose of use	Compounds	NP production method	Particle size (nm)	Modification(s)	Ref.
Heavy metal removal	CsNPs, Fe_3O_4	Co-precipitation (for Fe_3O_4 NP production) and crosslinking	~30	Conjunction with magnetic particles	[88]
	CsNPs, acrylic acid, lead (II) nitrate ($Pb(NO_3)_2$)	Crosslinking	50–200	Direct grafting	[90]
	CsNPs, ALG	Ionic gelation (separately for each compound)	396.1	Crosslinking between CS NPs and ALG NPs	[91]
Dye removal	CsNPs, β-cyclodextrin, Fe_3O_4	Co-precipitation (for Fe_3O_4 NP production), Emulsification and crosslinking	~100	Conjunction with magnetic particles	[92]
	CsNPs, ethylenediamine, Fe_3O_4	Co-precipitation (for Fe_3O_4 NP production), Emulsification and crosslinking	15–40	Conjunction with magnetic particles and amination	[93]
	CsNPs, Fe_3O_4	Co-precipitation	78.82	Conjunction with magnetic particles	[94]
		Crosslinking with TPP	68.74		
		Crosslinking with glutaraldehyde	55.93		

* *No information was found related to the surface charges of chitosan NPs.*

These facts have peaked the interest of researchers and different industries, and have made them consider biodegradable plastics that are defined as materials able to completely degrade to water and carbon dioxide by naturally occurring activities of microorganisms such as bacteria, fungi, and algae [110]. Although there is an increasing trend in the production and use of biodegradable plastics; the share of biodegradable plastics was less than 1% of the total plastic production in 2019 [111].

Polylactic acid (PLA), a biodegradable and renewable thermoplastic polyester which is obtained from lactic acid or lactide, seems one of the most promising materials that may be able to replace conventional petrol-based plastics due to its production from renewable sources, the high tensile strength, UV-blocking property, excellent aroma barrier and the processability in standard plastic production lines [112–115]. Interestingly, neat PLA possesses better or comparable O_2 permeability than some conventional plastics such as polystyrene or polyethylene derivatives. Although the price of PLA (2000€/tonne) is still very high compared to conventional plastics (PET: 850–1050€/tonne, HDPE: 1200–1500€/tonne), experts predict that PLA can compete with its petrol-based counterparts in terms of price in the near future considering fluctuating prices of petrol and its finite nature [115].

Currently, PLA-based food packaging materials are for single-use applications: beverage and yoghurt cups, daily meal containers, shopping bags, and cutlery products. Improvements in mechanical and

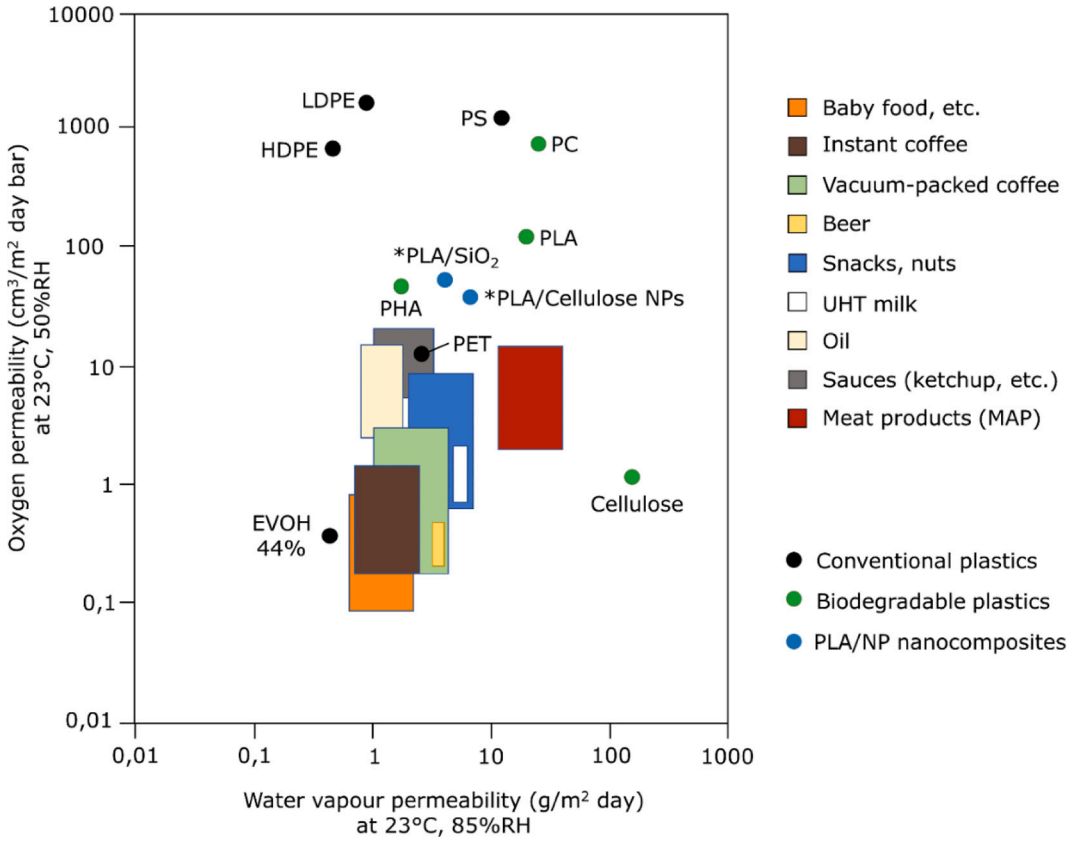

Fig. 5. *Overview of the barrier properties of packaging materials and requirements that need to be met for selected foods* [118–121]
**Approximate values due to different conditions and unit conversion*
LDPE: Low-density polyethylene, HDPE: High-density polyethylene, PS: Polystyrene, PET: Polyethylene terephthalate, EVOH: Ethylene vinyl alcohol, PC: Polycarbonate, PHA: Polyhydroxyalkanoate.

barrier properties are required for the widespread use of PLA in food packaging to become a reality. More specifically water vapor permeability, and brittleness need to be reduced [116,117], as illustrated in Fig. 5 in which an overview is shown of the various materials used in food packaging in the relation of oxygen and water permeability.

Inorganic NPs such as carbon nanomaterials, silica NPs, or metal NPs were extensively studied in the field of nanocomposites [122–125]. These particles can also greatly improve the properties of packaging materials as can be seen in Fig. 5 where nanocomposite properties are very close to the requirements that need to be met for specific food product categories, and quite improved compared to neat PLA for example. The use of inorganic NPs meets growing concerns in terms of environmental pollution; therefore, polysaccharide NPs such as cellulose, starch, chitin, and chitosan are thought to be more suitable fillers for PLA-based nanocomposites. These particles are more or less compatible with the base polymer and have been suggested to improve the mechanical, thermal and barrier properties [126,127]. Furthermore, natural polysaccharide NPs are biodegradable, renewable, versatile, and abundantly available. Besides, some of the polysaccharide NPs such as chitosan possess additional features such as antimicrobial and antioxidant activity, and options for chemical modification due to the presence of primary amino groups [128,129]. Table 5 shows how polysaccharide NPs affect the properties of biodegradable plastics, especially PLA. In general, it can be said that while the thermal properties do not change, the mechanical and barrier properties are improved.

As mentioned before, the compatibility of polysaccharide particles and the base polymer is not always ideal, therefore options to modify they surface properties are of great relevance since the specific surface area of the NPs is very high [135]. It has been suggested that a small amount of NPs (1–5%) will be sufficient for improvements to the matrix, and for this a homogenous distribution of particles inside the nanocomposite is a challenge that needs to be met.

For many nanocomposites, the thermodynamic equilibrium state is that of NPs in the form of aggregates [136,137]. Aggregation between NPs is mostly caused by Van der Waals forces or chemical bonds, and starts just after formation depending on concentration, size, and surface charge. Increasing concentration and smaller size promote aggregation while high surface charge provides more stable NPs [138,139]. Furthermore, (in)compatibility with the polymer matrix may induce aggregation. Keeping aggregation at the lowest levels or ensuring the disruption of aggregates during nanocomposite production is crucial for achieving desired product properties.

Different strategies have been using to manage the aggregation, one of them is breaking up aggregates during extrusion/melt mixing. It has been suggested that the specific mechanical energy (SME: energy given to the system during the process) is an indicative measure for this [140]. On the other hand, the energy that would be needed to break up certain aggregates can be extremely high [141,142], and it would be doubtful if an extruder can generate such energy input, which highlights the importance of surface modification methods to reduce the interaction energy in aggregates. Furthermore, it is good to mention that increasing temperature and long process times can lead to the degradation of PLA.

As mentioned, surface modification is an important tool to increase the hydrophobicity of chitosan NPs and thus facilitate homogeneous distribution in the polymer matrix. Various options are known from [143,144], such as fatty acids and aromatic acids that can be coupled to the amine group of chitosan. Besides, additional features can also be acquired, for instance by phenolic acid grafting that leads to improvements in biological activities (antimicrobial, antioxidant, antitumor, and anti-allergic) [145–147]. In a pioneering study by Salaberria et al. [133], the chloride form of a fatty acid (dodecanoyl chloride acid) was bonded to chitin NPs via acylation. These modified NPs had increased hydrophobicity, and mixed with PLA by extrusion showed higher antifungal activity, which we consider an interesting lead for further development of nanoparticle reinforce packaging materials.

Fig. 6 illustrates the whole production, functionalization, and degradation steps of PLA nanocomposites reinforced with chitosan NPs. Significant progress has been made in industry and academia, especially on PLA production. Moreover, NP production and various functionalization techniques are discussed in detail above, which is illustrative for further functionalization options. However, considering industrial production, some points need to be raised in relation to the origin and characterization of chitosan. Nowadays, shrimp and other crustaceans are the main sources of chitosan, which are allergenic for a part of the world population. The current chitosan production routes include a sequence of treatments for, especially deproteination that is expected to decrease greatly or even omit allergenicity effects. Although there are not many studies in the literature on this subject, no allergic reaction is

Table 5
Studies on polysaccharide NPs reinforced PLA and/or some other biodegradable plastic nanocomposites.

Filler	Matrix	NP preparation method	NP modification	NC preparation method	Main findings	Ref.
Cellulose NPs	•PLA •PLA, PHB •PLA, PHB and plasticizer	•Acid hydrolysis	•Surfactant grafting •L-lactide grafting	Solution casting or melt mixing	+Improvements in oxygen and water vapor permeabilities +Higher UV protection ±Similar thermal behavior -Decrease in transparency -Acceleration in disintegration -Higher stiffness with PLLA grafting	[122–125,127]
Starch NPs	•PLA	•Acid hydrolysis	•Crosslinking with ECH •PLA grafting	Solution casting	+Lower water vapor permeability ±Similar thermal behavior with PLA grafted SNPs -Lower glass transition temperature with epichlorohydrin crosslinked SNPs	[130,131]
Chitin NPs	•PLA •PLA and plasticizer •PLA, PEG and plasticizer	•Acid hydrolysis	•L-lactide grafting	Solution casting or melt mixing	+Higher tensile strength and Young's modulus +Lower water vapor permeability +The use of PEG reduced the aggregation of NPs ±Increase in biodegradability of chitin NPs reinforced PLA film ±Similar thermal behavior -Lower elongation at break (%) values	[126,132–134]
Chitosan NPs	•PLA	•Radical polymerization •SCASA •Unknown	•SMA grafting	Melt mixing	+Better miscibility between SMA-grafted CsNPs and PLA +Slight increase in elongation of PLA until 3% CsNPs content ±Similar thermal behavior	[10–12]

PHB: Polyhydroxy butyrate, PEG: Polyethylene glycol, ECH: Epichlorohydrin, SMA: Stearyl methacrylate.

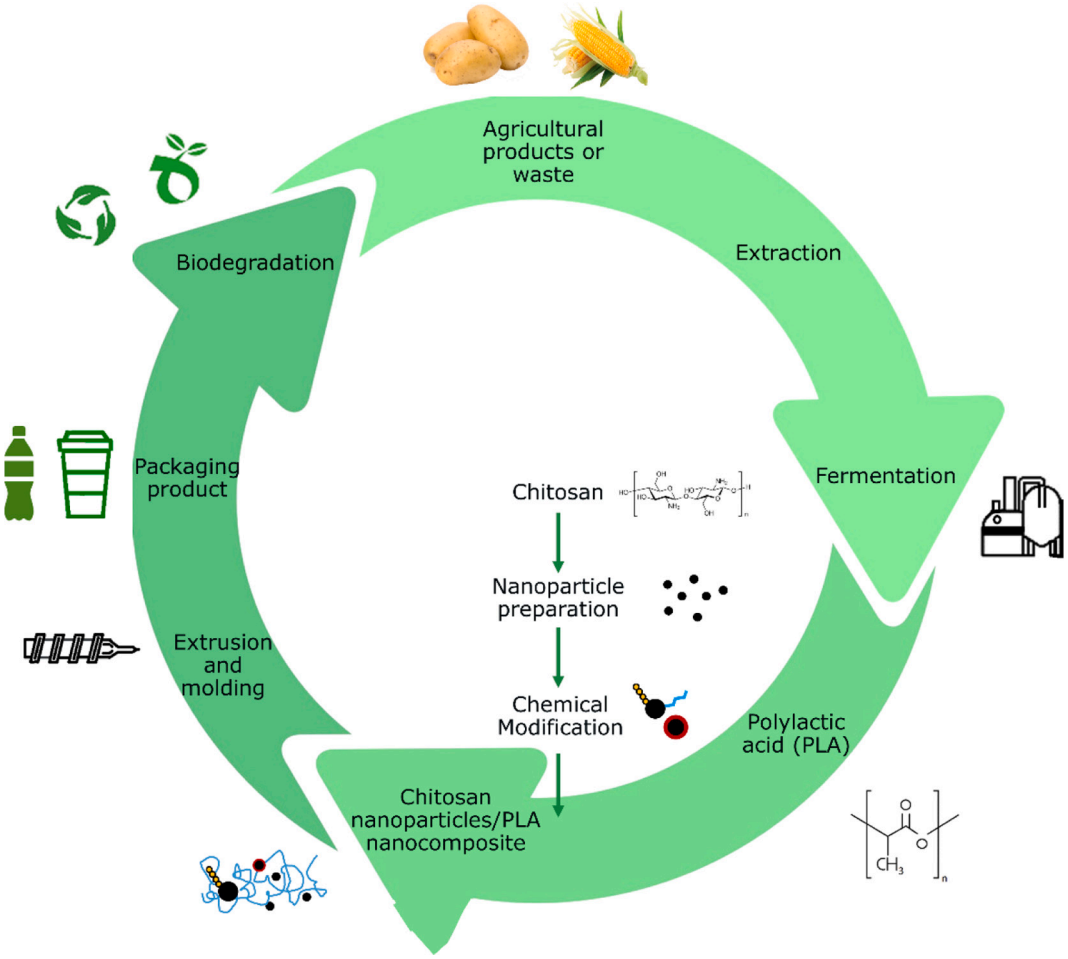

Fig. 6. The envisioned life cycle of chitosan NPs reinforced polylactic acid nanocomposites.

observed in wines processed with the chitosan-based film [148] or after the use of chitosan bandages [149]. The use of fungi-based chitosan could be an alternative to that obtained from crustaceans, although possible residue of mycotoxins and spores may then be a drawback that should be considered carefully [150,151]. On the other hand, different origin and preparation routes can lead to variable properties in bio-based molecules like chitosan, e.g. degree of deacetylation or molecular weight, which, as explained earlier is a promising route to further functionalization. Therefore, chitosan characterization for standard and reproducible material production is a critical point for industrial applications. Furthermore, determining the biodegradation and composting properties of NPs and packaging material will be needed.

5. Conclusions

Chitosan NPs have established an important place in different industries and scientific fields after their first description approximately two decades ago. Numerous preparation and modification methods have developed, including greener preparation methods that do not include harmful or toxic chemicals, for example, spray drying and supercritical-CO_2 assisted methods. The application of chitosan NPs is mostly to realize sustained release and high loading capacity of drugs or active ingredients in various fields such as pharma and agriculture.

Despite the relative ease of preparation, only a few studies are geared toward the application of chitosan NPs in biodegradable plastics. Preliminary studies show the great potential of chitosan NPs to upgrade PLA film properties (oxygen and water transfer) bringing them close to what is needed for food application. Further, NPs can be used to, directly or indirectly after surface modification, create additional features such as antioxidant and antimicrobial effects, which form a lead toward future active packaging options.

The authors anticipate that chitosan NP enriched PLA nanocomposites will play a significant role in biodegradable food packaging. These plastics have properties comparable to petrol-based polymers and may be given additional functionality, e.g. antimicrobial and antioxidant. Furthermore, they comply with societal aspects such as increased awareness of plastic waste and its' effects on nature and human life, and the demand for sustainable development of materials. So both from technical and societal points of view, this nanoparticle reinforced biodegradable food packaging will be in high demand.

Author statements

Murat Yanat: Writing & Editing.
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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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ALTERNATIVE METHODS OF SULFUR DIOXIDE USED IN WINE PRODUCTION

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ABSTRACT

Sulfur dioxide (SO_2) is the most common additive used in winemaking for many years. This compound is important for wine producers and consumers due to its antiseptic and antioxidant properties. However, excessive sulfites caused some symptoms such as a headache, nausea, stomach irritation and respiratory distress in asthmatic patients. Additionally, excessive SO_2 in winemaking process leads to organoleptic changes of final product. For these reasons, the maximum SO_2 concentrations allowed in wines were gradually reduced. In the wine industry it is essential to reduce or even eliminate SO_2 especially in the production of organic wines. These obstacles lead to requirements of new healthier and safety strategies for reduction of SO_2. Up to know have been discussed the priorities of SO_2 used in wine making. Recently some authors evaluated studies with chemical and non-thermal alternatives of SO_2. Some other authors reviewed the side effects of SO_2 used in wines, but none of them have comprised the effects of new techniques in grape, must, wine and pomace. This review discussed effects of different alternatives (thermal, non-thermal, chemical and natural additives) techniques demonstrated in grape, must, wine and pomace as possible alternative to SO_2 in comprehensive manner. The antioxidant, antimicrobial and sensory properties of tannin, oak and vine shoot extracts are also discussed as new alternatives of SO_2. The studies demonstrated that SO_2 could be lowered and even changed by using the development of new methods.

Keywords: sulfur dioxide (SO_2), natural alternatives, plant extracts, physical treatments

INTRODUCTION

SO_2 is a chemical preservative that has been used in the preservation of dry fruits and vegetables, canned fruits and vegetables, tomato paste and jam production in the food industry for many years (**Taylor *et al.*, 1986**). Although the historical process of usage SO_2 dates back to ancient times, it is thought that the first use of SO_2 in the food industry was primarily begun in the early 18th century (**Pasteur, 1866**). Subsequently, the use of this preservative has been followed by foods, especially with low pH such as fruit juices and fermented products. (**Ribereau-Gayon *et al.*, 2006**).

Phenolic compounds in red and white wines are primary substrates for oxidation. During wine aging, there is a gradual loss of phenolic compounds due to their participation in a number of chemical reactions such as oxidation (with polysaccharides and tannins) and formation of other stable anthocyanin-derived pigments. All these reactions could result in marked changes in the color, mouth feel and flavor properties of red wines (**Fulcrand *et al,* 2006**). SO_2 is the most common additive in winemaking, because of its multifunctional properties; inhibition of unwanted microorganisms, preventing oxidation and inhibition of enzymatic and non-enzymatic browning reactions and contribution to wine quality (**Cabaroglu and Canbas, 1993; Bakker *et al.*, 1998**). Even these advantages, negative effects of SO_2 on human health have been subject to researches for many years (**Vally *et al.*, 2009**). Excessive SO_2 leads to organoleptic changes in the final product as well as resulting in toxic effects on human health. With increasing health concerns and narrowing legal limits on chemical protectors, consumers are increasingly demanding to consume foods that contain non-chemical additives. As a result, there is an increasing tendency to reduce the use of SO_2 in winemaking and to use it in combination with additional alternative methodologies. For these reasons, current studies focused on compared effects of SO_2 against its new alternatives in wine production. A number of studies have been conducted as an alternative of SO_2. Some of them included non-thermal processes; some of them proposed using of new chemicals. Recently, the uses of natural preservatives, which may be an alternative to SO_2 and the effects on the final product, have been currently tested.

Many alternatives have been proposed as promising tools for replacement of SO_2 in wine but nowadays it is still do not completely possible to find a wine without preservative in the global market stores. However, there is a need for further review in which are discussed in a more comprehensive manner with current existing alternative techniques available to fully or considerably replace of SO_2 in wine making.

Until today, the authors have discussed various techniques that have the potential to be used in the wine production for preservation as an alternative method of SO_2 (**Crapisi *et al.*, 1988; Santos *et al.*, 2012; Morata *et al.*, 2017; Lisanti *et al.*, 2019**). Most of the studies have focused on chemical or non-thermal methods able to replace the SO_2. Despite the number of literature reviews as alternative methods to SO_2 in wine industry, it is surprising that there has been scarcely absence of a fully comprehensive overview of the current state-of-the-art about deeply discussed their influence, contribution and advantages/ disadvantages on the wine quality. In this review article various groups of chemical, physical and natural alternative methods have been discussed for this purpose: non-thermal treatments such as high hydrostatic pressure, high power ultrasound, ultraviolet, pulsed electric field and low electric application; chemical treatments such as dimethyl dicarbonate, lysozyme, chitosan, colloidal silver complex; treatments with bacteriocins and killer toxins; application of natural plant extracts such as grape based phenolic extracts, wood and grapevine shoot extracts, olive-based extracts and other plant extracts. In this sense, it is also important to remark thermal treatments' effects on wine quality. The latest investigation of thermal processing techniques on grape, must and wine focusing on the transformation of polyphenols and the fluctuation of antioxidative activities upon various processing. This technique currently reported to reduce the addition of SO_2 to wine. However, the effect of the thermal treatments on the composition and sensory content of wines were also reported. Therefore, we reviewed provides hints for the future processing of grapes and wines to reduce the use of SO_2.

In addition this review will thus help the reader to identify and evaluation of these new techniques reported up to now for wine industry and specifically focus on: (i) discussed the potential applications of methods underlying their ability to control unwanted microorganisms, (ii) understanding the effect of their antioxidant activity, their mechanism of action and (iii) their stability and contribution to sensory properties of wines.

