ARTICLE

LOW SO_2 WINEMAKING — MICROBIAL CONTROL POST-FERMENTATION

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In a recent article ('Low SO_2 winemaking – bioprotection for microbial control pre-fermentation', published in the Autumn 2019 issue of the Wine & Viticulture Journal) pre-fermentation conditions affecting fermentation kinetics, volatile acidity and ethyl acetate production were discussed. The following article is Part 2 with specific application to using less SO_2 post fermentation whilst maintaining the same high quality.

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CONTROLLING POPULATIONS DURING FERMENTATION

By controlling microbial populations during alcoholic fermentation and malolactic fermentation via inoculating quickly with desired strains of both *Saccharomyces cerevisiae* and lactic acid bacteria, there is minimal opportunity for spoilage microorganisms to grow. This ensures that the optimal species and strains are able to quickly and efficiently metabolise sugar and produce alcohol (*Saccharomyces cerervisiae*), and convert L-malic acid to L-Lactic acid (lactic acid bacteria), thus minimising the growth of potential spoilage microorganisms (Berbegal et al. 2017, Berbegal et al. 2018).

MICROBIAL POPULATIONS POST FERMENTATION AND DISCUSSION AROUND INFLUENCING FACTORS

Post fermentation, the two species of principal concern are *Brettanomyces bruxellensis* (yeast) and *Acetobacter spp* (bacteria).

B. bruxellensis is literally the dark horse of the wine industry. We know a lot more than we did a couple of decades ago, but its mechanisms still present new learnings. Recent studies have demonstrated the now increased SO_2 tolerance that *B. bruxellensis* presents in modern winemaking (Barata et al. 2008, Curtin et al. 2012, Agnolucci et al. 2014). Where once the presence of volatile phenols due to the presence of *B. bruxellensis* was considered 'terroir' and 'funk', purchasers are becoming savvy at understanding what the problem is and how it might affect the longevity of the wines.

B. bruxellensis is able to tolerate high levels of alcohol and increasing levels of SO_2 (Barata et al. 2008, Curtin et al. 2012, Agnolucci et al. 2014). Wines that have a higher pH will have less molecular SO_2 (the state of SO_2 that has anti-microbial function) (Ribéreau - Gayon et al. 2006). *B. bruxellensis* is suited to wine pH, is able to grow in both anaerobic and aerobic conditions and can adapt to very low levels of glucose and fructose by using alternative carbon sources (Curtin and Pretorius 2014, Crauwels et al. 2015). It is of principal concern due to its role in the production of phenolic off flavours 4-ethyl phenol, 4 ethyl-guaicol and 4 ethyl-catecol. These were described as 'barnyard', 'horse sweat', 'sweaty', 'bandaid' and 'iodine' aromas (Chatonnet et al. 1992).

Species of acetic acid bacteria (AAB) including *A. aceti* and *A. pasteurianus* are present in most wine that has not been sterile filtered at levels of up to 1000 cells/mL. These species grow much quicker than *Saccharomyces Cerevisiae* and much quicker than *B. bruxellensis*. They can metabolise alcohol and convert it to acetic acid which causes an increase in VA (Drysdale and Fleet 1988). They can proliferate when tanks are left on ullage or without SO_2 post fermentation. Practices such as high pH, lower SO_2 regimes and the absence of sterile filtration may promote their proliferation both in tank and bottle (Bartowsky et al. 2003).

CHITIN VS CHITOSAN

Chitosan is a non-allergenic polysaccharide derived from the *Aspergillus spp*. for winemaking applications. It is also a major component of the skeletal structure in crustaceans. Chitin is a major component of yeast cell

walls that is responsible for cell wall rigidity. The primary difference between chitin and chitosan is the acetylation/deacetylation level (an acetyl group is removed to chitin thus becoming chitosan compound), but there are other factors including molecular weight (hence polymerisation level) and deacetylation function distribution. Figure 1 demonstrates the change in molecular structure when going from chitin to chitosan (Rabea et al. 2003).

