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Shellfish chitosan potential in wine clarification

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Chitosan extracted from crustacean raw material could represent a new, high- efficient tool for wine clarification.

Abstract: Chitosan is a chitin-derived fiber, extracted from the shellfish shells, a by-product of fish industry, or from fungi grown in bioreactors. In oenology, it is used for the control of *Brettanomyces spp.*, for the prevention of ferric, copper and protein casse and for clarification. The International Organisation of Vine and Wine established the exclusive utilization of fungal chitosan to avoid the eventuality of allergic reactions. This work focuses on the differences between two chitosan categories, fungal and animal chitosan, characterizing several samples in terms of chitin content and degree of deacetylation. In addition, different acids were used to dissolve chitosans, and their effect on viscosity and on the efficacy in wine clarification were observed. Results demonstrated that, even if fungal and animal chitosans shared similar chemical properties (deacetylation degree and chitin content), they showed different viscosity depending on the acid used to dissolve them. A significant difference was discovered on their fining properties, as animal chitosans showed a faster and greater sedimentation compared to the fungal, independently from the acid used for their dissolution. This suggests that physic-chemical differences in the molecular structure occur between the two chitosan categories and that this affect significantly their technologic (oenological) properties.

Keywords: Fungal chitosan; animal chitosan; wine clarification; dissolving acid comparison.

1. Introduction

Chitin is the most abundant polysaccharide on earth after cellulose. Chitin is composed by 2-acetamido-2-deoxy-b-D-glucose (N-acetylglucosamine) units linked by $\beta(1\rightarrow 4)$ bounds and it is organized in layers of polysaccharide sheets. The sheets are composed by multiple parallel chitosan chains that could assume three different crystalline forms (α, β, γ). However, chitin is synthesized by a large number of living organisms, such as arthropods and insects (exoskeletons), crustacean (shells), algae, plants and fungi (cell walls) [1] mainly in its α-form, i. e. it is organized in parallel chitin chains structured in anti-parallel sheet. Differently, β-chitin, composed by chitin chains arranged in parallel sheet, and γ-chitin, a mixture of the previous two forms, are quite rare. For the extraction of chitin and its derivatives at the industrial scale, two principal sources of α-chitin are suitable, such as shellfish and fungi. Annually the seafood industry produced about 106 Tons of waste [2], most of which is destined to composting or to the conversion into low value products, namely animal feed or fertilizers [3]. As alternative, by-products as the shellfish shell could be directed to the component recovery, and chitosan (the deacetylated form of chitin, CTS) represents one of the best possibilities for their re-qualification. Concerning that, approximately 2000 Tons of chitosan is produced every year and its principal sources of extraction are shrimp and crab shell residues [3].

Besides, fungi represent an alternative abundant source of chitin and chitosan that could be extracted from both mycelium and spores [4]. Elsoud and El Kady [4] reported the first

attempts to begin a multiple added-value compounds production from fungi that involves chitin and other compounds. It was estimated that more than 60% of the biotech industries use fungi in different processes such as brewing and baking, as well food, antibiotics, pharmaceuticals, organic acid, and enzyme production, and that, only for citric acid production, *Aspergillus niger* cultivation results in an annual waste of ~80 kTon of mycelium [5]. In the choice of the source, it should be considered that the chitin structure, its percentage and purity vary in reason of the anatomical structure in which it is located. As example, the exoskeleton of shellfish is composed by chitin (20–30% *w/w)*, proteins (20–40% *w/w*) minerals (30–60% *w/w*) [2], and by pigments and lipid in trace [6]. Insects, instead, present chitin both in the exoskeleton and in inner parts, such as tracheal system, that contain catecholamines –*o*-quinones allowing cross-link between protein and chitin (36–62% *w/w* dry weight of chitin [2]). Instead, the fungal cellular wall consists of chitin (15 to 18%), β-glucans (37%), lipids (19%) and several other sugars (8 to 15%, Figure 1) [7]. However it was demonstrate that these percentages could vary among species and life stage [8].

Figure 1

chitin

membrane

β-glucans   β-glucan synthase   mannoprotein   chitin synthase
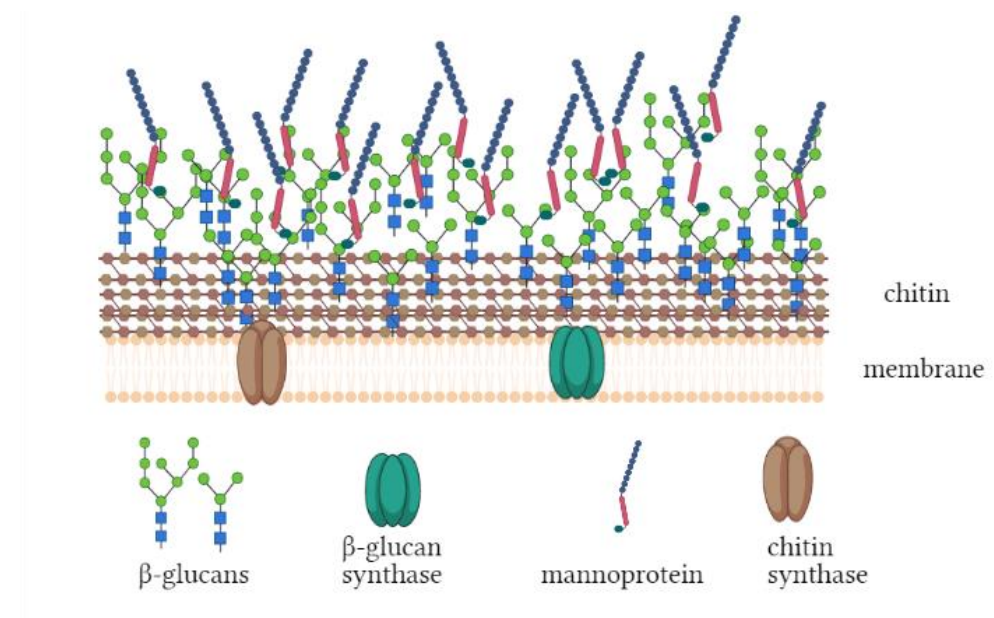
Chitin isolation from natural material follows three steps: the first- which could be called "pre-treatment"-consists in the raw material washing, drying, and smashing. In the case of shellfish, in this step minerals are removed by an acid washing (generally sulfuric acid, hydrochloric acid, acetic acid, nitric acid, and formic acid is used), natural pigments are eliminated by means of organic or inorganic solvents such as acetone, sodium hypochlorite or hydrogen peroxide and sodium hydroxide is used to remove proteins, glycoproteins and branched polysaccharides. Instead, for the fungal chitosan extraction an enzymatic pre-treatment of the raw materials to hydrolyze the β-glucans or, alternatively, an optimized alkaline hydrolysis at low alkali concentration were suggested. Sietsma and colleagues [8] demonstrated that the β-glucans composing fungal cell wall are organized in three main structures, which are soluble in water or in alkaline solution, or not soluble at all. The second step, called "deacetylation", is performed with low amount of substrate (1:30-1:40 *w/v*) at high alkali concentration (NaOH 1–4 M), high temperature (80–121°C) and for a short contact times (15 min–3 hours) in order to remove acetyl group from the chitin chains. Deacetylation carries to a polysaccharide backbone chain characterized by free amine groups (–NH$_2$). This confers a charge to CTS and thus the possibility to be dissolved into acid solution and the acquisition of specific properties, principally the possibility to bound molecules in solution In fungi, Sietsma *et al.* [8] reported that

