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Review

Chitin and Chitosan in the Alcoholic and Non-Alcoholic Beverage Industry: An Overview

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Abstract: The natural biopolymer chitin and its deacetylated derivative chitosan are abundant in nature. They are obtained from different sources, including the crustacean shells and the cell wall of fungi. Chitin and chitosan have various applications in the beverage industry, such as a flocculent to improve the clarification process, for the reduction of metals and contaminates, and to extend shelf-life. They are also used as material for the immobilization of microorganisms and enzymes, which allows the development of bioprocesses that preserve the quality of alcoholic and non-alcoholic beverages. Therefore, the main purpose of this overview is to consolidate some of the current practical applications of chitin and chitosan in the alcoholic and non-alcoholic beverage industry and to reveal new perspectives.

Keywords: clarification agent; metals reduction; contaminants reduction; antioxidant activity; antimicrobial activity; immobilization

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1. Introduction

Chitosan (deacetylated chitin) has been gaining increasing attention due to its biodegradability, renewability, nontoxicity, and non-allergenic properties in the case of fungal chitosan [1]. Chitin is synthesized by a vast quantity of living organisms and occurs as a building material in the cell walls of fungi and yeast or the exoskeleton of arthropods, crustaceans (shrimp and crabs), insects (beetles, ants, brachiopods, scorpions, and cockroaches) as well as in algae (green algae and brown algae) [2,3]. Chitosan is considered to be generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). Chitosan has been described as having several applications in the beverage industry (alcoholic and non-alcoholic beverages), such as a chelating agent for metal ions, antioxidant, antimicrobial, flocculant, and as a clarifying agent [4–6]. Chitosan and chitin have been applied as flocculants [7] and can be used for clarification of non-alcoholic and alcoholic beverages, such as fruit juices [8–11] and beer [4], since these increase the suspended particles sedimentation rate and remove particles that could, for example, impact in color and limpidity. Only fungal chitosan is authorized by the European authorities and by the International Organisation of Vine and Wine to be used in wines as a fining agent for clarification, reduction of heavy metals, prevention of iron and copper haze, reduction of the contaminant contents (particularly ochratoxin A), and for antimicrobial action (namely, *Brettanomyces* spp.) [12]. Several authors have studied the application of chitosan in wine, for example, to wine protein stabilization [13–16], for avoiding wine oxidation [17–20], as an antimicrobial agent [21–25], and for removing volatile phenolic compounds from red wine [26–28]. Recently, Castro-Marín et al. [29] published a review article concerning the different applications of chitosan in winemaking and summarizing the chemical mechanisms underlying its action.

The amino groups of chitosan are protonated in acidic environments, and chitosan is expected to show a performance characteristic of a polyelectrolyte [4]. Electrostatic interaction between chitosan (positively charged) and the acidic protein pepsin (enzyme) has been shown to be dependent on pH values [30]. Moreover, the interaction between

chitosan and pectin has been studied by Marudova et al. [31], describing the action of chitosan as an effective crosslinker at pH 5.6, and exhibiting gel behavior dependent on the pectin esterification degree. It has also been shown that the complexation of chitosan with alginate, pectin, or carrageenan produces coagulating agents with enhanced protein adsorption and greater limpidity increase than only chitosan application [32].

This overview summarizes the scientific publications reporting the application of chitin and chitosan in the alcoholic and non-alcoholic beverage industry, such as a clarification and chelating agent, as well as to extend the quality and shelf-life of beverages, and as a material for the immobilization of enzymes and microorganisms.

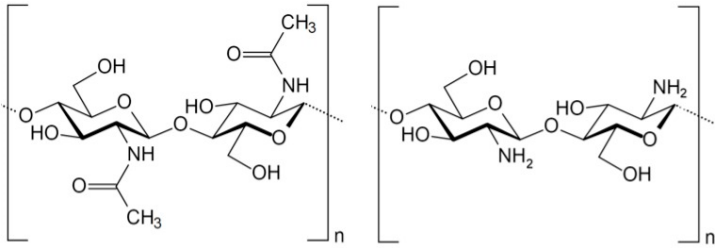
2. Chitin and Chitosan Structural Properties

Chitosan is a natural polysaccharide containing glucosamine and N-acetylglucosamine monomeric units. Chitin is produced by chemical treatments involving the extraction by acid treatment to dissolve the calcium carbonate, named demineralization, followed by the alkaline solution to dissolve proteins, called deproteinization [2,33]. The chitin extraction could also be performed by biological treatments using enzymes and microorganisms [34], namely proteolytic enzymes to digest the proteins or a fermentation process using microorganisms, which permits digestion of both proteins and minerals [35,36]. A decolorization process is also frequently performed to eliminate pigments to obtain colorless pure chitin [33]. This process should be adjusted according to the chitin source, due to the diversity in the ultrastructure of the chitin material obtained from the different sources [33]. Chitosan can then be obtained by partial deacetylation of chitin through hydrolysis by a chitin deacetylase [37–39] or by a chemical procedure [40]. From a chemical point of view, both acids and alkalis can be used to deacetylate chitin; nevertheless, glycosidic bonds are highly vulnerable to an acid; thus, alkali deacetylation is more frequently used [41]. At an industrial scale, deacetylation is usually a nonenzymatic process whereby chitosan is obtained by removing R-NHCOCH$_3$ residue by treating it with strong alkali (sodium hydroxide solution 40–50%) at high temperatures (100 °C) [40].

