Effect of a fungal chitosan preparation on *Brettanomyces bruxellensis*, a wine contaminant

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Keywords
antimicrobial, chitosan, mechanisms of action, wine spoilage, yeast.

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Abstract

Aim: To investigate the action mechanisms of a specific fungal origin chitosan preparation on *Brettanomyces bruxellensis*.

Methods and Results: Different approaches in a wine-model synthetic medium were carried out: optical and electronic microscopy, flow cytometry, ATP flow measurements and zeta potential characterization. The inactivation effect was confirmed. Moreover, fungal origin chitosan induced both physical and biological effects on *B. bruxellensis* cells. Physical effect led to aggregation of cells with chitosan likely due to charge interactions. At the same time, a biological effect induced a leakage of ATP and thus a viability loss of *B. bruxellensis* cells.

Conclusions: The antimicrobial action mode of chitosan against *B. bruxellensis* is not a simple mechanism but the result of several mechanisms acting together.

Significance and Impact of the Study: *Brettanomyces bruxellensis*, a yeast responsible for the production of undesirable aromatic compounds (volatile phenols), is a permanent threat to wine quality. Today, different means are implemented to fight against *B. bruxellensis*, but are not always sufficient. The chitosan of fungal origin is introduced as a new tool to control *B. bruxellensis* in winemaking and has poorly been studied before for this application.

Introduction

Among many potential applications (Xia *et al.* 2011), chitosan, a hydrophilic biopolymer industrially obtained by N-deacetylation of chitin, can be used as an antimicrobial agent (Goy *et al.* 2009; Kong *et al.* 2010). Numerous commercial applications of chitosan in various different sectors benefit from its antimicrobial activity. Namely, its application is described for food preservation (Shahidi *et al.* 1999; Rhoades and Roller 2000; Tsai *et al.* 2002; Devlieghere *et al.* 2004), manufacture of wound dressings (Ueno *et al.* 2001) and antimicrobial finished textiles (Takai *et al.* 2002). Chitosan preparations have been investigated as antimicrobial material against a wide range of targeted micro-organisms such as bacteria, yeast and fungi in experiments involving *in vivo* and *in vitro* interactions with chitosan presented under different forms (Coma *et al.* 2003; Dutta *et al.* 2009; Goy *et al.* 2009).

Antimicrobial activity of chitosan depends on various intrinsic and extrinsic factors such as the molecular weight, the deacetylation degree and the medium pH (Rabea *et al.* 2003; Zheng and Zhu 2003; Goy *et al.* 2009; Kong *et al.*, 2010). Therefore, studies that evaluated the minimum inhibitory concentration (MIC) for chitosan gave different results according to the microbial strains and to the external conditions used (Goy *et al.* 2009). For example, reported MIC values for bacteria varied from 20 ppm for *Escherichia coli* (Liu *et al.* 2001) to

2000 ppm for *Salmonella enterica* (Barzegar *et al.* 2008). Moreover, due to the fact that during wine storage most micro-organisms enter the state of viable but not culturable (VBNC) as shown by Millet and Lonvaud-Funel (2000), the usual methods for MIC determination are not appropriate to the case of wine contamination by *B. bruxellensis*.

Similarly to bacteria, some studies have been carried out on yeasts and moulds associated with food and plant spoilage. Generally, chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth in fungi (El Ghaouth *et al.* 1992a; Sashai and Manocha 1993). For yeasts associated with food spoilage, the concentration of chitosan inhibiting growth varies from 0·1 to 5 g l^{-1} in apple juice at 25°C (Roller and Covill 1999).

Nowadays, commercial chitin and chitosan formulations are mainly produced from biowastes coming from the seafood industry (Kurita 2006). More recently, a preparation of fungal chitosan has been developed by the KitoZyme company. Fungal chitosan can be more easily obtained under a controlled environment all year round from *Aspergillus niger* cultures. To the authors' knowledge, the interest of using such a chitosan in oenology particularly for the elimination of *B. bruxellensis* has been poorly documented (Gomez-Rivas *et al.* 2004; Ferreira *et al.* 2013) despite the fact that *B. bruxellensis* has for long been recognized as a problematic contaminant of both industrial bioethanol production (Gadaga *et al.* 2002; Teoh *et al.* 2004; Loureiro and Malfeito-Ferreira 2006) and fermented beverages, responsible for the production of bad flavours such as mousy taint and horse sweat taste in contaminated wines (Kheir *et al.* 2013). These yeasts can persist throughout the whole winemaking process and have in recent years become a major oenological concern worldwide. Several strategies (molecular SO_2 management, management of alcoholic and malolactic fermentations, lees management and barrel sanitation) are useful to control *Brettanomyces* and its development in musts and wines. However, these strategies are not always sufficient.

