

CHITOSAN OF FUNGAL ORIGIN AS A NATURAL TOOL TO FIGHT *BRETTANOMYCES*

JOSÉ MARÍA HERAS
LALLEMAND OENOLOGY

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WINE
YEASTS



WINE
BACTERIA



NUTRIENTS
/PROTECTORS



SPECIFIC
INACTIVATED YEASTS



ENZYMES



CHITOSAN



VINEYARD
SOLUTIONS



LALLEMAND OENOLOGY

Original *by culture*

Introduction

Brettanomyces bruxellensis constitutes a permanent threat to the quality of wines. These wine alteration yeasts can develop in difficult environments and at any time during the life of a wine, but particularly during the aging phase. Different means are currently used to fight against *Brettanomyces*, with varying degrees of success. However, none of them are entirely satisfactory because none of the currently available tools can satisfactorily eliminate all these spoilage microorganisms. The use of fungal-origin chitosan, an oenological practice admitted by the OIV in July 2009 and the European Union in December 2010, represents an innovative and effective tool in the fight against *Brettanomyces*. Several scientific publications from different laboratories have shown the effectiveness of chitosan, and since 2008, countless winery trials and uses have confirmed its effectiveness in many different conditions. The combined knowledge acquired from the results of these trials has helped winemakers to refine their methods in order to monitor the effectiveness of these treatments, and has contributed to our global understanding of the mechanism of action of chitosan upon *Brettanomyces* cells.

The origin of the chitosan used in oenology

Chitosan is a molecule derived from chitin. Chitin and chitosan are among the most widespread naturally occurring polymers on earth, second only to cellulose. Chitin is found especially in the exoskeletons of crustaceans and insects, as well as in the cell walls of certain fungi. A deacetylated derivative of chitin, chitosan, is a linear β -1-4 copolymer of N-acetyl D-glucosamine and D-glucosamine (Figure 1), which is obtained by hydrolysis of the acetyl groups ($\text{CH}_3\text{-CO}$). This operation liberates the primary amino groups (R-NH_2) and confers the chitosan with 'cationic' properties.

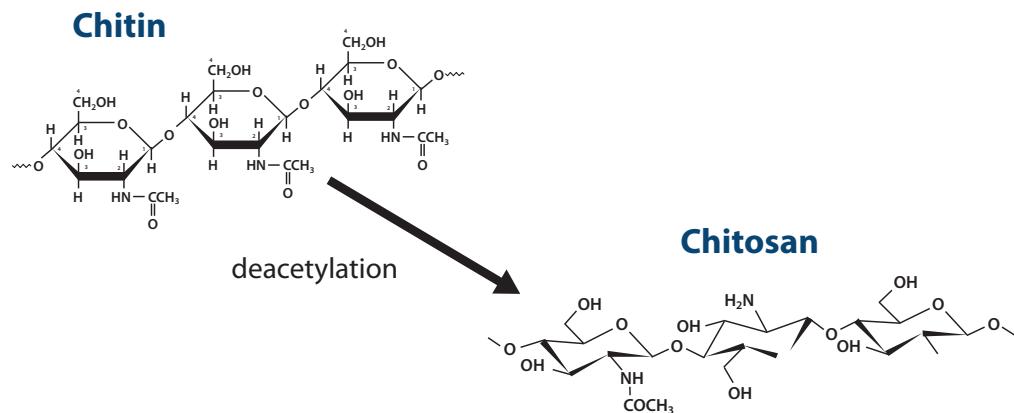


Figure 1: Chitosan, a polymer derived from chitin by deacetylation.

Indeed, there is not just one, but several chitosan variants, because variations in the degree of deacetylation, molecular weight, and preparation of the formulations (granulometry, in particular) result in molecules with a variety of different properties and actions. The innovation that led to the approval for the use of chitosan in oenology was the invention of the process used to obtain chitin from a non-animal fungal source, *Aspergillus niger*. This process, which was patented by Kitozyme, provides a natural source of chitosan that is biodegradable and non-allergenic.

