# EFFECTIVENESS OF CHITOSAN PREPARATIONS AGAINST *BRETTANOMYCES BRUXELLENSIS* GROWN IN CULTURE MEDIA AND RED WINES

Biljana PETROVA[1], Zachary M. CARTWRIGHT[2] and Charles G. EDWARDS[2*]

1 : INOZY, 34093 Montpellier cedex 5, France
2 : School of Food Science, Washington State University, Pullman, WA, USA, 99164-6376

**Abstract**

**Aim** : The antimicrobial properties of chitosan from different sources (fungal, crab shell, and lactate-forms) against *Brettanomyces bruxellensis* in culture media and red wines were investigated.

**Methods and results** : While concentrations of 4 to 8 g/hL were needed for crab shell or lactate-forms of chitosan to reduce yeast viability in liquid media, fungal chitosan did not exhibit antimicrobial activities no matter the concentration. *B. bruxellensis* E1 and I1a were inoculated into Cabernet-Sauvignon wine at $10^6$ cfu/mL and treated with 0, 4, 8 and 12 g/hL fungal chitosan. In contrast to previous results with media, addition of fungal chitosan to a red wine resulted in a three-log reduction of culturability. Addition of fungal chitosan also reduced the viability of *B. bruxellensis* growing in oak barrels containing Merlot wine from $10^5$ cfu/mL down to $\approx 10^2$ cfu/mL.

**Conclusion** : Depending on concentration, all preparations of chitosan added to red wines greatly reduced populations of *B. bruxellensis*. However, wines were not completely stable after treatment as populations eventually increased.

**Significance and impact of the study** : As *B. bruxellensis* is considered to be a worldwide threat to wine quality, it is crucial to improve knowledge of alternative control methods and strategies such as chitosan that winemakers can apply.

**Key words** : chitosan, *Brettanomyces bruxellensis*, spoilage

**Résumé**

**Objectif** : Les propriétés antimicrobiennes de chitosanes de différentes sources (fongique, de crustacés et sous forme de lactate) ont été étudiées contre *Brettanomyces bruxellensis* dans des milieux synthétiques et des vins rouges.

**Méthodes et résultats** : Bien que des concentrations de 4 à 8 g/hL de chitosane de crustacés ou sous forme de lactate ont causé une réduction de la viabilité des levures dans des milieux de culture liquides, le chitosane fongique n'a pas présenté d'effet antimicrobien, quelle que soit la concentration. Deux souches de *B. bruxellensis*, E1 et I1a, ont été inoculées dans un vin de Cabernet-Sauvignon à $10^6$ UFC/mL et traitées avec 0, 4, 8 et 12 g/hL de chitosane d'origine fongique. Contrairement aux résultats précédents sur milieu de culture, l'addition de chitosane d'origine fongique dans un vin rouge a entraîné une réduction de la cultivabilité de 3 logs. L'addition de chitosane fongique a également réduit de$10^5$ UFC/mL jusqu'à $\approx 10^2$ UFC/mL la viabilité de *B. bruxellensis* présent dans un vin de Merlot en fût de chêne.

**Conclusion** : Selon la concentration, toutes les préparations de chitosane ajoutées sur vin rouge réduisent fortement la population de *B. bruxellensis*. Cependant, les vins ne sont pas complètement stables après le traitement, car les populations levuriennes se sont finalement redéveloppées.

**Signification et impact de l'étude** : *B. bruxellensis* étant considérée comme une menace pour la qualité du vin dans le monde entier, il est essentiel d'améliorer la connaissance des méthodes et des stratégies alternatives de contrôle que les vignerons peuvent appliquer, telles que le chitosane.

**Mots clés** : chitosane, *Brettanomyces bruxellensis,* altération

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*Corresponding author : edwardsc@wsu.edu



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## INTRODUCTION

Limiting or eliminating the spoilage yeast, *Brettanomyces (Dekkera) bruxellensis*, from red wines is not an easy task for winemakers. While researchers have studied the use of dimethyl dicarbonate (Costa *et al.*, 2008; Zuehlke *et al.*, 2015), maintaining low temperatures (Barata *et al.,* 2008b), membrane filtration (Umiker *et al.*, 2013), thermal inactivation (Couto *et al.*, 2005), and other approaches, $SO_2$ continues to be the primary method of control in a winery (Barata *et al.*, 2008a; Agnolucci *et al.*, 2010). However, du Toit *et al.* (2005) and Zuehlke and Edwards (2013) suggested that *B. bruxellensis* might enter a viable-but-non-culturable (VBNC) state in the presence of $SO_2$, making detection by conventional microbiological methods (*e.g.*, plating) difficult. Sulfites are also known to cause human allergic reactions (Vally and Thompson, 2001), thereby necessitating the need to develop alternative strategies. One such approach could be the use of chitosan, a compound that has antimicrobial properties against a variety of bacteria and yeast species (Roller and Covill, 1999; Martín-Diana *et al.*, 2009).