β-glucans removal was more efficient if chitin was deacetylated before the alkaline wash. The third step, called "post-treatment", generally occurs as a low concentration acid washing (HCl, H_2SO_4, or, frequently, acetic acid at a concentration of 0.5–2% *v/v* for fungi and 2–10% *v/v* for crustaceans) that permits the recovery of deacetylated chitin (chitosan) leaving behind insoluble chitin. This extraction is performed at (60–95°C) over 3–16 hours. In fungi, the residual chitin is typically associated to the β–glucans through covalent bonds that make difficult its recover without degradation. The amount of insoluble chitin-glucan complex could easily reach 16% of the total β-glucans [8]. After the first washing, the chitosan- acid solution is brought to pH 10 to precipitate the CTS. Finally, the precipitate is washed, commonly with a mixture of water, ethanol or acetone, and dried. However, several variants of this general protocol could be found in literature [2–4] according to the producers manufacturing process. Chitosan demonstrates high plasticity and thus it can be prepared in different forms, namely as films, gels, beads and nanoparticles [9,1,10]. CTS could be used in several sectors, such as medicine, cosmetics, agricultural application and food [1,11] in the light of the high number of its valuable properties, such as its biodegradability, biocompatibility and low toxicity. CTS exhibits high potential as antimicrobial and antioxidant agent as well, it could be used in the preparation of films that act as barrier against chemical-physic change and the properties that possesses by itself could be further enhanced through the combination with other useful compounds (i.e. silver, catechins or organic acids) [11]. In winemaking, CTS spread as fining agent for different purposes, i.e. regulation of iron and copper excess, reduction of heavy metals or possible contaminants (as example ochratoxin), inhibition of unwanted microbial growth, especially against *Brettanomyces* spp., and protein stabilization. Chitosan has been admitted by the European Commission only recently [12], while International Organisation Of Vine And Wine (OIV) authorized chitosan utilization since 2009 [13, 14]. Today, OIV admits only the use of fungal chitosan (from *A. niger*), in order to avoid allergenic reaction due to the crustacean material, even if the functionality and the structure of the chitosan derived from crustaceans and fungi are declared identical by the producers. Several studies tried to define the details for the optimization of chitosan extraction [15,16, 5]. The most determinant chemical characters for chitosan are the deacetylation degree and the molecular weight. Previous studies discovered that the acid (organic or inorganic) used for chitosan dissolution manifests an effect on chitosan properties, such as the viscosity, mainly through the interaction with $-NH_2$ charged residue [17]. The acids used for dissolution were supposed to contribute in different way to other chitosan properties, enhancing its antifungal activity [18] or the interaction with bounded compound [19] as example. In this work the efficiency of chitosan on wine clarification has been evaluated comparing animal and fungal chitosans. As first, an overall of 10 commercial samples have been characterized for degree of deacetylation and chitosan purity. Moreover, samples have been dissolved into four different acid solutions with the aim to define whether and how this could influence viscosity and chitosan abilities in the wine fining.

2. Materials and Methods

2.1. Chemicals and reagents

Hydrochloric acid, acetic acid, malic and succinic acid, sodium hydroxide were purchased from Sigma-Aldrich (Milano, Italy). Water of HPLC grade was obtained by a Milli-Q system (Millipore Filter, Bedford, MA, USA).

2.2. Chitosan samples

Ten chitosans (CTS) were used for the comparison. Samples belong to two distinguished groups based on their origin, i.e. "MC" identified chitosans obtained from *Aspergillus niger* culture (samples F1, F2, F3, F4) while "SC" identified chitosan derived from shrimp shells (samples A5, A6, A8, A9) and crab shell (A7). MC were selected among chitosan

commercial products used for oenology purpose and were furnished from different suppliers, while SC were obtained from Sigma-Aldrich (Milano, Italy).

2.3. Chitosan deacetylation degree

The deacetylation degree was determined by titration as described by [20], titration method I. Chitosan (0.2 g) was dissolved into 20 mL of HCl 0.1 N and 25 mL of distilled water keeping the sample shaken at room temperature for 30 min. Then, other 25 mL of water were added and the sample was kept at the same condition for additional 30 min. Finally, sample solution was titrated adding NaOH 0.1 N by automatic titrator (Hanna Instrument, Villafranca Padovana, Italy). The degree of deacetylation (DDA) was determined by the equation:

$$DDA(\%) = 2.03*(V_2-V_1)/[m+0.0042*(V_2-V_1)]$$

where V_2 and V_1 are volumes of NaOH corresponding to the two inflection points. Each titration curve has been determined 3 times.

2.4. Chitosan content

CTS content of all the samples was determined *ex novo* as described by [21]. Five milligrams of chitosan powder were added to 400 μL of 10% *v/v* $NaNO_2$ and 10% *v/v* $KHSO_4$ (in the ratio of 1:1) and kept at room temperature for 15 min. After 3-Methyl-2-benzothiazolinone hydrazone (MBTH) 0.5% m/v addition and sample boiling, 500 μL of $FeCl_3·6H_2O$ 0.83% *m/v* were promptly added. Samples were then cooled at room temperature and 100 μL of each sample was transferred to a well of a 96-well microplate for the quantification at 650 nm in microplate reader (Molecular Devices, Menlo Park, CA, USA) and expressed as glucosamine equivalent. Data were expressed as percentage of chitosan on effective weight and quantification repeated 3 times per sample.

2.5. Viscosity

Viscosity was chosen as parameter to evaluate chitosan molecular weight [22]. Analysis was performed using Ubbelohde Viscometer type 1C (3-60 cS). Samples (1% chitosan *w/v*) were diluted 20 times in the selected buffer (acetic acid, succinic acid, malic acid and hydrochloric acid at 1% *v/v*) before starting the measurement, in order to assure that the efflux time remain below 300 sec. Samples were placed in thermostatic bath at 25°C until thermal equilibrium and then the time required for the efflux was measured in 2 replicates.

2.5. Wine clarification

Clarification was performed on Glera base wine furnished by Scuola di Enologia di Conegliano "G.B. Cerletti" (Conegliano, Italy). Chitosans were dissolved into four 1% *v/v* organic acids (malic, acetic, succinic and hydrochloric acid) at the 1% *w/v* concentration and let homogenize for 2 h stirring at room temperature. Wine was divided in 500 mL bottles in which 5 g/hL of chitosan were added singularly to the bottles, in 3 independent replications. Clarification was monitored measuring turbidity of the samples (nephelometer HI 83749, Hanna Instrument, Villafranca Padovana, Italy) after 30 min, 2, 4 and 24 h after the chitosan addition collecting 10 mL of treated wine from the bottle center.

2.6. Statistical analyses

R software (R version 3.0.1) was used for statistical analysis. Differences were evaluated by One-way ANOVA, Welch-ANOVA and Kruskal–Wallis H test depending on data distribution. Post-hoc analyses Tukey HSD test and Games-Howell test were used for ANOVA and Welch-ANOVA respectively, while Dunn test with Holm correction was chosen as Kruskal–Wallis post hoc test. Statistical significance was attributed with p-value <0.05 or confidence interval of 0.95.

3. Results and discussion

3.1 Chitosan deacetylation

The degree of deacetylation (DDA) is a useful tool for identifying chitosan structural rigidity and its polymer conformation, in addition it is directly connected to chitosan (CTS) number of positive charges [22] and thus to its cross-linking attitude [23]. The high number of charged amino groups arranged on the chitosan surface facilitates its dissolution in acid solutions and guarantees a general greater functionality, i.e. the control of microorganism, the binding of lipids, the improving of immune response and the cytotoxic activity [24]. Nevertheless, the DDA is strongly affected by the CTS production method in the light of the variation in the extraction protocols [4] that acquired even more importance when chitosans derived from different original material are considered as the case of the samples here studied. Nevertheless, the identification of original raw material cannot be sufficient to describe chitosan deacetylation and therefore, as first, selected chitosan underwent a preliminary test which define their deacetylation degrees.