MECHANISM OF ACTION AND IMPORTANCE OF SO_2 IN WINE INDUSTRY

Nowadays, SO_2 is known as the most effective chemical additive in winemaking. In case of development of new alternative methodologies to SO_2, the effects of this additive on wine properties and the mechanism of action in wine should be well understood (**Guerrero and Cantos-Villar, 2015**).

In general, SO_2 is mostly liquid (5% v/v diluted solution) form during wine production. Other common sulfur-containing salts are sodium metabisulfite ($Na_2S_2O_5$) or potassium metabisulfite ($K_2S_2O_5$), potassium bisulfite ($KHSO_3$), sodium bisulfite ($NaHSO_3$), calcium bisulfite ($CaHSO_3$), and sodium sulfite (Na_2SO_3) (**Aktan and Yıldırım, 2014**). After addition to the wine, sulfide-containing compounds are dissolved into their sulfide ions (HSO_3^-, SO_3^-) and sulfurous acid (H_2SO_3). These ions react directly with the oxygen before the wine polyphenols and are converted into sulfate (SO_4^{-2}) and sulfuric acid (H_2SO_4) forms (**Ribéreau-Gayon *et al*., 2000; Clarke and Bakker, 2004**). These reactions could be presented by following reaction (Reaction 1) (**Navarre, 1988; Cabaroğlu and Canbaş, 1993**).

$$2HSO_3^- + O_2 \leftrightarrow 2H^+ + 2SO_4^{-2} \text{ (Reaction 1)}$$

In a different view in explaining the mechanism of action of SO_2, it was concluded that SO_2 acts as an antioxidant by direct oxygen scavenging, by reacting with hydrogen peroxide produced by oxidation of polyphenols in wine and by reducing the quinones formed during the oxidation process back to their phenol form (**Boulton *et al*., 1996; Oliveira *et al*., 2011**). The sulfurous acid (H_2SO_3) which is the one SO_2 form, combines with acetaldehyde (CH_3CHO) to form aldehyde sulfurous acid ($CH_3CH(OH).CH_3H$). Herewith, SO_2 competes for hydrogen peroxide to prevent the formation of aldehyde and prevent unwanted acetaldehyde flavor in the wine (**Elias and Waterhouse, 2010**).

Besides its antioxidant effect, SO_2 plays an important role as an antimicrobial additive against unwanted microorganisms such as wild yeast and acetic acid bacteria in wine (**Yıldırım and Altındisli, 2015; Şener and Yıldırım, 2013**). Especially yeasts are very sensitive to SO_2. Bacteria become inactive in the amount of 40-50 mg/L SO_2, while wine yeasts are resistant to 150-400 mg/L of SO_2 (**Erich, 1977; Gomez-Plaza and Bautista-Ortin, 2019**).

Preservation of oxidation is great importance both for taste and color of wines. Polyphenols are oxidized by polyphenol oxidase (tyrosinase) and laccase enzymes that cause changes of color and flavor of wine (**Aktan and Yıldırım, 2012,2014**). SO_2 inhibits enzymes such as polyphenol oxidase, peroxidase, protease and inhibits Maillard reactions that lead to browning of wine (**Garde-Cerdan *et al*., 2008; Mayen *et al*., 1996; Ribéreau-Gayon *et al*., 2006**).

ADVERSE EFFECTS OF SO_2 AND LEGAL REGULATIONS

In addition to all the positive effects of SO_2, the negative effects on human health have been the subject of research for many years. Consumers observed different adverse effect level against SO_2 (most of the sulfide-sensitive individuals are affected in amounts of SO_2 ranging from 20 to 50 mg), which are associated with the many health risk such as angioedema, diarrhea, abdominal pain, bronchoconstriction and anaphylaxis (**Guerrero and Cantos-Villar, 2015**). Some other disorders are associated with SO_2 such as asthma, allergic reactions, headache, fatigue, itching (**Vally and Thompson, 2001,2003**). SO_2 and its derivatives may cause activation of proto-oncogenes and inactivation of tumor suppressor genes. It may even play a role in the pathogenesis of SO_2-related lung cancer (**Qin and Meng, 2009; OIV 2016**)

As a result of various studies, the daily intake of sulfite was assumed to be 43 mg / g on average for an individual weighing 60 kg (**Taylor *et al*., 1986**). The World Health Organization (WHO) has set limits on daily intake of sulfite as 0.7 mg/kg body weight (**WHO, 2009**). Considering this value, for an individual consumer weighing 60-80 kg, the acceptable daily dose is between 42 and 56 mg per liter/day. It should be kept in mind that a consumer who drinks only half a liter of wine can easily overcome this value.

With the understanding of the adverse effects of SO_2, legal regulations and standards have been introduced in national / international legislation related to SO_2 used in wines. In the European Union legislation, the manufacturers required to specify in the label that "contains sulfites", in the case of sulfite content higher than 10 mg/L in foods (**Regulation EC No 203/2012**). According to International Organization of Vine and Wine (OIV), these limits are 150 mg/L for wines with sugar content <5 g / L; 200 mg/L for wines with a sugar content ≥ 5 g/L (**OIV, 2017**). OIV and European Union regulations have gradually reduced the use of SO_2 to 100 ppm for "Organic Wines" (Table 1). According to U.S. Department of Agriculture (USDA), this limit is 100 ppm for wines labeled as "produced organic grapes" (**USDA, 2019**). SO_2 maximum limits may be higher based on the type of wine and sugar content in the regulations.

Table 1 Maximum limits of SO_2 according to sugar content of wines

Wine types	SO_2 limits for conventional wine Categories as in Regulation EC No 606/2009	SO_2 limits for conventional wine as in Canada (**CFIA, 2011**) and in USA (27 CFR 4.22(b)(1)) (**USDA, 2019**)	SO_2 limits for organic wine as in Regulation EC No 203/2012 (**IFOAM, 2013**)	SO_2 limits for organic wine as in Canada and USA (**CFIA, 2011; USDA, 2019**)
Red wines residual sugar < 5g/L	150 mg/L	350 ppm	100 mg/L residual sugar <2g/L 120 mg/L residual sugar >2g/L and < 5g/L	100 ppm
Red wines residual sugar ≥ 5g/L	200 mg/L		170 mg/L	
White & rosé wines residual sugar < 5g/L	200 mg/L		150 mg/L residual sugar <2g/L 170 mg/L residual sugar >2g/L and < 5g/L	
White & rosé wines residual sugar ≥ 5g/L	250 mg/L		220 mg/L	

ALTERNATIVE METHODS OF SO_2 USED IN WINE PRODUCTION

Many different methods have been tried to be an alternative of SO_2 used in wine production. Among these methods are chemical preservatives such as sorbic acid, ascorbic acid, dimethyl dicarbonate (DMDC). According to studied methods are bacteriocins (lacticin and nisin) (**Bauer *et al*., 2003; Rojo-Bezares *et al*., 2007**). In addition to these methods, non-thermal processes such as high hydrostatic pressure (HHP), pulsed electric field (PEF), ultraviolet irradiation (UV), high power ultrasound (HPU) and low electric current (LEC) have been studied as a potential alternatives of SO_2 to be used in winemaking (**Fredericks and Krügel, 2011; Morata *et al*., 2015; Delsart *et al*., 2015a,b; Costantini *et al*., 2015; Gracin *et al*., 2016; Briones-Labarca *et al*., 2017**). In addition to all these alternatives, natural alternatives continue to be tested currently such as eucalyptus and almond skin extracts (**Garcia-Ruiz *et al*., 2013b**), stilbenes extracts (**Raposo *et al*., 2016a, 2018**), thyme essential oil (**Freidman *et al*., 2017**), grape and wood tannins (**Sonni *et al*., 2009; Alamo-Sanza et al., 2019; Sánchez-Palomo *et al*., 2017**), hydroxytyrosol and oleuropein (**Raposo *et al*., 2016b,c**), glutathione (**Hosry *et al*., 2009**). All these tested methodologies are listed in Table 2.

The key proposed alternatives for development of alternatives of SO_2 during wine production should meet some topics summarized as follows;

- Human health should not adversely affected;
- It should be easily available and cheap;
- Must have antimicrobial and antioxidant properties;
- Substances or techniques should not cause very large changes in quality of wine;

Experimental studies in laboratory scale should also be applicable in the industry;

THERMAL PROCESSES

Thermal treatments such as pasteurization and sterilization are effective to inactivate undesirable microorganisms and enzyme, and thus are commonly used by the food industry. Thermal treatments in the wine-making process are very important for the final quality of the wine. Even if it cannot replace all functions it can complement the effect of sulfur dioxide (SO_2) with combination with other effective alternative techniques (**Lambri *et al*., 2015**). These technologies individually or in combination have shown great potential not just for sterilization also for extraction of anthocyanins and other polyphenols from grape to wine at fermentation step (**El Darra *et al*., 2013a; Corrales *et al*., 2009**). Moreover, heat treatment prevents browning due to inhibit oxidizing enzymes, such as laccase and polyphenol oxidase (**Clarke and Bakker, 2011**). This chapter summarizes the recent advances of thermal processing technologies in winery including heating and freezing grapes, must and wines. Contributions of

these methods to wine about their content and compositional change during processing were highlighted, as well as the primarily studies of the underlying heat conditions. The advantage and limitation of these technologies are also discussed along with the perspective insight of their future development.

Table 2 Some alternatives of SO_2 used during wine production

A. Chemical Additives	B. Non-thermal Applications	C. Phenolic Compounds and Plant Extracts	D. Killer Toxins and Bacteriocins	E. Combine Methods
DMDC (**Costa and Loureiro, 2008**)	High Hydrostatic Pressure (HHP) (**Briones-Labarca *et al*., 2017**)	Eucalyptus and almond skin extracts (**Garcia-Ruiz *et al*., 2013b**)	*Kluyveromyces phaffii* DBCG 6076 (**Ciani *et al*., 2001**)	Glutathione and cafeic acid or gallic acid (**Roussis and Sergianitis, 2008**)
Lysozyme (**Azzolini *et al*., 2010**)	Pulsed Electric Field (PEF) (**Delsart *et al*., 2015a,b**)	Stilbens extracts (**Raposo *et al*., 2016b**)	Pediocin PA-1 (**Diez *et al*., 2012**)	Glutathione and/ or elligatannis (**Panero *et al*., 2015**)
Ascorbic acid (**Sonni *et al*., 2011**)	Ultraviolet Irradiation (UV-C) (**Fredericks and Krügel 2011**)	Thyme essential oil (**Freidman *et al*., 2017**)	Nisin (**Rojo-bezares *et al*., 2007**)	Glutathione and ascorbic acid(**Comuzzo *et al*., 2015**)
Ethanethiol (**Dias *et al*., 2013**)	Ultrasound (HPU) (**Jiranek *et al*., 2008; Gracin *et al*., 2016**)	Grape and wood tannins (**Sonni *et al*., 2009; Alamo-Sanza *et al*., 2019;Sánchez-Palomo *et al*., 2017**)	Killer toxins CpKT1 andCpKT2 (**Mehlomakulu *et al*., 2014**)	Lysozyme and polyphenols (**Chen *et al*., 2015**)
NaOCl (sodium hypochlorite) (**Yoo *et al*., 2011**)	Dry Ice Application (**Costantini *et al*., 2015**)	Hydroxytyrosol and oleuropein (**Raposo *et al*., 2016a,b**)	Lacticin 3147 (**Garcia-Ruiz *et al*., 2013a)**	Lysozyme and tannins (**Sonni *et al*. ,2009**)
Chitosan (**Chinnici *et al*., 2014; Elmacı *et al*., 2015**)		Glutathione (**Hosry *et al*.,2009a,b**)		
Collaidal silver complex (CAgS) (**Izquierdo-cañas *et al*., 2012; Garcia-Ruiz *et al*., 2015)**				

Thermal treatment is often used for the processing of grapes to eliminate future bacterial contamination (**Li *et al*., 2017**). **Boban *et al.* (2010)** were indicated that thermally treated red wine at 75 and 125 ºC for 45 min effective against two common foodborn pathogens, *Salmonella enterica* serovar Enteritidis and *Escherichia coli*. Beyond the microbiological impact, the heat application on grapes or must has been proposed as pre-fermentative treatments to reduce the enzymatic activity and enrich composition of wines (**Ševcech *et al*., 2015**; **El Darra *et al*., 2013a**; **Corrales *et al*.,** 2009; **Clarke and Bakker, 2011**). Thermal treatment at 70°C, 10 or 20 minute allows the extraction of phenolic compounds in aqueous phase, mainly anthocyanins and aromatic compounds but a lesser extent tannins in the grape mash (**Girard *et al*., 1997**; **Ševcech *et al*., 2015**). However, heating young wine together with grape mash to 35–40°C causes an increase of tannin content and released of colour pigments with disrupted by the action of heating grape berry cells (**Ševcech *et al*., 2015**). However long periods over 80 ºC could result in cooked flavour in winemaking (**Rankine, 1973**).Even though, thermal processing is the most widely used process to inhibit microorganisms in the food industry, application of excessive heat to achieve lethality against specific food borne pathogens also degrades the quality and sensory attributes of products (**Li *et al*., 2014**; Wu, et al., 2014). However, they might have adverse effect on heat-sensitive polyphenols and other bioactive components (**Može Bornšek *et al*., 2015; Rodríguez *et al*., 2016**). Thus, wine components may be significantly affected by heating and phenolic compounds are subject to thermal degradation with corresponding significant changes in their antioxidant activity by application of excessive heat (**Larrauri *et al*., 1998; Pinelo *et al*., 2004, 2005; Sadilova *et al*., 2007; Volf *et al*., 2014**). For higher temperatures, the treatments are shorter because the release of phenolics in the must and wine is faster (**El Darra *et al*., 2013b**).

Freezing temperature is another choice to inactivated enzyme activity and inhibited pathogenic microorganisms. Cold soak and freezing (i.e. using dry ice) of grape mash were reported as a method for this purpose (**Ortega-Heras *et al*., 2012; Li *et al*., 2017**). Freezing have been reported to contribution to total poly-phenol extractability due to break tannin-containing cells of the seeds while cold soak have a low effect on the phenolic composition compared to freezing the mash (**Peinado *et al*., 2004; Sacchi *et al*., 2005**). Freezing of grape mash has been also reported to release anthocyanins due to break cell walls (**Ševcech *et al*., 2015; Sacchi *et al*., 2005**). For these reasons, applying this technique on grapes or must could be eliminate some microbiological degradation of wines and thus combination with other techniques help to reduce SO_2 concentration in winemaking.

NON-THERMAL PROCESSES

Especially in the last two decades, the importance of non-thermal processes has increased in the food industry and even in winemaking; these technologies have been tried by many researchers. The common characteristics of these technologies, in contrast to thermal processes don't cause the greatest changes in the colour, smell, taste, quality of the wine by reducing the effect of temperature. In the winery, non-thermal processes such as high hydrostatic pressure, pulsed electric field, ultrasound and ultraviolet radiation were mainly applied. These technologies have been observed that have positive effects on wine quality. Below, non-thermal processes which can be used in winemaking are mentioned.

High hydrostatic pressure (HHP)

HHP is a method of inhibition of microorganisms and inactivation of enzymes in packaged or unpackaged food by applying pressure with different parameters (about 200-800 MPa). Over the past decade, the use of HHP for food preservation and modification has increased significantly. This technology has been proposed for pasteurization of grape juice (**Buzrul, 2012**). HHP is the most offered technology in must and wine as non-thermal process. Recently, researchers have been demonstrated that pressure treatments affect the physicochemical and sensory properties of red wine. For this reason, the use of HHP technology to in wine industry could be used for non-aged wines. Although this practice has been suggested to be used with wine, it has not yet been applicable in wine industry.

Current studies demonstrated the effect of HHP application and how it changes aroma compounds as well as the sensory and quality characteristics of young wines (**Briones-Labarca *et al*., 2017**). Some researchers claimed that very high pressure (≥650 MPa) applied over a long period time (≥2 hours) could affect the red wine colour and reduce the amount of phenolic material (**Tao *et al*., 2012**). In the mentioned conditions, the HHP application could significantly affected the chromatic properties and phenolic composition of the *Nero D'avola Syrah* red wine ($p<0.05$). Meanwhile, sensory analyses demonstrated that the processing of HHP for 2 hours significantly reduced the severity of wine sour and fruity smell. On the other hand, **Santos *et al.* (2013a)** reported that HHP applied to red wine without SO_2 had better sensory properties than the SO_2 added wine. Same author showed that colour, taste and other sensory characteristics changes were not observed in red wine after application of HHP method some changes after 6 months storage of wine might occur (**Santos *et al.* 2013a**). In a different study, HHP technology applied on red wines at pressures of 500 and 600 MPa (5 and 20 minutes) and results showed a lower content of monomeric anthocyanins (13 to 14%), phenolic acids (8 to 11%), and flavonols (14 to 19%) when compared to the unpressurized wine, respectively (**Santos *et al.* 2016**). During 5 mount of storage, these HHP wines showed better sensory characteristics with less bending, higher cooked fruit flavour and lower density of fruity notes compared to unpressurized wine (**Santos *et al.* 2016**). Unlikely, **Briones-Labarca *et al.* (2017**) showed that, HHP treatment did not affect the physicochemical parameters of white wine, total phenols and flavonoid content after. Also, sensory properties such as taste, smell and overall quality were not affected by the HHP process at 300 MPa. **Santos *et al.* (2013b**) reported that HHP applied in white wine without SO_2 leaded to formation of brownish colour and had fewer phenolic substances with processing between 425 and 500 MPa pressures (5 minute) than the SO_2 added and untreated white wines after one year of storage in the bottle. According to these studies, HHP application is accelerating the Maillard reaction in white wine.

There are many reports that HHP application can be used to inactivate unwanted microorganisms in wine and must such as acetic acid bacteria, lactic acid bacteria, molds and yeast while providing positive contributions to wine quality (**Puig *et al*., 2003; Tonello *et al*., 1998; Buzrul, 2012; Mok *et al*., 2006; Delfini *et al*., 1995**). **Buzrul *et al* (2008)** reported that HHP treatment inactivated *E. coli* and *L. innocua* in kiwifruit and pineapple juices at lower pressure values at room temperature than the conditions used in commercial applications (>400 MPa). Studies have shown that HHP application in white wine able to decrease yeast count 3 $\log_{10}$ with 250 MPa pressure and 20 minute holding parameters; when

these parameters were tested with higher pressure (300-400 MPa and 15-20 minute), the yeasts were completely inactivated. HHP treatments were also tested for red and rose wines and results showed that pressure application to the wines between 300-600 MPa (3-6 minute) had a strong antimicrobial effect on different unwanted microorganisms (such as molds, yeasts, acetic and lactic acid bacteria) (**Tonello *et al*., 1996a,b, 1998**).

High power ultrasound (HPU)

Ultrasound application is a method that inhibits the microorganisms in the food products by using the sound waves (>14-16 kHz). Currently, there are many types of researches on the potential of use of HPU technology in wine production. As an alternative of SO_2, HPU can be used to control wine spoilage (**Luo *et al*., 2012**). HPU technology was successfully used for inactivating the yeast *S. cerevisiae* in red grape juice (**Bermúdez-Aguirre and Barbosa-Cánovas, 2012**). The results indicated that HPU application did not cause any changes in the amount of anthocyanin in red grape juice (**Tiwari *et al*., 2010**). **Jiranek *et al.* (2008)** suggested that HPU technology could be used for inactivation of undesirable microorganisms without changing colour and taste of wine. Also, **Masuzawa *et al.* (2000)** reported that HPU process increases some phenolic compounds in red wines.

In some studies have been investigated that the effect of HPU in the continuous flow treatment have an effect on reducing the number of *Brettanomyces* yeasts and lactic acid bacteria (LAB) in wines (**Gracin *et al*., 2016**). **Gracin *et al*. (2016)** screened yeast cells and lactic acid bacteria for susceptibility to HPU application using an ultrasonic processor (400 W, 24 kHz, 100 lm amplitude) at two different wine temperatures (30 and 40 °C). HPU application in continuous flow leads to satisfactory reduction of *Brettanomisces* yeasts (89.1-99.7%) and lactic acid bacteria (71.8-99.3%). More care should be taken to maintain the sensory properties of the wine during HPU application.

Ultraviolet (UV) irradiation

UV irradiation is one of the techniques for the inactivation of microorganisms in liquid food products. This practice can be applied to reduce or even eliminate the use of SO_2 as preservative in wine production by using radiation with a wavelength of 100-400 nM (**Falguera *et al*., 2011,2013**). UV irradiation successfully inhibited microorganisms in red and white grape juice. It also, didn't cause any significant changes in grape juice quality parameters (**Pala and Toklucu, 2013**).

The effect of UV irradiation, as an alternative technology for inactivating microorganisms in grape juice and wine, has been investigated. **Fredericks and Krügel (2011)** studied with white and red wines product from Chardonnay and Pinotage grapes, red and white grape juices product from Shiraz and Chenin Blanc grapes and succeeded to inhibit lactic acid and acetic acid bacteria by using UV technology at 254 nm. UV irradiation has been tested as an innovative technology in white wine production (**Fredericks and Krügel, 2011; Rizzotti et al., 2015**). According to **Falguera *et al.* (2013)** reported that UV irradiation could prevent spoilage of wine at the same rate as SO_2 without altering other quality parameters such as pH, tartaric acid and alcohol content. Moreover, the changes in the wine colour parameters need to be optimized the irradiation process before it was applied. It was observed that microorganisms found in white grapes and wines were more easily inactivated than red grapes and wines by using UV irradiation (**Fredericks and Krügel, 2011**). It was emphasized that the red wines and grapes absorbed more UV light (**Fredericks and Krügel, 2011**).

Pulsed electric field technology (PEF)

PEF is another method that inhibits microorganisms in the food product such as HHP and HPU application. In this method, electrical impulses are applied to the product for a short time (μsec) placed between a series electrode (effect intensity up to 70 kV/cm). PEF has been extensively tested in wine-making compared to HPU and UV technologies. **Garde-Carden *et al.* (2008)** demonstrated that PEF technology could be an alternative for reducing of SO_2 in must and wine. Also, **Abca and Evrendilek (2015)**, demonstrates that the PEF has potential for processing red wine without negatively impacting key features of wines.