In this context, the international organization of vine and wine (OIV: Organisation Internationale de la Vigne et du Vin) and the European Union have approved respectively in July 2009 and December 2010 the use of fungal origin chitosan as a new practice in the oenological codex. This fungal chitosan is easy to use, biodegradable, nontoxic and nonallergenic.

The exact mechanism of antimicrobial action of chitosan on *Brettanomyces* is still hypothetical. However, five main mechanisms have been proposed for other micro-organisms in the literature as follows: (i) interactions between positively charged molecules of chitosan and negatively charged molecules of microbial cell walls lead to changes in cell membrane structure and permeability, inducing the leakage of proteinaceous and other intracellular constituents and thus challenging the biochemical and physiological ability of the bacteria leading to a loss of growth capacity and death (Shahidi *et al.* 1999); (ii) chitosan acts as a chelating agent that selectively binds trace metals and subsequently inhibits the microbial growth (Cuero *et al.* 1991); (iii) chitosan activates several defence processes in cells, acts as a water-binding agent and inhibits various enzymes (El Ghaouth *et al.* 1992b); (iv) chitosan penetrates the cytosol of the micro-organisms and binds with DNA, inducing an interference with the synthesis of mRNA and proteins (Hadwiger *et al.* 1986; Sudarshan *et al.* 1992); and (v) chitosan can form an impermeable polymeric layer on cell surface which alters the cell permeability and prevents nutrients from entering the cell (Tokura *et al.* 1997).

This work aimed at studying the specific mechanisms of antifungal action of fungal origin chitosan against *B. bruxellensis* strains isolated from wines. As the development of these contaminating yeasts in winemaking often occurs after the fermentations during wine ageing, a synthetic wine medium was used in this study to avoid the potential interaction with different wine compounds.

Materials and methods

Materials

Chitosan

Chitosan is a linear polysaccharide composed of two repeating units (D-glucosamine units (GlcN) and N-acetyl-D-glucosamine (GLcNAc) units) randomly distributed along the polymer chain and linked by β(1-4)-bonds.

The chitosan preparation used is a powder with particles whose diameter is lower that 50 μm, product of the deacetylation of chitin extracted from *A. niger* and produced by KitoZyme company (Herstal, Belgium): KiOfine B® or No Brett Inside® (commercial available products). The viscosity of 1% solution in acetic acid is around 4 mPa.s, and the degree of acetylation is <30 %.

Strains

One of *B. bruxellensis* strains used was obtained from the culture collection of the ICV (Institut Coopératif du Vin). This strain has been isolated in Languedoc-Roussillon area (France) and has been used for all experiments. The two other strains of *B. bruxellensis* (V1 and V2) were obtained from the culture collection of the Laboratory of Chemical Engineering (INP-ENSIACET) and had been isolated in Spain and only used for zeta potential experiment. Strains were maintained on YPDA agar

slants (yeast extract 10 g l^{-1}; peptone 20 g l^{-1}; dextrose 20 g l^{-1}; agar–agar 20 g l^{-1}), incubated at 30°C and stored at 4°C.

Culture medium for Brettanomyces bruxellensis inoculum
The culture medium was made with glucose 20 g l^{-1}; $(NH_4)SO_4$ 2 g l^{-1}; KH_2PO_4 5 g l^{-1}; $MgSO_4$ 0·4 g l^{-1}; citric acid 0·3 g l^{-1}; malic acid 3 g l^{-1}; tartaric acid 2 g l^{-1}; yeast extract 1 g l^{-1}; ethanol 5%.

