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ORIGINAL PAPER

# Effectiveness of chitosan against wine-related microorganisms

**Simel Bağder Elmacı · Gökşen Gülgör · Mehmet Tokatlı · Hüseyin Erten · Aslı İşci · Filiz Özçelik**

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**Abstract** The antimicrobial action of chitosan against wine related microorganisms, including *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, *Oeonococcus oeni*, *Lactobacillus hilgardii*, *Brettanomyces bruxellensis*, *Hanseniaspora uvarum* and *Zygosaccharomyces bailii* was examined in laboratory media. In order to assess the potential applicability of chitosan as a microbial control agent for wine, the effect of chitosan, applied individually and/or in combination with sulphur dioxide ($SO_2$), on the growth of microorganisms involved in various stages of winemaking and on the fermentative performance of *S. cerevisiae* was investigated. Of the seven wine-related microorganisms studied, *S. cerevisiae* exhibited the strongest resistance to antimicrobial action of chitosan in laboratory media with a minimum inhibitory concentration (MIC) greater than 2 g/L. *L. hilgardii*, *O. oeni* and *B. bruxellensis* were the most susceptible to chitosan since they were completely inactivated by chitosan at 0.2 g/L. The MIC of chitosan for *L. plantarum*, *H. uvarum* and *Z. bailii* was 2, 0.4 and 0.4 g/L, respectively. In wine experiments, it was found that chitosan had a retarding effect on alcoholic fermentation without significantly altering the viability and the fermentative performance of *S. cerevisiae*. With regard to non-*Saccharomyces* yeasts (*H. uvarum* and *Z. bailii*) involved in winemaking, the early deaths of these yeasts in mixed cultures with *S. cerevisiae* were not probably due to the antimicrobial action of chitosan but rather due to ethanol produced by the yeasts. The complex interactions between chitosan and wine ingredients as well as microbial interactions during wine fermentation considerably affect the efficacy of chitosan. It was concluded that chitosan was worthy of further investigation as an alternative or complementary preservative to $SO_2$ in wine industry.

**Keywords** Chitosan · $SO_2$ · Wine spoilage · Antimicrobial activity

S. Bağder Elmacı and G. Gülgör have contributed equally to this work.

S. Bağder Elmacı (✉) · A. İşci · F. Özçelik
Department of Food Engineering, Faculty of Engineering, Ankara University, Ankara, Turkey
e-mail: sbagder@eng.ankara.edu.tr

G. Gülgör
Department of Food Engineering, Faculty of Agriculture, Uludağ University, Bursa, Turkey

M. Tokatlı
Department of Food Engineering, Faculty of Natural Sciences and Engineering, Gaziosmanpaşa University, Tokat, Turkey

H. Erten
Department of Food Engineering, Faculty of Agriculture, Çukurova University, Adana, Turkey

## Introduction

Sulphur dioxide ($SO_2$) is a widely used chemical preservative in the wine industry to prevent the growth of spoilage microorganisms (du Toit and Pretorius 2000; Enrique et al. 2007; Renouf et al. 2008). However, there has been increasing trend towards minimizing $SO_2$ levels in wine industry since high levels of $SO_2$ can result in off-odours and flavors in the final product and may cause allergic responses in some consumers (Costantini et al. 2009; García-Ruiz et al. 2008; Renouf et al. 2008; Threlfall and Morris 2002). According to the European Union legislations, it is required to declare the presence of sulphites on wine label if it exceeds 10 mg/L (Costantini et al. 2009; García-Ruiz et al. 2008; Salaha et al. 2008). Furthermore, the use of $SO_2$ has also the following technological restrictions. Its effectiveness depends on the pH. At low pH values, $SO_2$ is mainly present as molecular $SO_2$ which is the only antimicrobial form of $SO_2$. However, at wine pH values (3.0–4.0), only 5 % of $SO_2$ exists in the active molecular form (du Toit and Pretorius 2000; Enrique et al. 2007; Fugelsang and Edwards 2007). $SO_2$ has a greater activity on bacteria than on yeasts (du Toit and Pretorius 2000). Some wine spoilage yeasts may have resistance to the maximum permitted levels of $SO_2$ (Enrique et al. 2007). In this context, several chemical and naturally occurring preservatives such as dimethyldicarbonate, sorbic acid, fumaric acid, benzoic acid, nisin, lysozyme, killer toxins or chitosan, and alternative technologies such as hydrostatic pressure, the application of low electric current or pulsed electric fields technology have been proposed to control microbial growth in enology (Divol et al. 2005; du Toit and Pretorius 2000; Fugelsang and Edwards 2007; Puértolas et al. 2009; Renouf et al. 2008). However, no new compound or strategy has been identified which could be a real substitute for $SO_2$ with all its oenological properties including the antioxidative and antiseptic action in winemaking (García-Ruiz et al. 2008; Suzzi et al. 1985).

In recent years, chitosan has received considerable attention due to its antimicrobial activity against a broad range of microorganisms, such as bacteria, yeast and moulds (Aider 2010; Shahidi et al. 1999). Chitosan is a cationic polysaccharide produced by the deacetylation of chitin which is a natural polymer found in the exoskeletons of crustaceans (e.g., shrimp, crab, lobster) and insects, and in the cell walls of certain fungi (Moussaa et al. 2013; Tang et al. 2010). The unique antimicrobial activity of chitosan has been attributed to its positive charges that would interfere with the negatively charged residues of macromolecules on the cell surface, rendering membrane leakage (Valencia-Chamorro et al. 2011). In contrast with $SO_2$, chitosan has been observed to affect more quickly on fungi than on bacteria (Goy et al. 2009). With regard to human health, it is important to note that chitosan, as a natural antimicrobial agent, is generally recognized as safe (GRAS) by the US FDA (Giatrakou et al. 2010). Additionally, it shows medically important properties, such as antitumor activities, immunostimulating effects, cholesterol-lowering effects, wound healing effects and free radical scavenging activities (Park et al. 2008). Chitosan is also economically advantageous over the other natural preservatives like lysozyme, nisin, killer toxins since chitin, the main constituent of chitosan, is the second most abundant polysaccharide found in nature after cellulose and is a waste product of aquaculture (Aider 2010).