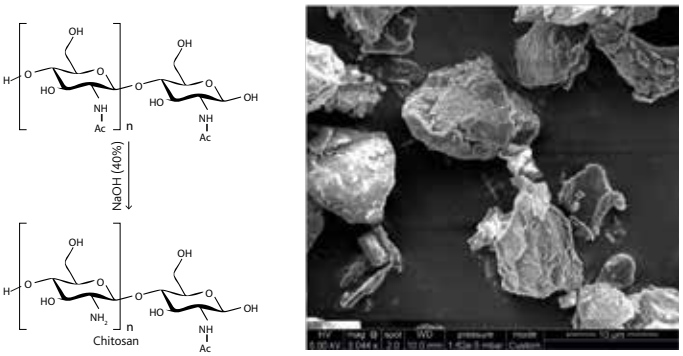

Figure 1. Preparation of chitosan from chitin (a). Sourced from Rabea et al. (2003) (b) chitosan in aqueous solution observed by SEM (microscopic studies conducted by **LAFFORT**® with the Bordeaux Imaging Centre).

Chitosan has been demonstrated to have an effect on membrane potential (the difference between the inside and outside of the cell) (Figure 2). However, other effects may also cause the death of *B. bruxellensis* due to its association with surface lipids, membrane permeability change and chelation of metal ions. At wine pH, chitosan is a positively charged molecule hence very reactive given its polycationic state (a molecule or a compound with multiple positive charges). Figure 2 demonstrates the effect of chitosan/ß-glucanase combinations on cell wall structure.

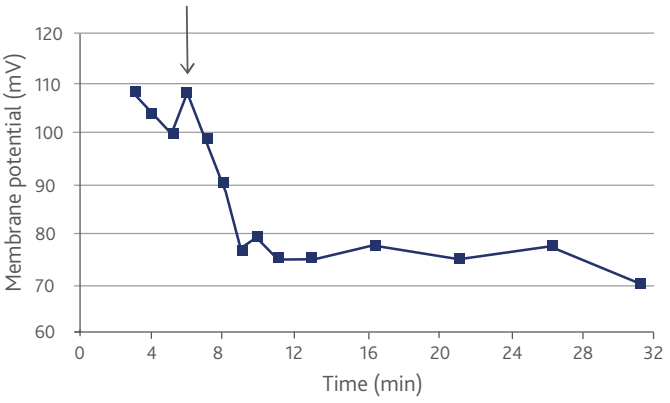

Figure 2. Difference in membrane potential Raafat et al. (2008) with an addition of chitosan of 10 ug/mL.

ADDING IN ENZYMES — THE KEY TO SUCCESS

Chitosan by itself can have an impact on cell viability (Figure 3). LAFFORT® has taken the concept of chitosan further and added ß-glucanase and pectinase activity, mainly promoting sedimentation and acting on the colloidal structure of the wine (ß-glucanase and pectinase activity). Trials conducted between LAFFORT®, BIOLAFFORT® and the Bordeaux Imaging Center investigated the impact of combined ß-glucanase and chitosan on the viability of *B. bruxellensis* cells, both lab cultured (Figure 3a) and spontaneously formed (Figure 3b) in wine (Nazaris et al. 2016). The combined effect is much greater on cell death than chitosan by itself (Figure 4).

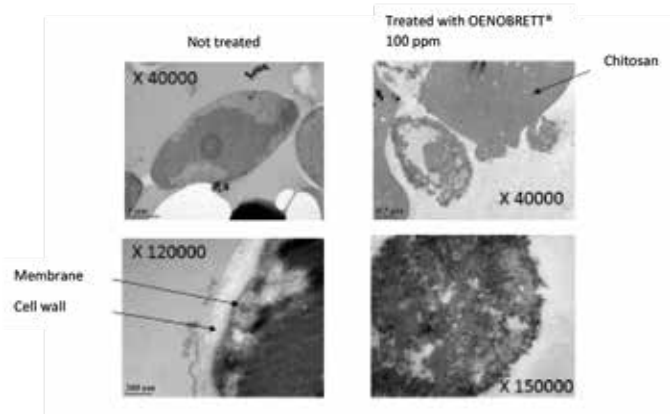

Figure 3a. Effect of **OENOBRETT**® on *Brettanomyces bruxellensis* cultivated on YPD media not treated (top and bottom left), treated with 100 ppm of **OENOBRETT**® after 8 days (top and bottom right).