The use of PEF technology is an alternative to the microbiological control system in winemaking process. This technology has been tested in must and wine and has succeeded in inactivating yeast and lactic acid bacteria. **Puertolas *et al.* (2009)** demonstrated that the PEF resistance of different wine degradation microorganisms such as *Dekkera anomala*, *Dekkera bruxellensis*, *Lactobacillus hilgardii* and *Lactobacillus plantarum* by applivation of this alternative at intervals of 16 to 31 kV/cm and at a temperature of 10 to 350 kJ/kg at 24 °C. As a result, the optimal was achieved at treatment of 186 kJ/kg at 29 kV/cm. It has been observed that the bacteria are more resistant both in wine and must than the yeast (**Puertolas *et al*., 2009**). Studies indicated that PEF treatment was inactivated *Brettanomyces/Dekkera* in long-term aged wine without sensory deviations (**González-Arenzana *et al*., 2019 a,b)**. Moreover, 31, 40 and 50 kV/cm treatments were resulted in *B. bruxellensis D* values of 181.8, 36.1 and 13.0 μs, respectively and at 50 kV/cm, a temperature rise determined of almost 10 °C, doubled inactivation to 3.0 log reductions indicated in red wines (cfu/mL) (**Wyk *et al*., 2019**).

PEF applications proposed as new techniques for the inactivation of yeasts in the sweet white wine are discussed. The results were compared with high-voltage electrical discharges (HVED) (**Delsart *et al*., 2015a,b**). The maximum yeast inactivations have been obtained with PEF and HVED of 3 and 4 log respectively. However, wine browning was less pronounced for samples treated by PEF compared to HEF and SO_2 treatments. PEF seems to be more suitable alternative technique for sulfide addition.

The advantages of the use of PEF technology in winemaking have been reported as reducing the maceration time and increasing the phenolic compounds (**Puertolas *et al*., 2009; Lopez *et al*., 2009; Puertolas *et al*., 2010a,b,2011**). On the other hand, it was observed that the colour intensity of PEF applied pink wine the amount of anthocyanin and the total polyphenol index were lower than non-applied PEF wine (**Puertolas *et al*., 2009**). The PEF application after the fermentation of red wine showed better colour characteristics with higher phenolic content (**Puertolas *et al*., 2010b**). It was also argued that PEF could affect of the ripening of wines (**Chen *et al*., 2009**). Regarding the positive effects of PEF treatment, studies showed that it was caused an increase in the colour intensity and was not altered the organoleptic wine quality. It is important to remark that, LAB were remained viable in wines six months after treatment (**González-Arenzana *et al*., 2019b**).

Low electric current (LEC)

LEC is another alternative method as potential to be used for reduction of SO_2 during wine production. Currently, this technique has been successfully applied to grape musts (200 mA, 16 days) (**Lustrato *et al*., 2006**). According to **Lustrato *et al.* (2006)**, although LEC method inhibits the yeast, it does not have an affect over growth of *S. cerevisiae*.

As an alternative to SO_2 in wine storage, LEC method was applied to *Montepulciano d'Abruzzo* red wine (**Lustrato *et al*., 2010**). **Lustrato *et al*. (2010)** studied for inactivation of selected yeast *Dekkera bruxellensis* strain (4481) by using LEC method (200 mA) for 60 days. The results showed that LEC significantly reduced viable living cells and increased the mortality rate of *D. bruxellensis* strains 4481 yeast.

CHEMICAL SUBSTANCES AS AN ALTERNATIVE OF SO_2 USED DURING WINE PRODUCTION

Many chemical preservatives have been identified to reducing the level of SO_2 that uses in wine production. It has recently been suggested that the use of chemical compounds such as dimethyldicarbonate, lysozyme, chitosan, and etc. can prevent the oxidation of wine and inhibit the unwanted microorganisms.

Dimethyl dicarbonate (DMDC)

DMDC is a chemical additive that inhibits the development of microorganisms such as SO_2 (**Ough *et al*, 1975; Divol *et al*., 2005**). DMDC also, acts by inhibiting alcohol-dehydrogenase and the glyceraldehydes-3-phosphate dehydrogenase enzymes (**Renouf *et al*., 2008**). The use of this additive up to 200 mg/L in wines has been approved by the European Union (EU) and the United States (USA) (**Santos *et al*., 2012**). In studies on the antimicrobial effect of DMDC have been reported that it is more effective on yeast than SO_2 (**Divol *et al*., 2005; Costa *et al*., 2008**). DMDC added stopped the growth of *B. bruxellensis* at different winemaking stages (**Renouf *et al*., 2008**). The concentration of 250-400 mg/L DMDC inhibits *Saccharomyces cerevisiae*, *Candida guilliermondii*, *Brettanomyces intermedius*, *Pichia membranaefaciens*, *Saccharomyces bayanus*, and *Saccharomyces uvarum* (**Delfini *et al*., 2002**). Morover, **Costa *et al.* (2008)** reported for the inoculums of 500 CFU/ml as the minimal inhibitory concentration (MIC) for the yeast species *Schizosaccharomyces pombe*, *Dekkera bruxellensis*, *Saccharomyces cerevisiae*, and *Pichia guilliermondii* was 100 mg/L of DMDC. The MIC for the most sensitive strains such as *Zygosaccharomyces bailii*, *Zygoascus hellenicus*, and *Lachancea thermotolerans* was 25 mg/L of DMDC (**Renouf *et al*., 2008**).

DMDC is less effective against bacteria when compared to SO_2. For this reason, in the practice of winery, the legally permissible maximum dose of DMDC is an effective preservative to control the low contamination rates of yeasts but is ineffective against lactic acid and acetic acid bacteria in wines (**Renouf *et al*., 2008**). On the other hand, DMDC action is temporary, so it is not recommended to be used for wine storage (**Delfini *et al*., 2002**).

Mixtures of SO_2 in different concentrations (25 and 50 mg /L) with lysozyme and DMDC favoured the formation of volatile compounds and biogenic amines in the wines**. Ancín-Azpilicueta *et al.* (2016)** indicated the effects of lysozyme and dimethyl dicarbonate mixtures on reduction of SO_2 level during wine making and remarked; i) Mixing low concentrations of SO_2 with lysozyme and DMDC reduced the concentration of biogenic amines (histamine, tyramine, putrescine, cadaverine, phenylethylamine + spermidine and spermine); ii) the total concentration of volatile amines (dimethylamine, isopropylamine, isobutylamine, pyrrolidine, ethylamine, diethylamine, amylamine and hexylamine) had been

determined higher in the sample fermented only with SO_2; iii) concentrations of amines with secondary amino groups (dimethylamine, diethylamine, pyrrolidine) have been determined higher in the sample only fermented with SO_2 than those fermented with DMDC and lysozyme or with a mixture of preservatives; iv) lysozyme by itself, and lysozyme mixed with SO_2, both reduced the formation of biogenic amines and the preservative mixture was seemed more advisable.

Lysozyme

Lysozyme is a protein that has been show to have an antimicrobial effects on many foods derived from white egg (**Azzolini *et al*., 2010; Delfini *et al*., 2004**). It is active at pH values in the range of 2.8-4.2 (**Delfini *et al*., 2004**). Studies reported that lysozyme is effective on many microorganisms in wine, especially on some lactic acid bacteria strains in wines (**Azzolini *et al*., 2010; Delfini *et al*., 2004; Chung and Hancock, 2000; Bartowsky *et al*., 2004; Gao *et al*., 2002; Gerbaux *et al*., 1997**). However, *Lactobacillus* and *Pediococcus* strain survived at higher concentrations of lysozyme (**Delfini et al., 2004**).

Today, practical methods and comparison experiments have been developed and validated to reduce the content of SO_2 during the wine ageing process. Current experiments indicate that a combined antibacterial system with lysozyme can be used to stabilize the wine during the ageing process, to reduce the SO_2 concentration and effectively prevent contamination from the dangerous LAB (**Chen *et al*., 2015; Sonni *et al*., 2011; Cejudo-Bastante *et al*., 2010**). **Sonni *et al.* (2011)** were tested the effects on the volatile composition of white wines during fermentation with lysozyme and tannin for replacement of SO_2. The data suggest that the addition of lysozyme and oenological tannins during alcohol fermentation may represent a promising alternative to the use of SO_2 and the production of wines with low SO_2 content. **Cejudo-Bastonte *et al*. (2010)** added at different doses (25 and 50 mg/L) lysozyme, DMDC and their mixtures with SO_2. In general, the finding that mixture of lysozyme and DMDC and SO_2 are advantageous for the formation of volatile compounds in wines. The wines obtained from mixtures of lysozyme and DMDC with 25 mg/L SO_2 had a better sensory quality than the wines obtained with 50 mg/L as the only preservative used.

Although OIV has allowed lysozyme to be used up to 500 mg/L in wines many years ago, this substance is not highly preferred by wine producers because of the over prays (enzyme use, clarification and fining procedure) (**Azzolini *et al*., 2010**). Also, its use in wine production could present a risk for consumers allergic to hen's egg (**Santos *et al*., 2012; Mainente *et al*., 2017a**). It's necessary to be mention containing lysozyme in the wine bottle labels. However, **Mainente *et al.* (2017a)** were described risk of the accidental presence of lysozyme in alcoholic beverages. In studies were determined traces of hen egg white lysozyme in 12 samples without label declaration. Moreover, **Mainente *et al.* (2017b)** highlighted that, mistranslations and misinterpretations because of the complexity of the regulations and lack of information in the EU Regulations caused the problems in the comprehension of the regulations.

Chitosan

Chitosan is a biopolymer approved by European authorities and by OIV for use as a purification and antimicrobial agent for wines (**Gómez-Rivas *et al*., 2004**). In winemaking, it can be used as helping to prevent bacterial spoilage. Fungal source chitosan has shown an increase reduction of oxidized polyphenolics in juice and wine and control of the spoilage yeast *Brettanomyces* (**Chorniak, 2007**).

The inactivation of acetic acid bacteria has been investigated in artificially contaminated wines. **Valera *et al.* (2017)** compared chitosan and SO_2 effects and both molecules reduced the metabolic activity of acetic acid bacteria strains treated in wines.

In addition, **Chinnici *et al.* (2014)** have been investigated protective effects of sulfides and chitosan additives against oxidative degradation of varietal thiols. Thiol oxidation had been significantly reduced by chitosan. **Chinnici *et al.* (2014)** suggested that this additive may contribute to preserving the diverse character of wines from aromatic grapes and reduced sulfide levels.

Chitosan-genipin films were used to produce white wines without addition of sulfur dioxide as a preservative (**Nunes *et al*., 2016**). It was observed that these wines were less susceptible to browning than organoleptic properties prepared using sulfur dioxide. In addition, the formation of iron-tartrate-chitosan complexes had been shown to inhibit oxidation reactions as well as microbial growth, reducing oxidation reactions by reducing the availability of iron and other metals. The use of chitosan-genipin films in wine production was proposed as an environmentally friendly and easy technique that can be preserved wines with required organoleptic qualities.

Colloidal silver complex (CSC)

Silver, which is widely used for water purification and medicine, has been used for many years due to its antimicrobial properties (**Silver *et al*., 2006; Pradeep and Anshup, 2009**). Current studies have shown that silver nonmaterial play role as an antimicrobial agent against a large scale of Gram-negative (Gr-) and Gram-positive (Gr+) bacteria (**Marambio-Jones and Hoek, 2010**). In the last decade, researchers examine the effects of replacing sulfur dioxide with a colloidal silver complex (CSC) during the production of wines. The legal limits established by the OIV for silver content in the final CSC wines were demonstrated as 100 mg/L (**OIV, 145/2009**).

CSC has been tested alone and in combination with small amounts of SO_2 in wines. **Garde-Carden *et al.* (2014)** determined that the red wines treatment with colloidal silver had similar physicochemical, aromatic and sensory properties to the control group but contained lower alcohol. As a result of this study, although the colour intensity of the wines produced with colloidal silver that stored 4 months was found to be higher, both anthocyanins and total polyphenol concentrations were lower. In addition, it was found that the concentration of bioamine and similar aromatic components were higher than the control group (**Garde-Carden *et al*., 2014**). Furthermore, **Izquierdo-Canas *et al*. (2012)** investigated the effectiveness of a CSC as an antimicrobial agent instead of SO_2 in both white and red wine. The CSC at 1 g/kg grape dose was shown to be an effective antimicrobial treatment to control the growth of acetic acid and lactic acid bacteria. Moreover, although red and white wines produced with CSC displayed chemical and sensory characteristics that were very similar to those obtained using the SO_2, the white wines were significantly affected by oxidation compared to those produced with SO_2. The silver concentration of white and red wines, 18.4 mg/L and 6.5 mg/L, respectively, were below the legal limits (**Izquierdo-Canas *et al*., 2012**). Therewithal, **Gil-Sanchez *et al.* (2019)** were studied effects of two silver nanoparticles coated with biocompatible materials (polyethylene glycol and reduced glutathione) and reported both silver nanoparticles were effective against the different microbial population present in tested wines. Regarding their in vitro digestion, the size and shape of the nanoparticles were determined almost unaltered in the case of silver nanoparticles coated with reduced glutathione, while in coated with polyethylene glycol some particle agglomeration was observed.

These results confirm the potential of CSC to be used in wine production. However, the wine composition was slightly affected with CSC treatment. CSC wines had a lower alcohol grade and acetaldehyde content than SO_2 wines (**Izquierdo-Canas *et al*., 2012**). Furthermore, these results indicate that the use of CSC for white wine production would require more studies of its probable combination with other antioxidant additives, such as ascorbic acid, to evaluate their effects in the final products.

BACTERIOCINS AND KILLER TOXINS

Bacteriocins are extracellular substances produced by different types of bacteria, including both Gram-positive (Gr+) and Gram-negative (Gr-) species (**Daw and Falkiner, 1996**). Bacteriocins are peptides with antimicrobial activity that prevent bacterial spoilage of foods. The two most commonly used bacteriocins in the food industry are nisin and pediocin produced by the specific LAB. Nisin is the only bacteriocin approved by the US Food and Drug Agency as a food additive (**Cotter *et al*., 2005; Bartowsky, 2009**). Studies are presented showing the effect of bacteriocins alone and in combination with SO_2 to preserve wine during the ageing and storage process. **Rojo-Bezares *et al.* (2007)** have studied the effect of nisin on the growth of 64 lactic acid bacteria, 23 acetic acid bacteria and 20 yeasts. Results demonstrated that nisin is an effective antimicrobial agent against wine LAB. **Fernández-Pérez *et al.* (2018)** also reported the inhibition effect of LAB by the use of nisin in combination with sulphur dioxide and obtained nisin by the natural producer *Lactococcus lactis* LM29 under enological conditions. They demonstrated that *L. lactis* LM29 produced nisin in the presence of 2 % and 4 % ethanol (v/v), while higher concentrations of ethanol fully inhibited the production of nisin. Finally, these results of wine ageing under winery conditions demonstrated that the use of 50 mg/L nisin decreased 4-fold the concentration of sulphur dioxide required to prevent LAB growth in the wines (**Fernández-Pérez *et al*., 2018**). In addition *Oenococcus oeni* demonstrated a much higher sensitivity to nisin, with MIC of the 0.024 g/mL. On the other hand, nisin demonstrated poor effect on the yeast strains, with a MIC value higher than the 400 lg/mL.

Khan *et al.* (2015) tested the antimicrobial activity of nisin with disodium ethylenediaminetetraacetate (Na-EDTA) in a broad pH range against selected gram-negative (*Escherichia coli* and *Salmonella typhimurium*) and gram-positive (*Listeria monocytogenes*) bacteria. Results showed that nisin concentration of 125-150 mg/mL with a Na-EDTA concentration of 20-30 mM and a pH of 5-6 was found to inhibit all the three selected bacteria.

Studies also described the effect of pediocin (another antimicrobial bacteriocin) on the growth of bacteria and yeast. **Diez *et al.* (2012)** observed inhibitory effects of pediocin PA-1 and either sulfur dioxide or ethanol with the combination on LAB growth. *Oenococcus oeni* was to be more sensitive to pediocin PA-1 (IC50=19 ng/ml) than the other LAB species (IC50=312 ng/ml). However, it has been reported that pediocin produced by the LAB is not effective for yeast (**Bauer *et al*., 2003**). No adverse effects related to any possible toxicity of the pediocin have been observed (**Delves-Broughton, 2011**).

Recently researchers have discovered new bacteriocins that have an increasing potential for food industry, namely lacticin 3147 (**Guinane *et al*., 2005; Martínez-Cuesta *et al*., 2001,2010**). In a study, bacteriocin Lacticin 3147

produced by *L. lactis* 1FPL105 and its mutant *L. lactis* 1FPL1053 was evaluated (**Garcia-Ruiz *et al*., 2013**). **Garcia-Ruiz *et al.* (2013)** indicated that lactisin 3147 and its combinations with potassium metabisulfite and eucalyptus extract may ultimately be effective in minimizing the SO_2 during wine production.

Killer toxins could be proposed as fungicidal biocontrol agents in winemaking to control the development of unwanted yeasts such as *Brettanomyces*/ *Dekkera* found in wine. **Oro *et al*. (2016)** investigated antimicrobial activity of Kwkt and Pikt killer toxins, two zymocins produced by *Kluyeromyces wickerhamii* and *Wickerhanomyces anomalus*, against *Brettanomyces*/ *Dekkera* wine spoilage yeast. These data support the potential use of zymosins to be used for reducing of SO_2. Two killer toxins, CpKT1 and CpKT2 from yeast *Candida pyralidae* showed a particularly lethal effect against several strains of *B. bruxellensis* found in grape juice (**Mehlomakulu *et al*., 2014**). Another killer toxin *Kluyveromyce phaffii* DBVPG 6076 demonstrated extensive anti-Hanseniaspora activity against strains isolated from grapes (**Ciani *et al*., 2001**).

NATURAL PLANT EXTRACTS

Today, consumers increasingly demand for foods that contain natural preservatives instead of chemical preservatives (**Amato *et al*., 2017**). One of the most promising natural alternatives to sulfides in wine production are using of natural plant extracts. Anti-oxidative and antimicrobial plant extracts rich in phenolic compounds have recently been proposed as a total or partial alternative to sulfides in wine production. The flavonoids, phenolic compounds and their derivatives, which are found in the structure of these extracts, have been shown to be effective in preventing auto oxidation (**Yıldırım, 2006, 2013; Yıldırım *et al*., 2007a,b**). It is stated that other phytochemicals (terpenes, alkaloids, lactones, etc.) found in the extract may contribute to the ant- oxidative properties of the extracts (**Yıldırım *et al*., 2015**). The mechanisms of action have been described as free radical scavenging, compounding with metal ions, inhibition or reduction of oxygen formation. In addition, these compounds inhibit the free radicals of the nutrients from being oxidized by giving hydrogen in the hydroxyl groups of aromatic rings (**Yıldırım *et al*., 2007a,b**). Current studies indicate that the growth of pathogenic and spoilage microorganisms can be strongly reduced or inhibited by certain plant extracts (**Xia *et al*., 2010; Bubonja-Sonje *et al*., 2011**). High antioxidant activities as well as effective antimicrobial activities make them a natural alternative method, instead of potentially synthetic preservatives. Then a rises the question, what are the effects of these compounds on wine quality properties. Natural preservatives that consumed in everyday life and tested in foods for being a alternative to chemical preservatives in the literature are only a small part of those found in nature. Nowadays, it needs to expand the current list of natural antimicrobial and antioxidant compounds that can be used as food preservatives.

The wines treated with these natural preservatives will be more competitive in the current global market. For this reason current studies are needed about effects of plant extracts on quality and sensory properties on the final product. It was reported that wines treated with these rich phenolic extracts prevent oxidation and cause sensory perception better than SO_2 (**Sonni *et al*., 2009**). Currently, these tests include; the addition of phenolic compounds (such as caffeic acid, catechin, tannins etc.) in wine (**Aleixandre-Tudó *et al*., 2013; Álvarez *et al*., 2009; Bimpilas *et al*., 2016; Canuti *et al*., 2012**), plant- based extracts (such as eucalyptus and almond skin extract) (**González-Rompinelli *et al*., 2013**) or wine-making by-products such as grape pomace (grape seed and skins), grapevine and oak wood extracts (**Cejudo-Bastante *et al*., 2017; Gordillo *et al*., 2014a,b, 2016; Jara-Palacios *et al*., 2014**).

The contribution of the addition of these compounds to the quality of wines and their antimicrobial and antioxidant activities are summarized in Table 3. Also these subjects presented and described in the following topics.

Table 3 Summary of natural protective alternatives tested in wines

Treatments	Contribution to wine quality	Antimicrobial activity	Disadvantages
Winemaking by-products (grape skins and seeds) extracts	Enzyme inhibition, Free radical scavenging activity, The fermentation process is not negatively affected, Better organoleptic character (**Sonni *et al*., 2009; Cejudo *et al*., 2010**)	*Bacillus cereus*, *Campylobacter jejuni*, *E. coli*, *L. monocytogenes*, *Salmonella enterica*, *S. aureus*, *Yersinia enterocolitica*, *Pseudomonas* spp., Lactic acid bacteria (**Baydar *et al*., 2006, 2004; García-Ruiz *et al*., 2011; Papdopoulou *et al*., 2005; Bartowsky 2009; Silva *et al*., 2018; Vaquero *et al*., 2007; Campos *et al*., 2009**)	The addition of enological tannins (gallotanen and procyanidin) to show a higher yellow color value in red wines (**Bautista-Ortin *et al*., 2005**).
Oak woods chips and extracts, grapevine shoots extracts	High antioxidant activity, The contribution of volatile components to sensory and aroma profile, High score in color and sensory scores (**Sánchez-Palomo *et al*., 2017; Pérez-Juan and Luque de Castro, 2015; Raposo *et al*., 2018**)	Acetic acid bacteria and pathogenic bacteria (**Alamo-Sanza *et al*., 2019; Alañón *et al*., 2014**)	Less effective in white wines than red wines (**Zhang *et al*., 2018**) The existence of a limited number of studies
Plant extracts (eucalyptus and almond skins, thyme essential oil, hydroxytyrosol)	Prevention of oxidation, Increase in aromatic composition (**Malayoglu, 2010; González-Rompinelli *et al*., 2013; Raposo *et al*., 2016b; Raposo *et al*., 2016c**)	*E. coli* O157:H7, *Salmonella enteriditis*, *L. monocytogenes*, *Salmonella poona*, *Bacillus cereus*, *Saccharomyces cerevisiae* and *Candida albicans*, Lactic acid bacteria (**Freidman *et al*., 2017; Serra *et al*., 2008; González-Rompinelli *et al*., 2013**).	Further studies are needed at different concentrations and longer storage conditions.