There are several studies dealing with the antimicrobial properties of chitosan in various food products including meat products (Aldemir and Bostan 2009; Ouattara et al. 2000), ready to cook chicken product (Giatrakou et al. 2010), shrimp (Huang et al. 2012), cheese (Gammariello et al. 2010), tomato juice (Giner et al. 2012), broccoli (Moreira et al. 2011), strawberry (Vu et al. 2011), pear (Xu et al. 2013). However, very few attempts have been made to assess the effectiveness of chitosan against wine-associated microorganisms in vitro or in wine conditions. Gómez-Rivas et al. (2004) investigated the effect of chitosan on *Saccharomyces cerevisiae*, *Brettanomyces bruxellensis*, and *B. intermedius*, both separately and in mixed cultivations. In another study, Portugal et al. (2014) evaluated the antimicrobial activity of chitosan against *Brettanomyces* both in the presence and in the absence of ethanol in vitro and under winery conditions. In both studies, it was only aimed to control the wine spoilage yeast *Brettanomyces*, not taking into consideration of other microflora involving in the wine production (e.g., non-*Saccharomyces* yeasts, lactic acid bacteria).

The objective of the present study was to test the antimicrobial activity of chitosan against a range of bacteria and yeasts of importance as fermentative organisms or wine spoilers. It was also aimed to

determine the effect of chitosan on some oenological parameters during different stages of winemaking, including alcoholic fermentation, malolactic fermentation and wine storage.

## Materials and methods

### Microbial strains

Seven wine related microorganisms, including *Lactobacillus plantarum*, *Saccharomyces cerevisiae*, *Oenococcus oeni*, *Lactobacillus hilgardii*, *B. bruxellensis*, *Hanseniaspora uvarum*, *Zygosaccharomyces bailii*, were tested for their susceptibility and sensitivity toward the treatment with chitosan. Indigenous wine yeast strain, *S. cerevisiae* Narince 3 (Bağder Elmacı et al. 2014) and *L. plantarum* MF513 (GenBank accession no. KJ855887) isolated from pickles were obtained from the Culture Collection of the Food Engineering Department of Ankara University. *O. oeni* DSM-20252, *L. hilgardii* DSM-20051 and *B. bruxellensis* DSM-70001 were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). *H. uvarum* JFO 1755 and *Z. bailii* JFO 0488 were kindly provided by Prof. Dr. Yeşim Özbaş from the Culture Collection of Hacettepe University. *O. oeni* Lalvin VP41 was used for the malolactic fermentation experiments. This strain was kindly provided from the Culture Collection of Çukurova University.

### Preparation of chitosan solution

Chitosan was purchased from Sigma-Aldrich (deacetylation 75–85 %, low molecular weight). A stock solution of chitosan was prepared by dissolving 10 g in 1 L of 1 % (w/v) glacial acetic acid (final chitosan concentration = 1 % w/v) and was then sterilized at 121 °C for 15 min. Chitosan is soluble in dilute aqueous acid solutions such as formic, acetic, lactic and oxalic acids. However, acetic acid is the most frequently used solvent of chitosan (Coma 2013).

### Determination of minimum inhibitory concentration (MIC) of chitosan

Sterilized stock solution, containing chitosan at levels ranging from 0.2 to 2 g/L, was added to YPG Broth (10 g/L yeast extract, 20 g/L peptone, 200 g/L glucose, pH 3.5) to a final volume of 10 mL to determine MIC of chitosan against the microbial strains of interest. Viable yeast and bacteria were inoculated at the initial concentration of approximately $10^6$ cells/mL. Chitosan-free medium inoculated with test strains, was used as a control. The chitosan treated cultures were then incubated at 28 °C for 6 days with the exception of the *S. cerevisiae* cultures which were incubated for 20 days. Viable microorganisms were enumerated on appropriate medium at certain intervals with spread plate method. The MIC was defined as the lowest concentration of chitosan that completely inhibited the microbial growth.

### Chitosan experiments

Experiments were set up to investigate the inhibitory effect of chitosan against *S. cerevisiae*, *H. uvarum, Z. bailii*, *O. oeni*, *B. bruxellensis* which are wine related microorganisms involving in different stages of winemaking. Wine fermentation and malolactic fermentation of musts were conducted with the addition of chitosan considering the minimum inhibition concentration (MIC) of chitosan for each strain. The antimicrobial activity of chitosan during wine storage was also investigated. All experiments were conducted in triplicate.

#### *Effect of chitosan on alcoholic fermentation*

Narince, a well-known white grape cultivar of Turkey, was used in this study and obtained from the trial vineyard of Ankara University, Faculty of Agriculture. The grapes were pressed manually, and filtered through a roughing filter. Narince must had a titratable acidity (as tartaric acid) of 6.3 g/L, pH of 3.3 and reducing sugar of 212 g/L. The must, pasteurized at 95 °C for 30 min, was used as fermentation medium. It was divided into eight batches to test for different experimental groups illustrated in Table 1.