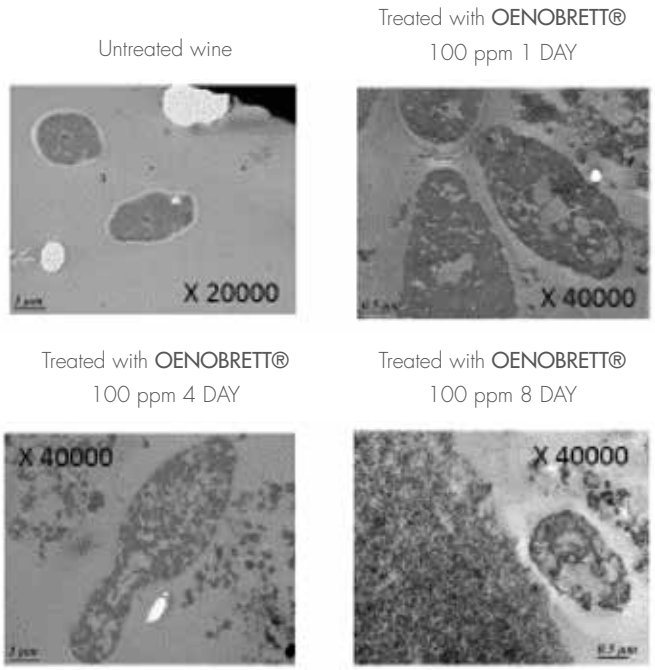

Figure 3b. *B. bruxellensis* cells in a wine naturally contaminated, with and without **OENOBRETT**® treatment 100ppm and observed after 1, 4 and 8 days of treatment.

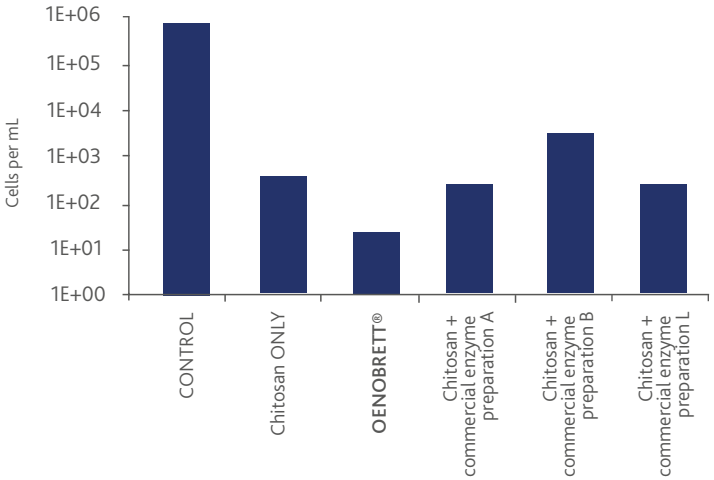

Figure 4. Effect of chitosan with and without enzymatic activities on viable *B. bruxellensis* cells.