The pH was adjusted to 3·5. Culture medium (200 ml) was then sterilized at 121°C for 15 min in Erlenmeyer flasks and then inoculated with *B. bruxellensis* cells from agar slants. Cells' incubation was carried out at 30°C with agitation at 150 rev min^{-1} during 65 h until sugars exhaustion. For the experiments with growing cells, the incubation was stopped at 48 h to get some sugar remaining and the cells still in the growth phase.

Conditions for chitosan treatment
The experiments with chitosan were carried out in a 250-ml medium (glycerol 6 g l^{-1}; tartaric acid 3 g l^{-1}; ethanol 13%) for which chemical characteristics were close to wine ones. The pH was adjusted to 3·7 with NaOH 10 mol l^{-1}. *Brettanomyces bruxellensis* was inoculated at about 5 to $20{\cdot}10^6$ cells ml^{-1} from a preculture defined above. Variable amounts of chitosan from 0·04 to 0·4 g l^{-1} were added prior to cell inoculation. For each experiment, a control was carried out with the same inoculum in a medium without any added chitosan. The experiments were carried out at 20°C, and flasks were stirred only just after inoculation and before sampling.

Methods

Analytical methods to follow the effect of chitosan on yeast
Brettanomyces bruxellensis population was characterized by two different analytical methods:
Cell counting using optical microscopy (Thoma Cell). Percentage of viable cells was measured using methylene blue staining (Bonora and Mares 1982). Experimental error was estimated to be <10% when total cells were counted above 150 (Lange *et al.* 1993).
Flow cytometry. Cells were collected from 1-ml samples by centrifugation. Cell pellets were suspended in 10 ml of Mac Ilvaine buffer. Volumes of 10 μl of cFDA (carboxyfluoresceindiacetate) and 5 μl of PI (propidium iodide), the fluorescent markers, were added to all samples. Before measurements on the flow cytometer, samples were incubated 10 min at 40°C and then centrifuged. cFDA-coloured yeasts were detected in green fluorescence and considered as alive, whereas PI-coloured cells were detected in red fluorescence and considered as dead. The yeast population detected for both fluorescence where considered as 'sublethal' yeast populations: cells with a modified membrane integrity (red) but with still active enzymatic activities (green).

Analytical methods to evaluate antifungal effect of fungal origin chitosan against Brettanomyces
The effect of fungal chitosan on *B. bruxellensis* growth was followed by three different analytical methods:
SEM (scanning electron microscopy). SEM MiniMEB® TM-3000 Hitachi was used to evaluate physical interaction between chitosan and cells. The electron microscope gives image of a sample, scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms of the sample. This interaction produces signals that contain information about the surface topography and other properties such as electrical conductivity. Liquid samples were prepared with a drop of the solution put on a carbon pastille and dried at least 24 h in a desiccator. Solid samples were crushed and put on a carbon pastille.
ATP measurements. The firefly luciferase system was used to determine the concentration of cellular ATP. The method is highly specific (MC Elroy and Green 1956) and based on the measurement of light emission produced during the oxidation of luciferin by molecular oxygen in the presence of ATP and magnesium ions. The light intensity is directly proportional to the concentration of ATP (Lundin and Thore 1975). The ATP kit from BioSyntec company was used with the luminator of EURALAM. A calibration curve was performed with five concentrations from 10^{-10} to 10^{-6} mol l^{-1} of ATP (R^2 = 0·9994).
Zeta potential measurement. Zeta potentials were measured in suspensions of *B. bruxellensis* strains ($20{\cdot}10^6$ cells ml^{-1}) and in a solution containing 0·40 g l^{-1} of chitosan using Zetasizer Nano ZS® apparatus (Malvern).

Statistical analysis

All experiments were carried out in duplicate, and results were reported as an average value of two replicates.

Results

In vitro survival studies

Due to the difficulty to determine MIC value in this context, in the first step of the work, two different analytical methods were compared to evaluate antifungal activity of chitosan against the ICV *B. bruxellensis* strain by assessing cells' concentration and viability during 20·5 h: microscopic counting on Thoma haemocytometer and flow cytometry. Concentration of chitosan used was 0·4 g l^{-1} for $20{\cdot}10^6$ cells ml^{-1} initial concentration of cells. The

results are presented on Fig. 1a for the control (without added chitosan) and on Fig. 1b for the treatment with added chitosan.