Fermentations were carried out in triplicate at 25 °C in 500 mL brown-colored fermentation flasks, containing 250 mL must adjusted to pH 3.5. The grape juice containing different concentrations of chitosan and/or $SO_2$ was inoculated with *S. cerevisiae* and a mixture of non-*Saccharomyces* species, *H. uvarum, Z. bailii* at the initial concentration of $10^6$ cells/mL for each species. The flasks were plugged with glass

**Table 1** Experimental groups in wine trials

| Experimental groups |
|---|
| Group A: negative control—no microorganism, no chitosan |
| Group B: positive control—no chitosan |
| Group C: 0.2 g/L chitosan |
| Group D: 0.4 g/L chitosan |
| Group E: 0.6 g/L chitosan |
| Group F: 0.2 g/L chitosan + 40 mg/L $SO_2$ |
| Group G: 0.4 g/L chitosan + 40 mg/L $SO_2$ |
| Group H: 40 mg/L $SO_2$ |

fermentation traps containing sterile distilled water to allow only $CO_2$ to emerge from the system. The fermentation was monitored by measuring the weight loss every 2 days until the end of fermentation during which the weight loss due to $CO_2$ production became constant. The fermentation rate (as g $CO_2$/Lh) was measured based on the weight loss within the second and fourth days of fermentation. Cell counts, concentrations of D-glucose/fructose and ethanol were also monitored throughout the fermentation experiments. Total yeast (TY) and non-*Saccharomyces* (NS) population were counted on YGC Agar and YGC Agar supplemented with cycloheximide (0.01 % w/v), respectively. The *S. cerevisiae* population was calculated by subtracting NS from TY. Glucose and fructose concentrations were determined enzymatically by Boehringer, R-Biopharm commercial kits (Cat. No. 10139106035). Ethanol content was analyzed by gas chromatography (Shimadzu GC-2010 Plus, Kyoto, Japan) equipped with a flame ionization detector (FID). Prior to injection, the samples were filtered through a 0.45 μm PVDF (polyvinylidene fluoride) filter (Millipore, Bedford, MA, USA). One microliter of samples was injected into the GC system which was equipped with a TRB-WAX capillary column (30 m long, internal diameter 0.25 mm). The column oven, injection and detector temperatures were maintained at 220, 250 and 230 °C, respectively. Nitrogen was used as carrier gas, and the fuel gas was a mixture of hydrogen and air.

*Effect of chitosan on malolactic fermentation (MLF)*

After the completion of alcoholic fermentation, the wines in Group B through H were inoculated with *O. oeni* Lalvin VP41 at a level of $10^6$ cells/mL to conduct MLF. LAB were counted on MRS Agar plates and the lactic acid production rate was determined enzymatically using Boehringer, R-Biopharm commercial kits (Cat. No. 10139084035) to monitor MLF and to evaluate the effect of chitosan and/or $SO_2$ addition on MLF. Malic acid was determined using a HPLC system (Shimadzu, Kyoto, Japan) which includes a pump (LC-20AD), an auto-sampler (SIL-20A HT), a degasser (DGU-20$A_3$), a column compartment (CTO-10AS VP) and a refractive index (RI) detector (RID-10A). The separation was performed in a Transgenomic IC Sep ICE-COREGEL-87H3 column operated at 35 °C with a flow rate of 0.5 mL/min using 5 mM $H_2SO_4$ as the mobile phase.

*Effect of chitosan during wine storage*

The finished wine was inoculated with *B. bruxellensis* DSM-70001 at the initial concentration of $10^4$ cells/mL and stored at 17–18 °C for 6 months in 200 mL hermetically capped glass bottles. The yeast population was counted every month on YGC Agar plates during storage.

Statistical analyses

Experimental data were analysed by using the Minitab statistical software, version 14 (Minitab Inc., State College, PA, USA). Statistical differences among means were determined by the Duncan's multiple range tests at the 5 % significance level.

## Results and discussion

The inhibitory effect of chitosan on the growth of *S. cerevisiae*

As shown in Fig. 1, at concentrations of 0.6–2 g/L chitosan, the initial inhibitory effect on *S. cerevisiae* was biocidal within the first 2 days of exposure, as the viable cell numbers were reduced to below detection limit of the plate count method. In the presence of 0.4 g/L, cell growth of *S. cerevisiae* decreased by approximately 1 log cycle within 2 days. However, the growth rate was not affected by the addition of 0.2 g/L chitosan as the growth pattern was similar to that of control for 20 days. It was also found that the chitosan concentration above 0.2 g/L, resulted in extended lag

phase (lag phase was 2 days for 0.4–1.8 g/L chitosan and 4 days for 2 g/L chitosan). The inhibitory effect of chitosan on *S. cerevisiae* by means of extending the lag phase has previously been reported (Gómez-Rivas et al. 2004; Roller and Covill 1999). After the extended lag phase, resumption of growth was observed in this concentration range. A similar growth pattern of *S. cerevisiae* in the presence of 0.1 and 0.4 g/L chitosan was also observed by Roller and Covill (1999). Although the presence of 0.4–2.0 g/L chitosan resulted in reversible inactivation of *S. cerevisiae*, a higher concentration was required for irreversible inactivation. Minimum chitosan concentration needed to inhibit the growth of *S. cerevisiae*, either partially or completely, was found to be higher than those found in other studies by Roller and Covill (1999) and Portugal et al. (2014) (0.1–0.4 g/L > 0.25 g/L, respectively). However, Gómez-Rivas et al. (2004) reported that chitosan concentrations above 4 g/L caused only a reduction in growth rate. These discrepancies could be attributable to the different media or concentration ranges used in the studies. In addition, the antimicrobial activity of chitosan appeared to be strain-dependent.