The antibacterial and antifungal properties of chitosan have been extensively studied and documented and are now well recognized. Numerous studies have demonstrated the antimicrobial action of this polymer against various bacteria and pathogens, but also against certain grapevine fungi such as *Botrytis* (Barka et al. 2004). However, only the study by Gómez-Rivas et al., (2004) referred to the potential usefulness of chitosan in fermentation media and, in particular, for inhibiting the growth of *Brettanomyces*.

Bornet and Teisseidre (2008) investigated the oenological properties of chitosan, especially in relation to *Brettanomyces bruxellensis*, first in the laboratory and later in Languedoc-Roussillon wines (Blateyron-Pic et al. 2011). Ten days after treatment with chitosan at a dose of 4 g/hL, *Brettanomyces* populations in contaminated wines were not usually detectable in selective culture media assays. In contrast, there was often an increase or the maintenance of these contaminating populations in untreated bottles of the same wines. Therefore, the effectiveness of chitosan, specifically in the fight against *Brettanomyces bruxellensis* in wine, was clearly demonstrated in this work.

The mechanism of action of chitosan against *Brettanomyces*

Studies carried out in 2011 by INP-ENSIACET in Toulouse and led by Patricia Taillandier, aimed to expand our knowledge of the mode of action of chitosan and the origin of its inhibition of the growth of *Brettanomyces* in wine. These experiments used synthetic media (tartaric acid 3 g/L; glycerin 6 g/L; ethanol 13% v/v; pH 3.7; with *Brettanomyces* 10^6 cells/mL) in order to control for the composition.

The lethal action of chitosan was analyzed by flow cytometry performed on these media at different time-points (T0, T5, and T24 hours) after the introduction of chitosan and were compared to a control sample that had not been subjected to any treatments. Moreover, flow cytometry made it possible to differentiate between dead and living cells. As shown in Figure 2, the lethal action of chitosan against *Brettanomyces* was rapid; at a dose of 4 g/hL, 5 hours were sufficient to obtain a lethal effect in 60% of the *Brettanomyces* population present in the synthetic medium.

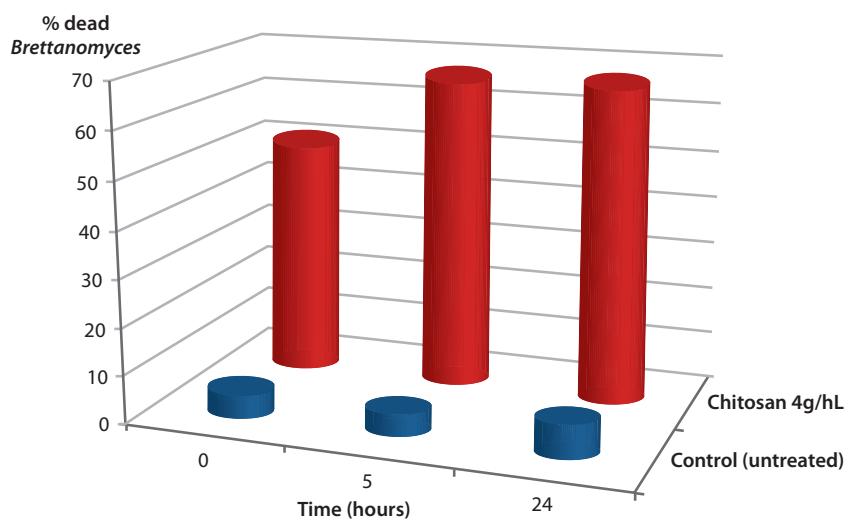


Figure 2: Evolution of *Brettanomyces* mortality measured by flow cytometry at the different timepoints studied.

This research also demonstrated how *Brettanomyces* in these conditions. Intracellular ATP flux measurements were performed over 2 hours to compare a control modality (absence of chitosan in the synthetic medium + *Brettanomyces* at a dose of 18×10^6 cells/mL) with the treatment modality (presence of chitosan at the dose of 10 g/hL in the synthetic medium + 18×10^6 cells/mL of *Brettanomyces*). As shown in Figure 3, the presence of chitosan resulted in the release of ATP of *Brettanomyces* into the culture medium. This phenomenon strongly disrupted the membrane permeability of *Brettanomyces*, which most likely caused the observed *Brettanomyces* cell death.