Grape-based phenolic extracts

Red wines contain more phenolic compounds than the white wine due to its fermentation with skins and seeds according to the winemaking technique. In addition, anthocyanins are significant group of phenolic compounds in red wines since they are responsible for the color characteristic of wine. Phenolic compounds are naturally found in wine and as well as in wine by-products (grape pomace, skins, seeds and stems). These compounds are very important because of their antioxidant, antimicrobial and anti-inflammatory effects (**Bianchini and Vainio, 2003; Revilla *et al*., 1998,2000; Cheynier, 2012; Parker *et al*., 2007**). The addition of only dried red and white grape seeds to white wines has been reported to provide approximately 380 mg/L Galic acid Equivalents (GAE) in the polyphenolic index compared to the control group wine (**Pedroza *et al*., 2011**).In studies are demonstrated that dried grape pomace addition to wine could play an important role on the yield of polyphenols in the wine compared to addition of fresh white grape skins (**De Torres *et al*., 2010; 2015; Pedroza *et al*., 2012,2013**). It is stated that, many different wine by-products such as grape / wine by-products can be utilized as food colorant and antifungal additive (**Cappa *et al*., 2015; Han *et al*., 2011; Lavelli *et al*., 2014; Torri *et al*., 2015**).

One of the reasons behind the potential of reutilization of the grape pomace in winery; It is caused by the extraction of a small amount of color pigments, fragrance compounds and phenolic compounds that found in abundance in the fruit, skins and seeds of pomace by fermentation into the wine (**Pinelo *et al*., 2006**). Thus, a significant amount of compound remains in grape waste.

Current studies demonstrated that the addition of grape pomace to the wines during fermentation increased the total phenolic compounds, catechin and dimeric procyanidin levels in the final product. The wine color and sensory scores remains the same or better than the control group wine (**Revilla *et al*., 1998**). Therefore, the addition of grape seed extracts rich in tannins to increase the wine quality has been suggested by many authors (**Harbertson *et al*., 2012; Neves *et al*., 2010**). It was reported that tannins addition to wine prevents from oxidation and caused better sensory properties than SO_2 added wines (**Sonni *et al*., 2009**). Furthermore, it is indicated that tannins can also be used to facilitate the clarification of must and wines (**Jiménez-Martínez *et al*., 2019**).

There are some studies demonstrating that the phenolic compounds found naturally in grape seed and pomace extracts have a high antimicrobial capacity against pathogenic bacteria that cause numerous deterioration in wine **(Sagdıc *et al*., 2011; Baydar *et al*., 2006,2004**). In addition to the main components such as polyphenols in grape seed extract, other phytochemicals (terpenes, alkaloids, lactones, etc.) found in the extract contribute to its antimicrobial property (**Tsuchiya *et al*., 1996; Cushnie and Lamb, 2005**). Recently, **Garcia-Ruiz *et al.* (2011)** reported a comparative study of the inhibitory potential of some phenolic acids, stilbenes and flavonoids on different LAB strains isolated from wines. IC50 values of most phenolics were higher than those of SO_2. Nevertheless, flavonoids and stilbenes showed the greatest inhibitory effects. Some of the authors indicate that these extracts inhibit the pathogenic microorganizymes such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Pseudomonas aeruginosa* and *Candida albicans* (**Papdopoulou *et al*., 2005; Silva *et al*., 2018**).

In another study, the antimicrobial effects of a common 54 different phenolic extracts on Merlot wines (produced in Spain in 2009) were evaluated. It has been found that grape seeds from these extracts have inhibitory effects on six different strains of lactic acid bacteria (*Lactobacillus hilgardii*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Pediococcus pentosaceus* and *Oenococcus oeni*) (**Sonni *et al*., 2009; Garcia-Ruiz *et al*., 2011**). It was also found that grape seed extract has a greater inhibitory effect against these bacterial strains than other phenolic plant extracts in the study. Although polyphenols are the main components in grape seed extract, it is stated that other phytochemicals (terpenes, alkaloids, lactones, etc.) found in the extract may contribute to the antimicrobial properties of the extracts. The by-product of the wine demonstrated an antimicrobial effects over same lactic acid and acetic acid bacteria (**Garcia-Ruiz *et al*., 2011**). It was observed that there was a linear correlation between the total phenol contents of the extracts and the oxygen-radical absorbance capacities of these compounds ($r = 0.9173$ and $p < 0.01$). These extracts are largely responsible for antioxidant properties due to their high levels of polyphenols contents (**Salaha *et al*., 2008; Galuska and Makris, 2013; Vaquero *et al*., 2007; Campos *et al*., 2009**).

Wood and grapevine shoot extracts

Red wine is particularly rich in tannins and therefore less lean to oxidation than white or rosé wines. These tannins can be obtained from tannin rich oak woods and grape seed or gallic acid and ellagic acid produced commercially. During the winemaking process, it is possible to enrich the wines with tannins obtained from grapes or oak and to obtain higher quality wines (**Versari *et al*., 2013**). **Pascual *et al*. (2017)** studied model wines to determine the oxygen-consumption capacities of enological tannins (such as ellagitannins) obtained from different sources and demonstrated that tannin addition was a good alternative of SO_2. In addition, condensed tannins are good antimicrobial agents acting by damaging the microorganisms' cell wall and inactivating binding enzymes (**Ya et al., 1988; Chung *et al*., 1998**). Treatment of wines with tannins is an enological practice permitted in many countries, including the EU and the United States.

Wine aging in oak barrels is a common practice for improving the wine quality due to the beneficial effects of wine on flavor, aromatic composition, color stabilization and astringency. Polyphenolic compounds, naturally occurring in the oak barrel, are particaly transferred to the wine during aging. With the diffusion of oxygen from the wood, different reactions occur between the anthocyanins and proanthocyanidins in the wine that stabilize wine color and astringency (**Zamora, 2019**). During this processes significant changes occur in the composition of the wine. With these changes, the final composition of the wine is enriched by flavor and aroma (**Pérez-Juan and Luque de Castro, 2015**). In a study investigating the effect of oak chips on the aroma profile of Verdejo white wines, 7 g/L oak chips were added to young wine during the alcoholic fermentation. As a result, volatile compounds, sensory and aromatic profiles of wine increased by oak chips addition of wine (**Sanchez-Palomo et al., 2017**). **Alamo-Sanza *et al*. (2019)** stated that there is a significant relationship between the phenolic content and antioxidant activity of the wood extract in the red wines aged 10 years with enological oak chips.

Similar to the approach in oak chips, vine shoots are also oenological materials with high potential due to their high antioxidant and antimicrobial properties (**Raposo *et al*. 2018**). Nowadays, this new alternative was tested in wines. **Raposo *et al*. (2016a)** tested addition of vine shoot extract containing 29% (w/v) stilbene compared to the control group (SO_2 added) wine and found that to the Syrah wines produced by using vine shoot extract demonstrated higher scores in color-related parameters and sensory scores than those treated with SO_2. **Cebrián-Tarancón *et al*. (2019)** tested 12g/L vine shoots in model wines and after 35 days of maceration ellagic acid, trans-resveratrol, vanillin and guaiacol values of samples were determined as higher than the normal wines. The results demonstrated to have a positive contribution for the functional properties of wines.

Olive-based extracts

Olive oil waste is a rich source of phenolic compounds. Almost half of the phenolic compounds found in olives and olive oils are hydroxytyrosols and its derivatives. Hydroxytyrosol is a low-cost bioactive compound with high antioxidant activity and good antimicrobial properties (**Anand and Sati, 2013**). Current studies focused on determining the potential capacity of hydroxytyrosol to reduce the amount of SO_2 in wine or model solutions. In Syrah wines, the hydroxytyrosol were obtained from olive wastes was proposed as an alternative of SO_2 (**Raposo *et al*. 2016b**). **Raposo *et al*. (2016b)** compared the white wines treated with hydroxytyrosol and with SO_2 during two winemaking stages (after bottled and stored in a bottle for 6 months). They observed that hydroxytyrosol improved colour as well as odours and tastes of the bottled wine. However, after storage for 6 months in the bottle, the hydroxytyrosol treated wines were more oxidized than the SO_2 wines (**Raposo *et al*. 2016c**).

In addition to hydroxrosol, olive wastes are rich sources of quercetin and oleuropein that show high antimicrobial and antioxidant activity (**Serra *et al*., 2008**). It is possible to observe the studies that tested the antimicrobial effect of these compounds (hydroxytyrosol and oleuropein) on many microorganism species (*Escherichia coli*, *Salmonella poona*, *Bacillus cereus*, *Saccharomyces cerevisiae* and *Candida albicans*) (**Serra *et al*., 2008**). Specifically, some phenolic compounds such as resveratrol, hydroxytyrosol, oleuropein, quercetin are reported to inhibit a variety of pathogenic microorganisms (**Aziz *et al*., 1998; Bisignano *et al*., 2010; Papdopoulou *et al*., 2005**). The results show that these extracts may have important applications as natural antimicrobial agents for the wine industry in the future.

Other plant extracts

Especially in recent years, phenolic compounds rich and aromatic plants such as sage, thyme, rosemary and carnation suggested as a natural preservatives in foods (**Malayoğlu, 2010**). Among them, rosemary has been studied extensively and today, this plant is the only commercial product that allowed as an antioxidant and antimicrobial additive in Europe and the US (**Bozin *et al*., 2007**). Current studies have focused on antibacterial, antioxidant and antiviral effects of rosemary. The *Rosmarinus officinalis* L. from Laminacae (Labiatae) family is an important medical and aromatic plant (**Gachkar *et al*., 2007**). In the literature, it is possible to find out that the protective effects of rosemary are widely tested on many foods, but there is no study with the treatment of rosemary extract for replacement of SO_2 in wine making. In a study, the protective effect of the almond shell and eucalyptus leaf extracts which have rich phenolic compounds on the barrel aged Verdejo wines were evaluated (**González-Rompinelli *et al*., 2013**). As a result of this study, it was observed that no significant difference was found in the sensory score and also, the aromatic composition and phenolic compounds changes were observed. In another study, red wines treated with thyme essential oil were determined as high antimicrobial effect on a food borne pathogen (*Escherichia coli* O157: H7) (**Freidman *et al*., 2017**). However, in a same study, low antimicrobial activity was observed in wines treated with powder mixture of apple peel, green tea and olives that is rich in phenolics (**Freidman *et al*., 2017**).

Glutathion (GSH) is another important natural compound tested for protective effects on wines. GSH is a tripeptide composed by glutamic acid, cysteine and glycine, and an important antioxidant that naturally present in many plants, animals, microorganisms and foods (**Meister, 1988; Yıldırım *et al*., 2007a,b**). GSH is naturally presents in wines in low concentrations (**Meister, 1988; Kritzinger *et al*., 2013**). It is known that GSH prevents the browning of white wine and protects against loss of flavour which occur due to oxidation in white wines (**Coetzee and du Toit, 2012; Vaimakis and Roussis, 1996; Roussis *et al*., 2007; Roussis and Sergianitis 2008; Li *et al*., 2008; Rodríguez-Bencomo *et al*., 2014; Hosry *et al*., 2009; Fracassetti *et al*., 2016**). The addition of GSH in must and wine up to a maximum of 20 mg/L was recently included among the oenological practices recommended by the OIV in 2016 (**OIV 2016; Webber et al., 2017**).

Currently, studies about the effects of the addition of GSH to the must or wine are being discussed. **Gambuti *et al*. (2015)** studied with Cabernet Sauvignon wines in a pilot scale for determining the protective effect of GSH and the results showed that anthocyanins were preserved in red wine containing high levels of GSH. However, **Gambuti *et al*. (2015)** indicate that GSH did not prevent colour stabilization in red wines while determined an increase in the degradation of malvidin 3-monoglucoside (**Gambuti *et al*,. 2017**). **Gambuti *et al*. (2017)** also reported that GSH is not effective enough in prevention of anthocyanins loss during red wine aging. **Webber *et al*. (2017)** assessed the effect of GSH addition (10, 20 and 30 mg/L) after storage of sparkling wines. The results indicated that although total GSH concentration gradually decreased during the storage, GSH reduced browning and acetaldehyde formation by up to 12 months. However, the presence of glutathione had little or no effect on the concentration of free SO_2, total phenolics, catechin, epicatechin, caffeic and coumaric acids (**Webber *et al*., 2017**). Researchers also, studied the effect of GSH and/or ellagitannins added to the bottle on the shelf life of a white wine with SO_2 content. **Panero *et al*. (2015)** observed that the addition of GSH and/or ellagitannins at a dose of 20 mg/L did not limit the oxidative evolution of bottled wines.

Further studies should be aimed by using a combination of different oenological methods with GSH for preventing wine oxidation. The effect of GSH for reducing the use of SO_2 in wines as an alternative should be studied with a higher concentration of GSH under low oxygen content bottling conditions. Therefore, in a study evaluating the quality parameters of Tempranillo and Albariño wines enriched with GSH, chitosan, DMDC and different combinations of hydrolyzable and condensed tannins, is stated that the combination of GSH and grape pomace tannins is the most effective method for increasing the sensory scores and shelf life of wines (**Ferrer-Gallego *et al*., 2017**). In another study to compare the antioxidant activity of GSH alone or with ascorbic acid in model wines, it was emphasized that the presence of ascorbic acid, high concentrations of glutathione may delay oxidative degradation of wine (**Sonni *et al*., 2011**).

CONCLUCION

In this review, it was discussed the main techniques that have potential to be used for wine preservation, as an alternative of SO_2. Taking above into account some of these methods could be proposed to use as a possible alternative of SO_2 during

wine production. Despite the promising results, the protective effects of these methods and the number of studies with industrial applications are still limited and further studies are needed in order to see the results for longer storage conditions (> 1 year), different varieties and concentrations, or in combination with existing alternatives. The cost of some non-thermal technique equipment should be considered by the wine producers. But it is also, possible to see that commercial wines with different characteristics that appeal to the market and to the demand of the consumer and which use these technologies and/or substances. These studies demonstrate the requirement of new experiments that could confirm the new alternatives of SO_2 to be used in wines.

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Review

The state-of-the-art research of the application of ultrasound to winemaking: A critical review

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ABSTRACT

As a promising non-thermal physical technology, ultrasound has attracted extensive attention in recent years, and has been applied to many food processing operation units, such as involving filtration, freezing, thawing, sterilization, cutting, extraction, aging, etc. It is also widely used in the processing of meat products, fruits and vegetables, and dairy products. With regard to its application in winemaking, most of the studies available in the literature are focused on the impact of ultrasound on a certain characteristic of wine, lacking of systematic sorting of these literatures. This review systematically summarizes and explores the current achievements and problems of the application of ultrasound to the different stages of winemaking, including extraction, fermentation, aging and sterilization. Summarizing the advantages and disadvantages of ultrasound application in winemaking and its development in future development.

1. Introduction

Wine refers to the fermented wine with certain alcohol content, which is made from fresh grapes or grape juice through partial or total fermentation. Generally, the main factors affecting winemaking include grape variety and quality, winemaking methods and fermentation strains. In recent years, with the development of science and technology, more and more novel technologies have begun to be integrated into the winemaking process in order to improve its efficiency and the quality of the resulting wine [1].

Ultrasound is a physical technology that has been used to accelerate the extraction, freezing, filtering, dehydration and sterilization processes in the field of food processing [2–5], which can enhance the performance of these processes and improve the quality characteristics (color, aroma and aroma substances, texture, nutritional value, security) and extend shelf life of the food products[6]. The ultrasound mechanism is attributed to the acoustic cavitation and its induced sonochemical effect. The acoustic cavitation consists of the formation, growth and violent collapse of small bubbles or voids in liquids. This violent bubble collapse is responsible for extreme localized pressure (up to 1000 atm) and temperature (up to 5000 °C), resulting in the formation of free radicals.

In the early stage, the reports about ultrasound application in wine were mainly focused on accelerating the ageing of Baijiu (distilled spirit), while the research in this field was not valued for a long time, due to the phenomenon of wine regeneration during storage after ultrasound irradiation [7–10]. In recent years, several applications of ultrasound in the production of wines and juices at laboratory scale have been reported. To be specific, ultrasound has been used to enhance heat transfer, to detect microbial contamination, to reduce membrane fouling in beverage clarification, to inactivate microorganisms, to clean equipment, to monitor processes, and to improve extraction and to accelerate reactions within beverages [11].

As a result, more and more attention has been paid to the application of ultrasound in winemaking with the in-depth understanding of ultrasonics sonochemistry by researchers. However, due to the differences of ultrasound equipment and grape varieties, the obtained results differ greatly among different research groups. Furthermore, most of these studies are mainly focused on the application of ultrasound to a certain part of the winemaking process, and there is a lack of systematic review of the literature related to its application to the whole winemaking process.

In this paper, a comprehensive review was conducted on references of ultrasound application in the four main stages of winemaking, i.e.

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extraction, fermentation, aging and sterilization, so as to provide some important basic data to wine producers for its potential industrial application.

2. Ultrasound and its application

Ultrasonic wave is a part of sound wave, inaudible to human ears, and which frequency is higher than 20 kHz [12]. When the sound wave passes through a medium, the ultrasound cavitation releases a large amount of energy, and causes localized, instantaneous high temperatures and high pressures, resulting in the microflow phenomenon and accelerating the mass transfer. Its basic working principle is attributed to the reflection and scattering of sound waves, similar to light waves [13]. Generally, ultrasound can be divided into low-intensity and high-intensity ultrasound according to its power and frequency, as shown in Fig. 1.

According to the working modes of ultrasound, it can be divided into dynamic ultrasonic equipment such as continuous and circulating type, and static ultrasonic equipment such as ultrasound baths and ultrasound probes [14], the static ultrasonic equipment being more commonly used in the laboratory. Ultrasound bath is a widely used and easy to be operated instrument, which is usually consisted of a container filled with a liquid (generally a water bath), a timer knob, a heater and at least one ultrasonic transducer, as shown in Fig. 2(a). The instrument usually uses a beaker, flask or test tube is used as sample container, which is semi-immersed in the liquid and fixed in a specific position to obtain the maximum cavitation effect [2]. An ultrasound probe usually consists of a generator, a line connected to the control panel, a transducer, and a sample container that can be fixed on the desired position, and sometimes also includes a stirring and a temperature control system, as shown in Fig. 2(b). Water bath type and probe type ultrasound have their advantages and disadvantages, as shown in Table 1.

3. Application of ultrasound in winemaking process

3.1. The winemaking process

Although the wine making process may vary in detail, the basic process is similar (Fig. 3). Firstly, the harvested grapes are crushed to break the skins and release the sugary colorless juice [1]. Then fermentation is carried out, which is divided into two stages: main fermentation and post fermentation. The main microorganisms affecting the wine fermentation process include *Saccharomyces* cerevisiae, non-*Saccharomyces* cerevisiae, lactic acid bacteria [16]. In the process of fermentation, to let stand grape juice along with grape skins is beneficial to the growth and reproduction of yeast, and a large amount of skin residue can be observed on the liquid surface, because the skin residue cannot release heat in time, the temperature is more conducive to fermentation at this time. During the main fermentation (alcoholic fermentation), as the nutrients in grape skins and seeds are transferred to the fermentation liquid, the flavor and bright color of the wine are increased, but the fermentation time should not be too long, otherwise it will lead to excessive tannin concentration, which will make the wine bitter and affect its taste [17]. Then the malolactic fermentation takes place, which reduces the acidity and roughness of raw wine, makes it soft and round, and also improves the sensory quality and biological stability of wine =. When the acidity of wine is relatively high, that is, when malic acid – lactic acid fermentation is most needed, this fermentation is more difficult to trigger. Under this condition, mild chemical acid reduction will increase the pH value to 3.2, which is conducive to the triggering of malic acid-lactic acid fermentation. As a result, fermentation gradually stops and aroma gradually increases. Then clarification and aging can be carried out (Fig. 4.).

Aging can be divided into maturation (oxidative aging) and bottle aging (reductive aging) [3]. The newly brewed wine contains many monomeric tannins. Aging in oak barrels (maturation) and bottles can reduce the bitterness of tannins and make the wine more full-bodied. Finally, the sterilization process of wine is mainly to kill harmful microorganisms in wine and prolong its shelf life.

3.2. Ultrasound as an extraction technique

In the process of winemaking, it is often involved in the migration of substances in grapes, skins and stems, and the enrichment and detection of substances in fermentation liquid. Conventional methods have problems such as time-consuming, require a large amount of solvents and have low efficiency [18]. Ultrasound extraction and separation technology, characterized by high efficiency and convenience, has been reported in many applications in winemaking [19,20]. During ultrasound-assisted extraction, the material produces microbubbles due to the action of ultrasonic cavitation, and their rupture produces several physical and thermal effects, which will eventually accelerate the transfer of compounds from grape skins and seeds to must and improve the extraction efficiency [21,22]. The application of ultrasonic waves in the grape juice making process can significantly promote the improvement of juice yield and color. The first possible mechanism is that the ultrasonic cavitation effect leads to the breaking of grape tissue cell wall, which is conducive to the full exudation of juice and pigments, thus improving juice yield and color. Second, ultrasonic mechanical vibration effect accelerates the transfer process of compounds, so that polyphenols can be quickly transferred from grape skins and seeds to juice. Ultrasound frequency, power density, amplitude and temperature are the most important parameters that affect the ultrasonic effect. Cavitation at low frequency produces larger bubbles, and with the increase of local temperatures and pressures, bubbles burst more violently. With the increase of frequency, more bubbles burst per time unit [21,23]. Ultrasound can accelerate the migration of phenols and other substances from grape skins and seeds to must during grape crushing, and the mass transfer follows the mass transfer kinetics model of Fick's second law [24,25].In terms of ultrasound application in grape juice making process, Palma et al. [26] studied the ultrasound-assisted extraction of organic acids in wine and wine by-products by using ultrasound probes

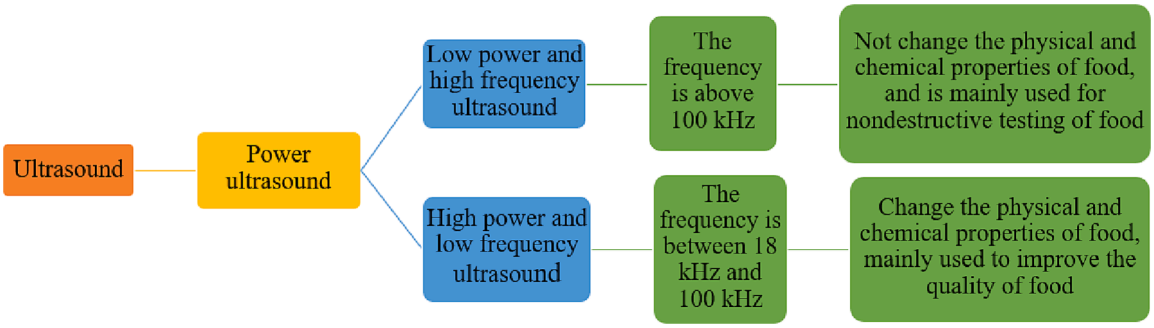

Fig. 1. Classification of ultrasound by the size of ultrasonic vibration radiation.