Recently, the use of chitosan of fungal origin was approved in Europe for use in wines by the Organisation Internationale de la Vigne et du Vin (O.I.V.). Chitosan can assist in must and wine clarification (Chagas *et al.*, 2012), remove heavy metals or ochratoxin A (Bornet and Teissedre, 2008), and reduce populations of undesirable microorganisms, most notably *B. bruxellensis* (Gómez-Rivas *et al.*, 2004; Ferreira *et al.*, 2013; Nardi *et al.*, 2014; Taillandier *et al.*, 2015). Gómez-Rivas *et al.* (2004) studied antimicrobial action of chitosan from crab shells against *Saccharomyces cerevisiae* and *B. intermedius* (*B. bruxellensis*). These authors observed addition of very high concentrations of chitosan (300 to 600 g/hL) resulted in inhibition of *B. bruxellensis* in a culture medium while *S. cerevisiae* was unaffected. In contrast, Ferreira *et al.* (2013) reported far lower amounts resulted in affecting the yeast where minimal inhibitory concentrations were determined to only be 30 to 32.5 g/hL. From a mechanistic point of view, chitosan appears to interact with cell walls/membranes resulting in leakage which triggers various stress responses (Rabea *et al.*, 2003; Liu *et al.*, 2004; Zakrzewska *et al.*, 2005; Park *et al.*, 2008). Zakrzewska *et al.* (2005) noted that application of chitosan to *S. cerevisiae* increased cellular resistance to β-1,3-glucanase, a characteristic of cell wall-stress. Recent work by Taillandier *et al.* (2015) concluded that the antimicrobial action against *B. bruxellensis* was the result of a number of mechanisms including cell aggregation and leakage of certain constituents.

As reviewed by Kong *et al.* (2010), chitosan is considered to be non-toxic, biodegradable, and has been used in food, pharmaceutical, agriculture, textile, water treatment, and cosmetic industries. Since chitosan is a derivative of chitin through deacetylation (Raafat and Sahl, 2009), preparations not only differ in original source but also in the degree of acetylation and molecular weight. Studying chitosan of different molecular weights (107 to 621 kDa), Ferreira *et al.* (2013) concluded the lower molecular weight preparations were more effective, with one strain of *B. bruxellensis* exhibiting a four-log reduction in population. However, Park *et al.* (2008) observed that larger molecular weights of the preparations studied (1 to 10 kDa) displayed stronger membrane-disrupting abilities against various yeasts other than *B. bruxellensis*.

As there is limited information related to inhibition by different preparations, the objective of this research was to evaluate and compare the effectiveness of fungal, crab shell, and lactate-forms of chitosan against *B. bruxellensis* in culture media and in red wine.

## MATERIALS AND METHODS

### 1. Yeasts

*B. bruxellensis* strains B1b, B5, E1, and I1a were originally isolated from commercial wines from Washington State (USA) as described by Jensen *et al.* (2009). Yeast cultures were suspended in glycerol for storage at -80 °C. For use, the strains were suspended in YM media (BD Diagnostic Systems, Sparks, MD, USA) at pH 7 and without ethanol before being streaked on solidified agar plates.

Starter cultures were prepared by removing a single colony and serially culturing in YM broth (pH 3.84) that contained increasingly higher concentrations of ethanol (0 % to 5 % to 10 % v/v). Ethanol was aseptically added to sterilized media upon cooling.

### 2. Chitosan

Three different chitosan preparations were obtained: fungal using *Aspergillus niger* (Lallemand, Montréal, Canada), crab shell (Sigma-Aldrich, St. Louis, MO, USA), and chitosan lactate (Sigma-Aldrich). Fungal chitosan (mw = 8 to 12 kDa; degree of acetylation = 10 to 15 %) and chitosan lactate (mw = 4 to 6 kDa; degree of acetylation = < 10 %) were prepared as 1 % w/w suspensions in distilled water while the same concentration of crab shell chitosan (mw = 50 to 190 kDa; degree of acetylation = 15 to 25 %) required suspension in 1 % v/v acetic acid.

### 3. Media experiments

Initially, *B. bruxellensis* B1b and B5 were inoculated into YM media (pH 3.8; 10 % ethanol) at approximately $10^3$ cfu/mL in 75-mL sterilized bottles. After inoculation, various preparations of chitosan were added at concentrations of 0, 2, 4, or 8 g/hL. Half of the bottles were shaken prior to sampling while the other half remained static (samples were removed at approximately mid-point at half volume height).