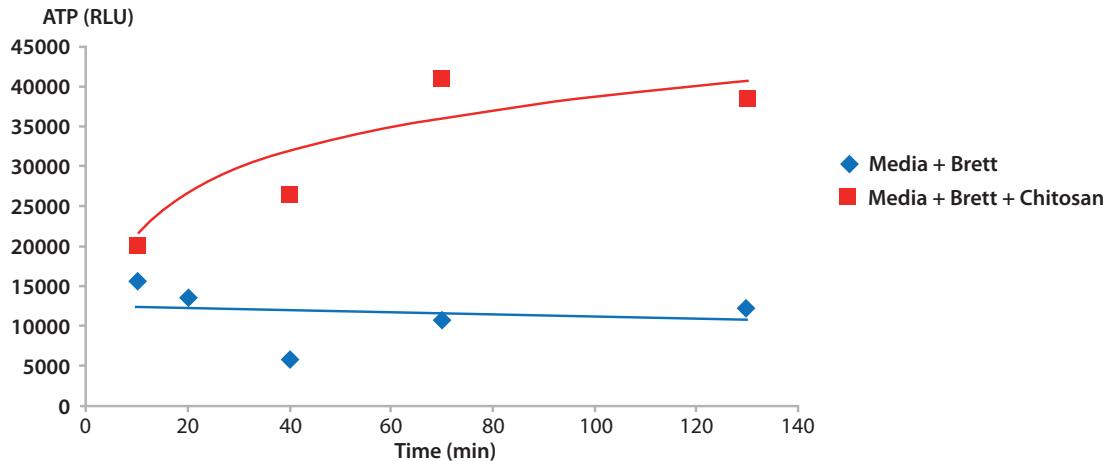


Figure 3: Monitoring the evolution of *Brettanomyces* ATP flux over time in the presence of chitosan 10 g/hL (red) or absence of chitosan (blue).

This work also highlighted the physical effect of chitosan against *Brettanomyces* cells. As shown in the conventional microscopy (photo 1A) and electron microscopy (photo 1B) micrographs, chitosan and *Brettanomyces* form aggregates—most likely the result of ionic charge interactions—thereby causing sedimentation of the *Brettanomyces* cells. It is important to note that chitosan is positively charged at the pH at which these studies were carried out (which emulated the pH of wine) while the surface of *Brettanomyces* yeast is negatively charged.

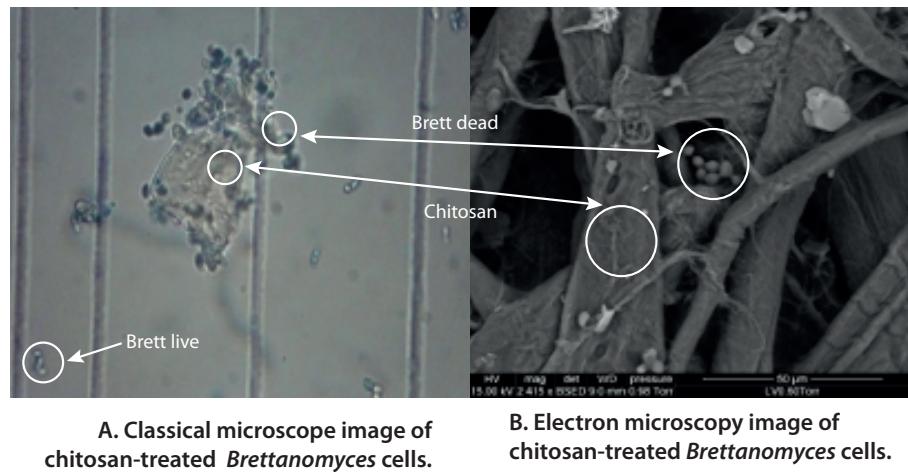


Photo 1: Observation of *Brettanomyces* treated with chitosan.