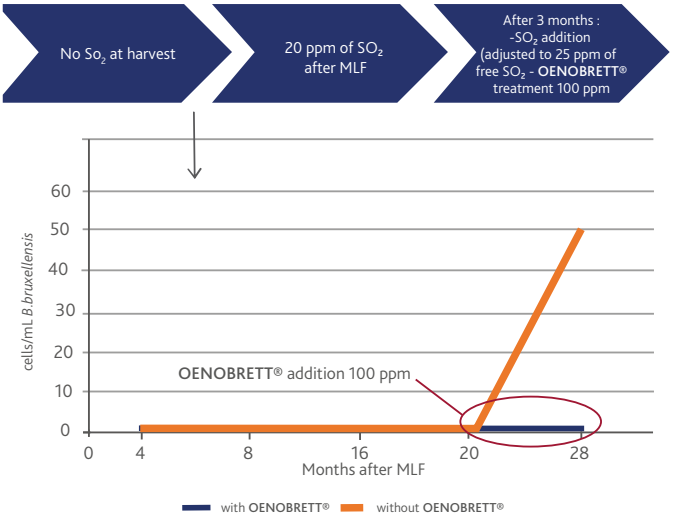

Figure 5. A red wine ex Margaux region with minimal SO_2, monitored for the growth of B. *bruxellensis* up to 5 months post MLF. (**LAFFORT®** and **EXCELL** laboratories, Bordeaux France).

PREVENTATIVE APPLICATION

Chitosan/ß-glucanase combinations may be used successfully as a preventative form of treatment before *B. bruxellensis* has the opportunity to proliferate. Because the combination affects cell viability, it will inhibit any growth before the cell mass is able to grow. Often the presence of *B. bruxellensis* is only determined by the winemaker when taint is detected sensorially. By this stage, the *B. bruxellensis* population has reached high cell numbers, in the order of 50 - 100 cells/mL. By preventing the growth in the first place, a winemaker is able to maintain a higher proportion of molecular SO_2 by ensuring there is a growth prevention strategy such as **OENOBRETT®** in place. This method is also much less dependent on personnel to monitor and detect a problem and becomes part of a standard operating procedure. Trials in 2013 on a red wine from Margaux was able to limit the growth of *B. bruxellensis* by using chitosan as a preventative treatment (Figure 5). Growth of this species in the control was recorded after four months of storage following primary and secondary fermentation. This preventative treatment can be used:

- On topping wine in case of barrel ageing.
- On wine lees.
- On red pressings (these contain a higher microbial load than the free run fraction).
- On wine with high pH (less available molecular SO_2).
- On wine with high micro-organisms population.

CURATIVE APPLICATION – OENOBRETT®

Application of chitosan/ß-glucanase based treatments is often more common once a problem has been detected. As we have seen, its application is able to effectively kill the yeast cell, literally destroying the cell structure. Trials conducted in 2013 demonstrated a complete reduction of population from $3x10^3$ cells/mL to not detected after the addition of 100 ppm of **OENOBRETT®** (Figure 6). The population required to commence producing 4-EP and 4-EG will vary depending on the wine in question, but often produces detectable levels in the $1x10^2$ to $1x10^3$ cells/mL range (Chatonnet et al. 1992). At this population level, SO_2 will be consumed very quickly — both free and total SO_2 will be greatly reduced here and unavailable from an antimicrobial point of view if added. At this cell level it is ultra-critical to reduce the microbial load in order to ensure a portion of molecular SO_2 to prevent further growth.

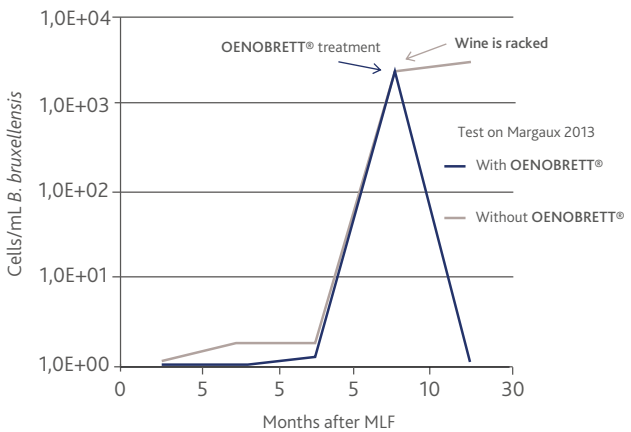

Figure 6. Use of **OENOBRETT®** on a red wine from Margaux after the detection of ***B. bruxellensis*** whereby viable cells are no longer detectable after treatment (**EXCELL** laboratories).