Figure 2
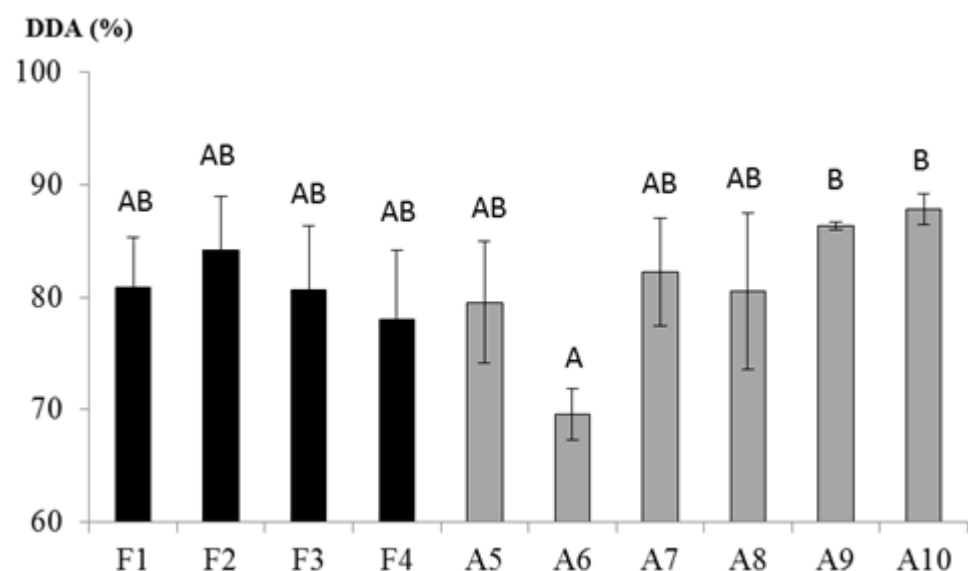
Overall, samples evidenced a degree of deacetylation varying between 70 to 95%, the common interval expected for commercial chitosan. CTSs could be categorized into three groups, i.e. "low" degree of deacetylation when DDA is ranging between 55– 70%, "medium" when comprise between 70–85% and "high" when achieve 85-95% of DDA [24]. So far, the "ultrahigh" degree of deacetylation -DDA above 95%- is difficult to reach through industrial process. Figure 2 show that A9 and A10 achieved the "high" value of DDA, with 86.3 and 87.7% respectively, while A6 evidenced the lowest level of deacetylation with 70% of DDA. The other samples were ascribed to the "medium" group. The comparison among samples highlighted a statistically significant difference between A6 and A9 and A10, with the latter grouped together ($F_{(9,19)}$=2.668, p=0.034). It should be noted that A6 was not completely dissolved in the buffer solution before the test, and that certainly influenced the result.

3.2 Chitosan purity

As previously stated, the origin of raw material determines chitosan physical properties. In fact, the choice of extraction protocol is based on the raw material origin and could change considerably the purity of the final extract [5]. As reported by Sietsma and colleagues [8], fungal CTS could present insoluble a percentage of β-glucan-chitin complex. Therefore, samples purity was determined by the depolymerization of chitosan into its

glucosamine monomers followed by their spectrometric quantification. The amount of monomers has been related to the initial sample mass and data express as percentage (Figure 3).
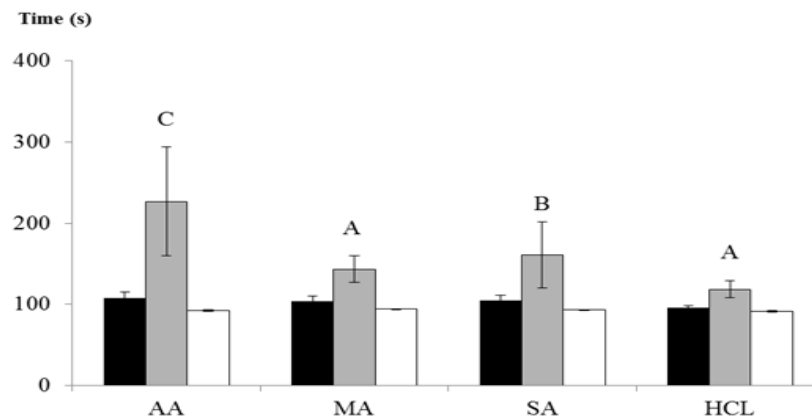
Figure 3
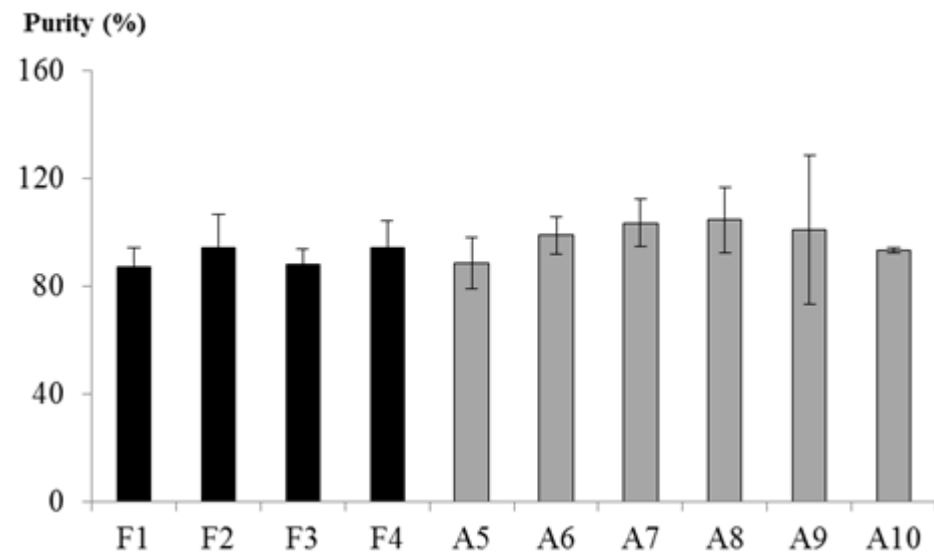
Data show that in all the cases sample purity was closed to 100%, with F1 and F3 as the loss pure at about 87%. Statistical analyses confirmed that there was no difference among samples neither between the two groups of fungal- (MC) and animal-derived- (SC) chitosans.
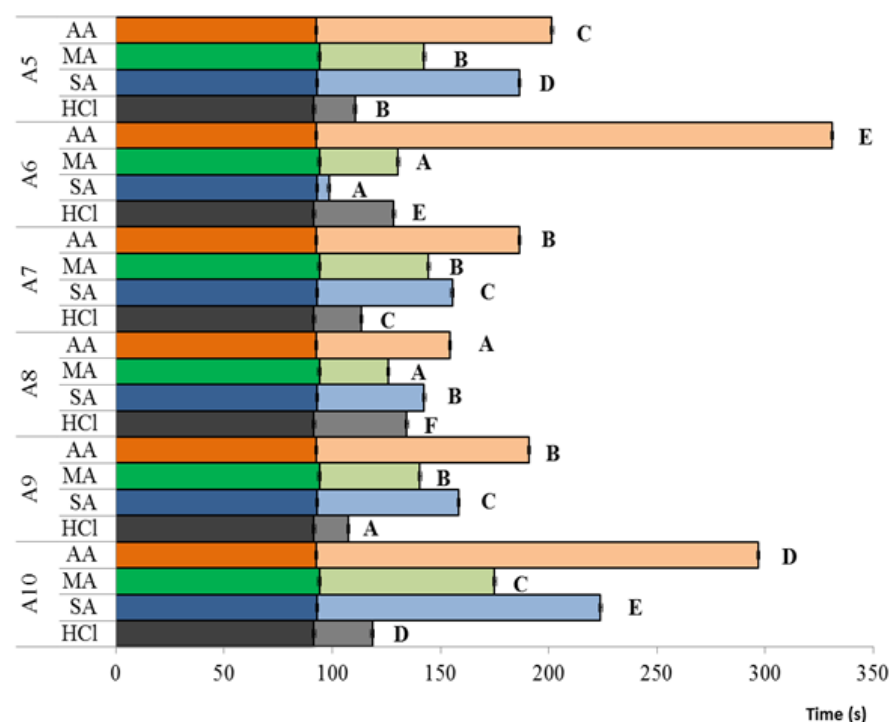
3.3. Chitosan viscosity

As known, viscosity reflects molecular characteristic of chitosan, namely the molar mass and the surface charge [22, 25]. As previously mentioned, DDA reveals a strict correlation with chitosan viscosity, but also the distribution of charges could play an important role, modifying conformational behavior of chitosan. New and colleagues [26] suggested that animal and fugal chitosan could differ for CTS charge distribution. Hence, studied samples were evaluated for the viscosities expressed when dissolved in four different acids, namely acetic, malic, succinic and hydrochloric acid. Previous experimental studies explored the effect that the dissolution acid could have on chitosan viscosity [18, 27], however these work did not compare several chitosan neither chitosans of different origin.

Figure 4

Statistical analyses revealed a significant difference among acid and categories, together with their interaction as evident by the Figure 4. Data highlights a different trend between animal and fungal chitosan at the acid change. Moreover, shell-derived CTSs manifested higher variability than the MC, which actually did not differ in viscosity from the respective control.