Recent studies which compared different molecular biology detection techniques, have highlighted the lethal effect of chitosan on *Brettanomyces* cells. The *Brettanomyces* population from a wine sample was analyzed by RT-PCR-PMAX (real-time polymerase chain reaction with PMAX, according to the technique adopted by Laboratorios Excell Ibérica in 2017).

PMAX is a dual dye technology developed by GenIUL. It exclusively quantifies living cells that have not suffered structural cell damage. By combining two dyes, the DNA of dead cells (either with damaged or undamaged membranes) can be eliminated from the analysis and therefore, only DNA from living cells will be detected. This product is highly effective in eliminating the PCR amplification of dead cell DNA and is the best method available to discriminate between live and dead microorganisms.

Chitosan was applied to an initial population of 1.5×10^3 *Brettanomyces* cells/mL at a dose of 4 g/hL, and the live cells were subsequently analyzed at 5 and 20 days after the treatment. As compared to the untreated control wine, the chitosan had had a lethal effect on the *Brettanomyces* cells after 5 days, and this effect was maintained at 20 days (Figure 4).

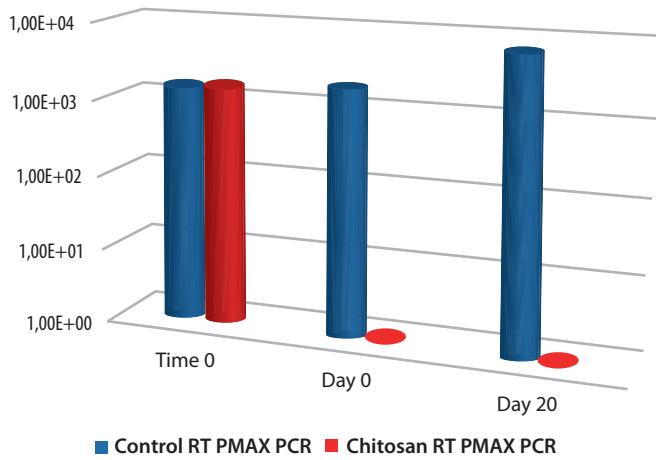


Figure 4. Follow-up by RT PCR PMAX of the evolution of living *Brettanomyces* cells over the time course studied.

These studies demonstrated that chitosan had two complementary effects against the *Brettanomyces* cells present in wines.

- A biological effect in which the interaction between chitosan and the *Brettanomyces* membrane caused the loss of cell viability.
- A physical effect whereby an ionic charge interaction between chitosan and the *Brettanomyces* cell walls caused aggregation and sedimentation of *Brettanomyces* cells.

Practical applications and perspectives

In cellar conditions, the effectiveness of this treatment has been shown in numerous trials in different wine matrices, varieties, and viticultural areas of the world among which, different regions in Spain. Some of these results are presented together in Figure 5, which shows the evolution of *Brettanomyces* populations after a 10-day treatment with No Brett Inside™'s chitosan at a dose of 4 g/hL followed by racking. These measurements were recorded both 10 days and 3 months after racking. In these industrial conditions where the recommendations for using fungal-origin chitosan are carefully followed, the efficacy of the treatment has been demonstrated for population levels exceeding 10⁴ CFU/mL.

