Relevance and perspectives of the use of chitosan in winemaking: a review

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REVIEW



# Relevance and perspectives of the use of chitosan in winemaking: a review

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**ABSTRACT**

Chitosan is a natural polymer that has quite recently been approved as an aid for microbial control, metal chelation, clarification, and reduction of contaminants in enology. In foods other than wine, chitosan has also been evidenced to have some other activities such as antioxidant and antiradical properties. Nevertheless, the actual extent of its activities in must and wines has not been fully established. This review aimed to gather and discuss the available scientific information on the efficacy of chitosan as a multifaceted aid in winemaking, including antimicrobial, chelating, clarifying and antioxidant activities, while summarizing the chemical mechanisms underlying its action. Attention has been specifically paid to those data obtained by using unmodified chitosan in wine or in conditions pertinent to its production, intentionally excluding functionalized polymers, not admitted in enology. Unconventional utilizations together with future perspectives and research needs targeting, for example, the use of chitosan from distinct sources, production strategies to increase its efficacy or the potential sensory impact of this polysaccharide, have also been outlined.

**KEYWORDS**

Wine; fining; chitosan; antimicrobial; chelation; antioxidant

## Introduction

Chitin is the second most abundant polysaccharide on earth, after cellulose. This biopolymer, composed of 2-acetamido-2-deoxy-$\beta$-D-glucose (N-acetylglucosamine) units linked by $\beta(1\longrightarrow 4)$ linkages (Figure 1), is synthesized in great amounts by a large number of living organisms, and forms the exoskeleton of arthropods and insects, the crustacean shells, and the cell walls of fungi and plants (Rinaudo 2006).

A main derivative of chitin is chitosan (KT), which can be industrially obtained by N-deacetylating chitin to varying extents (>50%) through a process involving deproteinization, demineralization, decolorization, and deacetylation (Aranaz et al. 2009). Deacetylation produces free amine groups ($-NH_2$) along the polysaccharide backbone. This confers to KT a polycationic character and, depending on the deacetylation degree (DD) and molecular weight (MW), changed solubility in acidic media (Friedman and Juneja 2010), and renders it a polymer that differs from other neutral or negatively charged natural polysaccharides. Chitosan can be prepared in different forms, such as films, gels, beads, nano/micro particles, and this possibility, together with its biodegradability, biocompatibility, and low toxicity makes it a versatile compound with a vast applicability in many fields, including food, medicine, cosmetics, and pharmaceutical sciences (Friedman and Juneja 2010; Kurita 1998; No et al. 2007).

In addition, the chemical structure of KT is highly eligible to be functionalized with a vast diversity of ligands by means of reactions such as carbonylation, alkylation, sulfonation, carboxymethylation, and quaternization, which enlarge enormously the potential applicability of the modified KT (Brasselet et al. 2019; Higueras et al. 2015; Rocha, Coimbra, and Nunes 2017).

Native KT is particularly of interest over synthetic polymers for application in food sector. Because of its versatility, KT has gathered the attention of both researchers and food technologists pursuing multiple objectives, such as protection against microbial spoilage, storage of fruits and vegetables, deacidification and clarification of juices, removal of solid material from water, and control of oxidation (Rinaudo 2006; Kong et al. 2010; Shahidi, Arachchi, and Jeon 1999). In addition, it 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, without objections from that administration (Food and Drug Administration 2011). In the last decade, KT has been accepted by the European Commission as a fining agent for the treatment of wines, for different purposes: prevention of iron and copper casses, reduction of heavy metals or possible contaminants, especially ochratoxin A, and inhibition of unwanted microbial growth, particularly *Brettanomyces* spp. (European Commission 2011). Despite its insolubility in must and wine, to avoid any potential concerns of allergenicity because of the crustacean raw material, only fungal KT (from *Aspergillus niger*) is admitted in winemaking, as the functionality and structure of the two chitosans are claimed to be identical (OIV 2009d). Thus, the distinct reactivity and versatility of KT is raising interest for

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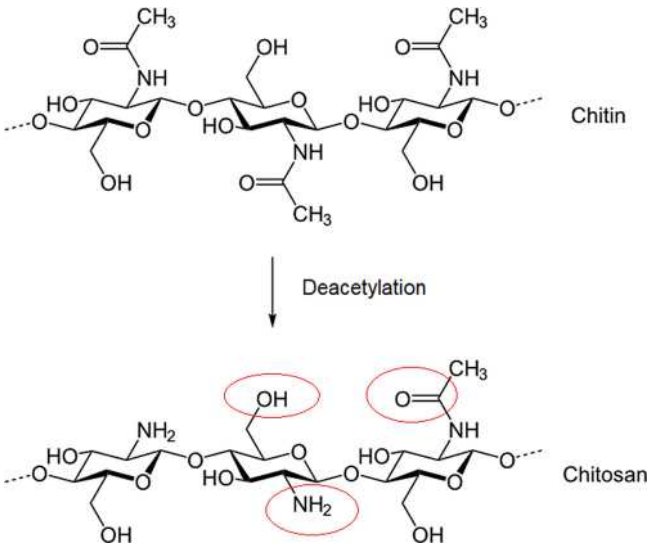


Figure 1. Chemical structures of chitin and KT. Ovals highlight the moieties that may contribute to reactivity and physico-chemical behavior of KT.

its utilization in enology. However, because of its recent introduction in winemaking, the actual range of applications and potential limitations of its use have not been fully elucidated yet and there is a lack of information about possible future developments.

The present review aimed to collect the advancements in the research on native (e.g. not chemically functionalized) KT and its use as an adjuvant, with specific focus on winemaking. Emphasis has been paid on those applications laid down by International Organization of Vine and Wine (OIV), that have already been practiced in enology, including, microbiological control, protein stabilization, metal chelation and ochratoxin removal. These have been individually discussed at first, following a priority given by their diffusion as a practice in winemaking or the abundance of studies. From paragraph 6 onwards, the antioxidant behavior and sensory influence together with other less common or potential utilizations of KT were outlined, also providing additional hints on future research subjects. Table 1 summarizes all the topics discussed in this paper and the main results pertinent to them.

## Antimicrobial activity

One of the main applications of KT in food and wine is linked to its versatile antimicrobial activity against a broad range of microorganisms such as gram-positive and negative bacteria, yeasts, and molds (Brasselet et al. 2019; Kong et al. 2010; Lisanti et al. 2019; Petrova, Cartwright, and Edwards 2016; Rinaudo 2006; Rocha, Coimbra, and Nunes 2017). In an alcoholic and acidic matrix as wine, microbial concerns not involve pathogens, and mainly relate to technological issues such as the correct management of *Saccharomyces* spp. and non-*Saccharomyces* spp. populations or the control of the development of unwanted bacteria, namely lactic or acetic acid bacteria. For these latter purposes, fungal KT has been approved by OIV at a maximum suggested dose of 10 g/hL (OIV 2009a).

### Activity on non-Saccharomyces spp. yeasts

Reportedly, among fermenting yeasts, KT generally manifests a higher inhibitory effect for wine related non-*Saccharomyces* species than for *S. cerevisiae* (Allan and Hadwiger 1979; Bağder Elmaci et al. 2015; Gómez-Rivas et al. 2004; Roller and Covill 1999) and this could help enologists in the correct management of alcoholic fermentation. Comparative trials demonstrated complete inactivation of *Hanseniaspora uvarum* and *Zygosaccharomyces bailii* at 0.4 g/L KT after 3 days of incubation, or *Candida* spp. and *Rodotorula* spp. at 0.3 g/L KT after 4 days of incubation (Rhoades and Roller 2000) even if, in red grape musts, its efficacy on non-Saccharomyces yeasts was found to be lower than that of 50 mg/L $SO_2$ (Picariello et al. 2020).

Much attention has been paid to the control of *Brettanomyces/Dekkera* spp., a problematic contaminating yeast responsible of "horse sweat" or "mousy taint" sensory notes (the so-called "brett" character), that sometimes develop during wood ageing and storage of red wines. In one investigation, *B. bruxellensis* was completely inhibited by 0.2 g/L KT (Bağder Elmaci et al. 2015). This minimal inhibitory concentration was also confirmed in a second study (Portugal et al. 2014), where 0.062 g/L stopped *B. bruxellensis* growth, but 0.25 g/L was necessary to kill 90% of the population ($MBC_{90}$). In this latter study, the authors, for the first time, compared the susceptibility to KT of 16 different *B. bruxellensis* strains, finding $MIC_{50}$ values spanning from 0.031 g/L to 0.062 g/L, and $MBC_{90}$ varying from 0.062 to >0.25 g/L. Similarly, in synthetic wine Taillandier et al. (2015) reported that 0.1 g/L KT had a lethal effect on 50% of *B. bruxellensis* cells after 24 h of contact, which was reduced to 3 h in the case of 0.4 g/L addition.