Deacetylation describes a reaction that removes an acetyl functional group. When the degree of deacetylation (expressed as a molar percentage) is higher than 50 mol%, the biopolymer becomes soluble in acidic aqueous solutions and is called chitosan and behaves as a cationic polyelectrolyte due to the protonation of amine groups in the presence of H^+ ions [42].

Therefore, chitosan—poly-β-(1,4)-D-glucosamine is a deacetylated cationic linear biopolymer of chitin—poly-β-(1,4)-N-acetyl-D-glucosamine (Figure 1), one of the most abundant natural polysaccharides found in the composition of fungi cell walls [43–45] and exoskeletons of insects [46–49], cephalopods, and crustaceans [49–54]. Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine and N-acetyl-D-glucosamine units. The ratio between these two monomeric units, expressed as the degree of acetylation or deacetylation, ranges from 40 to 99% (degree of deacetylation) and the molar weight ranged from 2 to more than 200 kDa [55]. The chitosan functional proprieties are dependent on the structural characteristics, for instance, degree of deacetylation, molecular weight, and purity. Various chitosans are available commercially, which differ primarily in the degree of deacetylation and molecular weight.

The acetylation degree and molecular weight has been reported to be important chemical characteristics of chitosan, which could significantly influence their proprieties in their several applications [56]. Furthermore, the free amino groups of chitosan are an important characteristic for physical properties, including solubility, chemical properties such as reactivity with other functional groups, and biological properties such as antioxidant and antimicrobial [57].



(**A**) (**B**)

Figure 1. Chitin (**A**) and chitosan (**B**) chemical structure.

3. Chitosan Applications in Beverages

3.1. Clarification Agent, Metals and Contaminants Reduction

In beverages such as fruit juice, clarification is an important step in the production process mainly to remove pectins and other compounds, which are present in the juice. Juice limpidity and homogeneity are the two major characteristics of clarified juices and are achieved by the complete removal of all suspended solids [58]. Chitosan coagulates the anionic suspended particles such as pectin and protein and consequently, their separation from beverages is fast and their turbidity decreases [6,10,59,60]. This behavior is related to the chitosan physicochemical properties associated with the presence of amine functions [31]. According to Rizzo et al. [61], the concentration of chitosan used in the clarification process and pH in addition to the initial juice turbidity to be treated are important variables for the coagulation process using chitosan and consequently to reach the desired limpidity. Domingues et al. [62] also showed that besides the chitosan concentration, the pH value is an essential factor for the juice turbidity to decrease, as all samples of passion fruit juice at pH 6 presented higher turbidity removal after the treatment with chitosan. Additionally, Rao et al. [6] showed that the optimal pH for green tea clarification using chitosan was at pH 5.5. It was observed a concentration-dependent, leading to higher turbidity reduction in juices when chitosan content was increased [60,62]. It was also shown that chitosan with a higher deacetylation degree is more effective in protein flocculating, this could be related to the augmented charge density from the free amino groups [63].

Several works have reported the positive effect of the application of chitosan as a clarifying and protein stabilization agent for apple, grape, lemon, orange, passion, açai juices, and green tea [6,59,60,62,64], and grape must and wine [13–16]. The application of fungal chitosan as a fining agent to facilitate settling and clarification, as well as a treatment to prevent protein haze in grape must and wine, is allowed by the International Organisation of Vine and Wine (OIV) [12], enology resolutions, OIV/OENO 336B/2009 and OIV/OENO 337A-2009, at a maximum dose of 100 g/hL.

Chitosan is mentioned in the literature as being a good clarification agent for grapefruit juice both with and without enzyme treatment [65] and a significantly effective clarification agent for apple juice [8]. More recently, Tastan and Baysal [10] showed that chitosan can be an alternative clarification agent to produce pomegranate juice with an excellent limpidity and with quality characteristics, such as pomegranate juice clarified with bentonite and gelatin. Chitosan has been shown to reduce the acidity of the fruit juices; according to Chatterjee et al. [59] the titratable acidity of grape juice clarified with bentonite–gelatin and chitosan were 3.24% and 3.10%, respectively. Furthermore, Martin-Diana et al. [66] showed that chitosan enriched juices had a higher pH value of 4.2 as compared to their original pH of 3.2; this effect could be due to the polycationic nature of chitosan and thus, its acid-binding properties. It was described that the turbidity of apple juice decreased 73.3% after the application of chitosan for 90 min. Rungsardthong et al. [60] likewise showed that apple juice clarified with fungal chitosan at 0.7 g/L and 40 °C achieved the maximum juice limpidity. Tastan and Baysal [11] showed also that apple juice clarification with traditional fining agents, such as bentonite and gelatin, was less effective than chitosan in decreasing the turbidity of apple juice; these results are following the previously shown by Chatterjee

et al. [59] who also described bentonite or gelatin to be less effective than chitosan during juice clarification. However, the high price of chitosan may be considered a disadvantage if compared to the bentonite and gelatin that are traditionally used in juice clarification [11].

Green tea beverage is colloidally unstable since it contains protein, phenolic compounds, free amino acids, polysaccharides, and pectin. Consequently, the limpidity of tea infusion could modify, producing the change of light scattering, the so-called tea cream [67]. The formation of complexes between monomeric or dimeric phenolic compounds and proteins to form a larger colloidal particle [68–70] during storage is responsible for the haze development in beverages Chitosan was able to coagulate the anionic compounds present in tea infusion, such as pectin and protein. Additionally, chelate metal ions selective and separate the suspended particles from beverages. Rao et al. [6] showed that chitosan was able to clarify green tea infusions by significantly decreasing the haze-active proteins without a significant effect on the composition of the phenolic compounds.