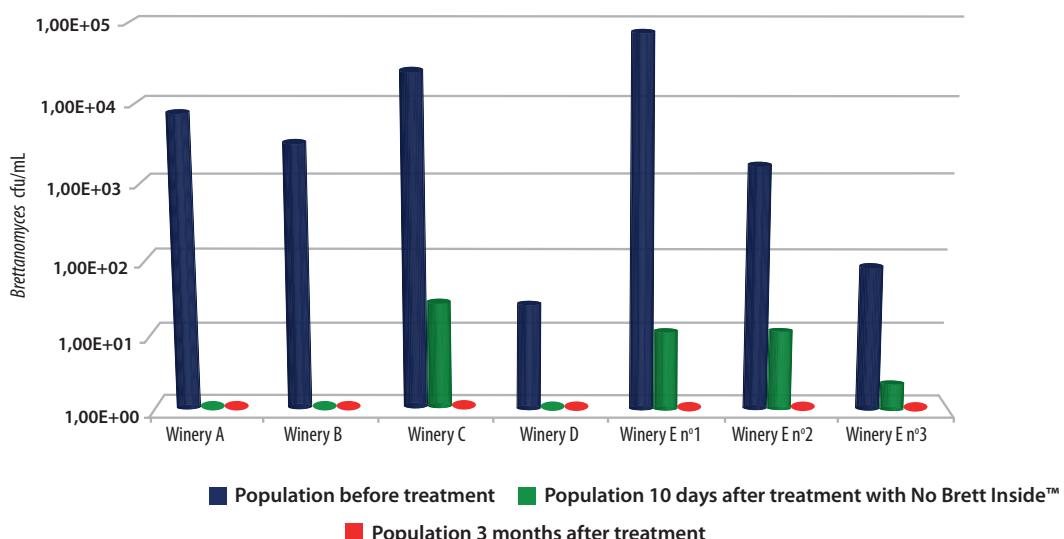


Figure 5. Winery trials in wineries after a 10-day treatment with No Brett Inside™ at a dose of 4 g/hL. The *Brettanomyces* population (CFU/mL) was measured by real-time polymerase chain reaction (RT-PCR).

In these trials, volatile phenol levels were analyzed at the three previously mentioned timepoints (before treatment, and 10 days and 3 months after treatment). No increases in the concentration of 4-ethylphenol and 4-ethylguaiacol were observed in the wine with chitosan. This result agrees with the findings presented above regarding the lethal effect of chitosan over a short period of time. Analytical controls of other oenological parameters were also carried out which verified that these treatments had not affected wine acidity, pH, volatile acidity, color, or total polyphenols. Moreover, from the sensory point of view, no changes were perceived in the aroma or taste of the wines.

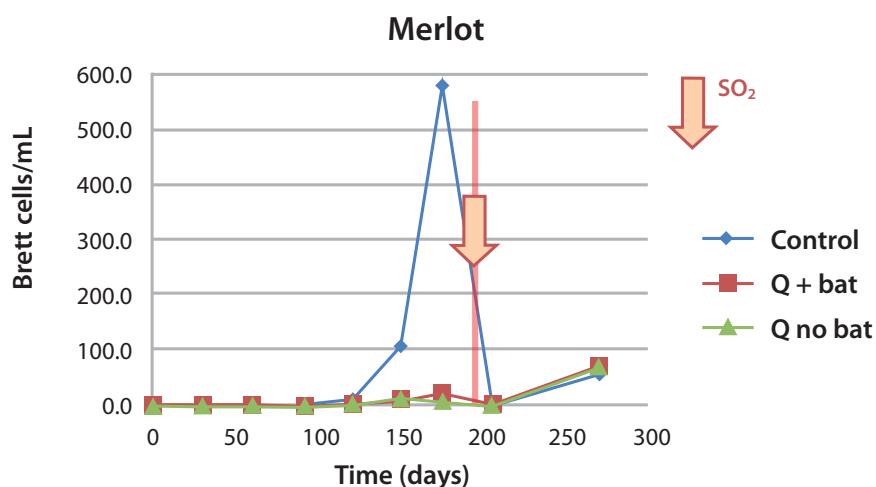
These experiences and the knowledge gained about the mechanism of action of chitosan have opened new horizons for scientific exploration. For example, when the wine is aged, the winemaker may want to incorporate a longer contact time with the chitosan, resulting in a racking delay of more than 10 days after incorporation. This would be doubly beneficial because:

- Instead of carrying out an unnecessary racking, the winemaker can take advantage of the classic racking procedure to eliminate the product.
- Above all, delaying racking could help protect wines from possible recontamination with *Brettanomyces*.