In a Cabernet Sauvignon red wine, 0.08 g/L of fungal KT allowed a 3-log reduction in population within 6–8 days, regardless of the tested strain (Petrova, Cartwright, and Edwards 2016). However, complete eradication was not achieved as longer monitoring in barrel ageing wines revealed eventual growth of up to $10^5$ CFU/mL at day 68 even in the presence of 0.1 g/L KT. In this regard, Nardi et al. (2014) stated that "*batonnage*", when done after KT addition, could be detrimental to wine protection, as instead of increasing the contact with the polysaccharide, it may promote the recovery of "brett" cells by resuspension and oxygen incorporation. At these dose levels, the authors claimed KT to be fungistatic rather than fungicidal.

Unexpected high amounts of KT were necessary to inhibit the growth of *B. bruxellensis* and *B. intermedius* in one of the first papers dealing with this subject (Gomez-Rivas et al. 2004). It was found that 6 g/L only lengthened the lag phase to 80 h without impeding a total recovery of cell vitality within 180 h. *B. intermedius* was more sensitive and was completely inhibited by 2 g/L KT.

According to some studies, ethanol seems not to affect *B. bruxellensis* sensitivity to KT (Bağder Elmaci et al. 2015; Petrova, Cartwright, and Edwards 2016; Portugal et al. 2014). However, growth inhibition has been shown to depend on the MW of the polysaccharide; a low MW KT (107 KDa) gave lower MIC (<0.15 g/L) when compared to

Q9 **Table 1.**

| FIELD OF USE (OIV code sheets 336-7-8 A/2009) | TARGET | MATRIX | TESTED DOSE (mg/L) | EFFECT | Reference |
|---|---|---|---|---|---|
| Reduction of undesirable microorganisms | Yeasts: *Brettanomyces spp.* | Red wine | 150–1500 | MIC and MLC varying from 150 to 500 mg/L depending on *Brettanomyces/Dekkera* strain and KT molecular weight. | Ferreira et al. 2013 |
| OIV maximum recommended dose: 100 mg/L | | Red wine in barrique | 40–100 | At the tested doses, KT demonstrated a fungistatic activity. | Petrova, Cartwright, and Edwards 2016 |
| | | | 40 | Fungistatic activity (prevention of the yeast development during elevage). Batonnage promoted yeast cell recovery. | Nardi et al. 2014 |
| | | Glucose, salts, and yeast extracts | 100–6000 | Lag-phase lengths >80 h. No biocidal activity against *B. bruxellensis*. Complete inhibition of *B. intermedius* at 2 g/L | Gomez-rivas et al., 2004 |
| | | YPG culture medium | 0.12–250 | $MIC_{90}$ = 62 mg/L and $MBC_{90}$ > 250 mg/L for 16 strains | Portugal et al. 2014 |
| | | Inoculum *in vitro* | 40–400 | Fungistatic activity (physical and biological effects on *Brettanomyces* cells). | Taillandier et al. 2015 |
| | *H. uvarum* and *Z. baili* | YPG culture medium | 100–400 | Growth inhibition at 0.3 or 0.4 g/L. | Bağder Elmacı et al., 2015 |
| | *S. cerevisiae* | Inoculum *in vitro* | 1000–6000 | Increase of the lag phase from 0 to 4 h depending on the concentration of KT. | Escudero-Abarca et al., 2004; Gómez-Rivas et al. 2004. |
| | | | 8–1000 | Minimum biocidal concentration >250 mg/L | Allan and Hadwiger., 1979 |
| | | Apple juice | 100 and 400 | Lag-phase extended by 2-3 days depending on the strains. Then, growth was recovered at levels similar to untreated samples | Roller and Covill 1999 |
| | | YPG culture medium | 0.12–250 | $MIC_{50}$ > 250 mg/L for 15 different strains. Ethanol enhance yeast sensitivity | Portugal et al. 2014 |
| | | | 600–2000 | Lag phase increase from 2 to 4 days depending on the concentration. | Bağder Elmacı et al., 2015 |
| | | Apple- elderflower juice | 300 | Better growth inhibition in juice (pH 3.3) than saline solution (pH 6.4) | Rhoades and Roller 2000 |
| | Lactic acid Bacteria *Unspecified LAB* | Apple-elderflower juice | 300 | Initial quick reduction in viable cells followed by restored growth after 8 days | Rhoades and Roller 2000 |
| | *O. oeni; L. hilgardi* | YPG culture medium | 200–2000 | Complete inactivation at 200 mg/L for at least 6 days. | Bağder Elmacı et al., 2015 |
| | *Pediococcus sp.* | Peptone water/Hopped malt extract | 10–1000 | In peptone water, growth was completely inhibited at 100 mg/L. In malt extract, the activity was only bacteriostatic. | Gil et al. 2004 |
| | *L. plantarum* | YPG culture medium MRS agar | 200–2000 30–1000 | Complete inactivation at 1200 mg/L for at least 6 days. *L. plantarum* was the most resistant LAB. MIC = 500–800 mg/L depending on KT deacetylation degree | Bağder Elmacı et al., 2015 Jung et al. 2010 |
| | | Peptone water/Hopped malt extract | 10–1000 | Only 1000 g/L prevented the development of bacteria. The effect of the pH and the matrix were also evaluated. | Gil et al. 2004 |
| | Acetic acid Bacteria: *A. malorum* and *A. pasteurianus* | Wine matrix | 200 | Growth inhibition (reduction of *Acetobacter spp*. activity; effects comparable to 60 mg/L of $SO_2$). | Valera et al. 2017 |
| Settling, clarification and prevention of protein haze | Protein removal | White wine | 1000 | At this concentration, KT cannot guarantee protein stability (comparative study with other oenological clarifying agents). | Chagas, Monteiro, and Ferreira 2012 |
| OIV maximum recommended dose: 1000 mg/L | | | 1000 | Haze-stable wines after heat tests at 50 °C and 56 °C. Reduction in total protein content by up to 14%. Specific reactivity toward chitinases. | Colangelo et al. 2018 |
| | | Fruit Juices | 2000 | Reduction of protein content by up to 30%. | Chatterjee et al. 2004 |
| | Clarification | Wine and must | 300 | Effective clarification only in de-pectinized musts. Diminution in amounts of phenols and caftaric acid. Significant reduction in contaminating viable cells. | Eder, 2012 |
| | | Beer | 5 | Surprising higher flocculating activity of KT (up to 97% clearer) with respect to stabifix + bentonite combined treatment. | Gassara et al. 2015 |
| | | Apple juice | 100–1000 | Highest flocculating activity and clearer juices at doses of 700 mg/L at 40 °C. | Rungsardthong et al. 2006 |
| | | | 100–1000 | Completely clear juices obtained with 700 mg/L of KT. Significant increase in the lightness of samples | Soto-Peralta, Muller, and Knoor 1989 |
| Reduction of heavy metal content | Cu, Fe | Model Wine | 1000 | Reduction of Fe and Cu contents by up to 80% and 56%, respectively. | Chinnici, Natali, and Riponi 2014 |
| OIV maximum recommended dose: 1000 mg/L | Cu, Fe, Pb, Cd | Wine | 100–2000 | Reduction of metal content by up to 90%, depending on the type of KT, wine, and pH. | Bornet and Teissedre 2008 |
| | | | 500–4000 | Removal of Fe and Cu up to 96% and 60% respectively, depending on the KT dose. Slightly lower efficacy in red wines with respect to white ones. | Magomedov and Dagestan 2014 |
| Reduction of contaminants | Ochratoxin A | Wine | 2000–5000 | Reduction of Ochratoxin A levels from 26–86% depending on wine and pH. | Bornet and Teissedre 2008 |

(*continued*)