When comparing the two methods for a single sample, we can note that sublethal cells detected by cytometry are counted as viable with methylene blue dying. The results for total cells' concentration were similar but generally somewhat lower with cytometry. It is noteworthy that aggregated cells are not taken into accounts in any method. With both methods in the presence of chitosan, the total concentration of cells decreased from $16{\cdot}5–19{\cdot}10^6$ to $12{\cdot}10^6$ cells ml^{-1} after 20·5 h. During the same time, the number of dead cells increased drastically up to 85% of the total population on the treatment with added chitosan, whereas the population remained mainly viable in the control.

In the second step, we tested the effect of the chitosan concentration between 0·04 and 0·4 g l^{-1} for the same initial *B. bruxellensis* concentration of $20{\cdot}10^6$ cells ml^{-1} during 24 h. The viability was determined by Thoma counting after methylene blue dying (Fig. 2).

The effect of chitosan on cells' viability was quicker for the highest concentration. For chitosan concentration of 0·4 g l^{-1}, 3 h was sufficient to observe a lethal effect on 50% of *B. bruxellensis* cells.

The action mode of chitosan

Physical effect: adsorption of chitosan on cell wall

According to several studies, most of the hypothesis in relation with the mechanism of action of chitosan implies a direct contact between the yeast's cell wall and the polysaccharide (Goy *et al.* 2009; Kong *et al.* 2010; Xia *et al.* 2011). Comparing treated and control samples by classical microscopic observations on Thoma haemocytometer,

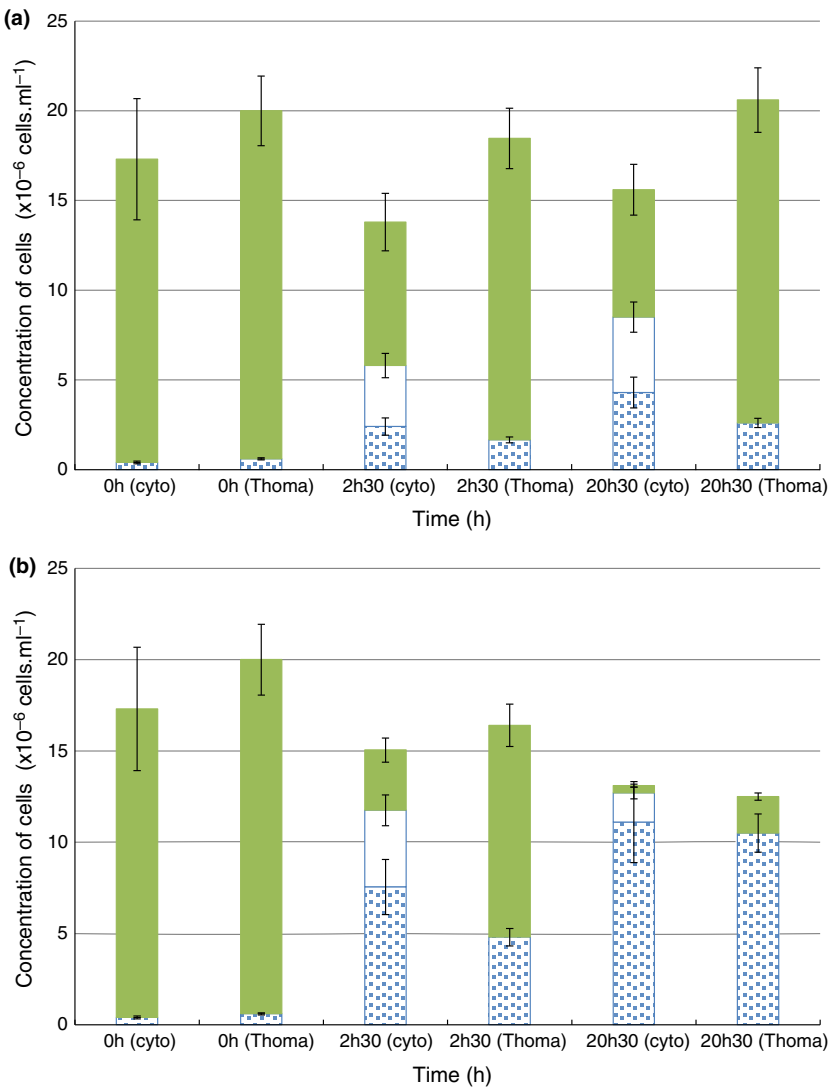

Figure 1 Evolution of *Brettanomyces bruxellensis* population in (a) the absence of chitosan and (b) the presence of 0·4 g l^{-1} of chitosan assessed by flux cytometry (cyto) and Thoma counting (Thoma). (▣) living cells; (□) sublethal cells and (⬚) dead cells.