## The inhibitory effect of chitosan on the growth of non-*Saccharomyces* yeasts

The chitosan exerted stronger inhibitory activity against non-*Saccharomyces* than *S. cerevisiae*. *H. uvarum* and *Z. bailii* were completely inactivated by chitosan at 0.4 g/L after 3 days of incubation and, unlike *S. cerevisiae*, no recovery of growth was observed after 6 days. There was no significant reduction in the numbers of *H. uvarum* and *Z. bailii* in the presence 0.2 g/L chitosan (Table 2). The MIC value (0.4 g/L) of *Z. bailii* was consistent with the findings of Roller and Covill (1999) who reported that *Z. bailii* was susceptible to chitosan at 0.1 and 0.4 g/L. The antimicrobial action of chitosan on *H. uvarum* has not been previously reported in literature.

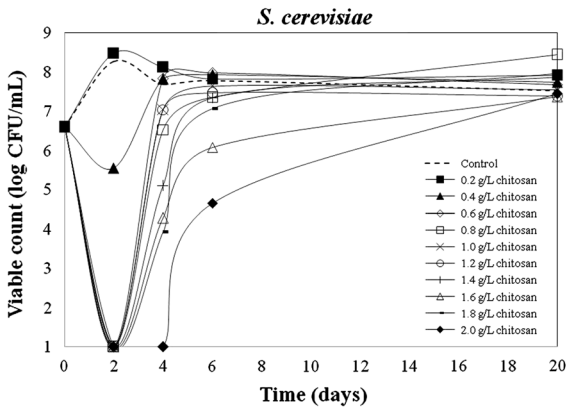



**Fig. 1** Growth of *S. cerevisiae* Narince 3 in YPG Broth (pH 3.5) in the presence of various concentrations of chitosan. The value "1" represented an undetectable number of cells (less than 10 CFU/mL)

## The inhibitory effect of chitosan on the growth of LAB

*L. plantarum* failed to grow with the addition of 2 g/L chitosan for up to 6 days (Table 2). In the presence of 0.8–2.0 g/L chitosan, the initial population of *L. plantarum* declined drastically on the third day of the experiment. The level of inactivation in the presence of 0.2 and 0.4–0.6 g/L chitosan were approximately 2 and 5 log CFU/mL, respectively. Following a sharp reduction in viable count at chitosan levels below 2 g/L, the growth of *L. plantarum* was resumed albeit at a slower rate than the control containing no chitosan. The antimicrobial activity of chitosan appeared to be more pronounced at chitosan levels ranging from 0.8 to 2.0 g/L. The MIC values of chitosan for *L. plantarum* found in this study, differed somewhat from those reported previously (Jung et al. 2010; Tantala et al. 2012). For instance, Jung et al. (2010) reported that low concentrations of acid-soluble or water-soluble chitosan (0.05–0.08 %) were able to inhibit the growth of *L. plantarum*. In addition, Tantala et al. (2012) reported that *L. plantarum* was susceptible to chitosan with MIC values of 0.008 % and MBC (minimum bactericidal concentration) values of 0.01 %. Of the seven wine-related microorganisms studied, *L. plantarum* was the second most chitosan-resistant organism after *S. cerevisiae*. By contrast, LAB strains, *L. hilgardii* and *O. oeni*, were completely inactivated by even the lowest chitosan concentration (0.2 g/L) (Table 2). As far as our literature survey could ascertain, no information was available on the antimicrobial effect of chitosan against *L. hilgardii* and *O. oeni*.

## The inhibitory effect of chitosan on the growth of *B. bruxellensis*

The effect of chitosan on *B. bruxellensis* was similar to that observed for *L. hilgardii*, *O. oeni*. Regardless of

**Table 2** Growth of wine-related microorganisms in YPG broth (pH 3.5) in the presence of various concentrations of chitosan

| Microorganism | Incubation time (days) | Viable count (log CFU/mL) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Chitosan concentration (g/L) | | | | | | | | | | |
| | | 0 | 0.2 | 0.4 | 0.6 | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 |
| *H. uvarum* JFO 1755 | 0 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 | 7.40 |
| | 3 | 8.07 | 7.81 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| | 6 | 7.86 | 7.90 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| *Z. bailii* JFO 0488 | 0 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 | 7.23 |
| | 3 | 7.94 | 7.62 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| | 6 | 7.60 | 6.68 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| *L. plantarum* MF513 | 0 | 8.74 | 8.74 | 8.74 | 8.74 | 8.74 | 8.74 | ND | ND | ND | ND | 8.74 |
| | 3 | 9.30 | 6.52 | 3.54 | 3.40 | <1.00 | <1.00 | ND | ND | ND | ND | <1.00 |
| | 6 | 9.41 | 9.31 | 7.94 | 7.34 | 5.64 | 6.06 | ND | ND | ND | ND | <1.00 |
| *L. hilgardii* DSM-20051 | 0 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 | 7.47 |
| | 3 | 6.74 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| | 6 | 6.28 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| *O. oeni* DSM-20252 | 0 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 | 8.98 |
| | 3 | 8.01 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| | 6 | 7.95 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| *B. bruxellensis* DSM-70001 | 0 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 | 5.24 |
| | 3 | 4.83 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |
| | 6 | 6.50 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 | <1.00 |

chitosan concentration, the growth of *B. bruxellensis* was completely inhibited throughout the experiment (6 days) (Table 2). Therefore, it was concluded that the MIC for chitosan against *B. bruxellensis* was 0.2 g/L. The MIC value we obtained for *B. bruxellensis* was in agreement with the findings of Portugal et al. (2014) who reported that chitosan levels higher than 250 mg/L was enough to prevent cell growth and kill 90 % of the initial inoculum. In contrast, Gómez-Rivas et al. (2004) indicated that the presence of chitosan ranging from 1 to 6 g/L prevented the growth of *B. bruxellensis* by extending the lag period.