To determine the impact of pH and ethanol on the effectiveness of chitosan, the pH of the YM medium was reduced to 3.0 prior to filtration through 0.22-µm filters. The pH of the medium was then adjusted to 3.7 or 4.0 using NaOH while the ethanol concentration was increased to 10 % v/v (pH 4.0) or 13.7 % v/v (pH 3.7). All media were aseptically distributed into sterile 100-mL milk dilution bottles and inoculated with *B. bruxellensis* E1 at $4 \times 10^4$ cfu/mL. Fungal chitosan was added to the bottles at concentration of 0, 4, 8, or 12 g/hL. Prior to sampling every other day, all bottles were inverted (shaken) twice. Samples were spiral plated on WL media and incubated at


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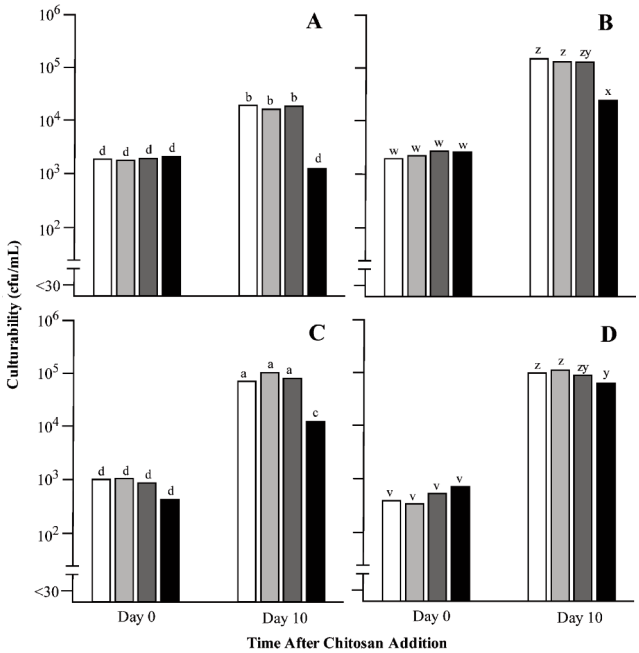



**Figure 1 - Culturability of *B. bruxellensis* strains B1b (A, C) or B5 (B, D) in media regularly shaken (A, B) or unshaken (C, D) after addition of fungal chitosan at concentrations of 0 (□), 2 (▒), 4 (▓), or 8 (■) g/hL. Within a given yeast strain, means with different superscripts ([a-d] strain B1b and [v-z] strain B5) were significantly different at $p \leq 0.05$.**

26 °C for 7 days. All treatments were replicated in triplicate.

### 4. Wine experiments

Commercially-prepared Cabernet-Sauvignon wine (pH 3.8, 13.5 % v/v alcohol) was sterile-filtered and inoculated with *B. bruxellensis* E1 or I1a to yield initial populations of 5 × $10^6$ cfu/mL. The wine was then distributed in 100-mL milk dilution bottles and treated with fungal chitosan, crab shell chitosan, or chitosan lactate at concentrations of 0, 4, 8, or 12 g/hL (three bottles per concentration). Bottles were shaken prior to sampling for culturability using WL media after incubation at 26 °C for 7 days.

A second wine, Merlot (pH 3.5, 13.4 % alcohol), was obtained from a commercial winery. Here, the concentration of free $SO_2$ was reduced to < 2 mg/L using $H_2O_2$ prior to filling Vadai Hungarian oak wine barrels (5.3 gallon) obtained from MoreWine! (Concord, CA, USA). Fourteen days after inoculation with *B. bruxellensis* E1, populations reached approximately $10^6$ cfu/mL. At this time, fungal chitosan was added at 0, 4, or 10 g/hL (three barrels per treatment) and thoroughly mixed in the barrel using a peristaltic pump. The barrels were left undisturbed for 10 days and then racked-off in five 1-gallon fractions, starting from the top of the barrel (fraction one) towards the bottom (fraction five). Wine samples were taken from each fraction and aliquots plated on WL. Colonies were counted after seven-day incubation at 26 °C. All collected wine fractions were subsequently stored at 18 °C for further microbial analysis.

### 5. Analyses

Culturabilities were determined by plating on WL agar (Fugelsang and Edwards, 2007) using an Autoplate 4000 spiral plater (Spiral Biotech, Bethesda, MD, USA). Two-way analysis of variance (ANOVA) and Tukey's HSD test were applied for mean separation using XLSTAT software (Addinsoft, New York, NY) with significance at $p \leq 0.05$. If mean populations were reported as "<30 cfu/mL", a population of 30 cfu/mL was assumed for statistical purposes only.

