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What kind of sanitation should be applied to remove *Brettanomyces bruxellensis* biofilms?

>>> The capacity for five *Brettanomyces bruxellensis* strains to form biofilm on stainless steel was confirmed, and the sanitation of these biofilms was tested using a solution of lactic acid and a reference method (a solution of foaming caustic soda and peroxide at 5 %). Different responses were observed depending on the strain: lactic acid solution induced a slight reduction in cell population, while the reference method resulted in the elimination of the adhered cells for three strains, but generated VBNC states for two others. The effects of sanitation on the biofilm formed is strain-dependent. <<<

Winery sanitation is at the heart of guidelines for controlling microbial contaminations and, in particular, contamination by *Brettanomyces bruxellensis*. Indeed, this spoilage yeast is able to survive in stressful conditions; for example, in the presence of sulphites[1, 2, 3]. The ability to form biofilm is a potential resistance strategy developed by some yeast[4], although little attention has been given to the case of *B. bruxellensis* so far. Biofilms are defined as a community of one or more types of microorganisms, which adhere to biotic and abiotic surfaces that can grow in all three dimensions, and which are embedded in a self-produced matrix called EPS (Extra Polymeric Substances)[5]. A recent study carried out at the IUVV (Dijon, France) described the biofilm of *B. bruxellensis* as a thin structure consisting of different cell morphologies, including yeast cells and pseudo-filamentous cells embedded in an extracellular matrix (Figure 1)[6]. Moreover, the persistence of biofilm cells on surfaces over time was demonstrated.
Currently, the most commonly used process for cleaning tanks is to apply a mixture of diluted chemical solutions containing alkaline detergents and acid solutions[7]. Eco-friendly alternatives that are effective in combatting all survival forms of *B. bruxellensis* are nevertheless sought to reduce the use of chemicals in the wine industry. In this context, we aimed to study the impact of two different methods of sanitation (a solution of foaming caustic soda and peroxide at 5 % and a solution of lactic acid at 5 %) on strains of *Brettanomyces* developed in biofilm.

■ Seeking removal of *Brettanomyces bruxellensis* biofilms

Five strains of *B. bruxellensis* from different origins were used (Table 1) and for each strain fourteen-day-old biofilms were formed on stainless steel chips in a synthetic medium (Yeast Extract- Peptone-Dextrose (YPD) medium), commonly used for growing yeasts in research laboratories, to evaluate two different sanitation methods. Before biofilm formation, starter cultures ensured that cells were in the same physiological state, allowing the accurate comparison of the ability of different strains to form biofilms. Indeed, growth kinetics is strain-dependent with strain BR17 being slower compared to the other strains (Table 1). On the other hand, strain GS04 was the fastest to grow. However, the ability of all investigated strains to adhere to and form a biofilm on stainless steel chips was confirmed with a starting population in the biofilm on the chip of around 106 cells/cm² for all the strains after 14 days of incubation (Figure 2).

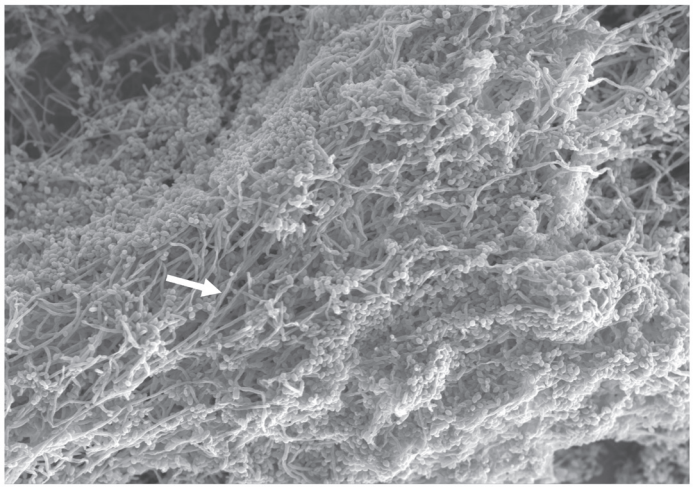

Figure 1. SEM observations (x 500) of one-month old biofilm of *Brettanomyces bruxellensis* developed on stainless steel chip on a YPD medium. The surface is covered in microcolonies containing yeast and filamentous cells (indicated by white arrow).

Table 1. Origins and growth parameters of *B. bruxellensis* strains.