Gassara et al. [4] showed that chitin and chitosan were able to flocculate colloidal particles from beer; however, chitosan was found to be most effective at a concentration of 5 mg/L, presenting a higher flocculation activity. This occurrence could be elucidated based on charge density, as chitosan has a high charge density, and it is known that polymer adsorption improves as the charge density increased [71]. The pH of beer samples was shown to be between 4.5 and 4.7, which could affect the chitosan behavior, as the amino groups are protonated (positively charged) in an acid solution [7], and the beer particles in suspension are negatively charged; the molecules bind to chitosan's negatively charged surface via ionic or hydrogen bonding.

Chitosan can bind with various metal ions i.e., chelating, such as cadmium, cobalt, manganese, copper, iron, zinc, lead, and mercury [72,73]. The intrinsic pKa value of chitosan rigorously depends upon the degree of deacetylation, ionic strength, and the charge neutralization of $–NH_2$ groups. For fully neutralized amine functions with a degree of acetylation of not more than 50%, the pKa value is always between 6.3 and 6.7 [74,75]. Therefore, at pH < 6.2, chitosan presented a positive surface charge due to protonation of the amine groups, which makes chitosan an excellent polymer to be used as a chelating agent [76], as protonation of the amine group leads to the sorption phenomenon of metal cations in acidic media because proton and metal cations compete for interaction with the amine group. Liu et al. [77] successfully applied chitosan to remove arsenic from *Laminaria japonica* Aresch juice. In wine, the reduction of the heavy metal contents with chitosan has been studied by several researchers and shown to be effective [20,73,78]. This practice is authorized by the OIV [12] to avoid wine iron and copper instability and to reduce heavy metals (iron, lead, cadmium, and copper) at a maximum dose of 100 g/hL.

Studies were also performed to reduce wine contaminants by the application of chitosan, such as for the reduction of ochratoxin A [73,79,80]. The OIV [12] also allows the application of chitosan to reduce possible wine contaminants, especially ochratoxin A, at a maximum dose of 500 g/hL.

It was also reported by Venkatachalapathy et al. [81] that chitosan removed efficiency pesticides (54–72% with 0.05% chitosan concentration and 86–98% with up to 0.5% chitosan concentration) from grape juice during the clarification process.

3.2. Extending the Shelf-Life

There is an increase in consumer demand for safe and healthier products; in this way, an increase in the consumption of freshly processed products has been observed, and the beverage industries need to search for compounds to extend the shelf life of these products by application of natural products. The antimicrobial and antioxidant activity of chitosan permits its application to extend the beverage shelf life, as shelf life is constrained by microbial spoilage and oxidation [57,82–84]. The antioxidant activity of chitosan is related to the scavenging effect on free radicals [83,85,86]. Chitosan deacetylation degree influenced its antioxidant activity, and this activity rises with unsubstituted amino groups [57]. Therefore, the antioxidant activities of chitosan increased with an increase

in deacetylation degree [87]. The molecular weight of chitosan has also been described to have a main effect on its antioxidant activities, with low molecular weight (16 to 190, 127 kDa), chitosans having more marked scavenging effects on superoxide and hydroxyl radicals than those with high molecular weight (>300 kDa), probably related to the compact structure of the chitosan with a high molecular weight that limited the antiradical activity of the hydroxyl and amino groups [88,89].

For antimicrobial activity, the most important factors are the type of microorganism [84,90–92], the chitosan charge density, concentration, molecular weight [84,92], hydrophilic/hydrophobic characteristics, chelating capacity, and the degree of deacetylation [84]. It has been suggested that the polycationic nature of chitosan that forms from acidic solutions below pH 6.5 is a crucial factor. A higher positive charge density leads to strong electrostatic interaction. Therein, the positive charge is associated with the deacetylation degree of chitosan [93]. The chitosan antifungal and antimicrobial activity against different fungi, Gram-positive and Gram-negative bacteria, is related to the chitosan cationic properties in an acidic media at pH values below chitosan pKa. As protonated amino groups bind to the negatively charged carboxyl groups, such as bacterial cell wall surface peptidoglycans, altering their barrier properties, leading to permeabilization and destruction of external membranes [94]. Therefore, Chitosan is most active at the fungi or bacteria cell surface leading to permeabilization [90,92,95–97], which results in leakage of intracellular material and consequently in cell death [96,98,99].

Challenges in the wine industry are preventing wine spoilage, maintaining wine color, and avoiding aromatic defects by limiting oxidation. Sulfur dioxide may be added to achieve these objectives; however, sulfur dioxide may have adverse effects on human health, such as pseudo-allergies [100,101], and winemakers are, therefore, trying to limit the use of sulfites (mainly sulfur dioxide) in the winemaking process [14,102,103]. Natural products such as metabolites produced by living organisms and/or those naturally occurring in nature have been studied, aiming to prevent microbial spoilage, and thus are an appropriate alternative to the use of synthetic products, such as sulfur dioxide [104]. Among the products studied, chitosan meets these requirements, as several activities have been demonstrated for different types of chitosan, namely as a preservative and an antimicrobial agent [17,21,85,105,106]. It has already been shown that chitosan has antimicrobial effects against bacteria (lactic acid bacteria and acetic acid bacteria), fungi, and spoilage yeasts, such as *Brettanomyces* sp., during wine storage [23,25,107]. However, it is permissive for the growth of *Saccharomyces* species [22,25,108]. In this way, OIV- OIV/OENO 338A/2009 resolution [12] allows the use of fungal chitosan to control the development of undesirable microorganisms, namely *Brettanomyces* sp at a maximum dose of 10 g/hL.