## Overall discussion of MICs

The MIC values of chitosan for *S. cerevisiae*, *L. plantarum*, *O. oeni*, *L. hilgardii*, *H. uvarum*, *Z. bailii* and *B. bruxellensis* were >2, 2, 0.2, 0.2, 0.4, 0.4 and 0.2 g/L, respectively. In order to mimic wine conditions and to standardize the effect of pH on the antimicrobial activity of chitosan, MIC assays were performed by using YPG Broth at pH 3.5. The standard methods of the Clinical and Laboratory Standards Institute (CLSI) were not followed for MIC testing. Since the aim of this study was to evaluate the possibility of using chitosan during winemaking process, MIC trials, performed with medium representing grape must conditions, constituted the preliminary step for further wine experiments. However, in the absence of chitosan, all tested organisms, except for *S. cerevisiae*, seemed to remain viable for the duration of the experiments, without increasing their population. This kind of behaviour was probably due to the fact that YPG Broth (pH 3.5) may not completely support the growth of organisms, besides *S. cerevisiae*.

Among the tested microorganisms associated with wine, *S. cerevisiae* showed the strongest resistance to antimicrobial action of chitosan. Similarly, Kiskó et al. (2005) reported that the growth of *S. cerevisiae* was sligtly delayed in apple juice treated with chitosan. *L. hilgardii*, *O. oeni* and *B. bruxellensis* were the most susceptible to chitosan with no viability observed after 3 days of incubation in the presence of 0.2 g/L chitosan. Non-*Saccharomyces* yeasts, *H. uvarum* and *Z. bailii*, were only able to resist 0.2 g/L

**Table 3** Effect of chitosan and/or $SO_2$ on some fermentation parameters

| Experimental groups | Fermentation rate (g $CO_2$/Lh) | Residual sugar* (D-glucose + D-fructose, g/L) | Ethanol** (g/100 mL) |
|---|---|---|---|
| Group A | 0.01 | 264.22 | 0.00 |
| Group B | $0.82 \pm 0.0281^{a,b}$ | $1.47 \pm 0.8488$ | $8.08 \pm 0.6324$ |
| Group C | $0.86 \pm 0.0015^{a}$ | $0.69 \pm 0.2039$ | $8.04 \pm 0.1032$ |
| Group D | $0.64 \pm 0.1415^{b}$ | $0.45 \pm 0.1978$ | $8.99 \pm 0.2851$ |
| Group E | $0.08 \pm 0.0121^{c}$ | $0.33 \pm 0.0469$ | $9.31 \pm 0.3827$ |
| Group F | $0.95 \pm 0.0566^{a}$ | $0.51 \pm 0.1143$ | $8.71 \pm 0.7700$ |
| Group G | $0.79 \pm 0.0700^{a,b}$ | $0.48 \pm 0.0395$ | $10.74 \pm 0.1788$ |
| Group H | $0.85 \pm 0.0224^{a}$ | $0.88 \pm 0.2174$ | $8.29 \pm 1.0346$ |

Values are expressed in mean ± SE

[a–c] Values with different letters indicate significant differences between groups treated with chitosan and/or $SO_2$ ($p < 0.05$)

*, ** No statistically significant difference was found between groups with respect to residual sugar and ethanol concentrations ($p > 0.05$)

chitosan. Since microorganisms other than *S. cerevisiae* were completely inhibited even after the 3rd or 6th day of incubation, the growth of these microorganisms was not followed for a longer time.

Based on the available literature information, bacteria were appeared to be more resistant than fungi to chitosan (Aider 2010; Goy et al. 2009; Kong et al. 2010). However, the results of our study did not confirm this evidence. The high tolerance of *S. cerevisiae* (the major yeast for the alcoholic fermentation) against chitosan indicated that chitosan could be applied in winemaking. It is also important to note that low levels of chitosan was able to inhibit *B. bruxellensis* which is considered as one of the wine spoilage species with high resistance due to its high tolerance to nutrient deprivation and high ethanol levels.

## Activity of chitosan in vinification

### *Effect of chitosan on alcoholic fermentation*

To determine the effect of chitosan alone or in combination with $SO_2$ on the fermentative activity, weight loss, concentrations of fermentable sugars and ethanol were monitored during the time course of fermentation experiments. There were significant differences in the fermentation rates of the different experimental groups (Group B–H) containing various levels of chitosan and/or $SO_2$ ($p < 0.05$). As shown in Table 3, Group E which was treated with the highest chitosan concentration (0.6 g/L) had the lowest fermentation rate (0.08 g $CO_2$/Lh). In contrast, no statistically significant differences were found between the experimental groups in terms of residual sugar and ethanol concentration at the end of alcoholic fermentations. In general, residual sugar content in the wine at the end of the alcoholic fermentation needs to be below 0.4 % (4 g/L, glucose + fructose) (Bisson 1999). Accordingly, virtually all fermentable sugars in the must were fermented in all groups. In all cases, the alcoholic fermentations were completed regardless of chitosan and/or $SO_2$ addition. These results revealed that chitosan had a retarding effect on alcoholic fermentation. Consequently, chitosan seemed not to alter the fermentative performance of the yeasts, demonstrating its potential applicability in wine.