At the end of the barrel experiments (>68 days), sediments containing chitosan and *B. bruxellensis* from the barrels were examined using scanning electron microscopy (SEM). Here, samples were fixed in 2 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 % cacodylate buffer and stored overnight at room temperature. The following day, samples were rinsed with 0.1 % cacodylate buffer (3 × 10 min each) and fixed with $OsO_4$ either overnight at 4 °C or for 2 hr at room temperature. The sediments were rinsed with buffer and dehydrated in series of increasing ethanol concentrations (30 %, 50 %, 70 %, 95 %, and finally 100 % v/v) for 10 min each. At 100 % ethanol, samples were dehydrated for 3 × 10 min Samples were then dried with HMDS (hexamethyldisilazane), sputter-coated with gold, and observed with an FEI electron microscope at high vacuum mode.

## RESULTS AND DISCUSSION

### 1. Media and wine experiments

Using a culture medium (YM), the chitosan preparations differed widely in terms of the inhibition of *B. bruxellensis*. In general, very limited inhibition was exerted against either strain B1b or B5 by fungal chitosan (Figure 1). Here, populations increased from $10^3$ cfu/mL to approximately $10^4$ cfu/mL by day 10 regardless of concentration of chitosan added, with or without regular shaking/mixing, or yeast strain. The one exception was strain B1b treated with 8 g/hL and regularly shaken where the population remained relatively constant. These results were in contrast to those for crab shell chitosan (Figure 2) or chitosan lactate (Figure 3). For instance, populations of B1b or B5 increased from $10^3$ cfu/mL up to $10^4$ to $10^5$ cfu/mL after 10 days but only in media containing 0 or 2 g/hL. In media containing 4 g/hL, populations either remained at $10^3$ to $10^4$ cfu/mL or decreased below limit of detection (<30 cfu/mL). At 8 g/hL chitosan lactate, neither B1b or B5 recovered when incubated under conditions of being regularly shaken or unshaken.

Crab shell and lactate-chitosan were found to be inhibitory to *B. bruxellensis* at concentrations far lower than those reported by Ferreira *et al.* (2013). While these authors reported minimal inhibitory concentrations of 20 to 50 g/hL, a concentration of 10 g/hL resulted in populations decreasing to undetectable levels in the present research. These results could be a function of molecular weight and/or degree of acetylation of the chitosan preparations as


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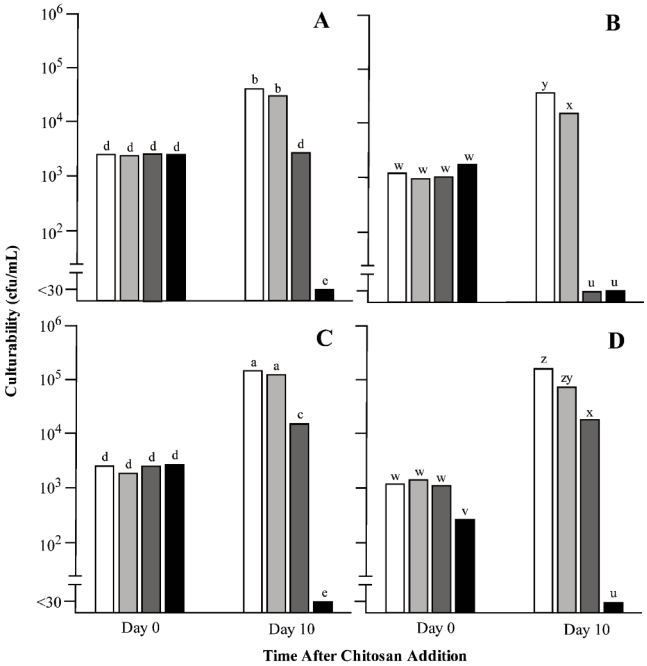



**Figure 2 - Culturability of *B. bruxellensis* strains B1b (A, C) or B5 (B, D) in media regularly shaken (A, B) or unshaken (C, D) after addition of crab shell chitosan at concentrations of 0 (□), 2 (▒), 4 (▓), or 8 (■) g/hL. Within a given yeast strain, means with different superscripts ([a-d] strain B1b and [v-z] strain B5) were significantly different at $p \leq 0.05$.**