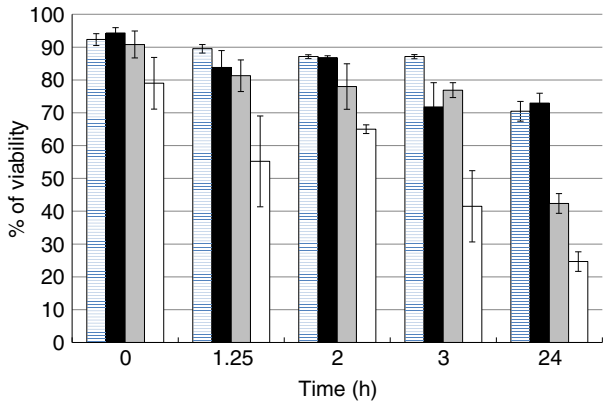

Figure 2 Evolution of *Brettanomyces bruxellensis* viability assessed by Thoma counting in the absence and the presence of different added chitosan concentrations. (▤) control— no chitosan; (■) 0·04 g l^{-1} of chitosan; (▥) 0·1 g l^{-1} of chitosan; (□) 0·4 g l^{-1} of chitosan.

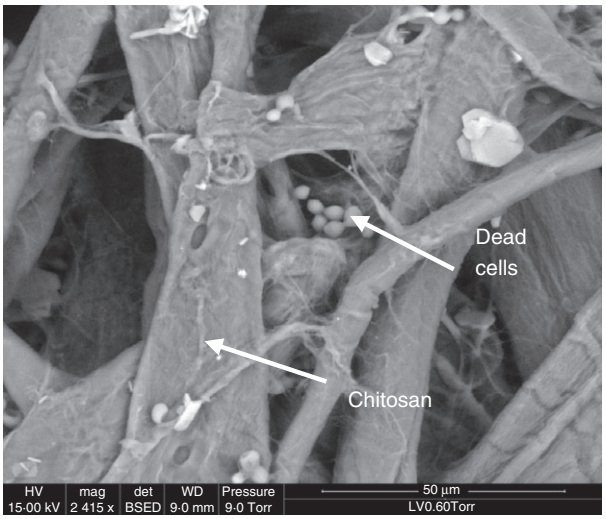

Figure 4 Scanning electron microscopy observation of ICV strain of *Brettanomyces bruxellensis* treated with chitosan (0·4 g l^{-1})

coupled with methylene blue coloration, clearly shows adsorption phenomenon (Fig. 3). This physical adsorption, apart from being responsible for other consequences, will also act in favour of sedimentation of cells that makes acceptable the idea of racking off the treated wine within a reasonable frame of time after chitosan addition.

The photography on Fig. 3a suggests a reversibility of this binding mechanism, at least for dead cells, as some dead cells are not aggregated on chitosan.

Electron microscopy observation (Fig. 4) confirms adsorption phenomena between chitosan and yeast. It is supposed that for pH < 6·3, the positive charge of $NH3^{+}$ groups of the glucosamine monomer allows interactions with negatively charged microbial cell walls that could lead to the leakage of intracellular constituents (Chung and Chen 2008; Goy *et al.* 2009; Kong *et al.*, 2010).

To go further in this adsorption mechanism, experiments were repeated with two other *B. bruxellensis* strains of our collection with initial cells' concentration of $15{\cdot}10^{6}$ cells ml^{-1} and 0·04 g l^{-1} of added chitosan. Zeta potential of both chitosan and *B. bruxellensis* strains were measured. Viability was measured after 24-h contact time by methylene blue staining. It was expressed as a percentage of the viability of the *B. bruxellensis* cells' control suspension without chitosan at the same time. *Brettanomyces bruxellensis* strains are negatively charged, whereas chitosan is positively charged (Table 1). It seems that the more negative the potential zeta of the strain was, the weaker the viability was. Thus, physical interactions between chitosan and cells may be first explained by electrostatic forces. This theory has already been proposed for bacterial cells (Chung *et al.* 2004; Raafat *et al.* 2008).