In orange juice, the application of chitosan showed a quality improvement in reducing enzymatic and non-enzymatic browning and controlling the spoilage during the storage period, avoiding the application of the traditional thermal treatments (pasteurization), which produces a negative impact on the nutritional value [66]. These authors showed that it is a necessary equilibrium between quality and nutritional values, as greater chitosan content enhanced quality; but it decreases vitamin C due to chitosan application related to the flocculation ability of chitosan, suggesting the application of lower chitosan doses in the orange juice. Moreover, Sapers [109] has already previously studied the effect of chitosan application on apple and pear juice enzymatic browning. The authors indicated that browning could be prevented in apple juice, for example, by the addition of at least 200 mg/L chitosan, independently of the type of chitosan applied (low or high molecular weight). The effectiveness of chitosan treatments to control enzymatic browning in apple and pear juices may be due to the capacity of the positively charged polymer to coagulate suspended solids to which polyphenol oxidase (PPO) is bound [109].

Apple juice research data has indicated that chitosan showed antifungal properties in juice [107,110], and as mentioned previously, has been shown that in apple juice, low-molecular-weight chitosan exhibited higher antioxidant and free radical scavenging effects than high-molecular-weight chitosan [88].

4. Chitosan Immobilization

Chitosan, like alginate, forms a gel by ionotropic gelation (or coacervation) and is a polymer with numerous applications in immobilization technology of enzymes and microorganisms, due to its nontoxic, biocompatible, biodegradable, and antimicrobial properties [111].

Chitosan-bearing protonated amino groups can interact with a broad variety of natural or synthetic anionic species, such as negatively charged proteins, DNA [112,113], and synthetic basic polymers, such as sodium tripolyphosphate (TPP) [114,115] to form ionic complexes. Ionotropic gelation has been used in the production of polymeric (micro and nan) particles for many applications, namely in biomedicine [116], and the pharmaceutical industry, including interferon [117–119] and antioxidant administration [120]. This technique is versatile and relatively simple. It is possible to produce particles in a wide range of sizes [121]. This ionic gelation method to prepare Chitosan/TPP nanoparticles presents the advantages of simple operation, low equipment requirements, low cost, good repeatability, environmentally friendly, and easy large-scale preparation [122,123], Figure 2.

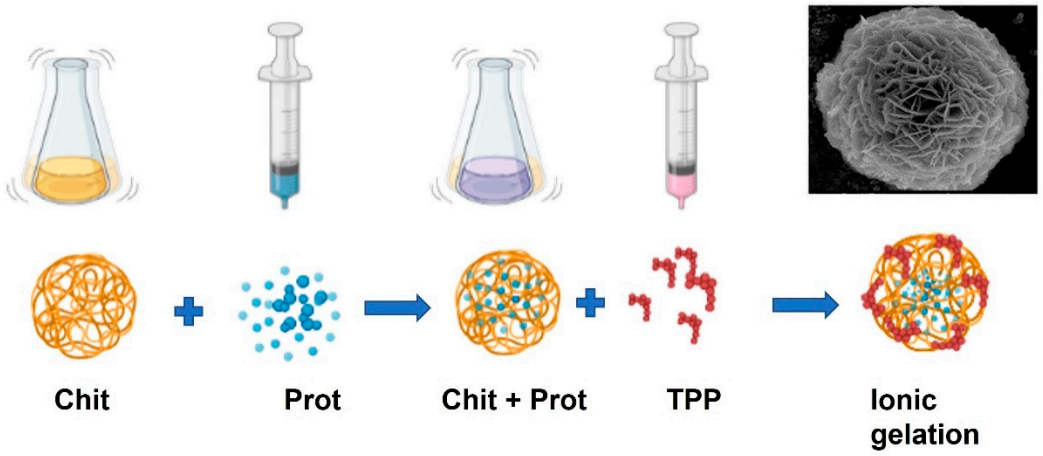


Figure 2. Schematic illustration of the preparation procedure for protein chitosan immobilization. Protein-loaded chitosan beads can be prepared by an ionic gelation technique. Chitosan (Chit) is firstly mixed with protein (Prot) to provide a homogeneous solution (Chit + Prot). Then interacts with sodium tripolyphosphate (TPP) solution to allow the protein to be encapsulated during the ionic gelation process.

In the literature, three other immobilization methods are mentioned: (i) solvent evaporation method, (ii) neutralization method, and (iii) crosslinking method [124]:

(i) Solvent evaporation method or its variant-spray drying method [125] is mostly used for the preparation of membranes and films, effective in the preparation of enzymatically active surfaces placed on tips of electrodes [124] or for the entrapment of the hydrophobic drugs [126,127].

(ii) The neutralization method is usually exploited to produce chitosan precipitates, membranes, fibers, and spherical beads of different sizes and porosities. Beads are obtained by dropping a chitosan solution into a solution of NaOH prepared in water-ethanol mixtures once ethanol facilitates the solidification of the beads [124]. Nowadays, this method is also used to produce chitosan films with favorable physical and barrier properties for improving the preservation of chilled meat [128].