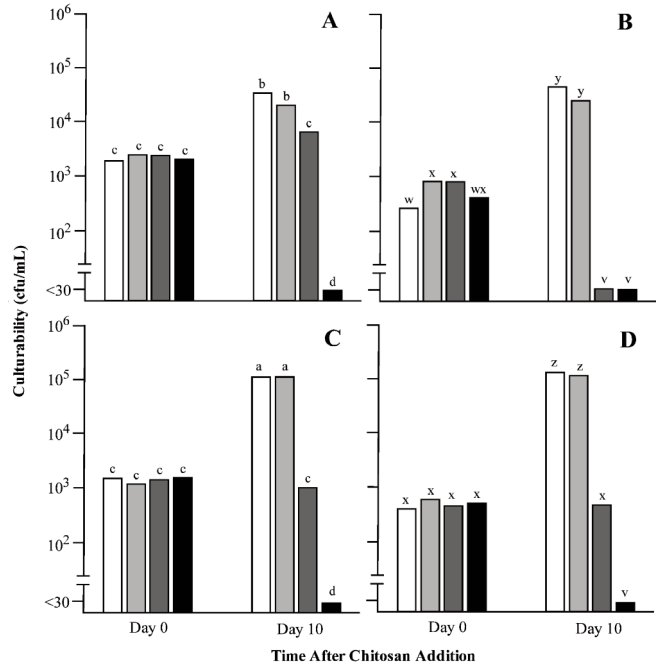



**Figure 3 - Culturability of *B. bruxellensis* strains B1b (A, C) or B5 (B, D) in media regularly shaken (A, B) or unshaken (C, D) after addition of of chitosan lactate at concentrations of 0 (□), 2 (▒), 4 (▓), or 8 (■) g/hL. Within a given yeast strain, means with different superscripts ([a-d] strain B1b and [v-z] strain B5) were significantly different at $p \leq 0.05$.**

differences have been noted (Park *et al.*, 2008; Ferreira *et al.*, 2013). While Park *et al.* (2008) utilized chitosan with molecular weights ranging from 1 to 10 KDa, the degrees of acetylation of these preparations were not reported.

Compared to media, the fungal chitosan preparation behaved differently when added to wine. Here, fungal chitosan provided similar results as crab shell or chitosan lactate when added to Cabernet-Sauvignon wine inoculated with *B. bruxellensis* strains E1 (Figure 4) or I1a (Figure 5). While untreated wines maintained $\geq 10^6$ cfu/mL, increases in chitosan concentrations from 4, 8, to 12 g/hL resulted in declines in culturability regardless of the preparation or strain examined. At the highest concentrations of chitosan, populations decreased from $10^6$ cfu/mL down to $10^2$ to $10^3$ cfu/mL. Differences in behaviors between media and red wine have not been previously reported but could be a function of number of factors such as pH and/or ethanol content.

To determine how some intrinsic factors affect the efficacy of the chitosan preparations, *B. bruxellensis* strain E1 was inoculated into YM media under two conditions: pH 4.0/10 % v/v ethanol and pH 3.7/13.7 % ethanol (Figure 6). Without chitosan added, populations of E1 increased from $5 \times 10^4$ cfu/mL to $\geq 10^7$ cfu/mL under conditions of pH 4.0/10 % v/v ethanol but only $10^5$ to $10^6$ cfu/mL in the conditions of pH 3.7/13.7 % v/v ethanol. With 4 g/hL either crab shell (Figure 6B) or chitosan lactate (Figure 6C), growth was only noted in pH 3.7/13.7 %. In contrast, the yeast grew under both conditions pH 4.0/10 % and pH 3.7/13.7 % regardless of concentration of fungal chitosan added (Figure 6A), with growth being retarded under lower pH/higher ethanol. As suggested by Park *et al.* (2008), affinities of low molecular weight/water soluble chitosan with the anionic components of the plasma membrane of the targeted yeast cell depend on relative positive charges. With $pK_a$ values approaching 6.3 to 6.5 (Zakrzewska *et al.*, 2005; 2007), more of the glucosamine residues would be cationic through protonation of amino groups in media or wine of lower pH. In addition, solubility of chitosan preparations varies widely, a factor which could impact inhibitory activity. For example, research by Qin *et al.* (2006) suggested that water-insoluble chitosan were far more inhibitory to *Candida albicans* compared to water-soluble preparations.

When inoculated into red wine being aged in the oak barrels, *B. bruxellensis* reached mean populations of $8.8 \times 10^5$ cfu/mL. However, eleven days after addition of fungal chitosan and subsequent racking, populations decreased depending on the concentration added (Figure 7). For instance, populations in those wines with 4 g/hL fungal chitosan declined to $10^2$ cfu/mL while those with 10 g/hL contained this population or less. By day 19 or 26, populations had reached undetectable levels in wines with 10 g/hL while small populations resided in wines with 4 g/hL chitosan. After initial decreases, populations gradually increased in all wines to eventually yield $10^3$ if not $10^4$ cfu/mL by day 68. By comparison, wines without