Biological effect: interaction between chitosan and cell membrane

The most likely hypothesis is a change of cell permeability due to interactions between positively charged chitosan and negatively charged microbial cell membranes (Chung and Chen 2008; Goy *et al.* 2009).

To evidence the membrane permeabilization activity of chitosan, extracellular ATP measurements were carried

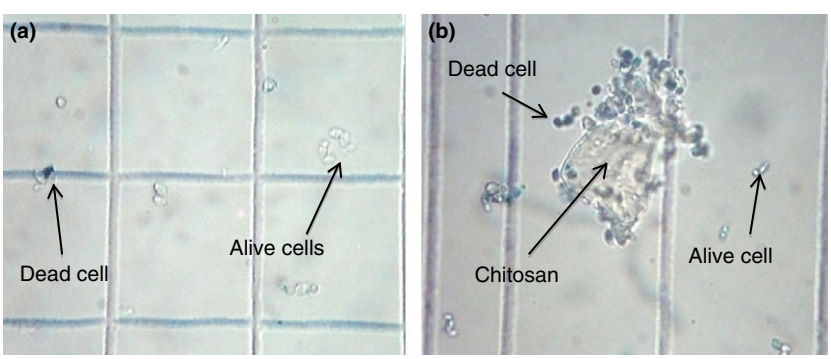

Figure 3 Comparative microscopic observations of yeasts cells in (a) the absence and (b) the presence of 0·4 g l^{-1} of chitosan after 20 min of incubation.

Table 1 Zeta potential of 3 *Brettanomyces bruxellensis* strains and chitosan suspensions and effect on the viability after 24-h contact time with chitosan

		Zeta potential (mV)	Viability in the presence of chitosan (expressed as % of the viability of the control without chitosan)
B. bruxellensis	V1	−3	50
Strain	V2	−3·3	33
	ICV	−7·8	25
Chitosan		+16·27	

out for 2 concentrations of polymer during 3-h contact time with ICV *B. bruxellensis* strain at $18{\cdot}10^6$ cells ml^{-1} (Fig. 5).

When the *B. bruxellensis* culture medium was treated with chitosan, a release of ATP was observed in the culture medium after 45 min (0·75 h) with 0·4 g l^{-1} and 2 h with 0·1 g l^{-1}. Furthermore, it appears that ATP release is chitosan concentration-dependent: the higher the chitosan concentration, the higher the ATP release. After 3 h, cells' viability was 80% and 52% respectively for 0·1 and 0·4 g l^{-1} of chitosan. Thus, the viability loss may appear with a delay compared to the release of ATP. We also measured the release of K^+ by conductivity and the release of protein by the Lowry method. No differences were observed between control and chitosan-treated cells (data not shown).

Effect of chitosan on growing cells

An experiment was carried out with growing cells of the ICV strain by inoculating the medium containing 0·04 or 0·4 g l^{-1} of chitosan with $4{\cdot}10^6$ cells ml^{-1} taken during growth phase (48 h and 5 g l^{-1} residual sugars) (5% inoculation volume). The contact time with chitosan was 168 h (7 days). The population viability was assessed by flow cytometry (Fig. 6).

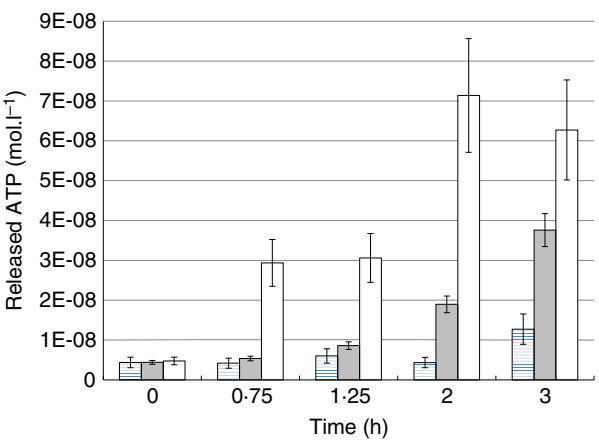

Figure 5 Evolution of released ATP for different chitosan concentrations in a wine-model medium contaminated with a *B. bruxellensis* population of $18{\cdot}10^6$ cells ml^{-1}. (▤) control—no chitosan; (▥) 0·1 g l^{-1} of chitosan; (□) 0·4 g l^{-1} of chitosan.