(iii) Crosslinking method where an acidic chitosan solution is exposed to straightforward crosslinking by mixing with a crosslinking agent, which results in gelling [124]. Recently, a new emulsification-crosslinking method was developed by Shi et al. [129] for the preparation of chitosan microspheres using an aqueous alkali–urea solution, as an alternative to the commonly used acidic solvents, to dissolve chitosan.

4.1. Chitosan Microorganism's Immobilization

Microorganisms' immobilization consists of the physical confinement of intact cells to a region of space with conservation of biological activity. The use of these methodologies for alcoholic fermentation offers many advantages over the use of the conventional free yeast cell method. The most studied methods for yeast immobilization include the use of organic supports, mainly alginate. Some advantages of the yeast-immobilization systems include high cell densities, product yield improvement, lowered risk of microbial contamination, better control, and reproducibility of the processes, as well as reuse of the immobilization system for batch fermentation and continuous fermentation technologies [130,131].

According to Martin and Etievant [132], for alcoholic beverage production, the cell carrier or immobilization matrix, must fulfill certain requirements: (i) Large surface, with chemical groups favoring cells to adhere; (ii) Easy to handle and regenerate; (iii) High and retained cell viability and operational stability; (iv) Does not affect catalytic activity; (v) Uniform and controllable porosity to allow the free exchange of substrates, products, cofactors, and gases; (vi) Mechanical, chemical, thermal, and biological stability; (vii) Easy to handle, cost-effective, and amenable to scale-up immobilization technique; (viii) Does not affect product quality.

Chitosan can be used in one single layer [133] or double-layer alginate–chitosan beads. Double layer beads have been used for the entrapment of bacterial and yeast cells in batch and continuous fermentation [134], alcoholic fermentations [135], sparkling wine fermentation by the traditional method [136], and in the reduction of the volatile acidity of acidic wines [137]. The presence of an outer chitosan layer improves the mechanical and chemical stabilities of the beads during fermentation and prevents cell leakage from the beads into the medium [137].

In a work performed by Vilela et al. [137] concerning the reduction of volatile acidity of acidic wines, immobilized *S. cerevisiae* cells (8.0×10^7 cells/mL; double-layer alginate–chitosan beads) were made by ionic gelation method and used in a biological deacidification process.

For the cell's immobilization procedure, pre-treated cells were centrifuged (5500 rpm at 4 °C for 10 min) and resuspended in an aqueous alginate solution (alginic acid sodium salt from brown algae, 2% w/v). The alginate cell suspension was then dropped into a $CaCl_2$ solution (0.18 M), with a needle attached to a 10-mL syringe, cooled at 4 °C, and homogenized by magnetic agitation (100 rpm). The beads remained in the $CaCl_2$ solution for 1 h (4 °C) and were then transferred to an Erlenmeyer flask (1000 mL) with 250 mL of low-molecular-weight chitosan (1 or 1.5% w/v) and incubated for 24 h at 25 °C with magnetic agitation (100 rpm). Lastly, beads were washed twice with sterilized water and transferred to 250-mL Erlenmeyer flasks. Approximately 100 beads were used in 230 mL of acidic wine. After a successful deacidification process, the beads were analyzed by scanning electron microscopy (SEM). It was possible to verify that the beads had an ellipsoidal shape (Figure 3A) and an average diameter of 500–2000 μm. SEM analysis also showed that the beads had an irregular surface, and some yeasts were observed near the bead surface, however, the external membrane maintained its integrity (Figure 3B) and there was no cell leakage to the deacidified wine [137]. Coating the alginate beads with chitosan (1.0% w/v) led to better external membrane integrity after the deacidification process. However, external chitosan integrity was pH-dependent. Total integrity was observed at pH 3.12 while at pH 3.50 some cell leakage occurred [137].

Wu et al. [138] immobilized *Rhodotorula mucilaginosa*, producing the ethyl carbamate (EC)-degrading enzyme, urethanase. The yeasts were able to remove 80% of EC when it was incubated with 5.0 g/L EC. Urethanase activity reached 4340.0 U/L in the optimal fermentation conditions. Cell immobilization of *R. mucilaginosa* in calcium alginate/chitosan was applied to improve cell resistance to environmental stresses. The immobilized cells removed 10 times more EC than that the free cells.