Table 1. Continued

| | | | | | |
|---|---|---|---|---|---|
| OIV maximum recommended dose: 5000 mg/L | | | 100–5000 | Removal of 67% of OTA at maximum levels of KT. Significant modification of pH and color | Quintela et al. 2012 |
| | | | 1000–4000 | 100% OTA reduction at 1000 mg/L. Significant removal of anthocyanins and polyphenols | Kurtbay et al. 2008 |
| Antioxidant activity | Reduction of browning | White wine | 4000 | Inhibition of browning and reduction of polyphenol content by up to 40%. Efficacy comparable with PVPP or caseinate | Spagna et al. 1996 |
| Use not provided for by OIV | | | 400 | Reduction of browning. Interactions between amine groups and polyphenols. Chitosan can be regenerated and reused after treatment. | Spagna, Barbagallo, and Pifferi 2000 |
| | | | film 100 $cm^2$ | Inhibition of oxidative browning. Removal of metallic ions and phenols | Nunes et al. 2016 |
| | | Model Wine | 1000 | Effective inhibition of browning. At 1 g/L same anti-browning power of 80 mg/L of $SO_2$ | Chinnici, Natali, and Riponi 2014 |
| | | Apple and pear juices | 100–1000 | Significant reduction of browning after filtration with kieselguhr. No direct adsorption of PPO. | Sapers, 1992 |
| | Antiradical | Model Wine | 200–2000 | Dose-dependent direct scavenging effect of ·OH radical by up to 98% at 2 g/L. Inhibition of generation of 1-hydroxyethyl radical. | Castro-Marin et al. et al., 2019 |
| | | Grape juice | 100–1000 | Scavenging of DPPH, ABTS, and $H_2O_2$, $O_2^{\cdot-}$ | Chien et al. 2013 |
| Other uncommon uses | Reduction of volatile phenols | Red Wine | 1000 | Reduction in the abundance of volatile phenols in the head space. | Milheiro et al. 2017 |
| | | | 1000 | Efficacy increased with DD and concentration up to 1 g /L. Crustacean KT was more effective than fungal. | Filipe-Ribeiro, Cosme, and Nunes 2018b |
| | $SO_2$ Free wines | White wine | 1000 | Effective in controlling the browning. Treated wines were richer in fatty acids and esters. | Castro-Marin et al. 2018 |
| | | | film 100 $cm^2$ | Microbial and chemical stability after 12 months of storage. Generation of positive aromas | Nunes et al. 2016 |
| | | White and red wines | 100 | KT avoided microbial spoilage and, in red wines, reduced the vegetal character. | Ferrer-Gallego et al. 2017 |

medium (310 KDa) and high (624 KDa) MW KT (MIC equal to 0.2 g/L and 0.5 g/L, respectively) (Ferreira et al. 2013).

## Activity on saccharomyces yeasts

The eventuality of the addition of KT in musts during the alcoholic fermentation to control, for instance, undesirable microbial development, may pose some concerns about potential interferences toward fermenting yeast belonging to the genus *Saccharomyces*. Actually, there is a common consensus on the relatively low effects of KT on the metabolism of *Saccharomyces* spp., at least at doses suggested by OIV for antimicrobial purposes even though some differences have been highlighted depending on the strain and dosages considered.

Bağder Elmaci et al. (2015) found concentrations of 0.6–2 g/L of KT to be biocidal to *S. cerevisiae,* whereas addition of 0.4 g/L caused a 1-log cycle of decrease in growth. However, concentrations of 0.20–0.25 g/L did not affect *S. cerevisiae* population (Allan and Hadwiger 1979). Similar results were obtained by Roller and Covill (1999) who reported an extension of the lag phase for up to 3 days at 0.1 and 0.4 g/L of KT for 2 out of 3 *S. cerevisiae* strains. The third strain was completely inactivated at the highest KT concentrations. All these studies seem to confirm that when chitosan is used at low or intermediate doses (<0.4 g/L), the extension of the lag phase is followed by a population regrowth at a comparable rate to that of untreated samples, proving that very high KT concentrations are required to obtain an irreversible inhibitory effect.

In another report, controversial results were obtained as concentrations of 1-4 g/L of KT elicited an increase in cell growth of *S. cerevisiae* population within 8 h (Gómez-Rivas et al. 2004). In that case, only massive additions (up to 6 g/L) were biocidal. The authors explained this behavior based on the augmentation of the nutrient matter by the hydrolysis of KT at lower dosages. Further, the same authors found that when grown in mixed cultures with *Brettanomyces bruxellensis*, *S. cerevisiae* increased the glucose consumption rate proportionally to the KT concentration (up to 6 g/L), but the reason for this fact remained unclear.

Alcohol was found to affect the in vitro sensitivity of *S. cerevisiae* to KT as the minimum inhibitory concentrations (MIC) for 15 strains (both commercial and isolated from wines) was at least 4-fold reduced by the presence of ethanol (at 12.5% v/v), which decreased $MIC_{50}$ from >0.25 g/L to 0.062 g/L (Portugal et al. 2014). This fact does not necessarily reflect an augmented inhibitory activity of KT in wine since in yeast extract–peptone–glycerol broth this activity was comparatively higher than in real wines (Bağder Elmaci et al. 2015).

As illustrated before, differences in sensitivity to KT among *S. cerevisiae* strains may sometimes emerge. This has been correlated to the amount of constitutive polyunsaturated fatty acids in the yeast cell membrane, which lend augmented permeability and fluidity to the membrane itself; strains with higher amounts of those compounds resulted in

more susceptibility, allowing KT to enter the cytoplasm more easily (Lopez-Moya and Lopez-Llorca 2016; Zakrzewska et al. 2007).

### Activity on lactic acid and acetic acid bacteria

Lactic acid bacteria are quite a large number of gram + microorganisms belonging to distinct genera, sharing the ability to produce lactic acid by fermenting sugars and/or malic acid. In wine, malo-lactic fermentation (MLF) is mainly carried out by *Oenococcus* spp., *Lactobacillus* spp., or *Pediococcus* spp. whose sensitivity to KT is expected to vary considerably, depending on the species.

One of the first reports on the application of KT to control lactic acid bacteria in beverages was from Rhoades and Roller (2000). They found a quick reduction in viable cells after the addition of 0.3 g/L of KT to an apple/elderflower juice (pH 3.8). However, after 8 days at 7 °C, the total count reached the same level as the untreated juice.

In culture media, the growth of 12 strains of *Lactobacillus brevis, L. casei, Pediococcus damnosus,* and *P. clausenii*, was reduced by 66–95% by 0.5 g/L KT after 9 days of incubation (Garg et al. 2010). *L. brevis* was found to be somewhat less sensitive than *L. casei* or *Pediococcus* species. Another study confirmed the relatively high resistance to KT of *Lactobacillus* sp. with respect to *Pediococcus* sp. (Gil et al. 2004). KT at 0.1 g/L completely inhibited the growth of *Pediococcus* bacteria in peptone water whereas for *L. plantarum*, doses as high as 1 g/L were necessary. As in other cases, the application in real matrixes revealed some distinct behavior given that, in hopped malt for beer production, KT showed only bacteriostatic effects.

Bağder Elmaci et al. (2015) found that KT concentrations of 0.8–2.0 g/L effectively inhibited the development and viability of *L. plantarum* inoculated at $10^8$ CFU, whereas *L. hilgardii* and *Oenoccocus oeni* were completely inactivated by 0.2 g/L KT. In another study (Jung et al. 2010), KT concentrations of 0.5–0.8 g/L turned out to be enough to inhibit the development of *L. plantarum*. The lowest MIC value was observed for the highest DD and the lowest MW, demonstrating that these are two determining factors for the antimicrobial activity of KT.

To our knowledge, the only paper deepening the efficacy of KT against acetic bacteria has recently been authored by Valera et al. (2017). Those authors reported a reduction in the *Acetobacter* spp. population of $10^2$ CFU after addition of 0.2 g/L of KT to a synthetic vinegar solution, at 3 and 6 months of treatment. It is worth noting that, in the same study, control samples added with 60 mg/L $SO_2$ showed similar cell counts and comparable volatile acidity to KT samples, indicating that KT was as effective as sulfites against acetic acid bacteria.