Figure 5

The Figure 5 represents the time requested for the solutions to throw the glass capillary, which means that high values correspond to high viscosities. Figure 5 is focused on the animal chitosan behavior because this category evidenced the major variability. In all the cases, CTSs revealed the highest density when dissolved in acetic acid and thus it was the most useful to distinguish chitosan by the specific molecular mass. It is known that viscosity directly depends on the chitosan molecular mass by the equation [22]:

$$[n]= KM_v{}^a \qquad\qquad (1)$$

where n is intrinsic viscosity, M_v the viscosity-average molecular weight and K and *a* are constants for given solute-solvent system and temperature. Two chitosans reported the highest values when compared with others, namely A6 and A10. This diversity is probably imputable to the specific production method [28]. Kasaai and colleagues [22] concluded that *a* is described by the equation:

$$a = [DA/(pH\ \mu)] \tag{2}$$

where DA is degree of acetylation, pH is the pH and (μ) the solution ionic strength, demonstrating a direct relationship between the dissolution media and the chitosan viscosity. The differences recorded from the comparison of chitosan viscosities among the four dissolving acids confirmed this interaction between CTS and the dissolution system. As a matter of fact, while deacetylation could be influenced by the chitosan manufacturing as reported by Bajaj and colleagues [28], and therefore it could explains the differences among chitosans, it should be assumed as constant when comparing the same chitosan sample dissolved in different acids. As explained by Kasaai *et al.* [22], low pH should lead to a higher degree of expansion of chitosan due to electrostatic repulsions, reducing the mobility of its structure, causing an increase in the viscosity. Unexpectedly, for each CTS, viscosities decreased following the pH lowering order (2.8, 2.6, 2.3, 0.6 for acetic, succinic, malic and HCl respectively). However, it was also demonstrated that the intrinsic viscosity decrease with the increase of the ionic strength, as the chain became more flexible and compact with a reduction of the repulsive potential owing to the masking effect of anions [29]. Hydrochloric acid possesses the highest ionic strength, followed by the two diprotic acids (succinic and malic acids) and by the acetic acid. According to that, CTSs showed a reduction in viscosity when dissolved into diprotic acids and even greater when dissolved into hydrochloric acid. Moreover, Figure 5 highlights an interesting variability among chitosans in the response to acid change, which could depend to the $-NH_2$ groups available on the CTS surface. According to Cho *et al.* [29], the viscosity decreases because of the shielding effect of anions on the positively charged amino groups, that in one hand induces a strong reduction of the repulsive potential but, on the other hand, increases the risk of flocculation and precipitation. In agreement with this, one of the studied samples (A6) showed an uncomplete dissolution in all the acids.

3.4 Wine clarification performance

Several works explored the effects that different solvents have on chitosan properties, testing, as example, antimicrobial activities against bacteria and mold [30, 18], CTS-membrane properties and hydrophobicity [31], CTS-film water vapor permeability [17], resistance and elasticity [32]. However, no studies explored whether and how the choice of the acid used for CTS dissolution influences wine clarification. Clarification is a process that occurs in nature, is linked to the flocculation and precipitation of suspended colloids and chitosan is known to enhance this process by the instability generated by the interaction between colloids and NH_2 residues of chitosan [33]. Chitosan physiochemical characteristics, such as degree of deacetylation and molecular mass as well, affect the clarification results [34]. Studied samples evidenced heterogeneities for both DDA and molecular mass and the viscosity test indicated that dissolution acid could affect chitosan molecular conformation. Therefore, a clarification test was performed comparing fungal and animal-derived chitosan dissolved into the four acids. Turbidity was recorded at 30 min, 2 h, 4 h and 24 h. After 24 h all the samples demonstrated a very low turbidity- lower than 100 NTU, including the control- and therefore that point was excluded from further considerations.

Figure 6
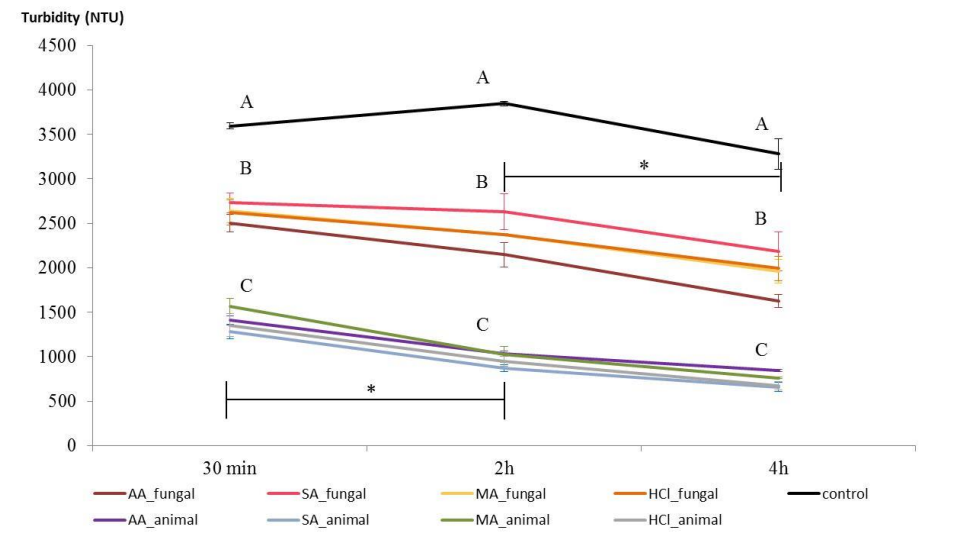
Data evidenced a clear distinction between chitosan categories ($\chi^2_{(2)}$ = 49.83, p<0.01), with fungal chitosan that reduce the wine turbidity already after 30 min -about 25%- and keep on lowering it in the successive hours (Figure 6). Nevertheless, animal chitosan showed a surprising clarification capacity by dropping the NTU value 30 min after the treatment of about 60%. Relationship between CTS and the dissolving acid was evaluated more specifically at two time point, namely after 4 h for fungal CTS and after 2 h for animal CTS, according to the significant statistical difference detected between two successive time points ($F_{(2, 141)}$= 15.3, p=<0.001 and $F_{(2, 213)}$=12.76, p= <0.001 respectively).

Figure 7
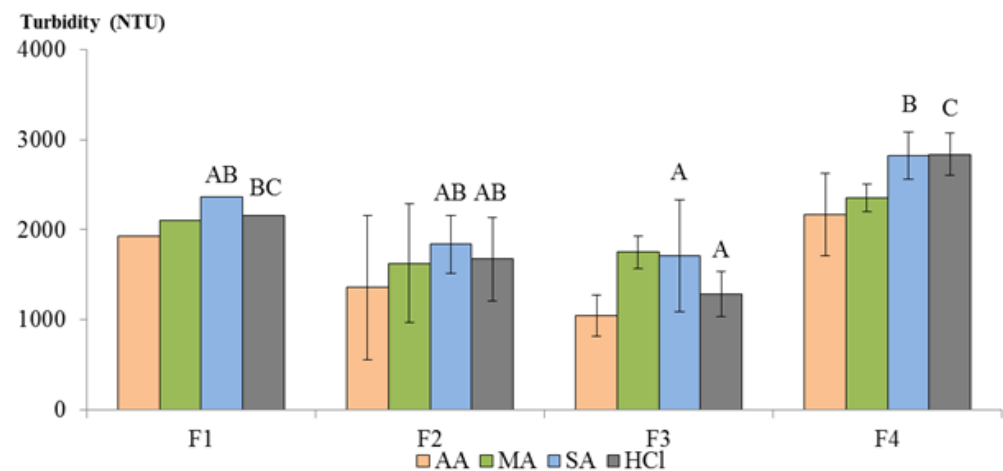
Figure 7 reports the turbidity values achieved from samples 4 hours after the treatment with MC. Statistical analyses revealed a significant sample effect, expressed by different letter posed above the histograms of succinic acid and hydrochloric acid, while the dissolving acid used for the chitosan preparation lost its effect. The importance of the dissolving acid reduced probably due to the sample dilution. However the difference among samples emerged only when the high ionic strength acids are associated to the samples, suggesting that sample variation concerning the mechanism of the primary amines protonation and probably the of CTS charge density [32].