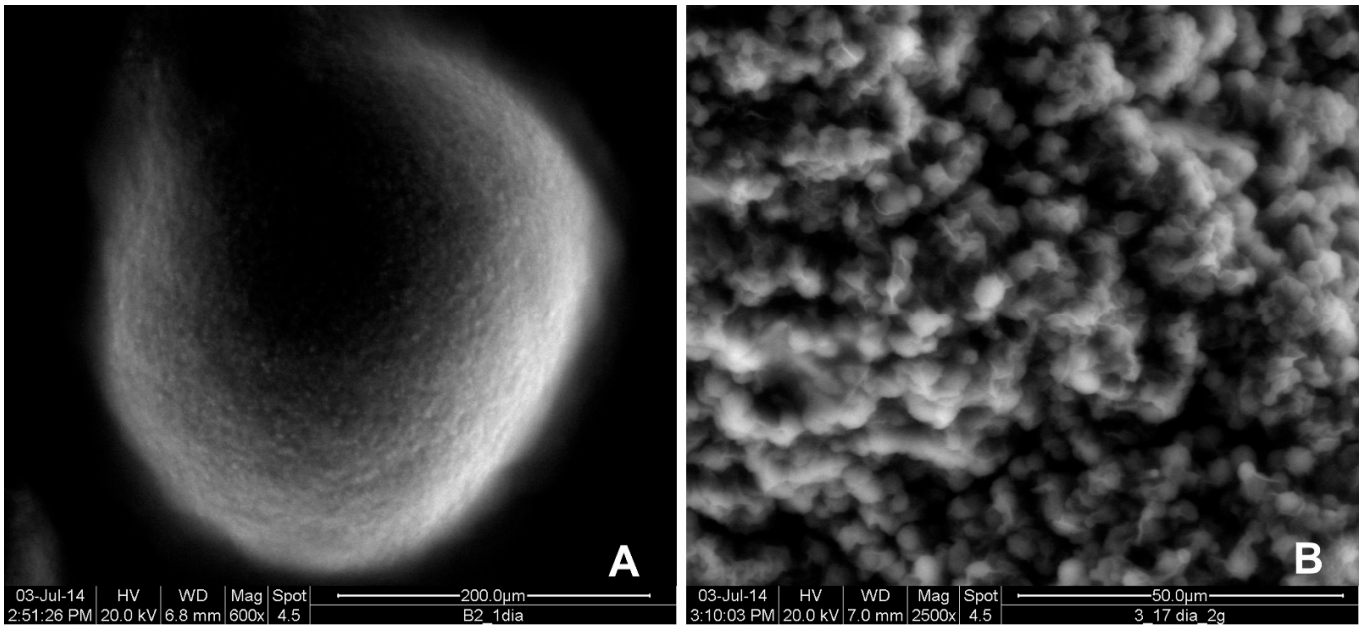


Figure 3. SEM image of *S. cerevisiae* cells, entrapped in double-layer alginate–chitosan beads: (**A**) double-layer bead with alginate 2% (*w*/*v*) and chitosan 1.0% (*w*/*v*) (×600); (**B**) external layer (chitosan 1.0% *w*/*v*) of a bead after deacidification, pH 3.12 (×2500). For SEM analysis, double-layer alginate–chitosan beads, were dehydrated (5 min in 70 % *v*/*v* ethanol followed by 5 min in absolute ethanol) and dried using critical point dehydration. Beads were then mounted into aluminum stubs and coated with a small amount of gold/palladium in a Polaron Range SC7620 Sputter Coater (Quorum Technologies Ltd., Ashford, Kent, UK). SEM analysis was performed in a Philips-FEI/Quanta 400 scanning electron microscope (FEI, Hillsboro, OR, USA).

Recently, Benucci et al. [139] developed a method to produce a Pale Ale beer fermented by a commercial yeast (*S. cerevisiae* Nottingham Ale), immobilized into chitosan–calcium alginate double-layer microcapsules. During the primary alcoholic fermentation, the consumption rate of fermentable sugars and dissolved O_2 was reduced in the beer obtained by encapsulated yeast in comparison to the free cell. Moreover, beer pH, alcohol content, color, and bitterness were not remarkably affected by the immobilized yeasts. However, the volatile profiles significantly differed in terms of terpenes, esters, and alcohol content showing a greater complexity of the aromatic profile concerning the beer fermented by encapsulated cells, thus proving that the yeast-inoculating form may typify the odor and flavor descriptors of the green beer.

4.2. Chitosan Enzyme Immobilization

Enzymes are functional biological macromolecules able to catalyze chemical reactions at very high rates and with high molecular precision. Enzymes are used by many winemakers to improve extraction, enhance aromas, and block malolactic fermentation (MLF). For instance, in white wines, monoterpenes are the most important compounds responsible for the aroma, most of these compounds are present in grapes as nonvolatile odorless glycoconjugates. Therefore, enzymatic hydrolysis has long been proposed as an alternative for the efficient release of aromatic compounds at the mild conditions of winemaking [140].

Commercial preparations of soluble enzymes from *Aspergillus niger* with α-L-arabinofuranosidase, α-L-rhamnosidase, and βG (β-D-glucosidase) activities have been used in alcoholic fermentation, usually added at the end of the fermentation stage [141]. These glycosidases are quickly inactivated in winemaking conditions media, making them less efficient. Moreover, catalyst residues remain in the final wine, thus requiring the addition of a fining agent, to stop the reaction [142]. Due to these disadvantages, the co-immobilization of glycosidases has been proposed, increasing the stability of the enzymes, and recovering the biocatalyst from the medium [143].

Cross-linked enzyme aggregates (CLEAs) allow for the immobilization of enzymes in their macromolecular matrix [144]. This technique has emerged in recent years as a substitute for carrier-bound enzymes. A relevant characteristic of this method is that it permits the co-immobilization of different enzymes, giving rise to the so-called combi-CLEAs that can be effectively applied in cascade reaction systems [144–146]. Good results have been obtained in the co-immobilization of glycosidases as combi-CLEAs for the enhancement of aromatic compounds in white wine [147]. But the small and variable particle size (5 to 50 μm) delays their recovery and prevents their application in industrial processes, namely winemaking. Therefore, the idea of entrapping the combi-CLEAs in a polymeric carrier will allow recovering the biocatalyst. Tavernini et al. [148] studied the effect of crosslinking reagents and crosslinking time on the specific activity and stability of entrapped combi-CLEAs of βG and α-L-arabinofuranosidase in polymeric chitosan beads, with an average diameter of 1.24 mm. The beads retained full activity after 91 days of incubation under winemaking conditions, making them suitable for aroma enhancement in wines. This interesting work of Tavernini et al. [148] shows that chitosan, due to its properties (high biodegradability, affinity to proteins, non-toxicity, inert) is suitable for food production and due to its electrical charges [in aqueous acidic media at pH < 6.5, is positively charged (NH_3^+ groups)], reacts with polyanionic compounds [149], offering exceptional gel-forming properties to be used for protein immobilization [150,151].