In this respect, Nardi and Sieczkowski (2014) carried out an experiment in Italy to test these hypotheses and to optimize the use of chitosan. A Merlot wine and a Sangiovese wine were inoculated with *Brettanomyces* (10^3 cells/mL) and each one was separated into three aliquots:

- An untreated control in which the settled lees were stirred back into the wine (bâtonnage) once a week.
- Two aliquots per wine treated with with No Brett Inside™ (4 g/hL), without racking, in which bâtonnage was carried out once a week in one but not in the other.

Each wine in the trial was analyzed over a period of 6 months. The sulfur dioxide (SO_2) in all the wines was readjusted after 200 days.



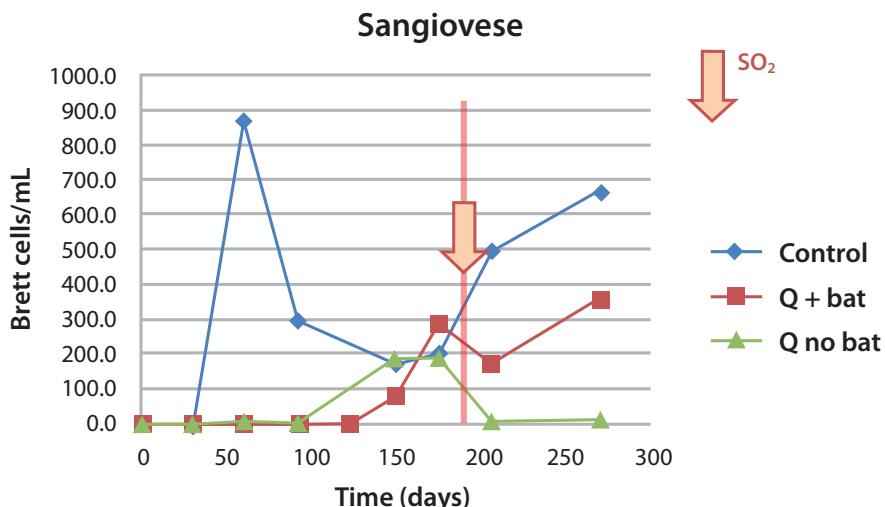


Figure 6. Effect on the development of *Brettanomyces* of prolonged contact with No Brett Inside™ during aging in wines in which the lees were or were not stirred back into the wine (bâtonnage) once a week.

The results (Figure 6) indicate the value of prolonged contact with chitosan while the wines were aged (for 6 and 9 months, respectively). This effect appeared to be more effective in the Sangiovese wine when bâtonnage was not implemented, although it did not encourage repeated contact between the product and the yeast cells. Unfortunately, these specific experiments did not allow us to reach a firm conclusion, although we can imagine two explanations for these results:

- The bâtonnage encouraged the regeneration of *Brettanomyces* by resuspending the lees and incorporating oxygen, etc.
- The bâtonnage biased the estimations of the concentration of *Brettanomyces* in the wines because it resuspended and homogenized the yeast cells.

We must also highlight the important role that SO₂ plays as a tool which acts in synergy with the action of chitosan and is still necessary to control spoilage in winemaking. Several other research projects are currently being conducted to explore different conditions in order to complete and refine these findings.

Conclusions

The trials reported by various research teams, as well as in laboratories and wineries, have confirmed that pure chitosan of fungal origin is an effective tool for fighting the contamination of wine with *Brettanomyces*. The homogeneous incorporation of chitosan into wine (which is important to guarantee its effectiveness), usually results in the complete destruction of *Brettanomyces* populations, or in certain cases, significantly reduces the populations of this contaminant.

When carrying out these experiments, it is important to incorporate a control protocol adapted to these treatments, to consider the existence of sublethal populations of *Brettanomyces*, and to use different means to detect false positives. Several studies have clarified the mechanism of action of chitosan against *Brettanomyces* and have also highlighted its speed of action, both in reducing the population and to prevent the appearance of volatile phenols.

In conclusion, chitosan derived from fungi is non-allergenic, of a natural origin, does not negatively impact the sensory qualities of wine, and is an innovative tool that can be used to prevent the wine taint and loss of quality caused by *Brettanomyces bruxellensis*.

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