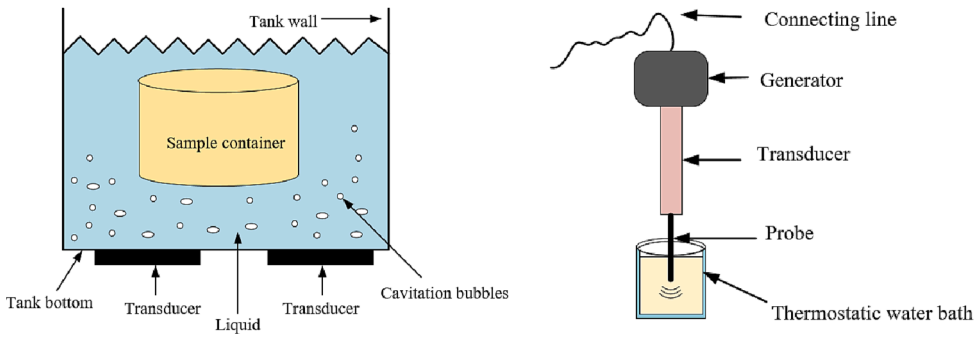

Fig. 2. Ultrasound equipment: (a) ultrasound bath mode (b) ultrasound probe mode [15].

Table 1
Comparison between ultrasound bath and ultrasound probe.

	Ultrasound bath	Ultrasound probe
Popularity	Easier to popularize	Difficult to popularize
Price	Lower	Expensive
Operability	Easy to operate	Difficult to control temperature
Intensity obtained	Lower	Higher
Contamination on reaction liquid	No	From corrosion of the probe surface
Processing scale	Relatively large	Relatively small

at 200 W, 24 kHz, amplitudes from 30 to 90%, and treatment times from 120 to 1500 s. The results showed that ultrasound-assisted extraction improved the contents of tartaric acid and malic acid in wine and wine by-products. Dujmic et al. [27] studied the extraction of polyphenols in wine by-products under the conditions of 400 W, 22 mm probe diameter, 90% amplitude and 25 min extraction time, and the results showed that the extraction rate was significantly improved. Plaza et al. [28] found that when ultrasound was applied to grape vinification process, it not only promoted the extraction of phenolic compounds from grapes, but also shortened the impregnation time, and obtained wine with good color characteristics. In the same line, Romero-Díez et al. [29] under the ultrasound conditions of amplitude of 55% and temperature of 25 ℃, obtained that ultrasound treatment improved the extraction rate of anthocyanin from wine lees and therefore shortened the extraction time. Ferraretto et al. [30] also studied the influence of ultrasound on grapes during in the vinification process. These authors found an improvement in the extraction of polyphenolic compounds from grapes due to the disruption of the cell wall caused by pressure alternance and cavitation provoked by ultrasound, resulting in a reduction in the length of classic maceration. Besides, the ultrasound-assisted yeast lysis released different fractions to the wine. Dalagnol et al. [31] studied the effects of ultrasound, mechanical agitation and nine industrial enzyme preparations on Cabernet Sauvignon grape juice production. Compared with mechanical agitation, ultrasound-assisted extraction improved the extraction rate of anthocyanins, and the combination of ultrasound, mechanical agitation and enzymatic hydrolysis showed a synergistic effect on the extraction of grape juice. Darra et al. and Carrera et al. [32,33] all assayed the ultrasound-assisted extraction of phenolic compounds from grapes and found that this method shortened extraction time and increased the content of phenolic compounds. Bautista-Ortín et al. [34] studied the influence of ultrasound treatment on the maceration stage, and determined the wine chromatic characteristics and the anthocyanins and tannin concentrations. The authors found that ultrasound treatment could significantly shorten the maceration time and increase the content of tannins and volatile compounds in the resulting red wine. Martínez-Pérez et al. [35] made a comparison between wines obtained by sonicated vinification from Monastrell red grapes (29.0 °Brix ripening level) and two wines obtained by classical vinification from Monastrell red grapes harvested with two different ripening levels (25.4 °Brix and 29.0 °Brix), and found that the total phenolic compounds and tannin contents of the two wines from the less mature grapes were similar, but the alcohol content of the wine produced by applying ultrasound was 15% lower. The authors concluded that the ultrasound-assisted-extraction of phenolic compounds from grapes, even when grape phenolic maturity is not complete, allows the production of

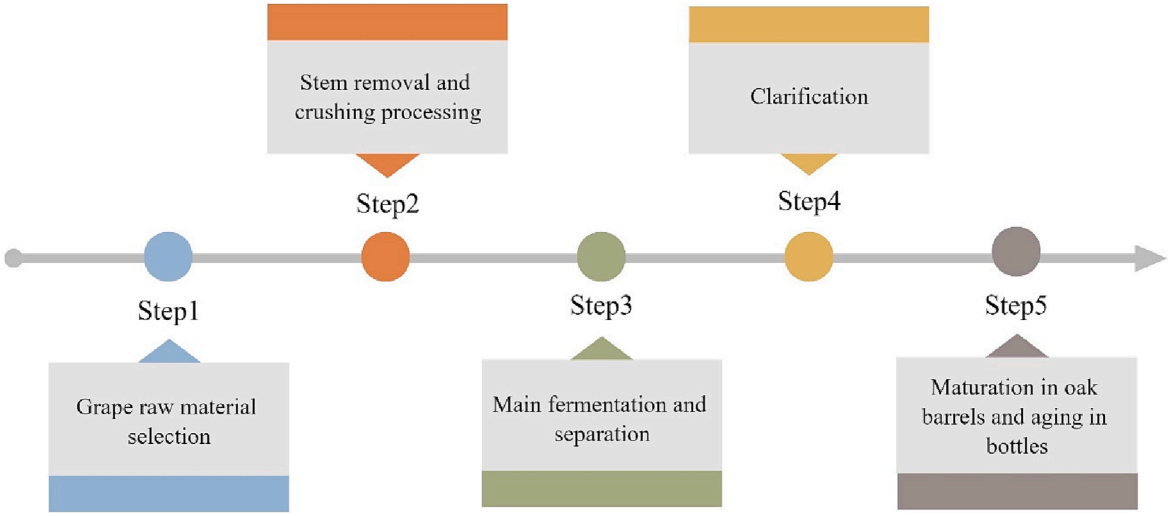

Fig. 3. Procedure of grape wine making [1].

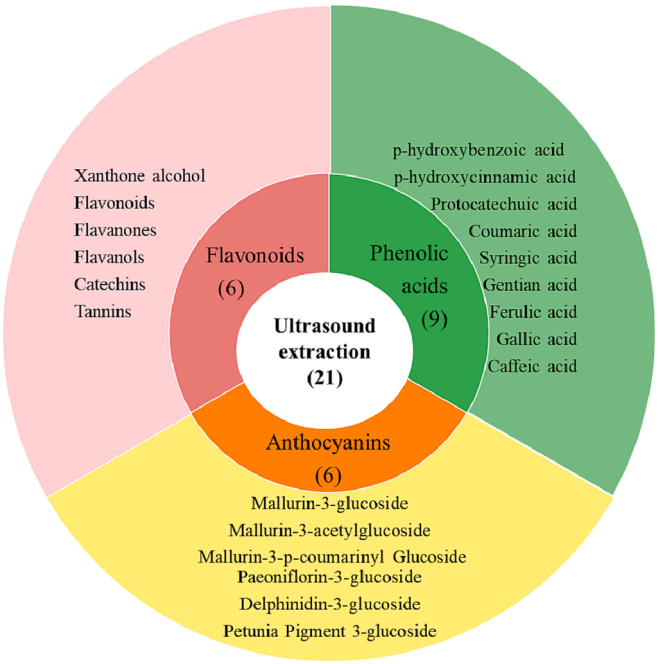

Fig. 4. Ultrasound assisted extraction of nutrients during winemaking.

quality wines with a reduced alcohol content (Fig. 5).

As can be seen from the above analysis (Table 2), ultrasound-assisted extraction has the advantages of high maceration efficiency and short extraction time. However, there are also the following disadvantages worth overcoming and solving: first, when the maceration container is large, the sound wave is not easy to radiate to every place, resulting in poor extraction effect; Second, ultrasound maceration energy consumption is relatively large; Third, the use of ultrasound probes is prone to cause pollution due to probe erosion and thus spoilage of wine by metal elements.

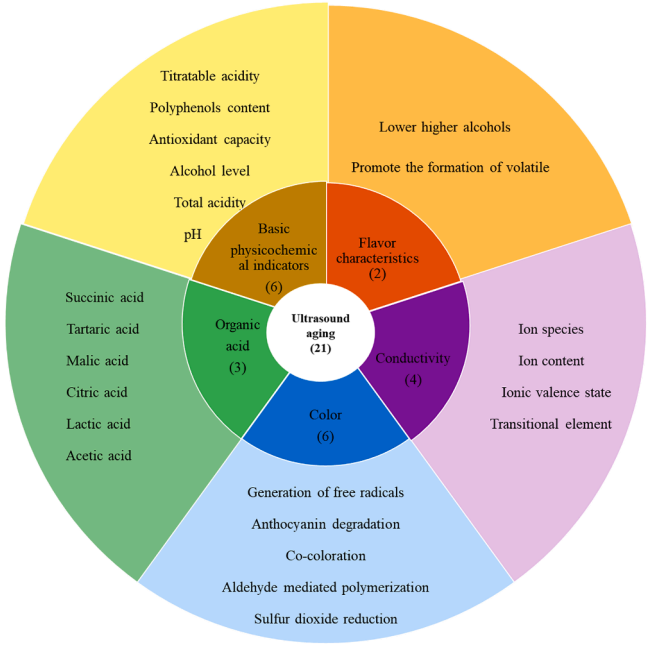

Fig. 5. Application of ultrasound in wine aging.

3.3. Research progress in application of ultrasound to wine fermentation

Fermentation is one of the key processes in winemaking, which can be divided into alcoholic fermentation and malolactic acid fermentation. During the alcoholic fermentation stage, yeasts, mainly *Saccharomyces cerevisiae*, transform sugars into ethanol, carbon dioxide and other substances. In the malolactic fermentation stage, alcohols can undergo esterification, redox and other reactions, improving wine taste and flavor. Over the fermentation process, nutrients in grape skins and seeds are transferred to the fermentation liquid, and the skin residue can float on the surface of grape mash due to the action of carbon dioxide to form a “skin cap”, thus affecting the fermentation performance. Stirring and other operations are often needed to destroy the residue cap and promote the full contact between the skin residue and the fermentation liquid. If ultrasound is applied, the turbulence provoked by cavitation can make grape skins and stalks oscillate rapidly in the fermentation liquid, and then accelerate the mass transfer process. The application of ultrasound in the process of microbial fermentation can instantly cause micro injury on the cell surface and local rupture of the cell wall, so as to change the permeability of the cell membrane and make the intracellular substances be released or the extracellular substances enter the cell [43].

In addition, the natural fermentation cycle of wine is generally long, easy to contaminate by miscellaneous bacteria. The equipment occupies a large area and the fermentation process requires relatively high labor intensity, which reduces the production efficiency of enterprises and increases the production cost [44,45]. In order to overcome the above problems, researchers began to try to use ultrasound in wine fermentation, and achieved certain results. When ultrasound is used in wine fermentation, on the one hand, it can improve the permeability and selectivity of yeast cell membrane, stimulate the secretion and metabolism of enzymes and improve the fermentation rate; on the other hand, it can kill miscellaneous bacteria and improve the quality of wine [46]. Changes in cell membranes are mainly attributed to mechanical oscillations and free radicals (e.g. H·and·OH) generated by the sonochemical and physical effects induced by ultrasound [47]. Japanese scholars have found that in the process of wine fermentation, the increase of carbon dioxide concentration hinders yeast growth and delay the generation of ethanol, making the fermentation cycle longer. Besides, carbon dioxide inhibits the formation of ester compounds, thus affecting the aroma characteristics of wine [48–50]. However, ultrasonic radiation can reduce the water-soluble carbon dioxide in the fermentation tank, promote the formation of aroma substances such as ethanol and esters, shorten the fermentation time and enhance the aroma of wine [51]. According to the different frequency of ultrasound, the role of ultrasound in the process of wine fermentation can be divided into two categories: one is to monitor the changes of substances in the fermentation process by high-frequency ultrasound, while the other is to use low-frequency ultrasound to extract compounds from grape skins and stalks and activate yeast metabolism or kill miscellaneous bacteria.

3.3.1. Application of high-frequency ultrasound in wine fermentation

The traditional monitoring method of the fermentation process is to take samples regularly to determine the changes of key fermentation parameters such as microbial growth, pH value, acidity, turbidity and chemical composition. This method is time-consuming and complex to operate. However, the use of high-frequency ultrasound can provide reliable information to characterize the real-time fermentation process of wine [52]. High-frequency ultrasound is usually used as a nondestructive analysis technique in wine fermentation to ensure product quality and monitor the fermentation process. High-frequency ultrasound using low power level, which is not enough to cause acoustic cavitation, generates sound waves through the material that produce zero or minimum physical and chemical changes. The ultrasound exposure time, power or frequency can be adjusted to obtain the desired sound energy into the wine and control cavitation, and so the technology

Table 2
Application of ultrasound-assisted maceration in wine and its by-products.

Sample	Ultrasound condition	Extracted compounds	Main result	Reference
Red wine lees	Power 400 W, the probe diameter 22 mm, the amplitude 90%, 25 min	Polyphenols	Increased maceration rate of polyphenols	[27]
Red wine	Frequency 28 kHz, power 2500 W	Anthocyanins, tannins	The content of tannin increased	[34]
Grape must and wine	Frequency 24 kHz, 37 kHz, power 20–75 W, 200 W	Polyphenols	Improvement of polyphenols maceration rate	[36]
Grape juice	Frequency 20 kHz 10 min, pulse treatment of 5 s on and 5 s off	Anthocyanins	The maceration rate of anthocyanins increased	[37]
Red wine lees	Frequency 20 kHz, power 200 W	Polysaccharide	Accelerated release of polysaccharides from wine lees	[38]
Red wine lees and model wine	Frequency 25 kHz, power 300 W	Polyphenols and tartrate esters	Improvement of polyphenols and tartrate esters extraction rate	[24]
Grape must and wine	Frequency 48 kHz for 10 min	Fruit acids and aromatic compounds	Extraction time shortened	[39]
Bio-products of wine making	Frequency 24 kHz, power 200 W	Tartaric acid and malic acid	The content of phenolic compounds increased significantly	[40]
Red wine	Frequency 35 kHz, power 2000 W, 15 min	Polyphenols	Ultrasound maceration did not favor polyphenols extraction from grape skins and the obtained wines were poor in anthocyanins and tannins	[41]
Grape pomace	Frequency 20 kHz, power 130 W, probe diameter 13 mm	Phenolic compounds	Shorten extraction time of phenolic compounds	[42]

can be used for wine fermentation monitoring analysis and quality control [52]. Measurement systems based on acoustics are non-invasive, hygienic, accurate, fast, low-cost and automatic [53,54]. Ultrasonic waves have been used in the fermentation process to monitor the change of substances and solution concentration [55,56]. Table 3 summarizes the available literatures on monitoring wine fermentation process by high-frequency ultrasound.

High-frequency ultrasound can be used to monitor the fermentation process of wine in real-time. Novoa-Díaz et al. [53] and Amer et al. [57] used ultrasound to monitor malolactic fermentation. The authors correlated the ultrasonic velocity to the conversion of malic acid into lactic acid and CO_2 by lactic acid bacteria. As malic acid is converted into lactic acid during fermentation, ultrasound propagation speed is accelerated, so that the ultrasound velocity could be used to predict the end of the malolactic fermentation process. Resa et al. [58] reported that the concentration of alcohol and sugar in the fermentation process could be predicted through the ultrasound speed of the fermenter.

3.3.2. *Application progress of low frequency ultrasound in wine fermentation*

Low-frequency ultrasound, also known as high-power ultrasound, uses high power to produce cavitation, and as a consequence, chemical reactions [48]. In the food industry, it can be used for degassing, homogenization and extraction [61,62]. In food fermentation, low-frequency ultrasound can activate enzymes and regulate microbial metabolism, eliminate foam, and improve product quality. At the same time, it can also play the role of antiseptic and sterilization.

Table 3
Application of high-frequency ultrasound in wine fermentation.

Sample	Ultrasound mode	Ultrasound frequency	Ultrasound effect	Reference
Wine	Pulsed ultrasound	1 MHz	Monitor the lactic acid fermentation in wine	[53]
Model wine	Pulsed ultrasound	2 MHz	Monitor the density and ultrasonic velocity during fermentation of a mixture of water, ethanol and sucrose	[58]
Model wine	Pulsed ultrasound	2 MHz	Monitor the concentration of malic and lactic acid during the malolactic fermentation in mixtures	[59]
Model wine	Pulsed ultrasound	2 MHz	Determin the concentration of yeast and maltose in the model solution	[60]

Wine producers cope with the problem of spoilage caused by contamination of miscellaneous bacteria. The traditional solution is the addition of sulfur dioxide and other chemicals [3,63–65], but these compounds have safety concerns. Therefore, the search for new substitutive materials or new techniques in this field has become a hot topic, and low-frequency ultrasound processing technology is one of them. The use of ultrasound in the fermentation process, because of its good bactericidal effect, can effectively reduce the amount of SO_2 that should be added in the fermentation liquid, while improving the quality of wine color, taste and so on. The mechanisms of its action are changes in the existing form of SO_2 in fermentation liquid by ultrasound cavitation, which increases the amount of combined sulfur dioxide, and produces sulfur-containing free radicals that undergo oxidative polymerization or degradation reaction with carbonyl compounds, phenolic compounds, etc. in wine, making the change of wine color.

Low-frequency ultrasound can activate the metabolism of *S. cerevisiae* and other yeasts during wine fermentation, and also increase yeast lysis thus releasing substances (mannoproteins, polysaccharides, etc.) to wine, which can speed up the wine fermentation rate and shorten the fermentation time (Table 4). Dai et al. [66] showed that the biomass growth of *S. cerevisiae* increased by 127.03%, compared with the control group, under optimal ultrasound conditions (ultrasound frequency 28 kHz, power 140 W/L and treatment time 1 h). Besides, ultrasound treatment enhanced the membrane permeability of *S. cerevisiae*, activated the wine yeasts, and accelerated the migration of nucleic acids and nutrients such as fructose-1,6-diphosphate from yeast cells to the fermentation medium. The fermentation time was shortened 36 h compared with the control group. Xiong [67] carried out alcohol fermentation tests with *S. cerevisiae* by using a small fermenter, and the results showed that low-frequency ultrasound could promote the proliferation and growth of *S. cerevisiae* cells and improve the fermentation efficiency of *S. cerevisiae*, and Logistic model could well predict ultrasound-assisted alcohol fermentation of *S. cerevisiae*. Hong et al. [68] reported that ultrasound treatment (ultrasound frequency 40 kHz, ultrasound power 50 W, temperature 30 ℃) can stimulate yeasts to produce by-products such as higher alcohols and probiotics, and meanwhile reduce the CO_2 produced in the metabolic process, thus promoting cell proliferation and biological metabolism, increasing enzyme production, accelerating biological metabolism and releasing compounds from the inner cell to the fermentation medium.Matsuura et al. [69] studied the influence of ultrasound treatment on the fermentation processes of wine, beer and sake, and found that ultrasound can reduce the level of dissolved carbon dioxide concentration in the fermentation process, increase the content of esters in wine, promote the formation of iso-amyl acetate, make the wine taste more mellow and shorten the fermentation

Table 4
Applications of low-frequency ultrasound to wine fermentation.

Sample	Ultrasound condition	Aim	Result	Reference
Red wine	Frequency 26 kHz, power 118 W, 20 min	Control the microbial spoilage during wine fermentation	Reduction of the cell survival rate of spoilage microorganisms in wine and improvement of the flavor and aroma characteristics	[65]
S. cerevisiae	Frequency 28 kHz, power 140 W/L, 1 h	Assess the effects of low-intensity ultrasound on *S. cerevisiae* at different growth stages were studied	Enhancement of the cell membrane permeability of *S. cerevisiae*, yeast activation, acceleration of the migration of nutrients from cells to fermentation medium, and shortening of the fermentation time	[66]
White wine	Frequency 40 kHz, 20 min	Assess the effects of different fermentation processes on microbial stability and quality of low-alcohol white wine	Ultrasound combined with SO_2 treatment greatly reduced the content of *S. cerevisiae* after fermentation, and improved the biological stability of wine	[71]
Red wine	Temperatures of 30℃ and 40℃, frequency 24 kHz, power 400 W, amplitude 100 μm, 60 s	Evaluate the effects of ultrasound on yeasts and lactic acid bacteria in wine	Yeast and lactic acid bacteria inhibition, reduction of the amount of SO_2 and preservatives	[72]

time. Qu et al. [44] found that ultrasound could affect the enzyme activity and metabolic pathway of yeasts, making the aroma and taste of wine significantly different. Simancas et al. [70] studied the influence of low-frequency ultrasound on fermentation products in wine, and the results showed that ultrasound had a positive impact, improving the content of volatile compounds related to sensory attributes and making the wine aroma stronger (Table 5).