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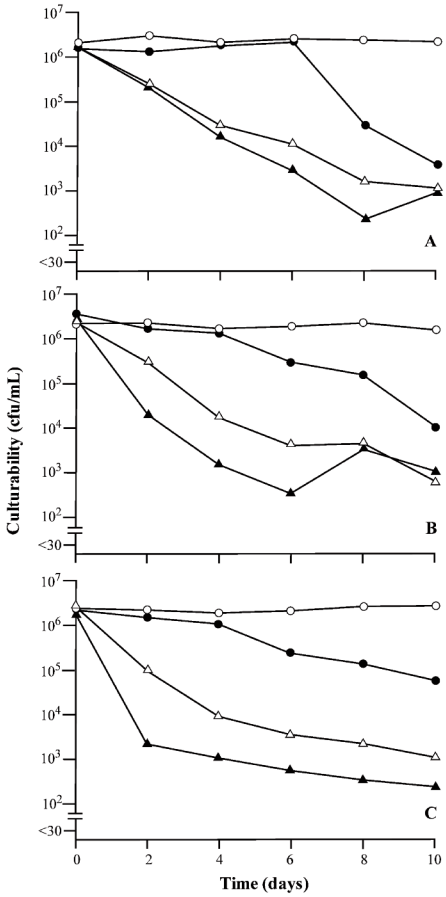



**Figure 4 - Culturability of *B. bruxellensis* strain E1 in Cabernet-Sauvignon wine with fungal chitosan (A), crab shell chitosan (B), or chitosan lactate (C) added at concentrations of 0 (○), 2 (●), 4 (△), or 8 (▲) g/hL.**

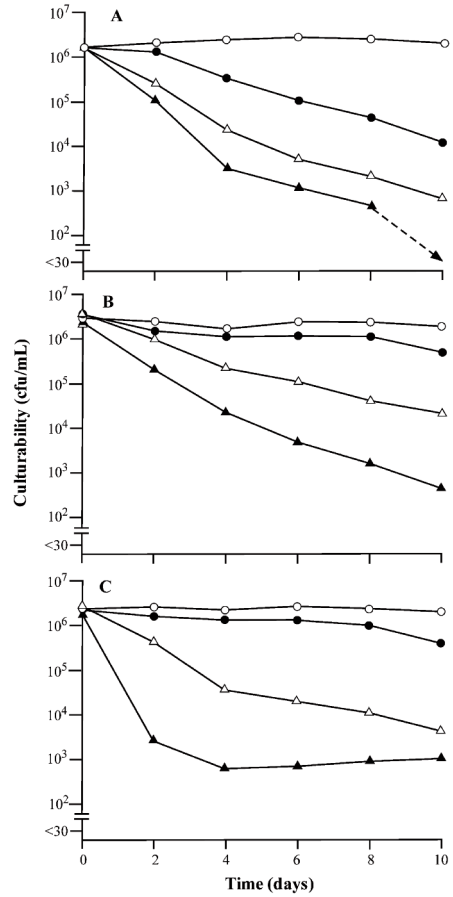



**Figure 5 - Culturability of *B. bruxellensis* strain I1a in Cabernet-Sauvignon wine with fungal chitosan (A), crab shell chitosan (B), or chitosan lactate (C) added at concentrations of 0 (○), 2 (●), 4 (△), or 8 (▲) g/hL.**

added chitosan yielded populations in excess of $10^6$ cfu/mL at the same time.

This study illustrated that while culturable populations initially declined to low levels after chitosan treatment, all fractions eventually grew to > $10^4$ cfu/mL. Thus, chitosan reduced, but did not necessarily eliminate, *B. bruxellensis* from barrel-stored wines although populations at the beginning of the study (*e.g.*, $8.8 \times 10^5$ cfu/mL) were far higher than commonly found in contaminated wines. In fact, Nardi *et al.* (2014) suggested that chitosan may allow > 9 months control when low populations of *B. bruxellensis* (<1000 cfu/mL) were present. However, Ferreira *et al.* (2013) and Taillandier *et al.* (2015) observed eventual increases in culturability of *B. bruxellensis* after chitosan treatment, in agreement with the current findings. In fact, Taillandier *et al.* (2015) recommended racking wines after a few days of contact with chitosan to avoid recovery of the cells. Thus, the impact of chitosan appears to be fungistatic, not fungicidal, as suggested by Goy *et al.* (2009).

As noted in Figure 7, few statistical differences were noted between the different fractions of wine sampled from the top to the bottom of the five-gallon oak barrels on any sampling day. Although population differences between top-to-bottom fractions was not observed in the five-gallon oak barrels, microbial stratification has been observed in larger tank volumes. Studying recovery of *S. cerevisiae* and *Oenococcus oeni* from various heights of commercial tanks (1,000 to 10,000 L), Porret *et al.* (2007) noted differences depending on the time within vinification (alcoholic or malolactic fermentation) as well as the microbial species. Although the wines were not analyzed for other yeasts (*e.g.*, *Brettanomyces*) or bacteria (*e.g.*, *Acetobacter*, *Lactobacillus*, or *Pediococcus*), the authors concluded that samples taken from the sampling valve of stainless steel fermentation tanks yielded representative samples.