Overall, the data on the antimicrobial activity demonstrate that KT may be a versatile tool to control a heterogeneous series of microorganisms. However, the maximum dose suggested by OIV (0.1 g/L) appears to be somehow inadequate as, in some circumstances, it only ensures a temporary growth inhibition, not impeding a successive recovery of microbial viability. At doses up to 0.4 g/L, KT can reduce the risk of unwanted growth of wild non-*Saccharomyces* yeasts during alcoholic fermentation, without great interferences to the *S. cerevisiae* population (apart from a lag-phase delay). Lactic acid bacteria were found to be variably sensitive to KT depending on the species, with *L. plantarum* being more resistant than *L. hilgardi, Oenococcus oeni,* and *Pediococcus sp.*

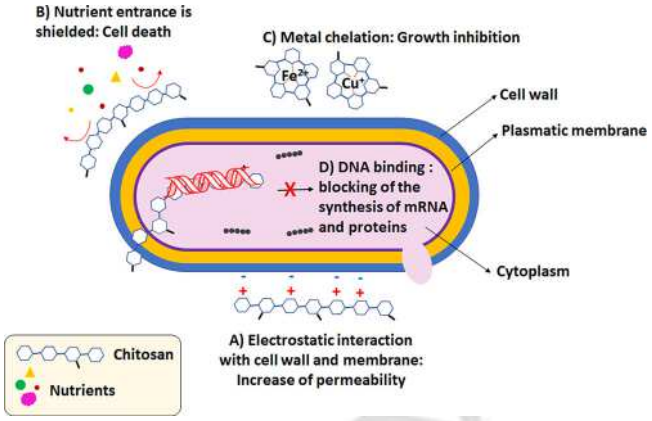


**Figure 2.** Proposed antimicrobial mechanisms of KT.

As far as the antimicrobial mechanisms are concerned, the differences in structure and metabolism among yeasts, bacteria, species, and strains should be considered. A list of reported modes of action is discussed below and is schematized in Figure 2:

1. As largely supported by literature, the polycationic behavior of KT in acidic media is of great importance for its antimicrobial efficacy. Comparative trials between KT and chitin, confirmed the decisive role played by amine group and DD for this activity (Allan and Hadwiger 1979). A high positive charge density leads to intense electrostatic interaction with negatively charged components of the cell surface, and thus weakening the membrane by increasing its permeability which leads to an osmotic and energetic imbalance, loss of growth capacity, and eventually cell death (Rabea et al. 2003; Taillandier et al. 2015; Verlee, Mincke, and Stevens 2017; Zakrzewska et al. 2007). In this respect, diversity in the cell structure of fungi and bacteria may justify varied sensitivity to KT. For instance, species that contain chitin in their cell wall (such as yeasts) have been found to be less susceptible (Allan and Hadwiger 1979). Further, the presence of negatively charged teichoic acid in the wall of gram + bacteria facilitate electrostatic interactions and sensitivity, which is not the case with gram– bacteria, where the binding of KT to lipopolysaccharides or proteins located in the outer membrane does not necessarily impairs the functionality of the cell wall underneath (Raafat and Sahl 2009). As mentioned before for yeasts, abundance of unsaturated fatty acids in the cell membrane play an additional role (Palma-Guerrero et al. 2010; Zakrzewska et al. 2007). This characteristic not only depends on yeast strains, but also on

the nutritional levels and oxygen availability during alcoholic fermentation.

2. Sudarshan, Hoover, and Knorr (1992) reported that once the cell membrane is weakened by KT it could penetrate the cytosol and bind with DNA, inhibiting the synthesis of mRNA and proteins. In yeasts, its entrance is thought to be both diffusive and ATP dependent, also as a function of MW of KT (Brasselet et al. 2019).
3. Chitosan, especially at high concentrations, could form a layer that envelopes the cell and prevents the uptake of nutrients from the medium (Ralston, Tracey, and Wrench 1964) or acts as an oxygen barrier inhibiting the development of aerobic microorganisms.
4. Chelation capacity plays an important role in antimicrobial action, as metal ions (Mg++, Ca++, Fe++ etc.) are important micronutrients that are crucial for the functionality of enzymes and stability of the cell wall (Kong et al. 2010; Raafat and Sahl 2009).

## Fining, clarification, and protein haze prevention

The practices of fining and clarification of musts and wines are common in enology. When dealing with fresh juices, clarification aims to reduce the amount of not only the suspended solids, including skins, stems or flesh particles, unwanted yeasts, and bacteria, but also proteinaceous and pectic substances that generate viscosity and cloudiness. However, the goal in winemaking is to favor the stability of the overall product over the entire producing and marketing processes. A stable wine is characterized by the absence of precipitates or haze at the time of bottling, through transport and storage, and till the time of consumption (Van Sluyter et al. 2015). Apart from microbial issues or precipitation of tartrate crystals, wine limpidity largely depends on colloidal phenomena, which involve some meta-stable molecules such as polyphenols, polysaccharides, metal ions, and proteins that under specific conditions may grow in size and flocculate (Ribereau-Gayon et al. 2001). In particular, white wine protein haze is thought to be caused by a two-step process where heat-unstable proteins such as chitinases or thaumatin-like (TL) proteins unfold and successively aggregate into light-dispersing particles (Waters, Wallace, and Williams 1992). During unfolding, the presence of constitutive phenols, metals or sulfate can increase the extent of flocculation and haze appearance (Van Sluyter et al. 2015).

Since 2003, KT has been included in Codex Alimentarius as a coagulating agent for fruit juices. The addition of fungal KT in musts and wines for fining purposes has been successively authorized by the OIV (OIV, 2009a; OIV, 2009b) to reduce turbidity by precipitating particles in suspension or excess of proteinaceous matter. The maximum recommended dose for this application is 100 g/hL. However, studies developed on the fining capacity of KT in winemaking, particularly in case of grape juice clarification are surprisingly scarce.

One single report, for instance, has been published in the last two decades targeting the clearing of fresh and cloudy grape juices with insoluble KT. In this work (Eder 2012), the efficacies of some common fining agents, including polyvinylpolypyrrolidone (PVPP), casein, gelatin-kieselsol, and KT, were compared for the clarification of white musts. It was found that, while giving the same amount of lees as casein and PVPP, KT (at doses of 0.3 g/L) had the highest efficacy in reducing the cloudiness of pectinase-treated grape juices, reaching a value as low as 7 nephelometric turbidimeter units (NTU). Enzyme activity seemed to be pivotal for KT activity, as in the must not treated with pectinases, the addition of KT resulted in increased cloudiness (even higher than the control). However, independent of enzyme addition, KT gave the highest reduction in contaminating viable cells (3-fold reduction as compared to that of the control sample), and thus contributing to lower the risks of unwanted microbial spoilage during alcoholic fermentation

Several studies have been conducted on other beverages or fruit juices. Gassara et al. (2015) studied the efficacy of chitin and KT as fining and protein stabilizing agents of beer. Results demonstrated higher flocculation activities for treatments with chitin and KT (96% and 97% reduction of turbidity, respectively) than the other conventional fining agents (Stabifix + bentonite), with 160 times lesser dosage (5 mg/L of KT). Moreover, analysis of total suspended solids showed a decrease of about 65% in samples treated with the two adjuvants. These results are in line with the work of Chatterjee et al. (2004) who also observed a significant reduction (30%) in protein content in different cloudy fruit juices after treatment with 2 g/L of KT.

In apple juices, addition of KT at 0.7 g/L afforded zero turbidity after 12 h at 20 °C, showing results comparable to the combined addition of gelatin (80 mg/L), silica sol (188 mg/L), and bentonite (1 g/L) (Soto-Peralta, Muller, and Knoor 1989). Other studies carried out with apple (Rungsardthong et al. 2006) and passion fruit juices (Domingues et al. 2012) demonstrated a dose-dependent behavior, leading to higher decreases in turbidity of juices when KT concentration was increased.

The influence of DD of KT on protein flocculation was evaluated (Gamage and Shahidi 2007). KT with the highest DD showed the best protein flocculating ability probably due to the increased charge density resulting from the additional free amino groups (Ariffin et al. 2005).

Similarly, acidic pH promotes extended protonation of the amino group, allowing KT to destabilize the colloids and promote flocculation. Pectin, for example, is a negatively charged polymer that contributes to stabilizing the protein colloidal suspensions by constituting a hydrophilic carbohydrate outer layer (Wang, Sun, and He 2017). KT was found to effectively act as a cross-linker of pectin network, resulting in increased flocculation and greater clarification speed because of electrostatic interaction with that "protecting" colloid (Taştan and Baysal 2017). This postulated mechanism is exemplified in Figure 3A.