Figure 8
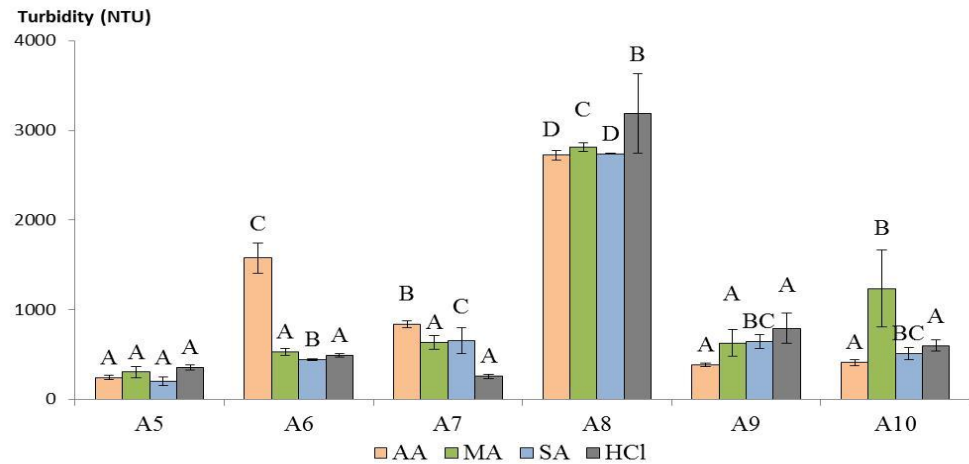
Animal chitosan allowed a greater clarification than MC at all the time points (Figure 6). Figure 8 shows the comparison of SC behavior after 2 h of treatment because after that point the chitosan clarification rate decreased. As expressed before, in animal CTS the manufacturing process varies in several steps, such as demineralization and deproteini-sation besides to deacetylation. Bajaj and colleagues [28] demonstrated that the alkaline deproteinization performed for 2 h could induced CTS backbone breaking even at 65°C, or less for longer treatment, while the comparison of the deacetylations revealed a less clear effect on CTS, confirming that the "pre-treatment" participates to define CTS molecular mass. However, in this work, no correlation between the viscosities expressed by the sample in acetic acid, parameter linked to the specific molecular mass, and the clarification capacity. This indicates that other factors are more relevant for determining CTS clarification property. Based on literature, two main parameters seem to strongly affect colloids- CTS interaction, namely the chitosan DDA and the pH of reaction [33]. However, A8 demonstrated a degree of deacetylation similar to A7 (Figure 2) while its clarification power is considerably lower. Concerning pH, this factor should be excluded because the experiment was carried out at the same pH value for all the chitosans as only one wine was used. The shellfish chitosans comparison registered a statistically significant effect of the dissolving acid, together with a significant interaction between sample and acid. This indicated that in several cases (A6, A7, A9 and A10) acids had an effect on CTS clarifying capacity but this influence was not consistent among chitosan samples and its degree was negligible. Probably the nature of this interaction could be attributed to the specific charge distribution on the chitosan surface.

4. Conclusions

Chitosan is a natural polymer that has been approved quite recently as a fining agent for microbial control, metal chelation, reduction of contaminants and clarification in oenology. Clarification property is strictly connected to chitosan property of binding colloids, such as protein, polyphenols, polysaccharides and metal ions. The origin of raw material and consequently the manufacturing process required for the chitosan extraction and purification, together with the efficiency in its deacetylation, are determining in the clarification results. To date, oenological codex permits only the use of chitosan derived from fungi that, as here demonstrated, possess low efficiency respect to the shellfish extracted chitosan. At present, no evidence of health risk in the use of animal-derived chitosan has been registered, while the recovery of useful molecule from industrial waste is generally recommended. Besides, it should be considered that chitosan from other sources, such as insect-derived chitosan, actually represent a potential source for a new generation of fining agents. Moreover, in this work was also evidenced that, differently from what registered for other application, the dissolving acid did not significantly influence the clarification efficiency.

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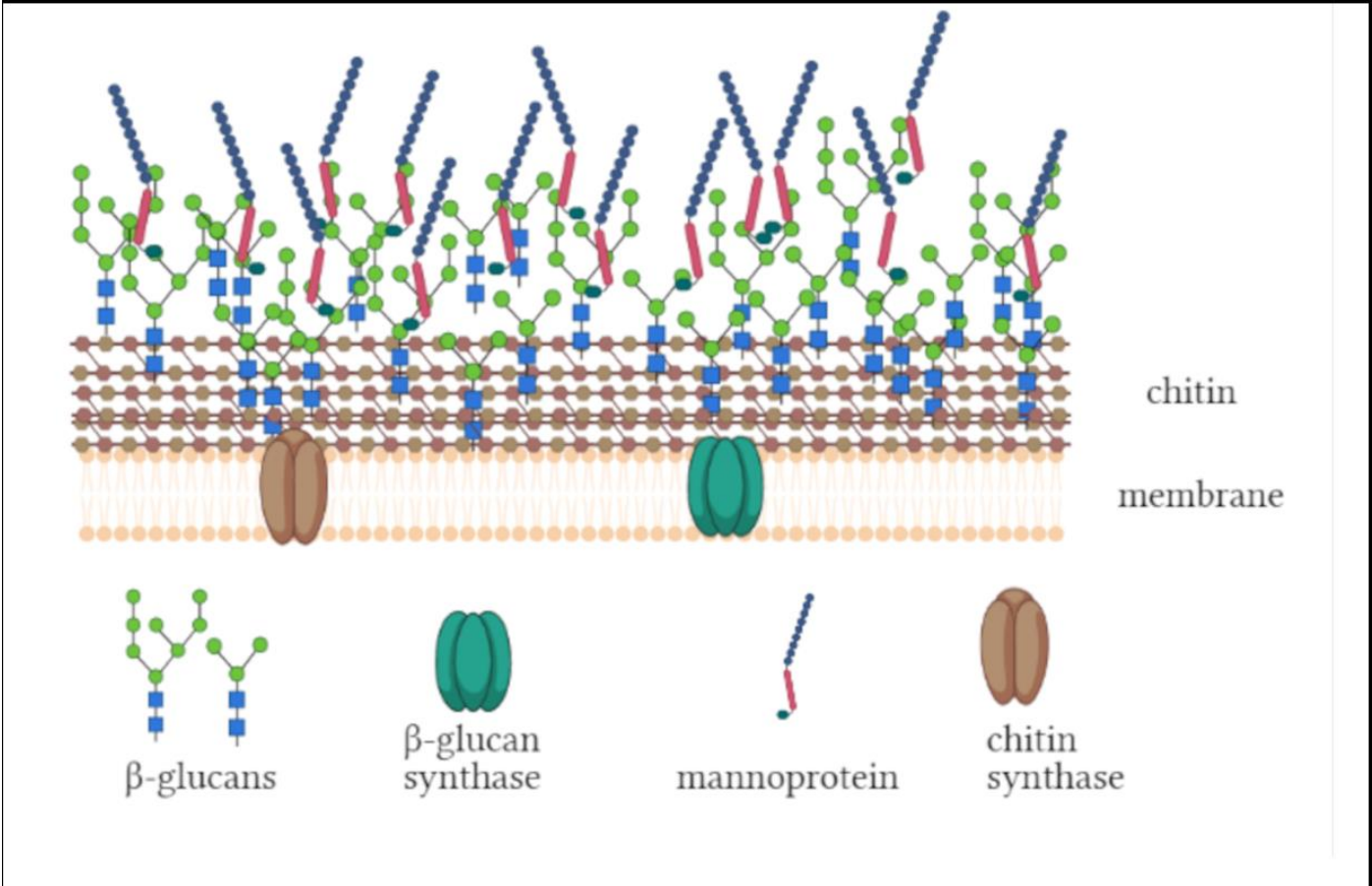
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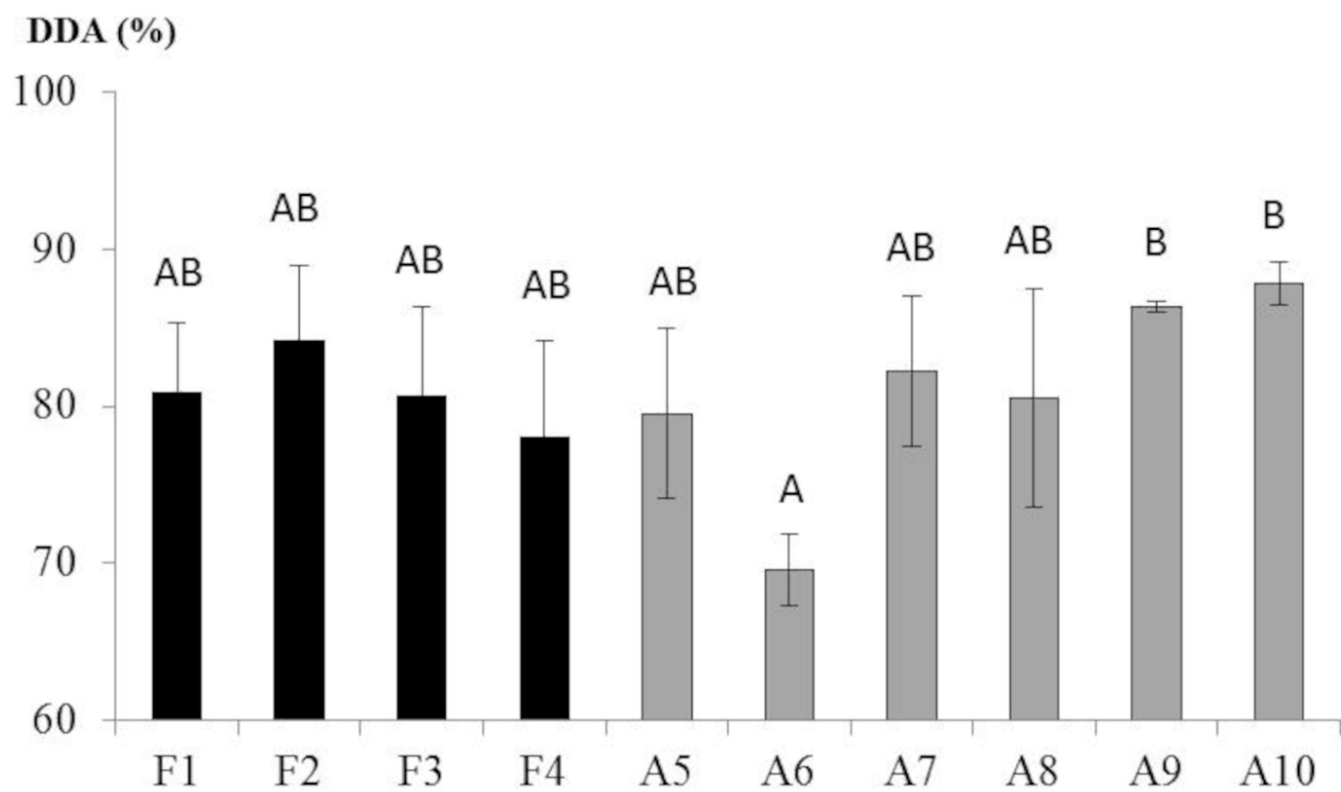**Figure 1**
    General structure of fungal cell wall.