Using flow cytometry, Taillandier *et al.* (2015) studied mechanisms of inhibition by fungal chitosan against *B. bruxellensis* using two fluorophores, one which detected active internal enzymes (carboxyfluoresceindiacetate or cFDA) while the other for compromised membranes (propidium iodide or PI). Similar to the findings of Zuehlke and Edwards (2013) using similar fluorophores and fluorescence microscopy, Taillandier *et al.* (2015) noted populations of cells which exhibited a


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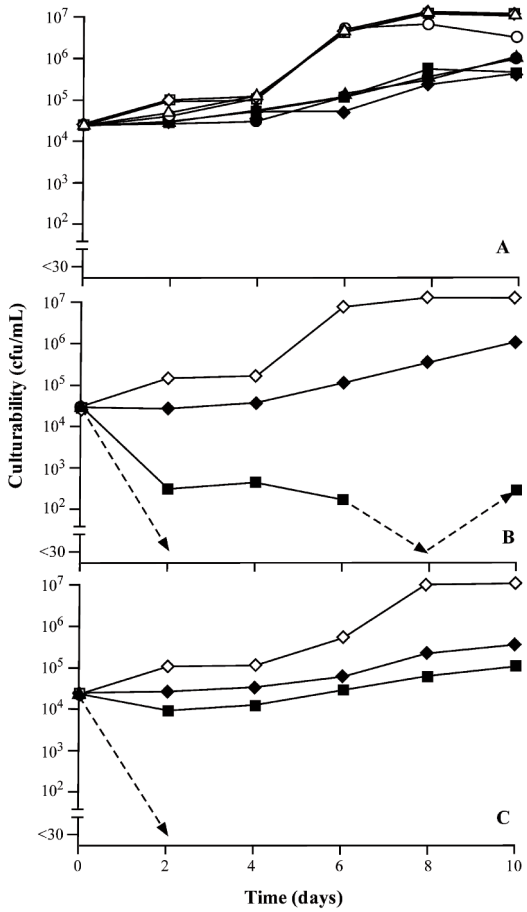



**Figure 6 - Culturability of *B. bruxellensis* strain E1 in media containing pH 4.0/10% ethanol (◇,□,△,○) or pH 3.7/13.7% ethanol (◆,■,▲,●) with fungal chitosan (A), crab shell chitosan (B), or chitosan lactate (C) added at concentrations of 0 (◇,◆), 4 (□,■), 8 (△,▲), or 12 (○,●) g/hL.**

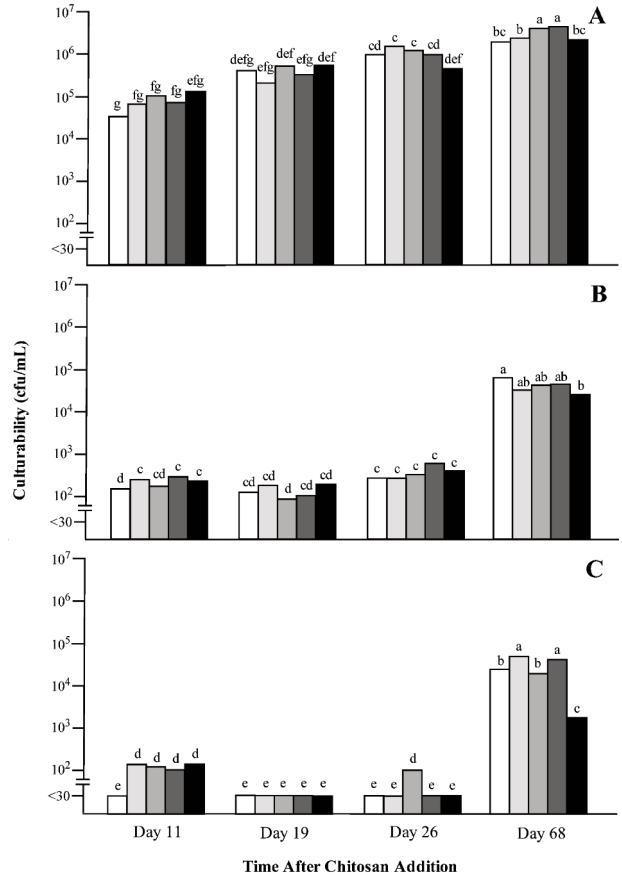



**Figure 7 - Culturability of *B. bruxellensis* strain E1 in Merlot wines treated with 0 (A), 4 (B), or 10 (C) g/hL fungal chitosan with one gallon samples taken from increasing depths within the five-gallon oak barrels; fraction 1 (top of the barrel;□), fraction 2 (▒), fraction 3 (▒), fraction 4 (▒), or fraction 5 (bottom of the barrel; ■). Means with different superscripts were significantly different at $p \leq 0.05$.**