For what concern wines, Chagas and coworkers (Chagas, Monteiro, and Ferreira 2012) investigated on the ability of 6 adjuvants, namely casein, egg albumin, isinglass, KT, chitin, and PVPP in stabilizing cv Muscat of Alexandria white wines against protein haze as compared to sodium

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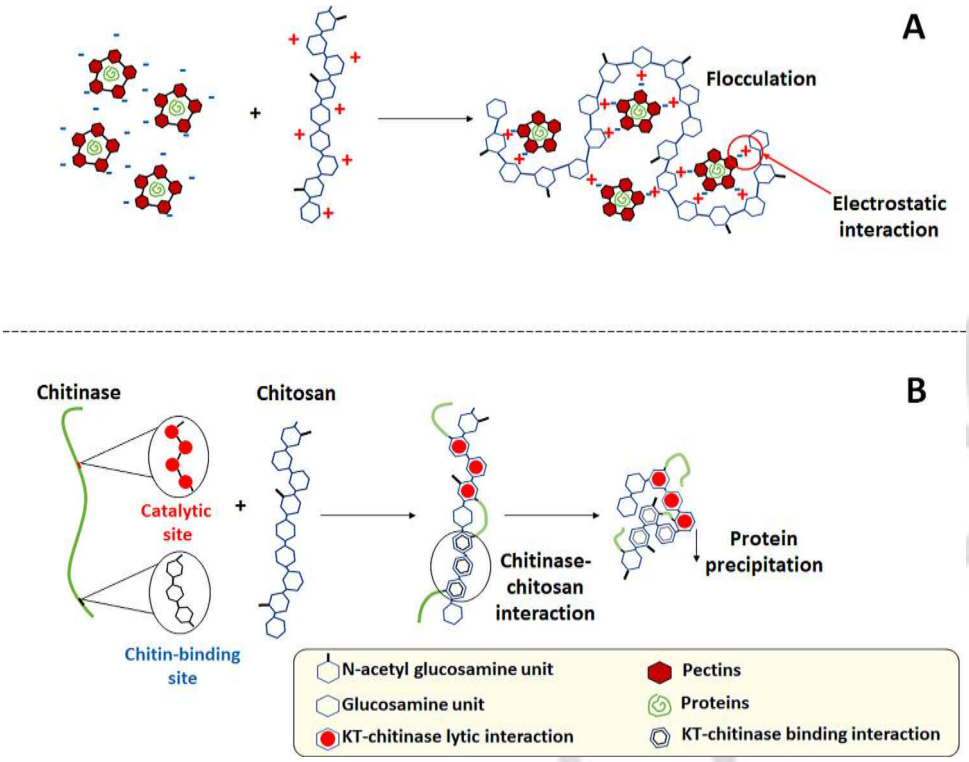


**Figure 3.** Postulated fining mechanisms of KT in juices and wines. A: Electrostatic interaction with pectins; B: Active site-binding with chitinase.

bentonite. The results demonstrated that among all the selected fining agents, only egg albumin and KT were able to decrease the wine haze-forming potential, although to an extent (about 10% reduction in turbidity after heat-stability test) that was not sufficient to permanently stabilize the wine. Addition of KT at 1 g/L did not remove significant amounts of dissolved proteins, but did induce a reduction of phenols, which the authors considered may have partially slowed down the flocculation.

A more detailed study on the interaction between KT and white wine haze-forming proteins was recently carried out by Colangelo et al. (2018). In contrast with the results cited above, in this work KT (1 g/L) reduced the total protein content of a white wine from cv. Moscato grapes by about 14%. Those wines were haze-stable after heat stability tests conducted at 50 °C and 56 °C, whereas a little instability was observed at 60 °C and 62 °C. This latter evidence suggested that observed haze were only because of the heat-resistant TL protein isoforms. As a confirmation, the authors found that KT almost totally removed wine chitinases, whereas the content of TL proteins was only slightly reduced by the treatment. Similar results were previously obtained with chitin that permitted wine haze reductions of up to 80% after heat test of fined samples, and a contextual removal of 29% of wine proteins (Gazzola et al. 2015). Authors claimed that chitin had a higher fining efficiency (ratio between percent reduction of haze and percent removal of proteins) when compared to that of bentonite. Further, it was elucidated that chitin could selectively interact with class IV chitinases, which are mainly responsible for the instability of white wine. In both studies, the inherent mechanism of interaction was postulated to be based on (i) the presence of a cysteine-rich chitin-binding domain in class IV chitinases, able to bind chitin and KT and supposed to be removed together with those insoluble polysaccharides (Figure 3B) and (ii) to a lesser extent, the decrease in phenolic compounds, available to participate in the haze-forming phenomena, because of their adsorption onto KT. It is worth mentioning that based upon the former mechanism, KT has been proposed as specific ligand for affinity precipitation and recovery of plant chitinases (Teotia, Lata, and Gupta 2004).

## Sorption of heavy metals

During the winemaking process and storage, the presence of high concentrations of transition metals may lead to the formation of insoluble precipitates, which is one of the causes of hazing in wine (Bornet and Teissedre 2008). Among others, iron and copper ions are the main contributors to this issue, which is particularly negative for the consumers. Moreover, it has been demonstrated that both metals are crucial catalysts of non-enzymatic oxidation of wine, even at trace levels, leading to oxidation of most compounds, such as ethanol, organic acids, and phenolic and volatile compounds, triggering wine browning and unwanted sensory changes (Danilewicz 2016).

Chitosan and its derivatives have demonstrated to chelate heavy metals in wine-like environment, and for this specific use, OIV has set a maximum dosage of 100 g/hl (OIV, 2009b).

In model wine solution, Chinnici, Natali, and Riponi (2014) observed a reduction of 70% for iron and 30% for copper after 21 days of treatment with 1 g/L of KT. In a

study performed by Bornet and Teissedre (2008), treatments with different doses of KT (0.2–2 g/L) achieved significant reductions in iron, lead, and cadmium (up to 90%, 74%, and 57% for red wines; 91%, 65%, and 23% for white wines and 90%, 84%, and 25% for sweet wines, respectively). Reported data confirmed a dose-dependent chelating effect, with larger metal removal at higher doses (2 g/L). In case of sweet wines, the presence of sugar seemed to decrease chelation. Furthermore, a slight reduction of sorption capacity in red wines compared to that in white wines was observed, and the reason probably is the higher presence of phenolic compounds that compete with metals for KT active sites (Magomedov and Dagestan 2014).

The adsorption behavior of KT against heavy metals in distinct environments and pH conditions, together with underlying mechanisms have already been reviewed (Guibal 2004; Wu, Tseng, and Juang 2010; Zhang, Zeng, and Cheng 2016). It is largely accepted that the presence of the free electron doublets of nitrogen atoms in amine moieties determine, in certain conditions, the complexation properties of KT (Guibal 2004). In nearly neutral (or mildly acidic) environments, transition metals with void d orbitals are selectively chelated by the polysaccharide via coordination complexes, whereas harmless alkaline or alkaline-heart cations (Ca, Na, K etc.) remain substantially unaffected by its presence. However, at pH <6.1, amines protonation results in increased electrostatic attraction with dissolved anions on the one hand, and in a corresponding decline of heavy metals complexation on the other (Gyliene et al. 2014). In addition, apart from the amine, at least in case of copper chelation, the hydroxyl groups at C3 position of the polymeric chain can further participate as ligand in the Cu-chitosan complex (Domard 1987). Hence, the metal sorption behavior of KT involves concurring mechanisms (chelation and/or electrostatic interaction) depending on a series of factors including pH and composition of dissolving solution, deacetylation degree of KT, and type and speciation of metals involved.