Figure 2
Deacetylation degree of chitosan. Mean of three replications (in percentage) and standard deviations are expressed. Black bars: fungal-derived chitosan, light grey bars: crustacean-derived chitosan. Capital letters represent statistical groups
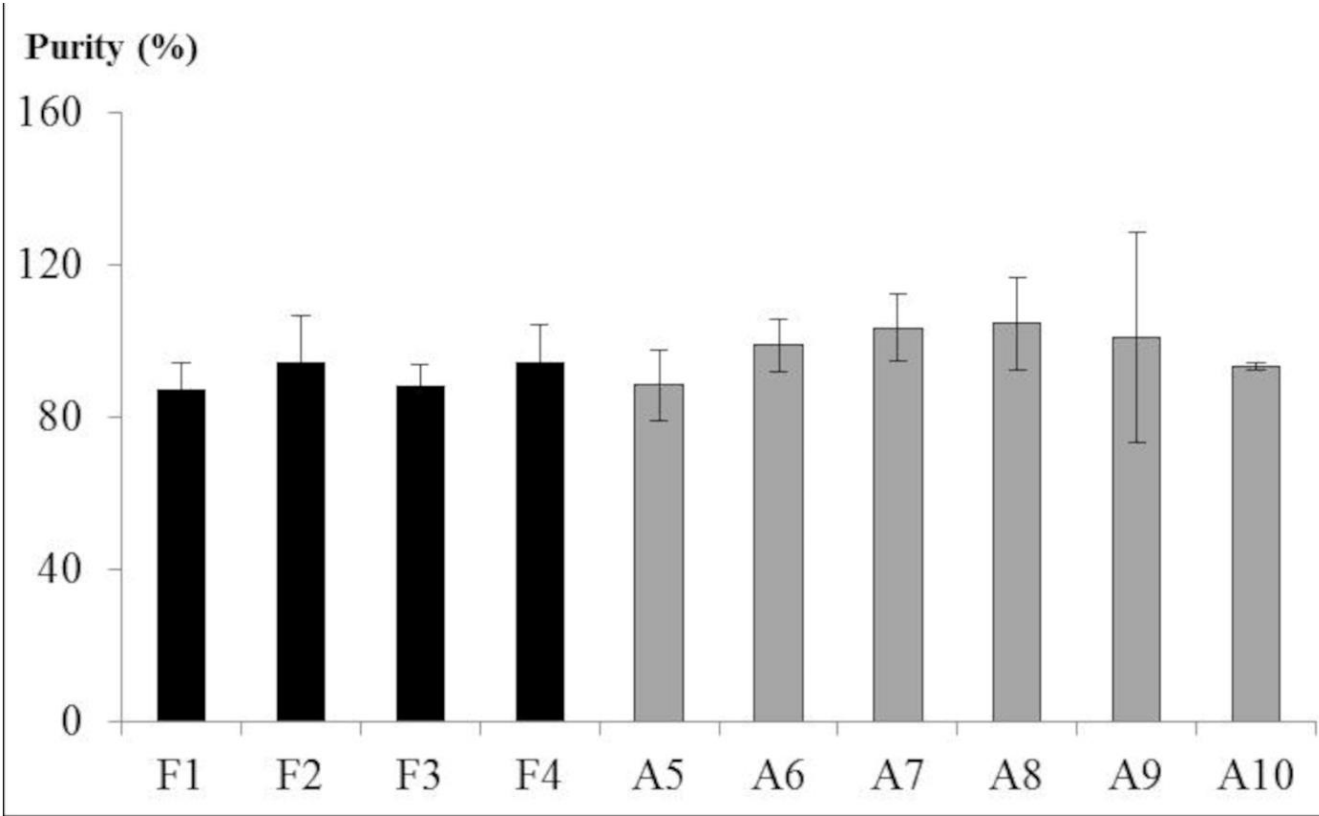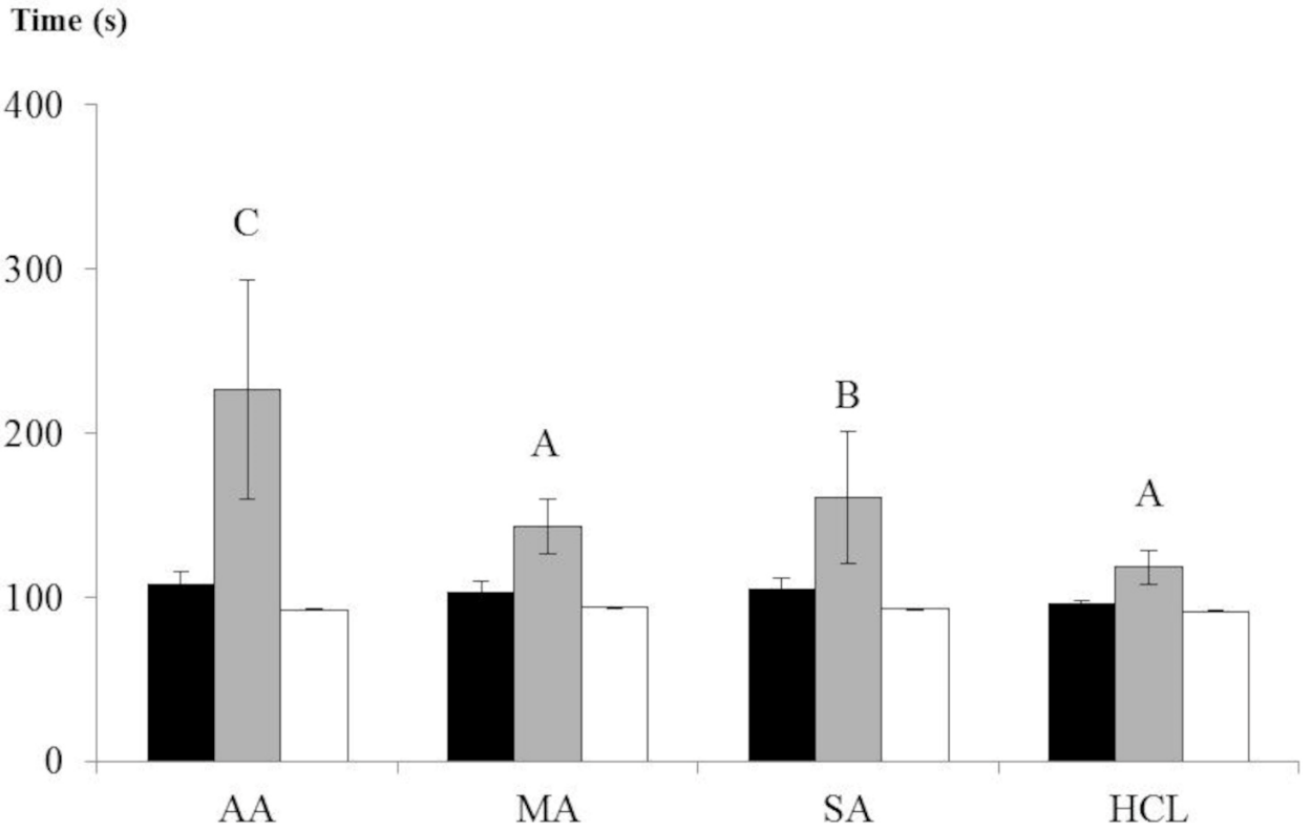
Figure 3