mixture of green (cFDA) and orange-red fluorescence (PI) upon treatment with chitosan. Although Taillandier *et al.* (2015) referred to these cells as being 'sublethal,' it remains unknown whether these populations were injured ('sublethal') or perhaps had entered a VBNC state observed by others (du Toit *et al.*, 2005; Agnolucci *et al.*, 2010; Zuehlke and Edwards, 2013). In either case, additional research is required to determine the effects of chitosan on the physiology of *B. bruxellensis*.

## 2. Changes to yeast morphology

Morphologies of yeast cells grown in oak barrels filled with red wine were altered several weeks after treatment with fungal chitosan. Although the width/length of cells remained similar before or after treatment, cells developed numerous small 'nodules' or 'bumps' not present in untreated wines (Figure 8). These nodules were small, < 0.5 µm in diameter, and appeared randomly over cell surfaces, more abundantly in yeasts treated with 10 g/hL but less so with addition of 4 g/hL. Furthermore, nodules were only observed in experiments conducted with oak barrels, not those with glass containers regardless of the substrate (media or wine) or type of chitosan (data not shown). While this phenomenon had not previously been reported, Park *et al.* (2008) noted chitosan disrupted cell surfaces of *Candida albicans* and *Fusarium oxysporum* which appeared uneven and irregular. More recently, Taillandier *et al.* (2015) noted adsorption between chitosan and *B. bruxellensis* although changes to cellular surfaces were not reported. As such, the nature or significance of these nodules towards potential changes in yeast physiology remains unknown.

## CONCLUSION

All three preparations of chitosan were effective in reducing populations of *B. bruxellensis* from wine, but not necessarily media. Although populations were greatly reduced, sometimes > 4 log, complete eradication was not achieved whereas populations eventually increased to > $10^3$ cfu/mL. As such, chitosan could be used a means to


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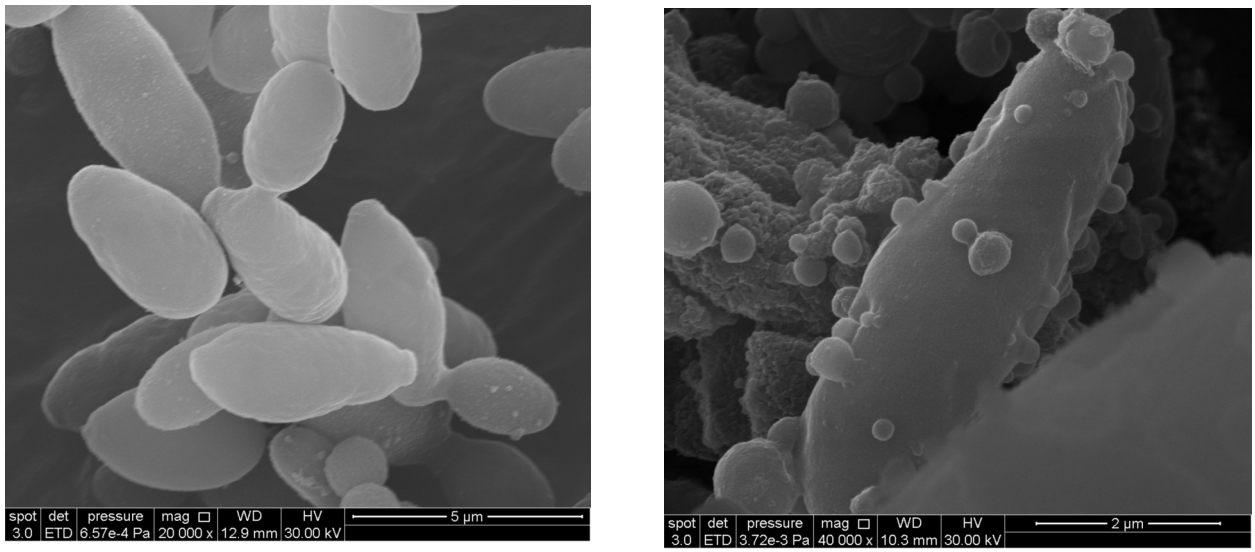



**Figure 8 - Scanning electron micrographs of *B. bruxellensis* E1 obtained from wine barrel sediment after addition of 0 g/hL (top) or 10 g/hL (bottom) fungal chitosan.**

reduce populations but not necessarily eliminate *B. bruxellensis* from aging red wines.

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