At pH values and composition relevant to wine, the proposed mechanisms can be summarized as below:

1. Only a partial, very limited removal of heavy metal ions may be because of the chelation of cations to the accessible surface of KT polymeric chain, with formation of a complex involving KT amine and hydroxyl groups (Figure 4A). At pH < 3.8, the amine groups of KT are almost completely protonated (Navarro et al. 2003), and electrostatic repulsions of cations largely dominate. For this residual chelating activity, the degree of deacetylation and the stereochemical distribution of the free amino groups determine the binding capacity of the ligand (Wu, Tseng, and Juang 2010).
2. Rather than direct chelation to amine groups, an alternative mechanism could be the deposition of metal hydroxide into the pores of crystallin KT particles (Park, Park, and Park 1984).
3. The presence of constitutive organic ligands in wine play a role in metal speciation and sorption efficacy of KT (Rocha et al. 2020). Depending on the pH, tartaric, malic, and citric acids may form ionic complexes with heavy metals. For example, in case of aqueous mixtures of copper/citric acid, at pH 3, about 35% of the metal is present in anionic complexes (in the form of Cu-citrate$^{-}$ and Cu(OH)-citrate$^{2-}$), whereas other species (Cu-H-citrate; Cu-$H_2$-citrate$^{+}$ and $Cu^{2+}$) represent the remaining 65% (Navarro et al. 2003). At higher pH values, the metal anion species rapidly approximate to 100% and could then be fully attracted by KT protonated amines via electrostatic interaction (Figure 4B). It is worth noting that for this mechanism, the presence of other anions such as unbound dissociated citrate or tartrate in solution may compete with metals for electrostatic attraction, decreasing the overall sorption efficiency of KT.
4. Gyliene and coworkers (Gyliene et al. (2014) investigated the specificity of iron sorption by KT at acidic pH. The authors reported that by treating the aqueous solutions of Fe(II) with 0.1–1 g/L of KT flakes, a significant metal ion uptake, together with a stoichiometric oxygen consumption was observed; however, this was not observed for the samples with Fe(III). They suggested that oxygen consumption depended on the oxidation of Fe(II) to Fe(III) that was catalyzed by KT, which can trap molecular oxygen onto its surface. According to these authors, in acidic solutions Fe(II) sorption is possible only after its oxidation to Fe(III) which, as discussed by Bornet and Teissedre (2007), can participate to the subsequent formation of a complex in the form of [CHI-$NH_2$-Fe]$^{3+}$ (Figure 4A) where the OH groups of the polymer chain seemed to be strongly involved.

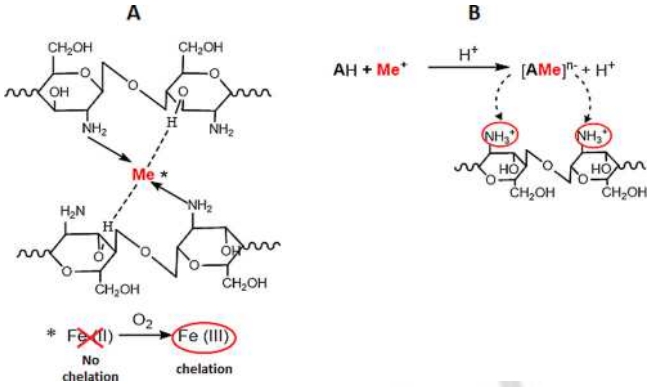


Figure 4. Chelation mechanisms of KT involving: A) chelation by amine (-$NH_2$) and hydroxyl (-OH) groups. Me: Metal. *: Mechanism by which Fe (II) is first oxidized to Fe (III) on KT surface and then chelated; B) electrostatic interaction between organic anion ligands and ammonium cations.

## Removal of ochratoxin A

Ochratoxin A (OTA) (Figure 5), is a mycotoxin produced by *Aspergillus sp.*, and is known for its nephrotoxicity and carcinogenicity in humans (Kurtbay et al. 2008). A wide variety of foods are susceptible to contamination by OTA as a result of fungal infection in the field during harvest and storage. Wine and grape juices are estimated to be the

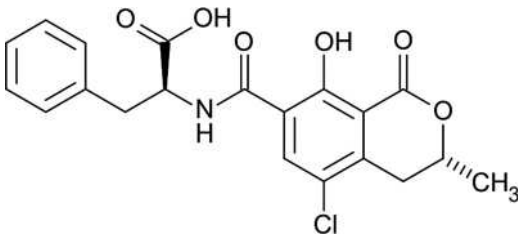


**Figure 5.** Chemical structure of Ochratoxin A.

second source of OTA in the diet after cereals, representing up to 10% of the total intake of this toxin (Quintela et al. 2012). Therefore, the maximum allowed concentration of OTA in European wine, must, and grape juice is fixed at 2 µg/L (European Commission 2005).

Chitosan has been proposed as an efficient tool for OTA removal from wines, based on the physico-chemical features of the mycotoxin. OTA is a weak acid because of the presence of carboxyl group on the phenylalanine moiety, with a pKa of 4.4 (Valenta 1998). Thus, it partially dissociates at wine pH, carrying a negative charge that can interact with a positively charged surface, like the one present in KT. Apart from this, Bornet and Teissedre (2007) evoked an additional mechanism suggesting that OTA may deposit into pores of the polysaccharide crystalline structure. For toxin removal, in 2009 OIV established a maximum admitted amount in wine of 500 g/L (OIV, 2009c).

In red wines fortified with 2.5 µg/L of OTA, Quintela et al. (2012) studied the efficacy of different doses (10, 50, 200, and 500 g/hl) of fungal chitin and KT for toxin removal. They observed a reduction of 29% and 67% for chitin and KT respectively at maximum dosage of 5 g/L, after 2 h of treatment. KT was the most efficient among the other tested fining agents including, bentonite, gelatin, albumin, and PVPP-plant protein complex but wine color and pH were drastically affected. Of all the adjuvants, and despite the lower removal ability, authors claimed chitin and plant-protein complexes to be the aidings of choice because of their limited impact on wine quality.

In another study, 2 g/L and 4 g/L of KT removed 78% and 100% OTA respectively, from a red wine containing 2.57 µg/L of mycotoxin (Kurtbay et al. 2008). In this case, contrary to bentonite and montmorillonite clays, KT quickly reached the adsorption equilibrium (estimated to be 90 min), whereas the specific adsorption increased with the doses, reaching the highest values (25 µg OTA/g KT) only after the addition of 100 mg of adjuvant. Once again, at those concentrations, KT remarkably affected wine composition especially with respect to anthocyanins and phenolics.

By using 5 g/L of 4 chitin derivatives (including chitin, KT, and chitin-glucan) with different DD, Bornet and Teissedre (2007) achieved the highest percentages of toxin reduction (84%) after treating red wines with KT for 48 h. For white and sweet wines, efficacy was significantly lower, but the reasons were not investigated. Removal of OTA by chitin and chitin-glucan were also considerable (up to 73% and 64%, respectively). Unfortunately, no information has been provided on the impact of those treatments on wine quality parameters.

Overall, the results suggested that in case of lightly contaminated wines the addition of 1 g/L or 2 g/L of KT could be an efficient treatment for OTA removal, without affecting the quality of wine. However, because of the requirement of elevated doses of KT, wines with higher concentration of OTA should be treated with less impacting adsorbents such as chitin or chitin-glucan, which likely avoid significant changes in wine composition.

## Antioxidant activity

The antioxidant capability of KT has been often claimed as one of the most promising features to be exploited in food technology and packaging (Chien et al. 2013; No et al. 2007; Schreiber et al. 2013; Shahidi, Arachchi, and Jeon 1999). Depending on the matrix and pH, KT is supposed to operate by means of a direct radical scavenging mechanism (Park, Je, and Kim 2004; Sun et al. 2007; Xing et al. 2005) or indirectly, via metal chelation, which would block the generation of radical species and initiation of lipid oxidation (Guibal 2004; Schreiber et al. 2013). However, it should be pointed out that the majority of scientific reports barely reproduce conditions applicable to winemaking, KT formulations (most of those papers deal with animal-derived or soluble modified KT), or with regard to the physico- chemical environment (pH, hydrophilicity, food matrix, or medium composition).

In wines, in presence of oxygen, ferric or cupric species catalyze the oxidation of *o*-diphenols to *o*-quinones, generating hydrogen peroxide, which, in turn, at acidic pH is decomposed to hydroxyl radical via oxidation of ferrous ions (the so-called Fenton reaction). This radical species can oxidize organic compounds (including ethanol, carboxylic acids, sugars, or thiols) at a rate proportional to their amount in the medium, generating aldehydes, ketones, or disulfides (Danilewicz 2016). Further reactions may involve *o*-quinones (electrophiles) and nucleophilic or reducing compounds present in wine such as sulfites, ascorbic acid, thiols, or polyphenols (Waterhouse et al. 2016).

If controlled, oxidation can be beneficial for red wines because of increased color stability and modulation of astringency; however, white wines are usually damaged by oxygen exposure due to the generation of adverse sensory attributes (browning or color changes, aromatic defects, and increase of astringency) and reduced nutritional properties. Hence, the exploitation of the antioxidant properties of KT during winemaking would present an interesting tool for producers, though its utilization as an oxidative scavenger has not been proposed and regulated by OIV yet.