The time courses for weight loss, sugar consumption and ethanol production during the alcoholic fermentation of the grape juice are presented in Fig. 2a–c. Based on the $CO_2$ evolution profiles, experimental groups could be ranked as follows: Group E (0.6 g/L chitosan) < Group D (0.4 g/L chitosan) < Group G (0.4 g/L chitosan + 40 mg/L $SO_2$) < Group C (0.2 g/L chitosan) < Group F (0.2 g/L chitosan + 40 mg/L $SO_2$) < Group B (0 g/L chitosan) = Group H (40 mg/L $SO_2$) (Fig. 2a). Accordingly, the fermentative activitiy of the yeasts was partially decreased proportional to the chitosan concentration. Group H, containing 40 mg/L $SO_2$, showed a similar $CO_2$ evolution profile with the positive control group (Group B), indicating that $SO_2$ alone had

no effect on the fermentative capacity of yeasts. Likewise, it can be concluded that the use of $SO_2$ did not intensify the effect of chitosan on fermentation kinetics. This could be related to the resistance of *S. cerevisiae*, the dominant yeast during fermentation, to the high levels of $SO_2$ in consequence of natural selection upon continuous exposure to $SO_2$ over the years (du Toit and Pretorius 2000). On the other hand, the final weight loss (approximately 21–22 g) was quite similar between the fermentation trials with or without chitosan and/or $SO_2$.

In all preservative-supplemented groups, the glucose and fructose contents of the grape juice remained relatively constant throughout the first 2 days of fermentation (Fig. 2b). Furthermore, in the presence of 0.6 g/L chitosan (Group E), the onset of sugar consumption was after the first 4 days. In parallel with the results of $CO_2$ evolution and sugar consumption, the ethanol production was delayed with the increase in chitosan concentration (Fig. 2c).

The evolution of viable cells of mixed culture fermentations of *S. cerevisiae*, *H. uvarum* and *Z. bailii* in the presence of chitosan and/or $SO_2$ is shown in Fig. 3, where non-*Saccharomyces* yeasts exhibited a reduced growth pattern compared to *S. cerevisiae*. As shown in Fig. 3a, the non-*Saccharomyces* population in the groups treated with 0.2, 0.4 and 0.6 g/L chitosan declined drastically on the 8th day of fermentation. However, when chitosan applied in combination with $SO_2$ (Group F and G), the complete inactivation of the non-*Saccharomyces* yeasts was achieved after 6 days, which was also observed in the control group. Of the treatments examined in this study, 40 mg/L $SO_2$ (Group H) were found to be the most effective for the inhibition of *H. uvarum* and *Z. bailii*, since the viable cell numbers were reduced to below detection limit on the 4th day of fermentation at this level of $SO_2$. However, it has been known that $SO_2$ alone is not sufficient to completely prevent yeast growth as many non-*Saccharomyces* yeasts resist to high concentrations (Fugelsang and Edwards 2007). Contrary to expectations, chitosan did not show a dose dependent inhibition of non-*Saccharomyces* yeasts during the alcoholic fermentation of the grape juice. Interesingly, even the control group exhibited a reduced growth profile when compared with the experimental groups containing chitosan alone and in combination with $SO_2$. Therefore, our results implies that the inhibition of *H. uvarum* and *Z. bailii* in mixed cultures with *S. cerevisiae* were not due to the antimicrobial action of chitosan but rather due to ethanol produced by the yeasts. The spontaneous fermentation of grape must is generally initiated by apiculate, non-*Saccharomyces* yeasts (*Hanseniaspora*, *Candida*, *Metschnikowia*, *Pichia*, *Issatchenkia*, *Kluyveromyces*) that predominate the first stages of fermentation. As the fermentation progresses, these yeasts start to die off giving way fermentative yeast, *S. cerevisiae* to complete the fermentation (Fleet 2003). The early deaths of the non-*Saccharomyces* yeasts were often attributed to their low ethanol tolerance (Fleet 2003; Pina et al. 2004). In our study, a modest inverse correlation was found between the ethanol content of wines and the ratio of viable cell numbers after 4 ($r = -0.64$) and 6 ($r = -0.49$) days of alcoholic fermentation to initial viable cell numbers of non-*Saccharomyces* (log n/log $n_0$). Portugal et al. (2014) reported that ethanol (12.5 %, v/v) did not significantly potentiate chitosan activity towards *B. bruxellensis*, as ethanol does not change the electric charge of chitosan. The antimicrobial activity of chitosan is often attributed to its polycationic structure which depends on pH and degree of deacetylation of chitosan (Kong et al. 2010). Contrary to non-*Saccharomyces*, *S. cerevisiae* maintained its viability until the end of fermentation experiments (Fig. 3b). With the highest chitosan concentration (0.6 g/L), a slight decrease in the viable cell numbers of *S. cerevisiae* and non-*Saccharomyces* yeasts were observed within the initial stages of alcoholic fermentation. The ranking made for the $CO_2$ evolution profiles of the experimental groups corresponded with the growth of *S. cerevisiae* at the onset of fermentation. This result indicates that the initial retarding effect of chitosan on fermentation kinetics could be related to the inhibitory effect of chitosan on the growth of *S. cerevisiae*. In the presence of 0.2 g/L chitosan (Group C), yeast–yeast interactions exhibited biphasic growth. On the 4th day of fermentation, the viable cell numbers of non-*Saccharomyces* yeasts decreased by approximately 2.5 log cycles whereas *S. cerevisiae* cells reached a maximum cell concentration of 8.6 log CFU/mL. Likewise, the population of *S. cerevisiae* declined drastically after 6 days of fermentation whereas the non-*Saccharomyces* yeasts reached a maximum cell concentration of 8.5 log CFU/mL. The biphasic growth of *S. cerevisiae* in mixed cultivations with *B. intermedius* and *B. bruxellensis* was also observed by Gómez-Rivas et al.