Enzyme immobilization can also be used for several other purposes. It can be used to protect wines and other fermented and non-fermented beverages from spoilage. Cappannella et al. [152] immobilized lysozyme from hen egg white (HEWL) on chitosan beads to develop a system for the continuous, and food-grade enzymatic lysis of lactic acid bacteria (*Oenococcus oeni*) in white and red wines, thus limiting the use of sulfur dioxide required to control malolactic fermentation (MLF). HEWL appeared more effective in the immobilized than in the free form, suggesting that covalent immobilization renders the enzyme less sensitive to the inhibitory effect of wine flavans. Another use is to protect consumers from undesirable fermentation by-products, such as higher alcohols (ethanol, 1-propanol, isobutanol, 1-butanol, isoamyl alcohol, and 1-hexanol) [153]. Han et al. [153] used apple crude enzymes immobilized in sodium-alginate and chitosan to decrease the higher alcohols content of Chinese liquors with 45–56% of ethanol. Significant degradation rates of higher alcohols were observed at different degrees. Guo et al. [154] immobilized triethylene tetramine-modified water-insoluble corn flour, in magnetic chitosan resin (TETA-WICF/MCR) aiming to eliminate the mycotoxin Patulin (biosynthesized by certain fungi that contaminate agricultural commodities) from apple juice to improve biocatalytic features. Such is the case of pectinases immobilized in chitosan. Pectinase has become more stable, robust, and can be recoverable [155,156].

In the work of Cacciotti et al. [157], a composite system, based on high amounts of montmorillonite and low amounts of chitosan, was proposed as potential alternatives to the free nano clay particles, commonly used as carriers for enzyme covalent immobilization. The chitosan was used to make the clay's appropriate supports for the protease bromelain and helped to maintain the clay particles together in a unique structure, acting as a binder. The same authors [158] also performed the immobilization of two enzymes (bromelain and a commercial pectinase-Pectinex® BE XXL) on chitosan beads and studied their application in pomegranate juice clarification. The enzymes were combined in a multi-enzymatic system and used in a fluidized bed reactor. At the end of the storage period, this technique allowed a significant reduction of haze-active molecules.

Besides the good properties achieved with enzymes chitosan immobilization, some cutbacks may occur. The low mechanical strength and crosslinking of chitosan sometimes require the use of agents to form more stable networks [159]. Glutaraldehyde is the compound most widely used for this purpose [160], yet this agent must be avoided in food and beverage products due to its cytotoxic effects at concentrations higher than 0.5 ppm [161]. Therefore, some studies have aimed to find natural agents with lower toxicity. One of those recently studied, is genipin (methyl (1S,2R,6S)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo

[4.3.0] nona-4,8-diene-5-carboxylate), a compound obtained from the fruits of Genipa americana and Gardenia jasminoides Ellis. Lima et al. [159] evaluated the immobilization of the enzyme β-galactosidase, produced by *Kluyveromyces* lactis, in genipin-activated chitosan support aiming to apply the enzymatic derivatives in the hydrolysis of ultra-high-temperature (UHT) diluted milk in lower temperatures. The immobilization proved to be a viable strategy for the biocatalysts' process. Genipin was efficient as an activating and crosslinking agent of the chitosan support, making the immobilized enzyme more stable and enabling mechanical resistance for the enzymatic derivatives.

5. Final Remarks

Chitosan is available in the market with different structural characteristics, mainly deacetylation degree and molecular weight. It could be used in the beverages industry for different purposes, as summarized in Table 1.

Its application in the beverage industry is to extend shelf-life due to its antimicrobial and antioxidant properties. It could also be applied as a clarification agent associated with its positive charge to reduce turbidity by precipitating particles in suspension. However, only fungal chitosan (from *Agaricus bisporus* or *Aspergillus niger*) is allowed to be used in the wine industry, as the crustaceous chitosan is restricted due to the allergenicity.

Chitosan can be used to immobilize microorganisms and enzymes, allowing high cell densities, product yield improvements, lowered risk of microbial contamination, better control, and reproducibility of the industrial processes, as well as the possibility of reusing the immobilized systems for batch fermentation and continuous fermentation technologies, while enzyme immobilization, besides improving enzymatic reactions by protecting enzymes from chemical degradation, may be used to protect beverages from spoilage and consumers from undesirable fermentation metabolites.

Aiming at achieving higher mechanical strength and crosslinking of chitosan beads, glutaraldehyde and genipin can be used. However, caution must be taken due to the possible health issues related to these kinds of compounds, namely glutaraldehyde.

Table 1. Chitosan origin and type, activity/advantages of application in beverages.