The first suggestions for the use of chitin-derived products for controlling wine browning dates to late '90's (Spagna et al. 1996; Spagna, Barbagallo, and Pifferi 2000). The starting hypothesis was based upon the capacity of KT to remove phenols, which would reduce the oxidizing potential of wines. At doses of 0.4–4 g/L, KT demonstrated an effective adsorption capacity, particularly toward hydroxycinnamic acids and procyanidins, achieving reductions up to 40% and 30%, respectively, in several Italian white wines.

This was reflected by a significant inhibition of browning tendency, not dissimilar from the one obtained with PVPP and casein (Spagna et al. 1996). Also when unconventionally used as a film in bottled wine, KT (100 $cm^2$/L) demonstrated its anti-browning behavior by reducing phenols quantity (about 15% of the initial amount) and chelation via complexation of tartrate-metals anions (Nunes et al. 2016).

Apart from native phenolics, KT can also adsorb already oxidized phenolic species such as the yellowish xanthylium cations or the carboxymethine-linked (+)-catechin dimer intermediates, further restraining the oxidative cascade and the browning expression (Chinnici, Natali, and Riponi 2014). The mechanisms involved in adsorption depend on the type of phenol implicated. For instance, catechin is linearly adsorbed as a monolayer up to the saturation point (at about 0.14 g/g KT) via hydrogen bonding. For hydroxycinnamic acids, the study of adsorption isotherm suggested a cooperative phenomenon involving KT protonated amines, $\pi$–$\pi$ stacking of planar hydroxycinnamic rings and competitive bonds with tartrate anions (Spagna, Barbagallo, and Pifferi 2000). For larger molecules such as procyanidins, steric hindrances and Van der Waals self-association forces may reduce the adsorption rates at high phenolic concentrations.

In cloudy apple and pear fresh juices, Sapers (1992) obtained notable prevention of enzymatic browning after addition of KT at 0.5–1 g/L and successive filtration with celite as aiding. He concluded that KT can inhibit enzymatic browning in unfermented juices by coagulating suspended solids to which polyphenol oxidases are bound, but excluded a direct adsorption of the enzyme itself onto KT.

In acidic media, the antiradical efficacy of native (e.g. not modified) KT raised some doubt in principle, because of the lack of easily donatable hydrogens and protonation of amines, which would hamper the transfer of the free electrons from N atoms (Schreiber et al. 2013). However, some attempts to estimate these features in wine relevant conditions have been made. It was reported, for instance, that the addition of 0.1–1 g/L KT increased the antiradical power of grape and apple juices by up to 4-fold against some natural oxidizing species, such as $O_2^-$ and $H_2O_2$ or synthetic reagents, such as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), demonstrating a hydrogen-donating capacity of KT (Chien et al. 2007; 2013). Castro et al. (2019) found that two distinct formulations of fungal KT (at 0.2–2 g/L) considerably reduced the accumulation 1-hydroxyethyl radical generated from ethanol oxidation in wine. Authors demonstrated that this result depended on a cascade that included i) the deactivation of the metal catalytic pool, mainly Fe(III)/Fe(II), by chelation, ii) the direct quenching of hydroxyl radical (·OH) by up to 90% at 2 g/L KT, and iii) the subsequent diminished oxidation rate of ethanol. Hydroxyl radical, in turn, is assumed to be scavenged after H abstraction from the amine residue on C2 position of KT and the successive molecular rearrangement of the polymer which breaks down into smaller oligomers and depolymerizes (Chang, Tai, and Cheng 2001). These antioxidant activities were found to increase with DD and decrease with MW (Chien et al. 2007; 2013; Dong, Xue, and Liu 2009; Sun et al. 2007).

The distinct anti browning mechanisms of KT are summarized in Figure 6. Overall, based on these data, KT may represent a green and environmentally friendly potential alternative to the use of traditional additives to prevent the development of oxidative spoilage in beverages, and may well deserve further studies to deepen the understanding regarding its behavior as an antioxidant in enology.

## Impact of chitosan on wine quality parameters

One concern regarding the use of KT in wine is certainly the evaluation of its impact on the overall quality of wines, including color, aroma, or other sensory features.

As already discussed, at dosages pertinent to the removal of OTA (up to 4–5 g/L), KT can negatively affect the red wine color (30–50% decrease in color density) because of the interaction with anthocyanins and procyanidins (Kurtbay et al. 2008; Quintela et al. 2012). However, such high doses should be regarded as an exception over the usual range of concentrations, which fall between 0.1 g/L and 1 g/L.

When added at <1 g/L, KT did not significantly reduce the color, anthocyanin content, or total phenolic index of red wines (Filipe-Ribeiro, Cosme, and Nunes 2018a, 2018b; Milheiro et al. 2017) even if at the highest amount some loss of caftaric and coutaric acids (up to 20% each) or flavanols (about 5%) have been recorded (Filipe-Ribeiro, Cosme, and Nunes 2018a). In white wines, because of the initial lower phenolic content, removal rates are higher and may reach values as high as 30% and 20% for hydroxycinnamic acids and flavanols, respectively, accounting for about 200 mg/L loss (Chinnici, Natali, and Riponi 2014; Spagna, Barbagallo, and Pifferi 2000).

Regarding volatile compounds, post-fermentative addition of 1 g/L KT slightly reduced the headspace aromatic abundance of red and white wines, particularly with regard to medium-chain fatty acid ethyl esters and terpenes (including rose-oxide, linalool, citronellol, and geraniol) (Colangelo et al. 2018; Filipe-Ribeiro, Cosme, and Nunes 2018a; Milheiro et al. 2017). The headspace of white wines bottled for 8 months in the presence of KT films was richer in fruity-scented compounds such as benzaldehyde, furfural, and ethyl pentanoate, some of them coming from Maillard reactions promoted by KT. Ketones, which originated from oxidation of alcohols or acids were lower; altogether, the wines were judged well balanced and bodied as the respective sulfite added counterparts (Nunes et al. 2016). However, those studies specifically evaluated the headspace composition (namely a way to estimate the relative concentration of volatiles in the gas phase likely reaching the nose during sniffing) and not the true concentration of those molecules in wines. Instead, in another investigation, wines added of KT during fermentation were compared with sulfited samples and analyzed for their actual volatile composition. The formers resulted to be richer in medium chain fatty acids and derived ethyl esters, reportedly because of the

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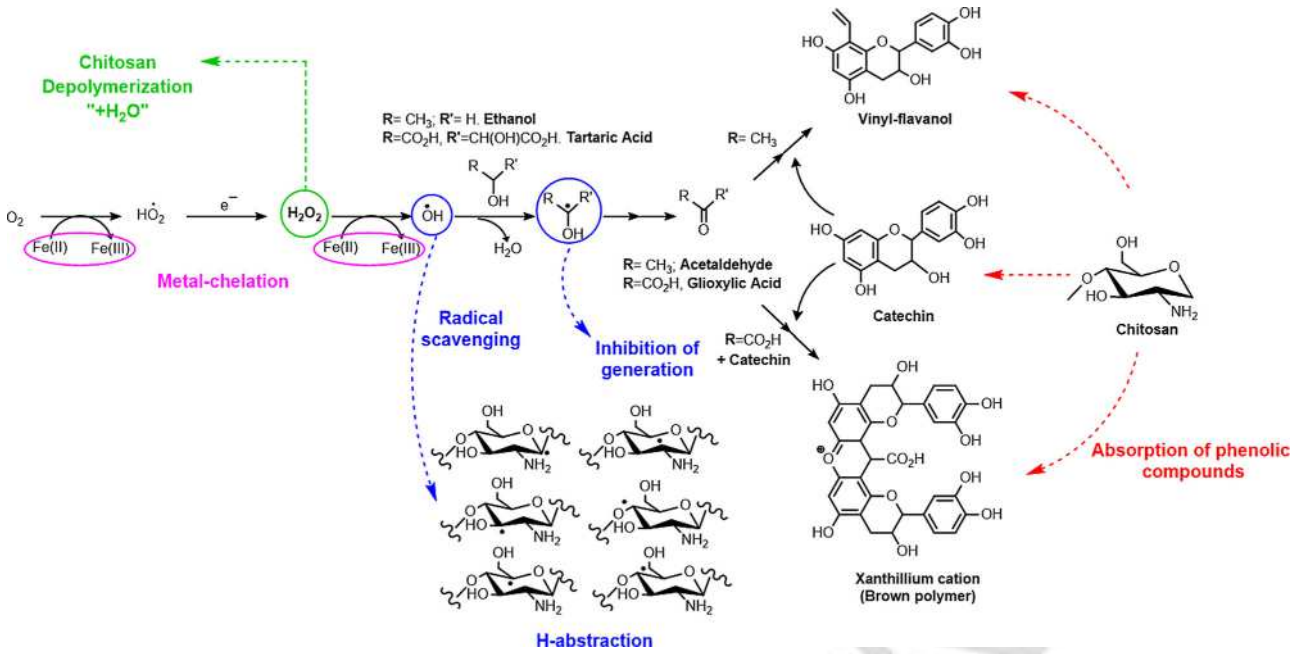


**Figure 6.** Established antioxidant mechanisms of KT in wine (modified from Castro-Marin et al. 2019).

modification of the yeast metabolism caused by the alterations in the cell membrane (Castro-Marin et al. 2018).