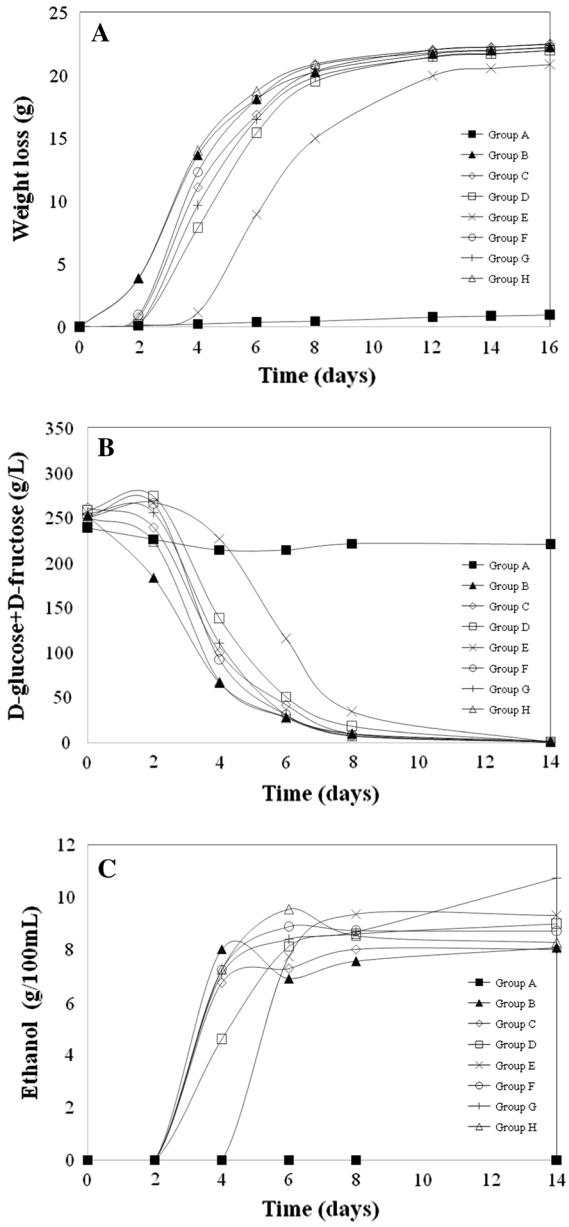



**Fig. 2** Time courses for weight loss (**a**), sugar consumption (**b**) and ethanol production (**c**) during the alcoholic fermentation of the grape juices containing various levels of chitosan and/or $SO_2$

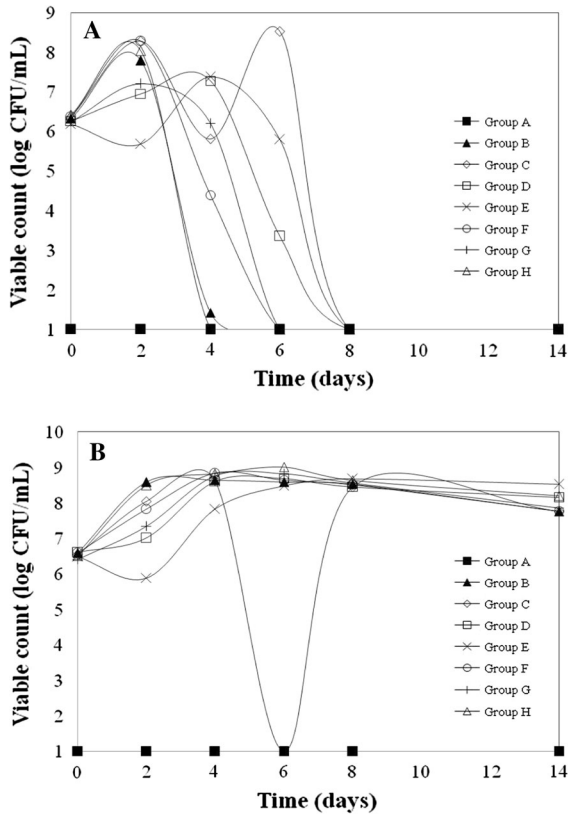



**Fig. 3** Growth of non-*Saccharomyces* yeasts (*H. uvarum* JFO 1755, *Z. bailii* JFO 0488) (**a**) and *S. cerevisiae* (**b**) during the alcoholic fermentation of the grape juices containing various levels of chitosan and/or $SO_2$. The value "1" represented an undetectable number of cells (less than 10 CFU/mL)

(2004) in the presence of 2 and 3 g/L chitosan, respectively.

In general, the antimicrobial effect of chitosan against *H. uvarum* and *Z. bailii* was reduced when tested in wine. This could be due to the interactions of chitosan with wine ingredients or the yeast–yeast interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts during alcoholic fermentations. The latter seemed to be principal factor that mask the antimicrobial effect of chitosan. It has been known that chitosan may interact with food components such as alginates, pectins, proteins, inorganic polyelectrolytes due to its polycationic structure (Aider 2010).