3.3.3. Current hindrances of the application of ultrasound in wine fermentation

The information illustrated in the previous sections which demonstrates the application of ultrasound technology in wine fermentation provides certain benefits to the winemaking process and, therefore, to wines. High-frequency ultrasound can dynamically monitor the changes during fermentation process in real-time, and low-frequency ultrasound can promote yeast growth and the production of ethanol, thus shortening the fermentation time, and enhance the aroma of wine. However, for low-frequency ultrasound, when the ultrasound power is too high and the treatment time too long, excessive cavitation can damage *S. cerevisiae* cells, thus delaying fermentation. At the same time, the degradation of polyphenols in the fermentation medium affects the flavor and quality of wine. Therefore, for the use of low-frequency ultrasonic waves in the wine fermentation process, the choice of ultrasound intensity, yeast concentration and processing time is of major importance to avoid these problems. In addition, in order to reduce the potential contamination from metal elements caused by ultrasonic cavitation erosion of stainless steel containers, it is recommended that ultrasound waves do not directly contact the wine, therefore, the use of ultrasound probes should be avoided.

Table 5
The advantages and disadvantages of ultrasonic applications in red wine processing.

Red wine processing	Advantages	Disadvantages
Ultrasound-assisted maceration	High maceration efficiency Shorten extraction time	Large maceration container will lead to poor extraction effect Ultrasound-assisted maceration energy consumption is relatively large
Fermentation	High-frequency ultrasound can dynamically monitor the changes during fermentation process in real-time Low-frequency ultrasound can promote yeast growth and the production of ethanol, shorten the fermentation time, and enhance the aroma of wine	Long treatment time will delay fermentation Ultrasound probe mode will cause wine pollution
Aging	Accelerate the maturation of wine Shorten the aging time Improve the taste and overall quality of wine	None
Sterilization	Shorten the sterilization time, reduce nutrient loss Improve product stability and extend shelf life	Only applicable to liquids or objects soaked in liquids The handling capacity should not be too large

4. Advances in the application of ultrasound in wine aging

4.1. Aging of wine

Wines can be matured or aged and these terms refer to different stages of development of the wine. Notwithstanding, these two terms are generally used rather confusingly [73]. Thus, it is widely accepted for most of winemakers and consumers that wine maturation in oak barrels is wine aging. As a result, it can be said that wine aging is composed of two stages: maturation (oxidative aging) and bottle aging (reductive aging) [3,74], although maturation is not regarded as an aging stage by wine experts.

Maturation takes place after fermentation, during which wine may undergo malolactic fermentation, be submitted to clarification processes or stored in oak barrels [3]. In general, maturation in the winery prepares wines for their useful lifetime, the time in which a wine is good to drink. The maturation process is intended to stabilize the wine and enable it to be drunk pleasantly and enjoyably on its commercial release [74].

Bottle aging (reductive aging) is usually related to the time wine spends in bottles in almost absence of oxygen and in this type of development wines can proceed from youth, through to maturity and, eventually, to a fading away of any worthwhile flavor [74]. The amount of dissolved oxygen in wines stored in bottles depends on the type of the closure and the materials of the bottle. Wines are usually stored in glass bottles with airtight caps to minimize oxygen exposure [75]. Bottle aging allows the development of more complex wine flavors over around 20 years or more.

Then comes a point in time at which the wine is deemed to be drinking at its best. It said that the wine has reached its “peak”, which can last for another 20, or 50 or even 100 years [73]. At this stage, the aroma components in the wine gradually increase and accumulate, and the taste is round, the wine body is harmonious, the color is bright, and

the quality is the best. Finally, there is a declining period, where the aromas are still intense at the beginning of this phase and then gradually fade [76].

Not all grapes lead to wines with potential to age. In some instances, it is because the grape cannot does not provide acidity or tannins enough to protect the wine but in the majority of cases it is because the general belief that wines from such grapes do not develop pleasing flavors with time [73]. Tannins, for example, react with the proteins present in saliva to produce a mouth-drying effect so that wines with high tannin level are regarded as astringent. During aging, tannins eventually reduce as polymerization in the bottle results in higher molecular-weight tannin complexes, which are unstable in wine and precipitate, forming the sediment in the bottle. Besides, the wine loses color over time as these complexes also contain anthocyanins [73].

A wine worthy to be aged must have a number of preservatives and be made in such a way as to be able to develop new flavors over time. White wines must have a low pH or high levels of residual sugars to help to protect them against all forms of microbial spoilage. Red wines have tannins as their main preservative, pH also being important [73]. In both cases, sulfur dioxide is commonly added to wine to manage spoilage organisms and for its good antioxidant properties, although the addition of SO_2 to wine can give a reductive aroma to wine [3]. The traditional method of wine maturation or oxidative aging is to store the wine in oak barrels for a period of time, where the esters, phenols and aldehydes in the oak are transferred to the wine to increase the complexity of the flavor. The special structure of oak makes it impermeable and breathable, which allows a certain amount of oxygen to enter the wine during aging in oak barrels, resulting in a moderate redox reaction, accelerating the maturation process and making the wine round and harmonious; at the same time, the tannins and pigments in the wine are combined into large molecules and precipitated, as aforementioned, changing the color of the wine from bright red to brick red.

However, the traditional method of aging wine in oak barrels has some drawbacks: first, it is time-consuming, and the maturation process in oak barrels usually takes several months to years; second, oak barrels are expensive, occupy a large area, and have to be replaced over time; third, the aging process is susceptible to microbial contamination and corruption [3]. In order to overcome the problems of the traditional maturation-aging process of wine, researchers have been exploring new alternative technologies.

4.2. Principles of ultrasound-assisted wine aging

New aging technologies have been reported for physical aging such as ultrasound treatment, pulsed electric field treatment, radiation treatment, and ultra-high pressure treatment [1,3,77–81]. In comparison, wine aging using pulsed electric fields and ultra-high pressure, which is currently more researched, more effective, and more promising for industrial applications. Notwithstanding, ultrasound, as a non-thermal physical processing method, has also received a lot of attention from researchers and is considered a promising new technology for accelerating wine aging [3,75,79,82].

In China, the research on ultrasound treatment of ripening wines started in the 1970s, and the main target of this research was white wines, but the technology did not receive much attention due to the problems of “rejuvenation” and the difficulty of controlling the conditions (Here a reference is needed). In recent years, there has been a “warming” phenomenon in the domestic research on the application of ultrasonic wave to the aging of wine [83].

The countries where ultrasound aging research was conducted earlier were the former Soviet Union and the United States. In the 1950s, several translations of Soviet books on the application of ultrasound in the food industry mentioned ultrasound aging of wine. In the 1960s, Singleton and Draper carried out research at the University of California related to the use of ultrasound to accelerate the maturation of red wine, but it was limited to the research conditions at that time, and the changes in the physicochemical properties of wine after ultrasound treatment were not significant and irregular. Besides, the resulting wine had a burnt taste, so the technology did not receive much attention at that time [74]. In Japan, research was also carried out in the last century on the effect of ultrasound on the formation mechanism of water clusters between ethanol and water molecules in wine and the quality of wine [84–86]. Since the 21st century (especially in the last 15 years), in China, several research institutes have carried out research on ultrasound-assisted wine aging and made a series of progress; as for the rest of the world, the main countries with more research on ultrasound-assisted wine aging are Italy, Spain and Argentina.

Ultrasound-assisted wine aging mainly occurs through the ultrasonic rupture of tiny bubbles in the liquid caused by the cavitation effect, which causes the local instantaneous temperature and pressure rise, generating free radicals and triggering a series of complex chemical reactions among the wine compounds, potentially accelerating chemical reactions related to wine aging. First, ultrasound can promote association and enhance the affinity between polar molecules, such as ethanol and water, and can even form larger, firmer polar molecules. Certain esters, acids and other wine components may also be involved in these bonds and form part of these larger molecules, so as to improve the softness and coordination of the wine. Second, ultrasound can reduce the activation energy of certain reactions. As a result, ultrasound can accelerate esterification, condensation, redox and degradation reactions within wine, and improve its alcohol-ester aroma and flavor. Third, the mechanical effect of ultrasound also can promote the volatilization of low-boiling-point components in wine and accelerate the extraction of flavor substances from oak barrels and yeasts. The above effects are regarded to accelerate the maturation of wine, shorten the aging time, and improve the taste and overall quality of wine [87].

4.2.1. Research progress in the application of ultrasound to wine aging

There are two main ways to apply ultrasound to age wines. One is to treat the wine directly with ultrasound to accelerate the chemical reactions associated with aging. The other is to use ultrasound in combination with oak barrels, oak chips, yeasts and fermentation residues to accelerate the release of flavor substances to wine and promote acoustic-chemical reactions, which ultimately allows the wine to mature and age rapidly and improve its quality [78,81,82,88]. Based on the effects and mechanisms of ultrasound on relevant compounds in wine, current research has focused on the following aspects:

(1) The effect of ultrasonic aging treatment on the basic physicochemical indicators of wine

Total acidity, titratable acidity, alcohol level, polyphenols content, color, conductivity, and antioxidant capacity of wine are all physicochemical properties of interest to wine producers, so whether these properties of wine are affected by ultrasound during rapid aging is also an important indicator for judging the feasibility of this technology.

Wine contains large amounts of phenolic compounds that are related to its antioxidant capacity. Hu et al. [89] demonstrated the strong antioxidant capacity of wine using electron paramagnetic resonance spectroscopy, and that it was related to its total phenols content. Fernandez- Pachón et al. [90] showed that the antioxidant capacity of red wine is related to phenolic and flavonoid substances. Acidity and pH are very important for the stability of wine, the main volatile acid in wine being acetic acid, which at high concentrations produces vinegary “off flavors” [3]. Studies have demonstrated that ultrasound of wine cannot significantly affect the pH and titratable acidity of white and red wines [91,92]. Shen et al. [91] determined the influence of ultrasound treatment on the phenolic content and free radical scavenging rate in red wine, and compared the ultrasound-treated wines with the group of wines without ultrasound treatment. The phenolic content, DPPH and hydroxyl radical scavenging rate were significantly increased, that is, ultrasound significantly improved the antioxidant properties of red wine, while it has no significant effect on wine quality parameters, such as alcohol content, total acid and titratable acid. Zhang et al. [77]

investigated the effects of ultrasound treatment under the condition of power of 100 W and frequency of 20 kHz on the color characteristics and changes in major phenolic compounds during wine aging, and the results showed that ultrasound treatment significantly improved the color of wine and accelerated the aging process. Shu et al. [92] reported that when intermittent sonication time was short and ultrasound power was low, ultrasound treatment enhanced anthocyanin content in wines, which promoted color presentation, accelerated the aging process, and made wines more harmonious. Celotti et al. [93] showed that the content of anthocyanins and phenolic compounds in wine increased continuously when increasing the amplitude and the sonication time. Li [94] used ultrasound probe to treat wine and showed that when the ultrasound power, the treatment time and the number of treatments were increased, and the chromaticity values of the wine increased, especially those that had a co-color effect on the wines. Zhang et al. [95] showed that ultrasound can improve the sensory characteristics of wine by modulating the interactions between phenolic compounds and proteins from yeast autolysis through a model wine. Liu et al. and García Martín et al. [96,97] showed that ultrasound can enhance yeast autolysis and increase the polysaccharide content in wine by increasing the rate and extent of yeast cell destruction without adversely affecting the sensory quality of wine. In addition, it could also promote the improvement of wine flavor to some extent. Tao et al. [88] studied the treatment of a model wine by ultrasound combined with oak chips. The results showed that the compounds released by oak chips enhanced the aroma of wine. Besides, ultrasound treatment increased the mass transfer and migration of phenols from oak chips to the model wine, and the content of total phenols in the model wine increased significantly after 150 min of ultrasound treatment. Juan et al. [98] showed a reduction in astringency, an increase in polysaccharide content and aromatic substances, an improvement in the harmonization of the wine and shortening of the aging time when red wine was treated with ultrasound in combination with oak chips and lees. Delgado-González et al. [99] found that the color intensity and phenolic compounds concentration of wine distillate showed an increasing trend when acoustic energy density was 40 W/L in their study on the effect of oak chips combined with ultrasound on wine aging. Yan et al. [100] showed that ultrasound has a significant effect on the electrical conductivity of wine, and the mechanism may be that ultrasound can affect the concentration, ionic valence and ionic strength of metal ions in wine. To be specific, ultrasound can oxidize Fe^{2+} to Fe^{3+}.

(2) Mechanisms and progress of ultrasonic treatment on wine color

The changes on wine color induced by ultrasound may be due to several factors, such as the generation of free radicals and their chain reactions (oxidative polymerization, condensation, etc.), co-color effects and reduction of sulfur dioxide in wine.

It was found that 1-hydroxyethyl free radical was produced in wine under ultrasound treatment, and this radical is a key radical in the maturation process of wine [101], which is the basis for the occurrence of related reactions during wine maturation; the mechanisms of its production may be due to the transient high temperatures and pressures generated locally by ultrasonic cavitation that cause the cleavage of water molecules in wine to produce hydroxyl radicals, which subsequently react with ethanol to form 1-hydroxyethyl radicals [4,102]. The 1- hydroxyethyl radical is then transformed into acetaldehyde, which acts as a bridge chain to mediate the polymerization of anthocyanins and flavan-3-ols to produce compounds responsible for color, thereby improving the color of the wine [83]. The color change caused by ultrasonic treatment of wine is also related to its auxochromatic effect: Li [94] showed that 20 kHz, 180 W ultrasound treatment for 20 min can enhance the auxochromatic effect by changing the structure of auxochromes (caffeic acid, etc.), which in turn accelerates the color change and the maturation of wine, the obtaining wines remaining stable after one month of storage without reversible changes. Xue et al. [103] reported the effect of 100 W power ultrasound treatment for 0 min, 14 min and 28 min on the co-color effect of caffeic acid and catechin in wine and model wine, and the results showed that ultrasound can not only affect the co-color effect of co-colorants, but also have significant differences on the co-color effect of different substances. Fu et al. [104] showed that 25 kHz, 500 W ultrasound treatment for 20 min could significantly promote the formation of pigment-like substances in model wines, thus improving the color quality of wines. In addition, Chen et al. [105] reported the effect and mechanism of sulfur-containing compounds on wine color in red wine and model wine under ultrasound (25 kHz, 500 W, and 20 min).

(3) Research progress on the influence of ultrasonic aging on wine flavor characteristics

There are many flavor-related substances in wine, some of them are volatile. Ultrasound treatment of wine affects its flavor to some extent. On the one hand, ultrasonic cavitation causes changes in flavor-related compounds in the wine, on the other hand, ultrasound can cause the loss of low-molecular-weight volatile compounds in the wine (degassing effect) [106]. The research of Sánchez-Córdoba et al. [107] showed that ultrasound treatment during pre-fermentative maceration of red wine did not modify the sensory profile of the wines while when applied over aging with oak chips, ultrasound extracted large amounts of volatile compounds from oak chips that provided red fruits, aromatic intensity and wood attributes, which was regarded by the authors as a positive trend at sensory level. Lukić et al. [108] studied the effect of ultrasound treatment on color and aroma compounds and found that ultrasound treatment significantly reduced the content of higher alcohols; however, the most suitable ultrasound conditions should be established to avoid excessive oxidation and degradation of phenolic compounds and compounds responsible for wine aromas. The authors also systematically studied the effects of ultrasonic aging on volatile flavor compounds and higher alcohols in wine. The results showed that ultrasound could significantly reduce the content of higher alcohols (40.44% reduction), and there were significant differences in the effects of different types of ultrasonic instruments (numerical control ultrasound cleaning machine, multi-frequency ultrasonic cleaner system and variable-amplitude ultrasound-rod cell disrupter). At the same time, ultrasound could also increase the types and content of volatile flavor compounds, indicating that ultrasound treatment can improve the taste and flavor characteristics of wine to some extent [109–111]. Chemat et al. [112] studied the relationship between ultrasound treatment and phenolic compounds degradation in wine, and the results showed that ultrasound treatment could prevent oxidative degradation of aromatic compounds. Lukić et al. [108] found that ultrasound frequency had the highest effect on the physicochemical parameters of red wine aging, followed by ultrasound temperature and amplitude. The lower the ultrasound frequency, the shorter the aging time, and the lower the amplitude and temperature, the more favorable the phenolic compounds, color, and aroma components in wine; the higher temperatures of water-bath ultrasound leads to the degradation of volatile compounds, while the larger probe diameter and the higher amplitude of ultrasound probe have less effect on phenols and volatile components. Cui et al. [113] showed that ultrasound treatment accelerated the cleavage of wine yeast more than microwave treatment, resulting in an increase in the content of aromatic compounds and esters and a decrease in the content of higher alcohols, and make the wine body more harmonious and color more stable.

4.2.2. Prospects of ultrasound wine aging technology

In summary, the available studies have shown that ultrasound, combined or not with oak chips, can indeed affect in a positive way the quality of wine to a certain extent and accelerate the aging of wine. The aim is to achieve wines that have the flavor and quality characteristics of wines traditionally aged but in a much shorter time.

However, the problems in the current studies include: First, most of the current research focus on the macroscopic description of the changes in physicochemical and sensory parameters of wine by ultrasound treatment, while research on the mechanism of ultrasound aging is rarely involved. Second, the ultrasound equipment used, the parameter

conditions assayed and the results obtained by the various research groups are very different, which cannot provide a reference for later research. Third, the available results in the literature cannot provide definitive information on the feasibility of the industrial scale-up of the aging of wine by ultrasound. In particular, the changes and mechanism of substances closely related to bitterness, astringency, browning and color in wine after ultrasonic treatment are still unclear. Changes in these compounds and attributes during the maturation and aging in barrels of wine are relatively clear, which is convenient to assess whether wines treated with ultrasound resembles to those naturally matured and aged.

Therefor it is suggested to conduct the first research by constructing a model wine system composed of the main compounds found in wine (or at least those related to aging) to eliminate the interferences from non-target compounds. In this way, the effect mechanism of ultrasound on the quality parameters and sensory characteristics of wine can be studied more deeply and systematically, providing theoretical basis for the commercial application of ultrasound aging.

4.2.3. Research progress of ultrasound for wine sterilization

This step of winemaking takes place after the completion of alcoholic fermentation and other fermentation processes. In order to stop the fermentation process in time and prevent the spoilage and pollution by other bacteria, sterilization or the addition of chemical preservatives (such as sulfur dioxide) are usually carried out. Ultrasound is regarded as a new type of physical sterilization process, and its mechanisms of action is the transient, local high pressures and high temperatures generated by ultrasonic cavitation bubbles when they rupture, which are sufficient to disrupt the cell wall structure of microorganisms and cause bacterial death [62]. Studies have shown that ultrasound has good sterilizing effects, which is helpful to avoid the adverse effects caused by traditional thermal and chemical sterilization processes, reduce processing time and nutritional loss, and maintain the original flavor of food [114–116]. In general, there are many factors that affect the performance of ultrasound sterilization, such as the size and morphology of microorganisms, ultrasound power, amplitude and frequency, and processing time and temperature [117]. Cells with larger surface area are more susceptible to ultrasound cavitation [118], and Gram-positive bacteria and spherical cells have stronger resistance to ultrasound than Gram-negative bacteria and rod-shaped cells. Budding spores and some heat-resistant microorganisms also have stronger resistance [119]. Joyce et al. [120] showed that at low frequency range (between 20 kHz and 38 kHz), the killing rate of *Bacillus* increased significantly with the increase of treatment time and ultrasound intensity. Pagán et al. [121] found that combined ultrasound and microwave treatments were more effective for inactivating *Listeria monocytogenes*. Gracin et al. [72] found that continuous high-power ultrasound treatment was effective in inhibiting *Brettanomyces* yeasts and lactic acid bacteria in wine up to 89.1–99.7% and 71.8–99.3%, respectively. Bevilacqua et al. [122,123] reported that ultrasound treatment at 20 kHz, 130 W, 20–60% amplitude and 2–6 s pulses for 2–6 min was effective in reducing the number of *Brettanomyces* yeasts and some harmful microorganisms, and extending the shelf life.

In the wine sterilization process, ultrasound can also be combined with other methods such as heat treatment and sulfur dioxide addition in order to shorten the sterilization time, reduce the loss of nutrient, and achieve better sterilization results. Cui et al. [71] carried out research on the treatment of white wine by ultrasound combined with sulfur dioxide. The results showed that ultrasound under the most suitable conditions (40 Hz ultrasound for 20 min and 40 mg/L SO_2) had higher total lethal rate of *S. cerevisiae*, and improved the biostability, the flavor and quality of wine. Finally, Lv et al. [124] combined high pressure electrostatic field, ultrasound and pasteurization, respectively, with traditional sulfite treatment for wine, and the results showed that ultrasound combined with sulfur dioxide treatment was the most effective, with characteristic aroma and flavor, and remained clear and transparent after 12 months of storage, improving product stability and extending shelf life.

5. Future prospects for research and industrial application

From the information provided in the previous sections, it can be concluded that ultrasound can be reasonably applied in the various steps of winemaking to accelerate mass transfer, activate yeasts to speed up fermentation metabolism, shorten aging time, reduce SO_2 consumption, improve wine quality characteristics, extend shelf life, and other beneficial effects. However, the following problems in the application of basic research and industrial application of the technology still remains to be overcome.

First, most of the current researches on ultrasonic treatment of wine are conducted under laboratory-scale conditions, and the results obtained are not consistent. Therefore, basic laboratory research should continue to be strengthened to clarify the basic requirements needed for the use of ultrasound in each step. In terms of ultrasound power, it is recommended the use of power density to describe the ultrasound conditions to unify the ultrasonic power.

Second, ultrasound used is mostly static ultrasound, which leads to relatively large energy consumption and strong corrosion to the container, which is not suitable for industrial mass production. Therefore, it is recommended the development of suitable ultrasound equipment for large-scale application.

Thirdly, the mechanisms of ultrasound working in the different steps of winemaking is not completely clear, which to a certain extent limits its commercial application. It is suggested to carry out interdisciplinary research in different fields to lay the theoretical basis for large-scale applications.

Fourth, it is recommended that ultrasound be combined with other methods. To be specific, in order to substitute the maturation in oak barrels, ultrasound could be combined with the addition of oak chips to obtain the tannins and volatile compounds from wood.