Sample purity. Mean (in percentage) and standard deviations of three replications are expressed. Black bars: fungal-derived chitosan, light grey bars: crustacean-derived chitosan.

Figure 4

Chitosan viscosity. Mean and standard deviations (three replications for each sample) are expressed. Black bars: fungal-derived chitosan, light grey bars: animal-derived chitosan, white bars: corresponding acid solution (control). Capital letters represent statistical significant differences among dissolving acids ($p<0.05$).
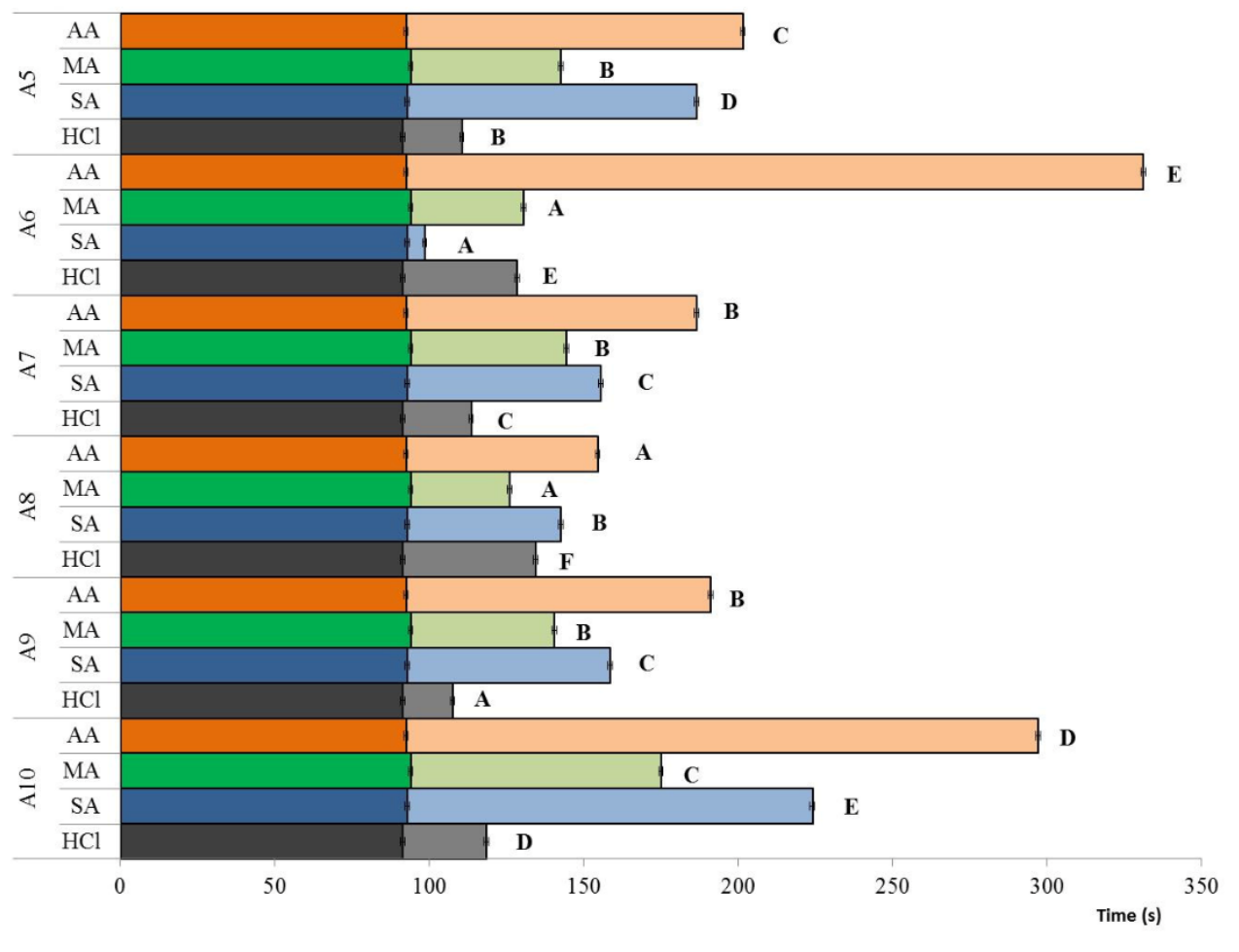

Figure 5

Animal-derived chitosan viscosities in four acids. Mean and standard deviations of two replications are expressed. Dark colors bars: acid viscosities, light colors bars: chitosan viscosities. Capital letters represent statistical significant differences among chitosans dissolved into the same acid ($p<0.05$).
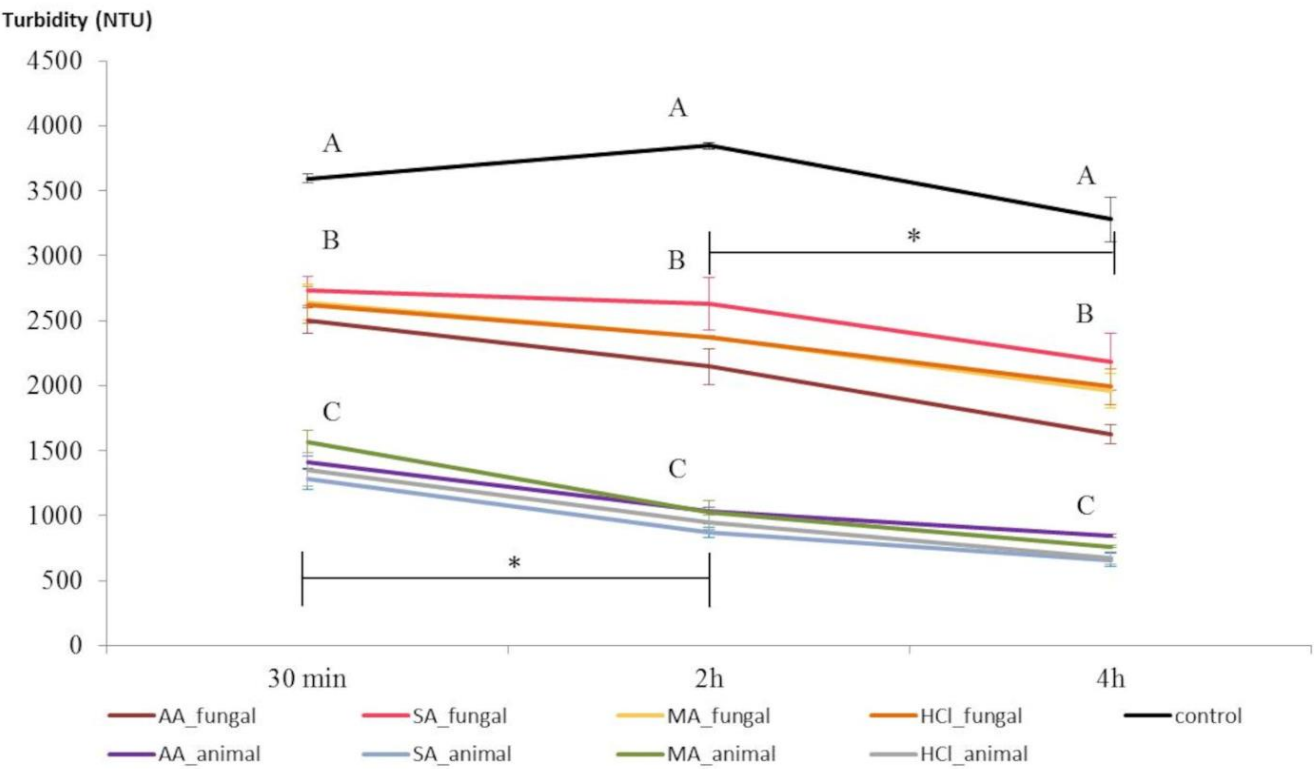
Figure 6