CONTROLLING BACTERIA

The control of both lactic acid bacteria and acetic acid bacteria also comes into question primarily post-alcoholic fermentation, but high pH can induce the proliferation of *Pediococcus spp.* and *Acetobacter spp.* pre-fermentation if the must is left unprotected. The primary role of lactic acid bacteria is to conduct malolactic fermentation (MLF) which is not always a desired outcome. Lysozyme alone may not be sufficient to kill the entire population of lactic acid bacteria, which can reach up to $1x10^8$ cells/mL during MLF, making it quite challenging to stop. The combination of lysozyme, chitosan and ß-glucanase has an effect not only on *Oenococcus oeni* cell membrane (principal bacteria responsible for MLF) but also *Pediococcus spp.* which produces a lot of ß-glucans, making it difficult to lyse with lysozyme alone. Figure 7 demonstrates the arrest in MLF in 2017 trials on base wine for cognac distillation when there is a high population of bacteria that has already started to conduct MLF with the use of a chitosan, ß-glucanase and lysozyme mix used at 200 ppm.
Acetic acid bacteria, on the other hand, is not affected by lysozyme and has limited impact from chitosan (Valera et al. 2017). These species are responsible for spoilage in the form of acetic acid production and are commonly found in levels of 10^3-10^4 cells/mL in unfiltered wines. Combinations of chitosan, ß-glucanase and potato protein may be used in wines where there is an unidentified microbial problem as a blanket strategy. Whilst the *Acetobacter spp.* may not damage the cell wall, **MICROCONTROL®** can drop out by means of fining/sedimentation a portion of the microbial load. Understanding that these populations are present and the risk factors associated with their proliferation after reaching critical mass is imperative to providing protection. Factors such as ullage, low molecular SO_2, high percentages of whole bunches (which can cause high levels of acetic acid bacteria) and unmanaged caps on red wine fermentations may cause proliferations of acetic acid bacteria.

OXIDATIVE PROTECTION

During alcoholic fermentation, the space is often protected due to complete saturation of CO_2 produced by fermentation. As fermentation slows down or goes through MLF, the level of CO_2 may drop and enable oxygen to contact the wine. Strategies to control microbial populations will not protect the wine from oxidation, but limiting the proliferation of spoilage microorganisms will enable the wine to retain more SO_2 in molecular form.

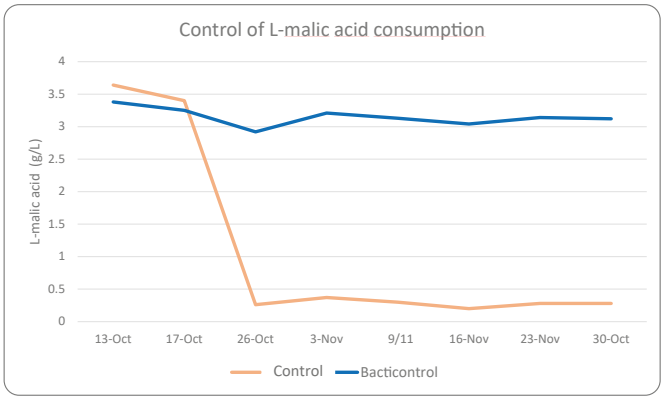

Figure 7. 2017 trials on Cognac base wine (EXCELL laboratories, Bordeaux).

CONCLUSIONS

Using lower rates of SO_2 post fermentation poses the risk of spoilage, primarily due to the proliferation of *B. bruxellensis* and *Acetobacter spp.* after primary and secondary fermentation. *B. bruxellensis* may cause taint due to the production of taint compounds 4-EP and 4-EG and *Acetobacter spp.* are able to metabolise ethanol to form acetic acid. By understanding which microorganisms pose a risk at this stage in production, it is possible to target their growth directly via the use of chitosan and ß-glucanase combinations that disrupt structural components in the cell membrane (chitin and glucans). The chitosan by itself does not display the same efficacy as chitosan and ß-glucanase together on *B. bruxellensis* cell death.

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