### *Effect of chitosan on malolactic fermentation*

Table 4 shows the effect of chitosan and $SO_2$, applied individually and/or in combination, on the viability of *O. oeni* Lalvin VP41 during the malolactic fermentation experiments of white wines. In the absence of chitosan (Group B), *O. oeni* cells did not proliferate, but remained viable for at least 4 days. A possible explanation for this result may be the nutrient depletion by *S. cerevisiae* in the previous alcoholic fermentation stage. As indicated in the "Effect of chitosan on alcoholic fermentation" section. Effect of

chitosan on alcoholic fermentation', nearly all fermentable sugars were exhausted at the end of fermentation experiments. Besides, wine yeasts can produce toxic metabolites inhibitory to malolactic bacteria including ethanol, $SO_2$, medium-chain fatty acids and antibacterial proteins/peptides (Fugelsang and Edwards 2007). In support, strong correlations were found between the ethanol content of wines at the end of alcoholic fermentation experiments and the ratio of viable cell numbers after 2 days of malolactic fermentation to initial viable cell numbers of *O. oeni* (log n/log $n_0$) ($r = -0.82$). As shown in Table 4, the lowest chitosan concentration (0.2 g/L), either alone or in combination with 40 mg/L $SO_2$, did not affect the growth of *O. oeni* significantly during the first 4 days of malolactic fermentation experiments. Similarly, at 40 mg/L $SO_2$, there was no noticeable inactivation effect on the bacteria. However, all of the treatment groups, except for the Group C, exhibited a reduced growth profile at the end of malolactic fermentation experiments when compared with the control group. In general, the inhibitory effect was greatest with 0.6 g/L chitosan and 0.4 g/L chitosan + 40 mg/L $SO_2$ treated groups (Group E and Group G). The effect of chitosan and/or $SO_2$ on the malolactic fermentation kinetic was not determined, since *O. oeni* did not grow at all in white wines. In any case, the conversion of malic acid to lactic acid did not occur, and the concentrations of malic and lactic acid (ca. 4.5, 0.1 g/L, respectively) were constant throughout the experiments (data not shown).

**Table 4** Effect of chitosan and/or $SO_2$ on inactivation of *O. oeni* Lalvin VP41 during the malolactic fermentation of the white wines

| Experimental groups | log n/log $n_0$ | |
|---|---|---|
| | Day = 2 | Day = 4 |
| Group B | $0.97 \pm 0.0174^{a,b}$ | $0.93 \pm 0.0104^{a}$ |
| Group C | $1.00 \pm 0.0013^{a}$ | $0.97 \pm 0.0129^{a}$ |
| Group D | $0.83 \pm 0.0010^{c}$ | $0.70 \pm 0.0089^{b}$ |
| Group E | $0.69 \pm 0.0340^{d}$ | $0.48 \pm 0.0055^{c}$ |
| Group F | $0.93 \pm 0.0121^{b}$ | $0.87 \pm 0.0097^{a}$ |
| Group G | $0.75 \pm 0.0017^{d}$ | $0.54 \pm 0.0428^{c}$ |
| Group H | $0.96 \pm 0.0062^{a,b}$ | $0.89 \pm 0.0903^{a}$ |

Values are expressed in mean ± SE

$n_0$ initial viable cell number, $n$ viable cell number after 2 and 4 days

[a–c] Values with different letters within a column indicate significant differences between groups treated with chitosan and/or $SO_2$ ($p < 0.05$)

*Effect of chitosan during storage of wine*

*B. bruxellensis* was not able to grow in the finished wines even after one month of storage probably due to nutrient deprivation similar to *O. oeni*. Therefore, the effect of chitosan during wine storage could not be determined.

## Conclusions

From the oenological point of view, it is worthy of note that the chitosan was able to inhibit the growth of the unwanted spoilage species (*B. bruxellensis*, *L. hilgardii*, *H. uvarum*, *Z. bailii*) to a greater extent compared to *S. cerevisiae* in laboratory media. However, the efficacy of chitosan was decreased under winemaking conditions, pointing out the necessity of evaluating antimicrobial activities in real food systems to obtain promising results for practical applications. It was important to note that the addition of chitosan did not significantly alter the viability and the fermentative performance of *S. cerevisiae*. This study has shown that chitosan had a retarding effect on alcoholic fermentation. With regard to non-*Saccharomyces* yeasts involved in winemaking, the early deaths of these yeasts were probably due to their inability to survive the increasing concentrations of ethanol produced in the fermentation. Therefore, higher chitosan concentrations (above 0.6 g/L) could be recommended for further studies. However, the increase of volatile acidity could be a concern at high chitosan concentrations due to increasing acetic acid concentration. It has generally been known that a high level of volatile acidity can result in a vinegary taint; an undesirable characteristic for wines and also wines having elevated concentrations of acetic acid (greater than 0.8 g/L) are regarded as defective (Bağder Elmacı et al. 2014). In the present study, the acetic acid concentration used for the preparation of the chitosan solution was not so high as to affect the sensory profile of the wine. Because of the nutrient depletion by the yeasts in the alcoholic fermentation stage, the effect of chitosan on malolactic fermentation and the growth of *B. bruxellensis* during wine storage could not be determined. This study constitutes the preliminary step in assessing the

effectiveness of chitosan against wine-associated microorganisms in vitro and in wine conditions. However, because of the several factors that affect the antimicrobial activity of chitosan including the complex interactions between chitosan and wine ingredients, microbial interactions during wine fermentation, etc., extensive future work should be conducted to consider chitosan as an alternative or complementary to $SO_2$ in wine industry.

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