Clarification of Glera wine. Turbidities of treated and untreated wine are compared. Category mean and standard deviations (three replications for each chitosan sample) are expressed. Capital letters represent statistical significant differences among dissolving acids and stars express statistical difference between successive time points ($p<0.05$).
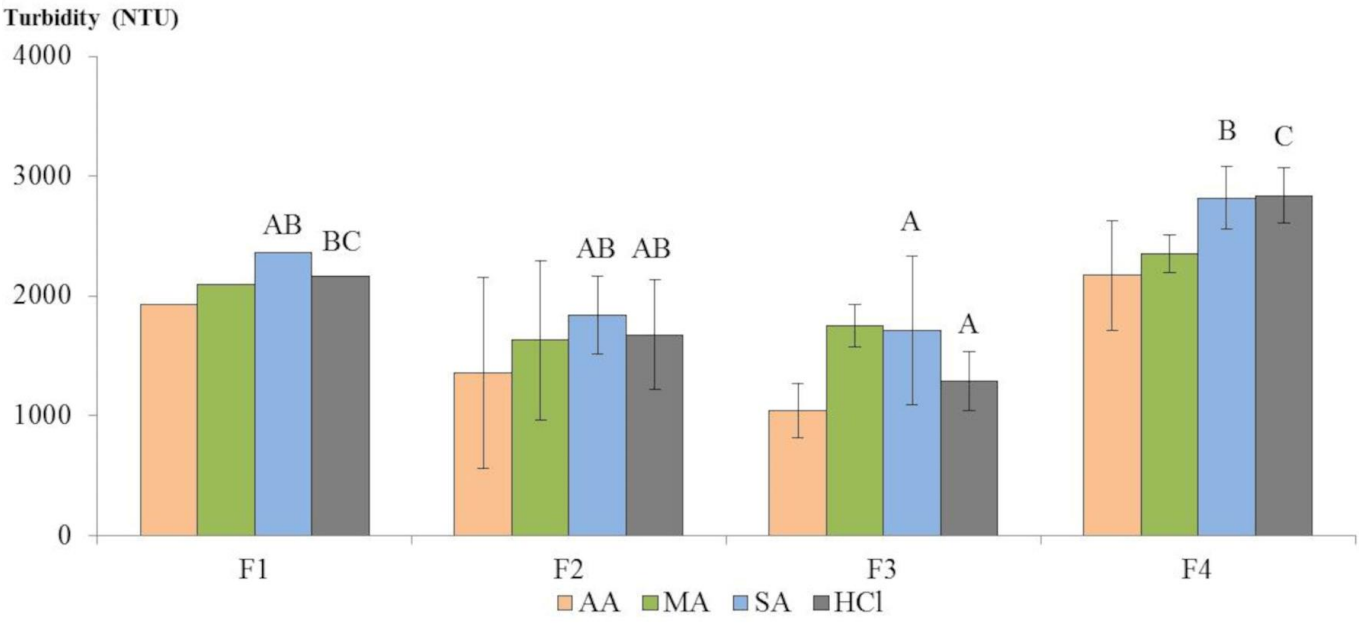

Figure 7

Fungal-derived chitosan clarification after 4 h. Mean and standard deviations of three replications are expressed. Capital letters represent statistical significant differences among samples dissolved into the same acid ($p<0.05$). AA: acetic acid, MA: malic acid, SA: succinic acid, HCl: hydrochloric acid.
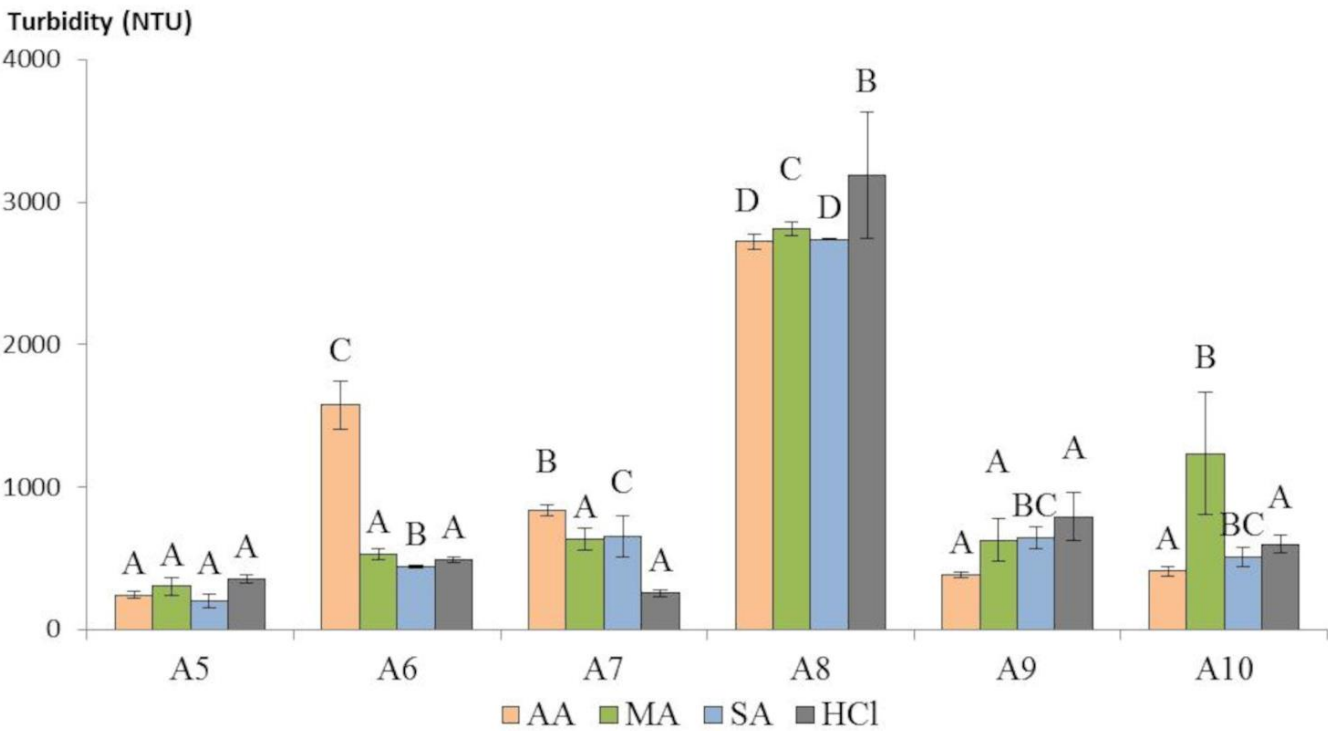
Figure 8

Animal-derived chitosan clarification after 2 h. Mean and standard deviations of three replications are expressed. Capital letters represent statistical significant differences among samples dissolved into the same acid ($p < 0.05$). AA: acetic acid, MA: malic acid, SA: succinic acid, HCl: hydrochloric acid.