After 24 h, almost no living cells remained in the presence of chitosan whatever its concentration is. After 168 h for the control, the population doubled and the viability was high: the dead cells represented only 12% of the total cells. When chitosan was added to the medium, the population increased only 1·7- and 1·33-fold, and the dead cells were 18 and 53% of the total cells respectively for concentrations of 0·04 and 0·4 g l^{-1} of added chitosan. Moreover, the living cells' proportion was very low in the presence of chitosan, respectively 30 and 2·2%. These results show that the chitosan has an effect on the growing rate of *B. bruxellensis* and also on its physiological state. Despite the presence of residual sugars, chitosan prevented the increase of living cells at the highest concentration.

Discussion

Our results showed that for *B. bruxellensis* yeast in stationary phase, the studied chitosan had a negative effect on their viability as it drastically decreased the living cell concentrations after 24 h. The effect was linked to added chitosan concentration. Chitosan activity should be related to adsorption phenomena of cells on the polymer. This has already being suggested by some authors, for others, yeasts from *Saccharomyces* genus. Zakrzewska *et al.* (2007) have suggested that adsorption occurred between chitosan and phospholipids of the wall and membrane.

The polycationic structure of chitosan is a prerequisite for antimicrobial activity (Liu *et al.* 2001). When pH was below the pKa of chitosan, electrostatic interactions between the polycationic structure of chitosan and the predominantly anionic components of the micro-organisms' surface play a primary role in antimicrobial activity.

This adsorption phenomenon was qualitatively visualized by optical microscopic and MEB observations and led to aggregation mechanisms. The assessment of zeta potential confirmed that the chitosan was positively charged because of the protonated NH^{3+} groups of the molecule (Goy *et al.* 2009), whereas yeast wall was negatively charged. The aggregation of cells we observed has also been reported by Savard *et al.* (2002) for yeast. Moreover, a close relation was found between zeta potential of yeasts and chitosan activity. A higher negatively charged surface of cells would result in greater amount of adsorbed chitosan and so to greater changes in the structure of the cell wall and in the permeability of the cell