As a polycation, KT could potentially interfere with the acidic pattern of wines by binding anions from organic acids (Bornet and Teissedre 2007). Despite some authors claiming no changes in pH or titratable acidity after the treatment (Nardi et al. 2014; Nunes et al. 2016), others did find some effects. Castro-Marin et al. (2018) using 1 g/L KT in white musts, reported a decrease in titratable acidity of 1.25 g/L and a 0.08 unit increase in pH because of the removal of tartaric and malic acids. Concordantly, the pH of a red wine added with 0.5 g/L and 2 g/L KT increased by 0.05 and 0.10 units, respectively (Quintela et al. 2012). In another study, model wine solutions were deprived to various extents of organic acids, mainly tartaric, malic, and acetic acid, after the addition of 1 g/L of KT (Colangelo et al. 2018). This behavior should be taken into consideration, so as not to affect the microbial stability and sensory features of wines.

Astringency is a further sensory aspect of KT that has been little investigated. Notwithstanding the lack of evidence about the change in tactile sensations of wines treated with KT, a direct correlation between astringency and DD of dissolved KT was demonstrated (Luck et al. 2015). This correlation was argued to be because of the charge density of the polymer, which in turn, affects its binding capacity toward salivary proteins at acidic pH (Luck et al., 2015). An 85% deacetylated KT from shrimps dissolved in aqueous acetic acid elicited a perceived astringent stimulus when added at concentrations >50 mg/L (Rodriguez et al. 2003). On the contrary, in milk, astringency was only affected after the addition of 450 mg/L of a nano powdered KT preparation (Seo et al. 2011). Further information on this specific sensory aspect in wine is needed. Even though KT should not remain in wine because of its insolubility, which would make its tactile impact improbable, there is some evidence that, depending on the dose, up to 5% of the polysaccharide may dissolve into the final product, potentially interacting with other compounds in the mouth (Filipe-Ribeiro, Cosme, and Nunes 2018b)

## Other uncommon utilizations in winemaking

Based on the already cited assumption that KT can interact with volatile compounds and reduce their partition coefficients to the gas phase, it has been recently proposed for the remediation of wines containing high concentrations of volatile phenols (VP) coming from unwanted proliferation of *Brettanomyces/Dekkera* yeast. When added at doses of 0.1–1 g/L to red wines spiked with VP (300 µg/L and 1500 µg/L for ethyl guaiacol, and ethyl phenol, respectively), KT induced a decrease of headspace VP by up to 36%, but did not change the total VP concentrations in wine (Filipe-Ribeiro, Cosme, and Nunes 2018a; Milheiro et al. 2017). This corresponded to a significant decrease in the perception of the negative phenolic attribute of those wines when compared to the spiked ones, even if this descriptor remained at levels higher than the unspiked wine (Filipe-Ribeiro, Cosme, and Nunes 2018b). The efficacy increased with the increasing degree of deacetylation and KT concentration of up to 1 g/L, whereas higher amounts (5 g/L KT) did not improve this result further (Filipe-Ribeiro, Cosme, and Nunes 2018a). In addition, the reported data suggested that crustacean KT could be more effective than fungal KT, probably because of the lower MW or higher presence of neutral sugars of the tested fungoid polymer (Filipe-Ribeiro, Cosme, and Nunes 2018b).

Another not common utilization of KT is to produce sulfite-free wines. The utilization of sulfites as food additives has raised some concerns among scientists and consumers because of the health concerns involved (risks of urticaria, asthma, and chronic diseases (Vally and Misso 2012), pressuring regulatory institutions to establish mandatory labeling

rules for food containing sulfites. Accordingly, in the wine industry, where sulfur dioxide is largely employed as an antioxidant and antiseptic, there is an increasing interest in finding the way to reduce (or eliminate) its use.

While some authors have suggested its usefulness to this aim in principle (Lisanti et al. 2019; Rocha, Coimbra, and Nunes 2017; Santos et al. 2012), others provided more detailed information on the effects of KT added in sulfite-free wines at distinct production steps.

Chitosan has been used during the alcoholic fermentation of sulfite-free white musts to manage fermentative course and browning (Castro-Marin et al. 2018). Results, some of which have already been discussed in the previous chapter, demonstrated that although KT (1 g/L) did not change most of the general parameters, yet it did affect the fixed acid content and volatile composition of wines. A higher production of fatty acids and related esters (bearing positive fruit-reminiscent notes) was observed. This fact was linked to the interaction of KT with the cell wall and cell membrane of *S. cerevisiae* and is expected to only occur when the former is present during fermentation. A 12-month period of storage showed that compositive peculiarities were maintained, and that oxidative processes were not significantly different between samples.

For white and red wines obtained in 2 consecutive vintages, $SO_2$-free samples with added KT (at 100 mg/L) after alcoholic (white wines) or malolactic fermentation (red wines) were found not to be significantly different in sensory features, from wines with added sulfites. In case of red wines, trained sensory panelists found that the KT samples reduced the green vegetal character and increased the balsamic notes (Ferrer-Gallego et al. 2017). Due to the absence of $SO_2$, sulfite-free red wines may contain higher portions of polymeric pigments which contribute to stabilize the color of products submitted to controlled oxygenation practices (Picariello et al. 2020).

In one study, KT was successfully used as preservative in the form of film (100 $cm^2$), to be inserted in bottled sulfite-free white wines (Nunes et al. 2016). According to the authors, KT film contributed to both chemical and microbial stability of wines during the 12 months of storage, as evidenced by lack of browning or volatiles decline. Furthermore, some positive aromas (benzaldehyde, furfural, or ethylpentanoate) were claimed to be generated because of Maillard or Strecker reactions promoted by the amine group of KT, which made those wines better appreciated with respect to sulfite added wines. Evidence of metal chelating and Fenton blocking activities exerted by KT were also provided.

These results, even if not, suggest that KT may be a promising tool for the reduction of sulfites in wine. It is one of the very few adjuvants that could combine both the antioxidant and antimicrobial properties together with the versatility to be used in different steps of the vinification process.

## Further research needs and technological perspectives

Because of the several properties and utilizations of KT, quite a large amount of information is still needed to fully characterize its usability in winemaking. Increased KT solubility should in principle positively affect its antimicrobial and clarifying efficacy, simultaneously permitting to reduce both the doses needed to reach the technical target and the cost of intervention. In sectors different from food, this has often been obtained by chemically modifying the molecule or by reducing its MW. For food purposes, such modification should not require harmful reactants that could impair the natural character of the molecule or raise health concerns. In addition, increasing levels of soluble KT may pose some technical concerns about wine stability and filterability or the need to define validated analytical methods for eventual KT remaining in the product.

The use of KT from sources other than fungi (e.g. crustacean or from insects) may contribute to further contain the overall cost of its addition. However, issues about allergenicity (in the case of seafoods) are still to be concretely dispelled, though some reports suggest a lack of actual risks (Amaral et al. 2016).

The employment of KT as an active packaging material seems to be promising as well. Its film-forming properties and the possibility for other naturally derived components (including grape phenolics or antioxidant wine byproducts) to be chemically bound to the backbone chain, greatly increase the range of possibilities, especially if alternative wine packaging such as bag in box or metal cans are considered.

Above all, additional studies aiming to deepen the impact of treatment on the sensory, compositive, and qualitative characteristics of wines are necessary, which can thoroughly evaluate all the outlined modes, timing, and duration of KT presence in the final product.

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