Chitosan Origin/Type	Beverage	Activity/Advantages	Reference
Chitin and chitosan	Beer	Application of chitin (5 mg/L) and chitosan (5 mg/L) for clarification and haze removal in beer. Chitin and chitosan showed the ability to flocculate beer colloidal particles, being chitosan's most effective.	[4]
Chitosan (deacetylated: 95%, molecular weight 100 kDa)	Green tea infusions	Chitosan coagulates the anionic compounds (pectin, proteins, etc.) from the tea infusion, and chelate metal ions selectively, separating the suspended particles from beverages. Being therefore effective to clarify green tea infusions	[6]
Commercial water soluble chitosan (Carboxymethyl chitosan, CAS No. 83512-85-0) deacetylation degree of 90–95%, medium molecular weight	Pomegranate juice	Clarifying agent	[10]
Chitosan from squid gladius (*Loligo vulgaris*) Deacetylation degree—71%	Apple juice	Application as a clarifying agent without affecting nutritional value. Chitosan (400 mg/mL) exhibited high antimicrobial activity against. *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Fusarium solani*, *Botrytis cinerea*, *Alternaria solani*.	[11]
Fungal chitosan (from *Agaricus bisporus* or *Aspergillus niger*)	Grape must and wine	Application as a fining agent to facilitate settling and clarification, as well as a treatment to prevent protein haze, allowed, at a maximum dose of 100 g/hL (OIV-OENO 336B/2009; OIV-OENO 337A-2009).	[12]
Fungal chitosan (from *Agaricus bisporus* or *Aspergillus niger*)	Wine	Application to avoid wine iron and copper instability and to reduce heavy metals (iron, lead, cadmium, and copper) at a maximum dose of 100 g/hL (OIV-OENO 338A/2009), to reduce possible wine contaminants, especially ochraoxin A, at a maximum dose of 500 g/hL (OIV-OENO 338A/2009) and to control the development of undesirable microorganisms, namely *Brettanomyces* sp. at a maximum dose of 10 g/hL (OIV-OENO 338A/2009).	[12]
Chitosan from shrimp shell	Fruit juice	Application of chitosan at low concentration showed to be effective in the clarification of different fruit juices (apple, grape, lemon, and orange juices).	[59]
Acid-soluble fungal chitosan (*A. glauca* var. *paradoxa*) Degree of deacetylation 86%.	Apple juice	The fungal chitosan is highly effective in reducing juice turbidity.	[60]
Chitosan from shrimp shells (Sigma-Aldrich, Iceland)	Passion fruit juice	Clarifying agent	[64]
Chitosan from crab shells (Sigma-Aldrich Ireland Ltd., Dublin, Ireland) (0 to 2 g/L)	Orange juices	Extending the shelf-life–it was shown that increasing chitosan concentration extended the quality of the orange juice, reducing enzymatic and non-enzymatic browning and controlling the spoilage during the storage time.	[66]

Table 1. *Cont.*

Chitosan Origin/Type	Beverage	Activity/Advantages	Reference
Chitosans with low molecular weight (LMWC, MW = 12 kDa), medium molecular weight (MMWC, MW = 95 kDa) and high molecular weight (HMWC, MW = 318 kDa)	Apple juice	LMWC exhibited stronger scavenging activity toward DPPH radicals, superoxide anion radicals and hydrogen peroxide., therefore could increase anti-oxidant activity in apple juice	[86]
Chitosan glutamate (Drammen, Norway). 42% glutamate; deacetylation degree range of 75–85%	Apple juice	Antifungal properties for foods prone to fungal spoilage	[110]
Chitosan hydrochloride (SEACURE CL 110) from FMC Biopolymer A/S-Drammen, Norway	Fermented milk	Chitosan coated alginate-beads reduced the final concentrations of free cells, the initial release of free cells, and the rate of lactate production in milk fermented batch-wise to a final pH of 4.7 in five consecutive batch fermentations.	[134]
Chitosan Sigma Aldrich (Milano, Italy)	Riesling sparkling wine	Application of encapsulated yeast in chitosan-alginate microcapsules, produced a sparkling wine having sensory properties like those produced by free yeasts (both adapted and non-adapted to ethanol) in terms of aroma, taste, and body.	[136]
Low-molecular-weight chitosan (Sigma-Aldrich-St. Louis, MO, USA)	Acidic wine	Significantly reduced the volatile acidity of an acidic wine	[137]
Not discriminated	Chinese Rice Wine	Cell immobilization in chitosan-alginate beads was applied to improve *R. mucilaginosa* cell resistance, and 51.6% of Ethyl Carbamate (EC) in commercial rice wine was removed by the immobilized cells. This was the first to remove EC by urethanase from *R. mucilaginosa.*	[138]
Chitosan (low molecular weight, 75–85% deacetylated)	Pale Ale beer	Application of a commercial brewing yeast (*S. cerevisiae* Nottingham Ale), entrapped into chitosan–calcium alginate double-layer microcapsules, to produce a Pale Ale beer with an improved flavor profile.	[139]
Chitosan from *A. niger*	Italian wines (Sauvignon blanc and Sangiovese)	Applying the immobilized HEWL (lysozyme from hen egg white) appeared more useful than the free form, in the continuous lysis of lactic bacteria in real white (Sauvignon blanc) and red (Sangiovese) wine.	[152]
Chitosan, with a deacetylation level of about 90%, from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China)	Apple juice.	The adsorption performance of the TETA-WICF/MCR obtained toward patulin, in apple juice, demonstrated that was depended on adsorbent dosage, contact time, temperature, and initial patulin concentration. It was also found that the TETA-WICF/MCR had good reusability without any adverse changes in apple juice.	[154]

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