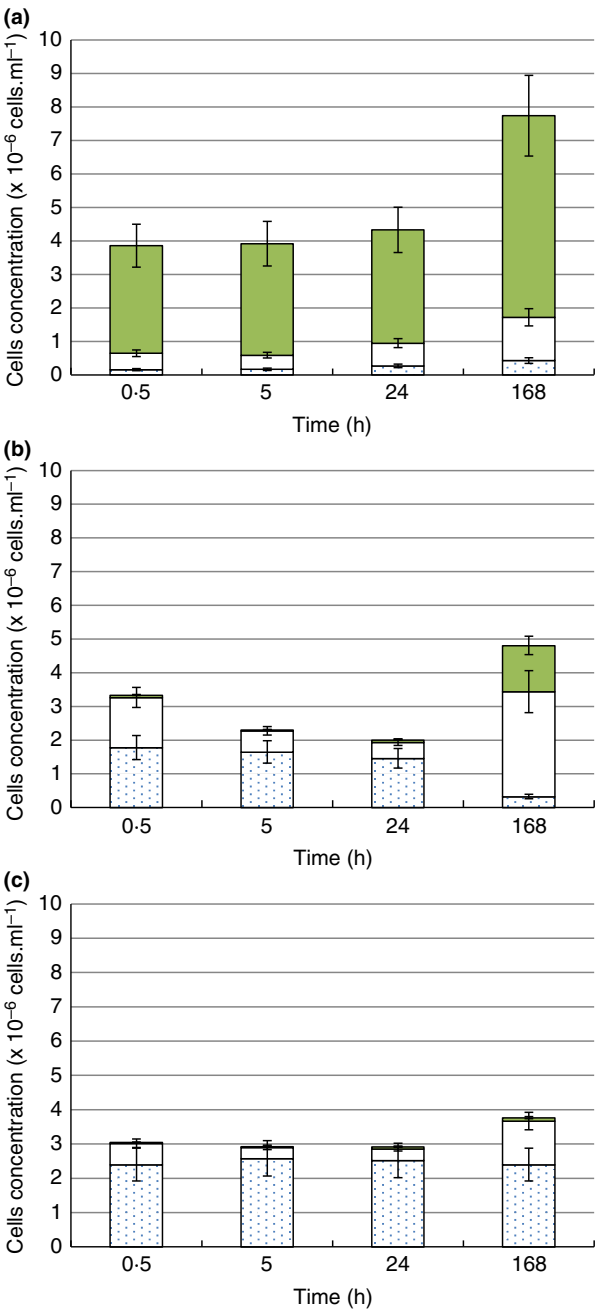

Figure 6 Concentration of cells as a function of time for (a) control (matrix alone: model wine + sugar 5 g l^{1}), (b) matrix + 0·04 g l^{-1} chitosan and (c) matrix + 0·4 g l^{-1} chitosan on a wine-model medium contaminated with a *Brettanomyces bruxellensis* population of $4{\cdot}10^{6}$ cells ml^{-1}. (▩) living cells; (□) sublethal cells; (▨) dead cells.

membrane. This phenomenon was already observed for bacterial cells (Chung *et al.* 2004).

Other studies reported the leakage of intracellular components as proteins and potassium as an effect of chitosan on bacteria cells (Chung and Chen 2008; Kong *et al.* 2010). In this study, only extracellular ATP was detected after at least 45 min of contact time with chitosan according to its concentration. These results suggest that chitosan increased membrane permeability quite rapidly. This effect is similar to the one observed by Alfenore *et al.* (2003) when they exposed *Saccharomyces cerevisiae* strain to the killer toxin K2. This observation suggests again a membrane structure pertubation by chitosan similar to the killer protein effect. Sublethal cells detected by flow cytometry after 2·5 h of contact time could be linked with the effect of chitosan on the membrane that may make possible the coloration by PI while the cells still had active enzymes.

For *B. bruxellensis* in growth phase and in the presence of residual sugars, after a viability loss measured during the first 24 h of contact time, the living cells started to increase slightly at 168 h but remained to a very low concentration compared to the control ($0{\cdot}8{\cdot}10^{6}$ *vs* $6{\cdot}10^{6}$ cells ml^{-1}) even for the lowest concentration of chitosan. Ferreira *et al.* (2013) reported that a chitosan concentration of 0·75 g l^{-1} was enough to inactivate a strain of *B. bruxellensis* in wine after 2-h contact time, but a concentration of 1·5 g l^{-1} only led to a 3-log reduction for another strain. Roller and Covill (1999) reported results for 7 yeasts from 4 genera tested in apple juice in the presence of chitosan glutamate: after a cellular death during the first 2 days of contact time, the yeast of some species started to grow if the concentration of chitosan was inferior to 0·5 g l^{-1}. They made the hypothesis of the recovery of injured cell after 6 days. In our case, the recovery is less important due to the medium which is less favourable than apple juice (alcohol and low sugar and nitrogen). Actually in real winemaking conditions, the situation could be controlled by racking off the wine after a few days of contact time before the possible recovery of yeast cells.

In conclusion, the results reported here demonstrate that fungal origin chitosan can induce both physical and biological effects on *B. bruxellensis* cells: adsorption phenomena due to electrostatic interactions leading to cells' aggregation and sedimentation and cell membrane damage leading to ATP leakage and so a drastic viability loss of *B. bruxellensis* cells. Thus, the antimicrobial action mode of chitosan against *B. bruxellensis* is not a simple mechanism but result of several mechanisms leading to a net decrease of the viable cells' concentration in the medium.

The impact of fungal origin chitosan on *B. bruxellensis* has also been tested under winemaking conditions at winery scale and was also efficient in reducing the concentration of viable cells (Pic *et al.* 2011).

Conflict of Interest

No conflict of interest declared.

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