CRediT authorship contribution statement

Qing-An Zhang: providing funding, supervision, revising and editing the manuscript. **Hongrong Zheng:** writing the original draft, drawing the figures and editing manuscript. **Junyan Lin:** drawing the figures and writing the original draft. **Guangmin Nie:** writing the original draft. **Xuehui Fan:** revising and editing the manuscript. **Juan Francisco García-Martín:** revising and editing the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Safer Chemical Ingredients List

Lista de sustancias químicas más seguras

On this page:

Related Information

For chemical manufacturers and raw material suppliers looking for information on how to list a chemical on the Safer Chemical Ingredients List (SCIL), visit our step-by-step guide. <https://epa.gov/saferchoice/how-list-chemical-safer-chemical-ingredients-list>

A downloadable spreadsheet of the Safer Chemical Ingredients List (xls) <https://www.epa.gov/sites/default/files/2015-09/safer_chemical_ingredients_list.xls> (458 KB) is also available. See the "Updates" tab in the Excel spreadsheet for recently added and/or updated chemicals.

Safer Chemical Ingredients List

- The listed chemicals are safer alternatives, grouped by their functional-use class <https://epa.gov/saferchoice/standard#tab-2>. †
- Chemicals are marked as a 🟢 green circle, 🌓 green half-circle, 🔺 yellow triangle, or ⬜ grey square. ‡
- This list includes many of the chemicals evaluated through the Safer Choice Program. It does not include confidential chemicals. There may be chemicals not included in this list that are also safer.
- Some of the chemicals listed in these functional use classes may not be on the TSCA inventory <https://epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory> and therefore may not be authorized/allowed for TSCA uses. Additionally, some of the chemicals listed in these functional use classes may not be authorized/allowed for FIFRA <https://epa.gov/laws-regulations/summary-federal-insecticide-fungicide-and-rodenticide-act> or other regulated uses. Persons considering TSCA, FIFRA, or other regulated uses for these substances should engage in appropriate diligence to ascertain whether such use is authorized.

Please Select: All Functional Use Classes

or Select a Functional Use Class:

- **Antimicrobial Actives**
- **Chelating Agents**
- **Colorants**
- **Defoamers**
- **Emollients**
- **Enzymes and Enzyme Stabilizers**
- **Oxidants and Oxidant Stabilizers**
- **Polymers**
- **Preservatives and Antioxidants**
- **Processing Aids and Additives**

- **Skin Conditioning Agents**
- **Solvents**
- **Specialized Industrial Chemicals**
- **Surfactants**
- **Uncategorized**

Search: Name chitosan **or CAS RN**

Clear Options

Show 25 entries

Code	Common Name	CAS Registry Number	Functional Use
	Chitosan	9012-76-4	Antimicrobial Actives <https://epa.gov/node/94583>; Preservatives and Antioxidants <https://epa.gov/node/44763>
	Chitosan acetate	87582-10-3	Processing Aids and Additives <https://epa.gov/node/44797>

Showing 1 to 2 of 2 entries (filtered from 957 total entries)

Previous 1 Next

Please note: Use of chemicals from the list in product formulations does not entitle the manufacturer to make any claims related to the EPA or the Safer Choice Program or label. Manufacturers may only make such claims after going through formal third-party Safer Choice review, qualifying for the Label based on both ingredient- and product-level criteria (addressing, for example, issues of ingredient

purity and physical form, potential synergistic effects, and performance), and entering an EPA Safer Choice partnership. For more information see Steps to Get the Safer Choice Label <https://epa.gov/saferchoice/steps-get-safer-choice-label-your-product>.

Overview of the Safer Chemical Ingredients List

The Safer Chemical Ingredients List (SCIL) is a list of chemical ingredients, arranged by functional-use class, that the Safer Choice Program has evaluated and determined to be safer than traditional chemical ingredients. This list is designed to help manufacturers find safer chemical alternatives that meet the criteria of the Safer Choice Program.

Before Safer Choice decides to include a chemical on the SCIL, a third-party profiler (i.e., NSF, International or ToxServices) gathers hazard information from a broad set of resources, including the identification and evaluation of all available toxicological and environmental fate data. The third party profiler submits a report to Safer Choice, with a recommendation on whether the chemical passes the Criteria for Safer Chemical Ingredients. Safer Choice staff performs due diligence by reviewing the submission for completeness, consistency, and compliance with the Safer Choice Criteria. If more than one third-party has evaluated the chemical, Safer Choice also checks for differences in the profiles and resolves any conflicts. In some cases, Safer Choice may also perform additional literature reviews and consider data from confidential sources, such as EPA's New Chemicals Program. Safer Choice does not typically examine primary literature (original studies) as part of its review and listing decisions.

The list is not intended to be exclusive. Chemicals may be submitted as part of a formulation that the program has yet to review or a chemical manufacturer may develop a chemical to meet the Safer Choice criteria. If these chemicals meet our criteria, they may be approved for use in Safer Choice-labeled products and added to the SCIL. Chemicals may be removed from the list or have their status changed based on new data or innovations that raise the safer-chemistry bar. Safer Choice will ensure that no confidential or trade secret information appears in this list.

About the List

Safer Choice ensures that no confidential or trade secret information appears in this list.

† The Safer Choice Standard <https://epa.gov/saferchoice/standard> and the Criteria for Safer Chemical Ingredients <https://epa.gov/saferchoice/standard#tab-2> are protective and address a broad range of potential toxicological effects, including:

- carcinogens, mutagens, reproductive or developmental toxicants;
- persistent, bioaccumulative and toxic chemicals;
- systemic or internal organ toxicants;
- asthmagens;
- sensitizers; and
- chemicals on authoritative lists of chemicals of concern.

Additionally:

- Chemicals that exhibit endocrine activity are closely evaluated. Those associated with toxicological hazards are not allowed.
- Impurities can be present in chemicals that are used in Safer Choice-certified products. Safer Choice limits impurities that do not meet its criteria to not more than 0.01 percent in the final product. The safer chemicals list does not include impurities.
- EPA does not list fragrances on the SCIL. Fragrances used in Safer Choice-certified products must meet the Safer Choice Criteria for Fragrances <https://epa.gov/saferchoice/safer-choice-criteria-fragrances>.

*****All chemicals in the listing are among the safest for their functional use.

Green circle - The chemical has been verified to be of low concern based on experimental and modeled data.

Green half-circle - The chemical is expected to be of low concern based on experimental and modeled data. Additional data would strengthen our confidence in the chemical's safer status.

⚠ **Yellow triangle** - The chemical has met Safer Choice Criteria for its functional ingredient-class, but has some hazard profile issues. Specifically, a chemical with this code is not associated with a low level of hazard concern for all human health and environmental endpoints. (See Safer Choice Criteria <https://epa.gov/saferchoice/standard#tab-2>). While it is a best-in-class chemical and among the safest available for a particular function, the function fulfilled by the chemical should be considered an area for safer chemistry innovation.

▢ **Grey square** - This chemical may not be acceptable for use in products that are candidates for the Safer Choice label and any currently certified products that contain it may need to reformulate per Safer Choice Compliance Schedules <https://epa.gov/saferchoice/safer-choice-implementation-and-compliance-schedules>. Manufacturers are invited to provide information to justify continued listing of this chemical on SCIL and use in Safer Choice-certified products. Unless information provided to EPA adequately justifies continued listing, this chemical will be removed 12 months after grey square designation.

Note: Some functional class listings contain both green circle and yellow triangle chemicals because the yellow triangle chemicals fill a functional need not met by the available green circle chemicals. For example, yellow triangle preservatives are needed to meet the full range of antimicrobial efficacy, and yellow triangle solvents are needed to meet restrictions on volatile organic compounds (VOCs) in certain product classes.

Additional Resources

See the following resources for additional information on the chemicals in cleaning products:

- ChemView <https://chemview.epa.gov/chemview>

  EPA's centralized database of information on chemicals subject to TSCA, including EPA assessments, regulatory actions, and health and safety data.

- CleanGredients® <http://www.cleangredients.org/>

  This database of safer chemicals supports the Safer Choice Program by providing a list of chemicals, arranged by component class, that meet the Safer Choice Criteria.

- Household and Commercial Products Association (HCPA) Ingredient Dictionary <https://www.thehcpa.org/resources/ingredient-dictionary/>

Last updated on September 30, 2024

EPA's Safer Choice Criteria for Colorants, Polymers, Preservatives, and Related Chemicals

The Safer Choice approach to product review and labeling focuses on identifying the safest possible chemical ingredients, within a functional class context, that are necessary for a product to perform well. The general requirements in the *Safer Choice Master Criteria for Safer Chemical Ingredients* serve as the reference set of benchmarks on which Safer Choice bases its chemical ingredient-specific criteria. The criteria also constitute a baseline set of toxicity parameters, with functionality-driven tailoring, that all ingredients without component-specific criteria must address to be considered for use in Safer Choice products.

While EPA has used the stringent and comprehensive elements of the Master Criteria to evaluate ingredient classes, its experience in implementing the Safer Choice Program has demonstrated that most functional classes require a tailored approach. For example, colorants, polymers, and certain preservatives have as part of their functionality the ability to resist degradation. They also typically lack a complete set of measured toxicity data, for which Safer Choice substitutes data based on predictive models, estimation techniques, and expert judgment. EPA has therefore adapted its criteria for colorants, polymers, preservatives, and related chemicals (e.g., defoamers) to accommodate specific functional-class characteristics, like persistence, permitting the listing of the safest chemicals in those classes.

Although modifying the Master Criteria to some extent, the provisions serve largely to clarify, elaborate on, and make more transparent the technical considerations involved in evaluating chemicals in the functional classes without tailored criteria. The approach Safer Choice has adopted retains the human health safety thresholds from the Master Criteria, but allows flexibility in environmental toxicity and fate endpoints, as appropriate to chemicals that persist as part of their functionality. To address the lack of data common to many of these chemicals, Safer Choice relies on a mix of estimated, measured and authoritative list-based data elements. (Please note that any modifications to the component-class criteria will not alter the prohibition on the use of listed carcinogens, mutagens or reproductive or developmental toxicants, or persistent, bioaccumulative and toxic chemicals in Safer Choice products.)

Colorants, Polymers, Preservatives, and Related Chemicals (extract from the Safer Choice Standard, section 5.8)

Colorants (including pigments and optical brighteners), polymers, and certain preservatives (including antioxidants) (and other chemicals referenced in section 5.14) include as part of their functionality the ability to resist degradation and be effective over long periods. They also can be complex molecules and mixtures and often lack

measured toxicity data. To identify the safest available chemicals in each class given their functional characteristics, the toxicity thresholds in the Master Criteria will be used to evaluate human health endpoints, and the thresholds in section 5.8.3 will be used for environmental endpoints. Data on these chemicals will be required as per 5.8.3, unless noted otherwise.

5.1 Polymers

To be acceptable for labeled products, polymers must have low-concern characteristics.[1] Also, the requirements of this section apply to the low molecular weight components of polymers (typically less than 1,000 daltons). Safer Choice encourages the use of degradable polymers whenever possible; only those that do not degrade into CMRs or PBTs will be allowed.

Special conditions for certain categories of polymer: In addition to the requirements in 5.8.3, polymers that are respirable or water-absorbing must be in solution. Anionic polymers used as chelating agents must meet the requirements in the Safer Choice Criteria for Chelating Agents, except section 5.9, Environmental Toxicity and Fate, which must be addressed as per 5.8.3. Perfluoroalkyl polymers, allowed only in floor finishes, must, at a minimum, be limited to fluorinated carbon-chain lengths of less than eight atoms.

5.2 Preservatives

Preservatives have biocidal properties and time-sensitive functionality. Safer Choice will allow use only at the lowest effective level. In addition to the CMR and PBT prohibitions in 5.2, preservatives that release CMRs or PBTs or whose reaction byproducts are CMRs or PBTs will not be allowed.

5.8.3 Special requirements

For colorants, polymers, and preservatives, the toxicological endpoints in the Master Criteria will be addressed as follows:

1) *For Acute Mammalian Toxicity (section 5.1 of the Master Criteria), Neurotoxicity (5.4), Repeated Dose Toxicity (5.5), and Skin Sensitization (5.8), the following apply*:

   Data requirements: Screen Authoritative Lists. Chemicals with new measured data not yet reviewed by authoritative bodies may be subject to review.

2) *For Carcinogenicity (section 5.2 of the Master Criteria), Genetic Toxicity (5.3),*

[1] Described in the Sustainable Futures' Interpretive Assistance Document for Assessment of Polymers (http://www.epa.gov/oppt/sf/pubs/iad_polymers_june2013.pdf).

and Reproductive and Developmental Toxicity (5.6), and Respiratory Sensitization (5.7), the following apply:

Data requirements: Screen specified R-Phrases and Authoritative Lists. Available data, measured and/or estimated, for the chemical and/or a suitable analog may be reviewed against the criteria using a weight-of-evidence approach.

3) *Environmental Toxicity and Fate*

Limitation on Persistent, Bioaccumulative and Toxic chemicals: Acceptable chemicals must not be persistent (half-life ≥ 60 days), bioaccumulative (BCF/BAF ≥ 1,000), and aquatically toxic* (LC/EC50 ≤ 10 mg/L or NOEC/LOEC ≤ 1 mg/L).

Limitation on very Persistent and very Bioaccumulative chemicals: Acceptable chemicals must not be very persistent (half-life > 180 days or recalcitrant) and very bioaccumulative (> 5,000).

Limitation on very Persistent and very Toxic chemicals: Acceptable chemicals must not be very persistent (half-life > 180 days or recalcitrant) and very aquatically toxic* (LC/EC50 < 1.0 mg/L or NOEC/LOEC < 0.1 mg/L).

Data requirements: Screen Authoritative Lists. Available data, measured and/or estimated, for the chemical and/or a suitable analog may be reviewed against the criteria using a weight-of-evidence approach.

*Excludes the algal shading effects of colorants.

David Brown
Chinova Bioworks Inc.
50 Crowther Lane, Suite 100
Fredericton, New Brunswick E3C 0J1
CANADA

Re: GRAS Notice No. GRN 000997

Dear Mr. Brown:

The Food and Drug Administration (FDA, we) completed our evaluation of GRN 000997. We received Chinova Bioworks Inc.'s (Chinova) notice on March 4, 2021, and filed it on July 13, 2021. Chinova submitted an amendment to the notice on July 21, 2021, adding the subcategory of egg substitutes. Additionally, Chinova submitted amendments dated September 9, 2021, November 8, 2021, and December 1, 2021, providing additional information regarding the intended uses, specifications, analytical methods, dietary exposure, and safety of the notified substance.

The subject of the notice is chitosan from white button mushrooms (*Agaricus bisporus*) for use as an antimicrobial at levels ranging from 0.015 g to 0.15 g per 100 g of food. The intended uses and use levels are shown in Table 1.[1] The notice informs us of Chinova's view that the use of chitosan is GRAS through scientific procedures.

Table 1. Proposed uses and use levels for chitosan

Food Category	Food Uses	Maximum Use Level (g/100 g)
Baked goods	Bagels and English muffins	0.06
	Bread (excluding sweet type)	0.06
	Cakes	0.06
	Cornbread, corn muffins, tortillas	0.06
	Muffins	0.04
	Pastries	0.05
Alcoholic beverages	Cocktail drinks	0.02
Beverages and beverage bases	Energy Drinks	0.01
	Flavored and carbonated waters	0.01
	Soft drinks	0.01
	Sport, electrolyte and fluid replacement	0.01
Cheese	Cheese-based sauces	0.1
	Cottage cheese	0.05

[1] Chinova states that chitosan is not intended for use in infant formula, infant foods, and in products under the jurisdiction of the United States Department of Agriculture.

	Cream cheese and cheese spreads	0.1
	Processed cheese and cheese mixtures	0.06
Coffee and tea	Ready-to-drink tea	0.01
Condiments and relishes	Relish	0.08
Confections and frostings	Coatings	0.1
	Frostings and icings	0.1
Dairy product analogs	Imitation cheese	0.15
Fats and oils	Fat-based sauces	0.05
	Margarine and margarine-like spreads	0.05
	Mayonnaise and mayonnaise-type dressings	0.06
	Salad dressings	0.08
Gelatins, puddings, and fillings	Flans, custards and egg-based desserts	0.08
Grain products	Energy, protein or meal-replacement bars	0.02
Gravies and sauces	Gravies	0.02
	White sauces	0.1
Jams and jellies	Jams, jellies, preserves, and marmalades	0.1
Milk Products	Yogurt	0.06
Processed fruit	Fruit drinks, ades and smoothies	0.02
	Fruit-based desserts	0.1
Plant protein products	Meat analogs	0.15
	Egg substitutes	0.08
Processed vegetables	Vegetable purees	0.04
Sugar substitutes	Sugar substitutes	0.1
Sweet sauces, toppings and syrups	Sweet sauces, syrups and toppings	0.06
	Cocoa syrups	0.1

Our use of the term “chitosan” in this letter is not our recommendation of that term as an appropriate common or usual name for declaring the substance in accordance with FDA’s labeling requirements. Under 21 CFR 101.4, each ingredient must be declared by its common or usual name. In addition, 21 CFR 102.5 outlines general principles to use when establishing common or usual names for nonstandardized foods. Issues associated with labeling and the common or usual name of a food ingredient are under the purview of the Office of Nutrition and Food Labeling (ONFL) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety (OFAS) did not consult with ONFL regarding the appropriate common or usual names for “chitosan.”

Chinova provides information on the identity and composition of chitosan [(1,4)-2-amino-2-desoxy-beta-D-glucan] extracted from white button mushrooms (*Agaricus bisporus*). They describe it as a white to beige powder containing 95% chitosan and up to 5% beta-1,3-D-glucans. The identity of the two components is confirmed using ^{1}H-NRM and infrared spectroscopy. Chinova states that chitosan is a linear polycationic polysaccharide composed of glucosamine and N-acetyl glucosamine monomers linked

with a 1,4-beta-linkage and having a molecular weight ranging from 10 to 400 kDa and a CAS registry number of 9012-76-4.

Chinova describes the manufacturing process for chitosan as a multistep extraction and purification from white button mushrooms. Chinova states that chitosan is manufactured in accordance with current good manufacturing practices and that all raw materials, processing aids and food contact articles used in the manufacturing are food grade and approved for their respective uses in accordance with an appropriate regulation, are the subject of an effective food contact notification, or are GRAS for that use. Chitosan is produced from white button mushrooms via thermal deacetylation with sodium hydroxide followed by collection of the biomass and repeated rinsing with water. The chitosan is separated from the biomass by extraction with vinegar. The remaining solids are removed by centrifugation and the chitosan is precipitated by adjusting the pH with sodium hydroxide. The precipitated substance is collected via centrifugation and repeatedly washed with water. The chitosan is then dried using a drum dryer and milled into a fine powder.

Chinova provides specifications for chitosan that include total chitosan (≥ 95 % w/w), beta-1,3-glucans (≤ 5 % w/w), degree of deacetylation (≥ 80 mol %), moisture (≤ 10 %), total ash (≤ 3 %), solubility (≥ 99.5 %), total arsenic (≤ 0.2 mg/kg), cadmium (≤ 0.2 mg/kg), mercury (≤ 0.2 mg/kg), lead (≤ 1 mg/kg) and limits for microorganisms including aerobic microbial count (≤ 100 colony forming units (CFU)/g), yeast and mold (≤ 100 CFU/g), *Escherichia coli* (absent in 10 g), and *Salmonella* (absent in 25 g). Chinova provides the results of five non-consecutive batch analyses to demonstrate that chitosan can be manufactured to meet the specifications.

Chinova provides the results of stability studies for three batches of chitosan stored at either 25 °C and 60% relative humidity (recommended storage conditions) or 40 °C and 70% relative humidity (accelerated storage conditions). Based on the results, Chinova notes that chitosan is stable for at least 9 months when stored under both the recommended and accelerated storage conditions and up to 24 months based on the results from the accelerated storage conditions.

Chinova states that the technical effect for chitosan is as an antimicrobial. Chinova provides results demonstrating this technical effect in beverages, baked goods, and dairy products.

Chinova discusses the estimated dietary exposure to chitosan for the intended uses and use levels described in Table 1. Chinova notes that chitosan's intended use as an antimicrobial is not additive to its use as flavoring agent. Based on food consumption data from the 2017-2018 National Health and Examination Survey (NHANES), Chinova estimates the mean and 90th percentile eaters-only dietary exposure to chitosan to be 0.11 g and 0.21 g/person (p)/day, respectively, for the U.S. population aged 2 years and older. Chinova states that the intended uses in GRN 000997 would be substitutional for the use as a flavoring agent and therefore, there would be no increase in the cumulative dietary exposure to chitosan.

Chinova states that chitosan is not digested by human digestive enzymes; absorption and systemic exposure to intact chitosan molecules consumed in the diet will not occur. However, chitosan is subjected to microbial fermentation in the gastrointestinal tract, as is seen for dietary fibers. Chinova discusses a published six-month study in rats where rats of both sexes were fed either control diets or diets containing seafood shell-derived chitosan at concentrations of 1%, 3%, or 9%. Chinova agrees with the study authors' conclusion that no effects were observed when chitosan was fed at less than 1% (approximately equivalent to 450 mg/kg bw) in male rats and 9% (approximately equivalent to 6,000 mg/kg bw) in female rats. Chinova states that Chitosan does not have any reproductive or developmental toxicity and is not mutagenic or genotoxic. Chinova discusses human studies in which subjects consumed shrimp or shellfish derived chitosan ranging from 0.5 to 6.75 g/day for 4 to 24 weeks. There was no decrease in serum fat soluble vitamins (A, E, D), α-carotene, and β-carotene, or changes in clinically relevant serum parameters.

Chinova includes the statement of a panel of individuals (Chinova's GRAS panel). Based on its review, Chinova's GRAS panel concluded that chitosan is safe under the conditions of its intended use.

Based on the totality of the data and information described above, Chinova concludes that chitosan is GRAS for its intended use.

Standards of Identity

In the notice, Chinova states its intention to use chitosan in several food categories, including foods for which standards of identity exist, located in Title 21 of the CFR. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FD&C Act)

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of Chinova's notice concluding that chitosan is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods containing chitosan. Accordingly, our response should not be construed to be a statement that foods containing chitosan, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Chinova provided, as well as other information available to FDA, we have no questions at this time regarding Chinova's conclusion that chitosan

is GRAS under its intended conditions of use. This letter is not an affirmation that chitosan is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to GRN 000997 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2022.02.28 14:14:45 -05'00'

Susan Carlson, Ph.D.
Director
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

Agency Response Letter GRAS Notice No. GRN 000397

Return to inventory listing: **GRAS Notice Inventory (https://wayback.archive-it.org/7993/20171031010838/http://www.fda.gov/grasnoticeinventory)**

See also **Generally Recognized as Safe (GRAS) (/7993/20171031010838/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm)** and **about the GRAS Notice Inventory (/7993/20171031010838/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**

CFSAN/Office of Food Additive Safety

December 19, 2011

Véronique Maquet, Ph.D.
KitoZyme S.A.
Rue Haute Claire, 4
Parc Industriel des Hauts-Sarts, Zone 2
BE-4040 Herstal BELGIUM

Re: GRAS Notice No. GRN 000397

Dear Dr. Maquet:

The Food and Drug Administration (FDA) is responding to the notice, dated July 28, 2011, that you submitted in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on August 3, 2011, filed it on August 8, 2011, and designated it as GRAS Notice No. GRN 000397.

The subject of the notice is chitosan from *Aspergillus niger* (chitosan). The notice informs FDA of the view of KitoZyme S.A. (KitoZyme) that chitosan from *A. niger* is GRAS, through scientific procedures, for use as a secondary direct food ingredient [1] in alcoholic beverage production at levels between 10 and 500 grams per hectoliter (100 liters).

As part of its notice, KitoZyme includes the report of a panel of individuals (KitoZyme's GRAS Panel) who evaluated the data and information that are the basis for KitoZyme's GRAS determination. KitoZyme considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. KitoZyme's GRAS panel discusses identity, specifications, method of manufacture, dietary exposure, and safety of chitosan, including the history and current regulatory status of *A. niger* and chitosan in food, and concludes that chitosan is GRAS under the intended conditions of use.

Chitosan is identified by the CAS registry number 9012-76-4. KitoZyme describes chitosan as an insoluble, non-digestible fiber derived from the post-fermentation biomass of non-viable *A. niger* used to manufacture food-grade citric acid. KitoZyme states that strains of *A. niger* used in the production of citric acid are nonpathogenic and nontoxigenic, and have a long history of safe use worldwide. Hydrolysis of the raw *A. niger* material produces chitosan, which is then washed, precipitated, concentrated, and dried. KitoZyme states the degree of acetylation for chitosan to be 0 to 30 mole percent (%). All materials and processing aids used in the manufacture of chitosan are food-grade. The notifier provides product specifications for chitosan, including microbiological limits, heavy metals, and chemical characterization. KitoZyme states that chitosan from *A. niger* is chemically equivalent to chitosan from shellfish, based on Fourier transform infrared spectroscopy and nuclear magnetic resonance analyses. KitoZyme noted the presence of beta-1,3-D-glucans (present in ~10 to 15% concentration on a weight by weight basis) that is absent from shellfish sources of chitosan.

KitoZyme reported on a published 13-week subchronic toxicity study in Wistar rats. Twenty rats per sex were fed *A. niger*-sourced chitin and beta-glucan in a 30:70 ratio. KitoZyme reported the No Observable Adverse Effect Level to be the highest dose tested, 6,589 and 7,002 milligrams/kilogram body weight/day for the male and female rats, respectively.

When chitosan is used in the production of alcoholic beverages, it is removed from the wine, must, beer, cider, or spirits at the end of the treatment using physical separation processes, such as racking, centrifugation, or filtering.

KitoZyme notes that chitosan preparations are insoluble in both water and ethanol, and because the material is removed from solution, intake modeling is not considered necessary. In addition, high performance liquid chromatography of wine processed with chitosan indicated that the final product was free from chitosan carry-over products at the limit of detection. In addition, KitoZyme notes that neither chitosan nor beta-1,3-D-glucans are digested by the human gastrointestinal tract, therefore, absorption and systemic exposure to chitosan would not occur.

Section 301(ll) of the Federal Food, Drug, and Cosmetic Act (FD&C Act)

The Food and Drug Administration Amendments Act of 2007, which was signed into law on September 27, 2007, amends the FD&C Act to, among other things, add section 301(ll). Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of KitoZyme's notice that chitosan is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing chitosan. Accordingly, this response should not be construed to be a statement that foods that contain chitosan if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusion

Based on the information provided by KitoZyme, as well as other information available to FDA, the agency has no questions at this time regarding KitoZyme's conclusion that chitosan from *A. niger* is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the

subject use of chitosan. As always, it is the continuing responsibility of KitoZyme to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000397, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at http://www.fda.gov/grasnoticeinventory.

Sincerely,
Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

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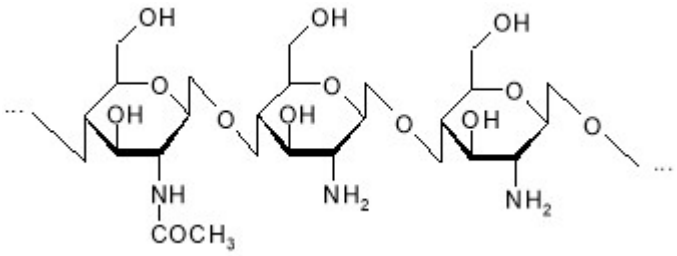
[1] 21 CFR 173: A secondary direct food additive has a technical effect in food during processing but not in the finished food (e.g., processing aid). The technical use of chitosan in GRN 000397 is for microbiological stabilization, removal of contaminants, and/or clarification of the alcoholic beverage.

More in GRAS Notice Inventory (/7993/20171031010838/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

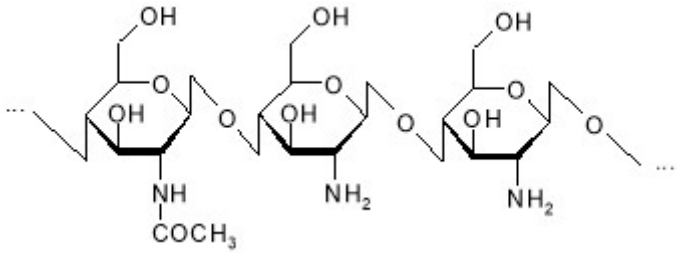
COEI-1-CHITOS Chitosan

$[C_6H_{11}NO_4]n$

CAS number Chitosan : [9012-76-4]



Chitosan



1. Purpose, origin and applicability

Chitosan, a natural polysaccharide prepared of fungal origin, is initially extracted and purified from reliable and abundant food or biotechnological fungal sources such as *Agaricus bisporus* or *Aspergillus niger*.

Chitosan is obtained by hydrolysis of a chitin-rich extract. Chitin is a polysaccharide composed of several N-acetyl-D-glucosamine units interconnected by □□ (1.4) type linkages.

Chitosan is composed of glucosamine sugar units (deacetylated units) and N-acetyl-D-glucosamine units (acetylated units) interconnected by □□ (1.4) type linkages.

It is used as a fining agent in the treatment of musts for flotation clarification to reduce cloudiness and the content of unstable colloids.

It is also used for stabilising wines. This polymer actually helps eliminate undesirable micro-organisms such as *Brettanomyces.*

2. Synonyms

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Poly(N-acetyl-D-glucosamine)-poly(D-glucose).

3. Labelling

The following information must be stated on the packaging label: exclusively fungal origin, product for oenological use, use and conservation conditions and use-by date.

4. Characters

4.1. Aspect and solubility

Chitosan comes in the form of a white, odourless and flavourless powder. Chitin-glucan is almost completely insoluble in aqueous or organic medium.

4.2. Purity and soluble residues

The purity of the product must be equal to or higher than 95 %.

Dissolve 5 g of chitin-glucan in 100 ml of bidistilled water and agitate for 2 minutes. Filter after cooling on a fine mesh filter or membrane.

Evaporate the filtrate and dry at 100-105 °C. The content of solubles should not be higher than 5 %.

5. Tests

5.1. Determination of the acetylation degree and chitosan origin

5.1.1. Determination of the acetylation degree

The acetylation degree is determined by potentiometric titration, using the method described in Appendix I.

5.1.2. Determination of the source

Chitosan, as a natural polymer, is extracted and purified from fungal sources; it is obtained by hydrolysis of a chitin-rich extract. This chitosan is considered identical to chitosan from shellfish in terms of structures and properties.

An identification of the origin of chitosan is made based on 3 characteristics: content of residual glucans (refer to method in annex II), viscosity of chitosan in solution 1 % and settled density (following settlement).

Only fungal origin chitosan has both contents of residual glucan > at 2 %, a settled density ≥ at 0,7 g/cm^3 and viscosity in solution 1 % in acetic acid 1 % < at 15 cPs

5.2. Loss during desiccation

In a glass cup, previously dried for 1 hour in an oven at 100-105 °C and cooled in a

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desiccator, place 10 g of the analyte. Allow to desiccate in

the drying oven at 100-105 °C to constant mass. Weigh the dry residue amount after cooling in the desiccator.

The weight loss must be lower than 10 %.

Note: all the limits stated below are reported in dry weight except for the microbiological analyses

5.3. Ashes

Incinerate without exceeding 600 °C the residue left from the determination of the loss during desiccation as described in 5.2. Allow to calcine for 6 hours. Allow the crucible to cool in a desiccator and weigh.

The total ash content should not be higher than 3 %.

5.4. Preparation of the test solution

Before determining the metals, the sample is dissolved by acid digestion (HNO_3, H_2O_2 and HCl). Mineralisation is performed in a closed microwave system. The sample undergoes neither crushing nor drying before mineralisation.

The reagents used for the mineralisation of chitosan are as follows: HNO_3 (65 %) (Suprapur), HCl (37 %) (Suprapur), H_2O_2 (35 %). The 0.5 to 2 g sample of chitosan is placed in a flask to which are added 25 ml of HNO_3, 2 ml of HCl and 3 ml of H_2O_2. This is submitted to microwave digestion with a maximum power of 1200 watts; Power of 60 % for 1 min, 30 % for 10 min, 15 % for 3 min, and 40 % for 15 min). The solution is diluted in a volumetric flask with bidistilled water to a final volume of 25.0 ml.

The metal contents can then be determined.

5.5. Lead

Lead is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The lead content must be lower than 1 mg/kg.

It is also possible to achieve lead determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.6. Mercury

Mercury is determined by atomic absorption spectrophotometry, using the method described in appendix II.

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The mercury content must be lower than 0.1 mg/kg.

It is also possible to achieve mercury determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.7. Arsenic

Arsenic is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The arsenic content must be lower than 1 mg/kg.

It is also possible to achieve arsenic determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.8. Cadmium

Cadmium is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The cadmium content must be lower than 1 mg/kg.

It is also possible to achieve cadmium determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.9. Chromium

Chromium is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The chromium content must be lower than 10 mg/kg.

It is also possible to achieve chromium determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.10. Zinc

Zinc is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The zinc content must be lower than 50 mg/kg.

It is also possible to achieve zinc determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.11. Iron

Iron is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The iron content must be lower than 100 mg/kg.

It is also possible to achieve iron determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

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5.12. Copper

Copper is determined by atomic absorption spectrophotometry, using the method described in appendix II.

The copper content must be lower than 30 mg/kg.

It is also possible to achieve copper determination by atomic absorption, using the method described in chapter II of the International Oenological Codex.

5.13. Microbiological control

5.13.1. Total bacteria count

The total bacteria count is performed according to the horizontal method by means of the colony count technique at 30 °C on the PCA medium described in appendix III.

Less than 1000 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

5.13.2. Enterobacteria

The enumeration of *Enterobacteria* is carried out according to the horizontal method by means of the colony count technique at 30 °C described in appendix IV.

Less than 10 CFU/g of preparation.

5.13.3. Salmonella

Carry out the enumeration as described in chapter II of the International Oenological Codex.

Absence checked on a 25 g sample.

5.13.4. Coliform bacteria

Carry out the enumeration as described in chapter II of the International Oenological Codex.

Less than 100 CFU/g of preparation.

5.13.5. Yeasts

The enumeration of yeasts is carried out according to the horizontal method by means of the colony count technique at 25 °C on the YGC medium described in appendix VI.

Less than 100 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

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5.13.6. Moulds

The enumeration of moulds is carried out according to the horizontal method by means of the colony count technique at 25 °C on the YGC medium described in appendix VII.

Less than 100 CFU/g of preparation.

It is also possible to carry out the enumeration as described in chapter II of the International Oenological Codex.

6. Ochratoxin A testing

Prepare an aqueous solution (distilled water) of chitosan at 1 % and agitate for 1 hour, then carry out determination using the method described in the Compendium of International Methods of Analysis of Wine and Musts.

Less than 5 µg/kg.

6.1. Storage

Keep container closed and store in a cool and dry place.

Appendix I: Determination of the acetylation degree

1. Principle

This method consists in determining the acetylation degree of chitosan by titration of the amino groups. The acetylation degree is the ratio of the number of N-acetyl-glucosamine units to the number of total monomers.

This method is based on the method described by Rinaudo et al., (1999).

The titration of a chitosan solution by means of NaOH at 0.1 M must be performed in order to identify two pH jumps from 0 to 14.

Chitosan is dissolved in 0.1M HCl, the amino groups (on the deacetylated glucosamine units (G)) are positively charged (HCl in excess)).

The chitosan solution (of known quantity) is titrated by NaOH of known concentration. In the first part of the reaction, the excess quantity of HCl is determined:

1.1. $HCl\ (excess) + NaOH + NH_3^+Cl^- \dashrightarrow NaCl + H_2O + NH_3^+Cl^-$

After the first pH jump, the quantity of charged amino groups is determined:

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1.2. $HCl + H_2O + NH_3^+Cl^- + NaOH$ --> $NH_2 + 2H_2O + 2NaCl$

1.3. After the second pH jump, the excess quantity of NaOH is measured.

The determination of the NaOH volume between the two jumps makes it possible to identify the quantity of charged amines.

2. Reagents and materials

2.1. Commercial preparation of chitosan

2.2. Distilled or deionised water

2.3. Chlorhydric acid 0,3 M

2.4. Sodium Hydroxide 0,1M

2.5. Glass cylindrical flasks, pipettes, burettes...

2.6. Magnetic mixer and stir bar

2.7. pH-meter with temperature sensor.

3. Samples preparation

Before determination, the samples are prepared according to the protocol described hereafter:

100 mg of chitosan are placed into a cylindrical flask to which 3 ml of 0.3 M HCl and 40 ml of water are added. Agitate for 12 hours.

4. Procedure

First introduce the pH electrode of the pH-meter as well as the temperature sensor into the cylindrical flask. Check that the pH value is lower than 3.

To bring to pH = 1, add a V1 volume (ml) of HCl 0.3 M and agitate.

Then to bring to pH = 7 with a V2 volume (ml) of 0.1 M NaOH

These operations can be carried out using an automatic titrator.

5. Expression of results

The acetylation degree of chitosan is expressed in %. This formula is the ratio of the mass of acetylated glucosamine (aG) units in g actually present in the sample, to the mass in g that would be present if all the groups were acetylated, where:

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$$Q = (V_{NaOH} \times 0.1)/(1000 \times M_{cs}) = \text{specific concentration in amino groups}$$

- Mcs: dry weight of chitosan in g

$$V_{NaOH} = V2 - V1 = \text{Volume of 0.1 M NaOH between 2 pH jumps in ml}$$

For a 1 g sample
With G = Glucosamine part; a = acetylated part
aG weight actually present (in g) =

$$1g - (\text{Number of moles of G groups/g}) \times \text{G molecular weight} = 1g - Q \times 162$$

aG weight if all the deacetylated groups were acetylated (in g) =

$$1g + (\text{Number of moles of G groups/g}) \times \text{molecular weight a} = 1g + Q \times 42$$

The acetylation degree will be equal to DA, where:

$$\mathbf{DA} = (1 - 162 \times Q)/(1 + 42 \times Q)$$

Bibliography

- Rinaudo, M., G. Pavlov and J. Desbrieres. 1999. Influenced of acetic acid concentration on the solubilization of chitosan. *Polym.* 40, 7029-7032.

Appendix II: Determination of the residual glucan content

1. Principle

This method consists in determining the content of residual glucans in chitosan by means of spectrophotometry.
This method is based on a colorimetric reaction with a response depending on the degradation of the starch hydrolysates by hot concentrated sulphuric acid.
This degradation gives a brown yellow compound with a colour intensity proportional to the content of residual glucans.

2. Reagents and materials

2.1. Glucan 97% (Société Mégazyme)
2.2. Commercial preparation of chitosan
2.3. Distilled or deionised water

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2.4. Ethanol
2.5. Acetic acid 1%
2.6. Solution of phenol 5%
2.7. Glacial acetic acid 100%
2.8. Glass cylindrical flasks, pipettes, volumetric flasks,...
2.9. Magnetic mixer and stir bar
2.10. Chronometer

3. Preparation of the standard range

A stock solution of glucan (glucan with a purity of 97 % is provided by the company Megazyme) is prepared according to the precise protocol described hereafter:

500 mg of glucan are introduced into a volumetric flask of 100 ml into which 6 ml of ethanol and 80 ml of distilled water are added.

Agitate and boil out to allow glucan dissolution

Allow to cool, adjust to the filling mark with water

Agitate for 30 minutes.

Pour 1 ml of this solution into a 50 ml volumetric flask and adjust to the filling mark with 1 % acetic acid.

The solution is ready to use to produce the standard range according to the protocol hereafter.

Stock solution V (ml)	Water V (ml)	Glucan M (µg)
0	1	0
0.1	0.9	10
0.3	0.7	30
0.5	0.5	50
0.7	0.3	70

4. Samples preparation

Before determination, the samples are prepared according to the protocol described hereafter:

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100 mg of chitosan are placed into a 50 ml volumetric flask to which 25 ml of 1 % acetic acid are added.

Agitate for 12 hours then adjust to the filling mark.

5. Procedure

In a test tube, add 1 ml of the analyte solution, 1 ml of phenol at 5 % and 5 ml of concentrated sulphuric acid.

Agitate this mixture using a vortex for 10 s, then allow to cool for 1 hour.

The absorbance A is measured at 490 nm.

6. Expression of the results

Determine the glucan content in µg/g from the calibration curve (0-70 µg). This content is expressed in µg/g of chitosan.

Appendix III: Metal determination by atomic emission spectroscopy

1. Principle

This method consists in measuring atomic emission by an optical spectroscopy technique.

2. Sample preparation

Before the determination of metals, the sample is dissolved by acid digestion (HNO_3, H_2O_2 and HCl). Mineralisation takes place in closed microwave system. The sample undergoes neither crushing nor drying before mineralisation.

The reagents used for the mineralisation of chitosan are as follows: HNO_3 (65 %) (Suprapur), HCl (37 %) (Suprapur), H_2O_2 (35 %). The 0.5 to 2 g sample of chitosan is placed in a flask to which are added 25 ml of HNO_3, 2 ml of HCl and 3 ml of H_2O_2. The whole is then submitted to microwave digestion (Power of 60 % for 1 min, 30 % for 10 min, 15 % for 3 min, and 40 % for 15 min). The solution is then diluted in a volumetric flask with bidistilled water to a final volume of 25.0 ml.

The metal contents can then be determined.

3. Procedure

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The dissolved samples are nebulised and the resulting aerosol is transported in a plasma torch induced by a high frequency electric field. The emission spectra are dispersed by a grating spectrometer and the line intensity is evaluated by a detector (photomultiplier). The detector signals are processed and controlled by a computer system. A background noise correction is applied to compensate for the background noise variations.

4. Expression of the results

The metal concentrations in chitosan are expressed in mg/kg.

Appendix IV: Total bacteria count by counting the colonies obtained at 30 °C

PCA medium

Composition:

Peptone	5.0 g
Yeast extract	2.5 g
Glucose	1.0 g
Agar-agar	15 g
Adjusted to	pH 7.0
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 30 °C in aerobiosis for 48 to 72 hours.

Count the CFU number.

Appendix V: Enumeration of *Enterobacteria* is carried out according to the horizontal method by means of the colony count technique at 30 °C

VRBG medium

Composition:

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Peptone	7 g
Yeast extract	3 g
Glucose	10 g
Sodium Chloride	5 g
Crystal Violet	0.002 g
Neutral Red	0.03 g
Agar-agar	13 g
Bile salts	1.5 g
Adjusted to	pH 7.4
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.
The Petri dishes are inoculated by pour plate method and spiral plating method.
After inoculation, they are incubated at 30 °C in aerobiosis for 18 to 24 hours.
Count the CFU number.

Appendix VI : Enumeration of yeasts by counting

YGC medium

Composition:

Yeast extract	5.0 g
D-glucose	20 g
Agar-agar	14.9 g
Choramphenicol	0.1 g
Adjusted to	pH 6.6
Water	complete to 1000 ml

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The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 25 °C in aerobiosis for 3 to 5 days without being turned over.

Count the number of yeasts.

Appendix VII : Enumeration of the moulds by counting

YGC medium

Composition:

Yeast extract	5.0 g
D-glucose	20 g
Agar-agar	14.9 g
Choramphenicol	0.1 g
Adjusted to	pH 6.6
Water	complete to 1000 ml

The medium is sterilised before use in an autoclave at 120 °C for 20 min.

The Petri dishes are inoculated by pour plate method and spiral plating method.

After inoculation, they are incubated at 25 °C in aerobiosis for 3 to 5 days without being turned over.

Count the number of moulds.

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II.2.1.22 Fining using chitosan

Classification:

- Chitosan: processing aid

Definition:

Addition of chitosan of fungal origin for the purpose of fining musts

Objectives:

- To facilitate settling and clarification
- To carry out a treatment to prevent protein haze

Prescriptions:

a) The doses to be used are determined after preliminary testing. The recommended dose used should be less than or equal to 100 g/hl.

b) Chitosan must comply with the requirements of the International Oenological Codex.

Recommendation of the OIV

Admitted

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II.3.2.12 Fining using chitosan

Classification :

- *Chitosan : processing aid*

Definition:

Addition of chitosan of fungal origin for the purpose of fining wines

Objectives:

a) To reduce turbidity by precipitating particles in suspension.

b) To carry out a treatment to prevent protein haze by the partial precipitation of excess proteinaceous matter.

Prescriptions*:*

a) The doses to be used are determined after preliminary testing. The maximum dose used must not exceed 100 g/hl.

b) Sediments are eliminated by physical procedures.

c) Chitosan of fungal origin may be used alone or together with other admitted products.

d) Chitosan must comply with the requirements of the International Oenological Codex.

Recommendation of the OIV

Admitted

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