Strain	Origins	Lag phase (h)	Generation time (h)	Growth rate μ (h^{-1})
S11	Burgundy wine (IUVV, Dijon)	40 ±8.021	11.36 ±4.867	0.097 ±0.042
S14	Burgundy wine (IUVV, Dijon)	27 ±1.732	18.47 ±4.265	0.056 ±0.013
BR17	Vallée du Rhône wine (IR, Orange)	100 ±0.000	29.76 ±4.066	0.034 ±0.005
GS04	Vallée du Rhône wine (IR, Orange)	27 ±0.000	12.15 ±0.156	0.08 ±0.001
GS12	Vallée du Rhône wine (IR, Orange)	29 ±0.000	22.67 ±1.365	0.044 ±0.003

Lag phase: period during cell number remains relatively constant prior to rapid growth
Generation time: time taken by the microorganism to double in number during a specified time period
Growth rate: amount by which a variable increases over a given period of time

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. The effect of the winery method

The winery method, or reference method used by the Inter Rhône cellar, consists of applying a solution of foaming caustic soda and peroxide at 5 %. Biofilms were therefore exposed to this solution for 15 min, and populations on the chip were determined on agar media (culturable population) and by flow cytometry (viable population) at the end of the cleaning process. No remaining cells on the chips were detected after the treatment of three strains S11, GS04 and GS12, thereby demonstrating the effectiveness of this method (Figure 2A). However, for BR17 and S14 strains, some viable cells were detected by flow cytometry, thereby revealing cells in a viable but non culturable (VBNC) state[3].

. The effect of lactic acid

To investigate an alternative cleaning procedure for winemaking equipment, an eco-friendly approach was tested. Biofilms were treated with a solution of 5 % lactic acid for 15 min, and populations on stainless steel chips were determined as previously described. The results show a significant reduction in the population of culturable cells on the chips after the lactic acid treatment for all strains, except for strain GS12 (Figure 2B). However, this treatment did not eliminate all the cells.
These assays demonstrate different strain- and treatment-dependent behaviour. Strain GS12 appears to be slightly more tolerant than the other strains to the lactic acid method. On the other hand, strains BR17 and S14 seem to be more tolerant to the winery method with the induction of the VBNC state, while populations of the other three strains were totally eliminated. This VBNC state could explain the resistance of these two strains to different chemical products and could be the cause of the year-on-year recontamination of tanks, as cells may resuscitate from the VBNC state when the stress conditions disappear.

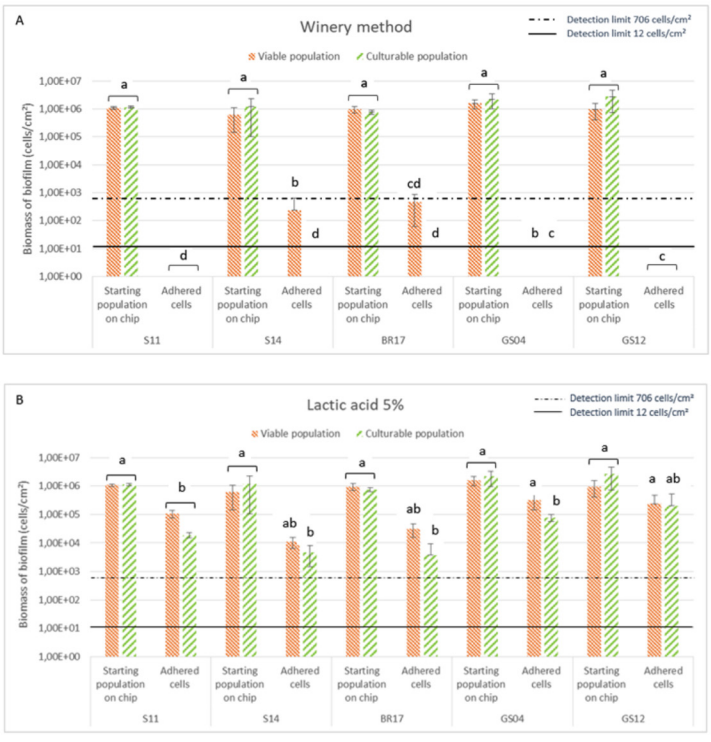

Figure 2. Concentration of viable or culturable cell populations (cells/cm²) of five *B. bruxellensis* strains over winery method (A) and lactic acid 5 % (B) sanitation process. The term "adhered cells" corresponds to the cells bound to the surface of the chip after treatment. Different letters represent significant difference (p-values ≤ 0.05) between cell population adhered to chips. Detection limit of the technics: 706 cells/cm² (cytometer: viable; dotted line), 12 cells/cm² (plate: culturable; full line).

■ Conclusion

All the tested strains, regardless of origin, were able to form biofilms on stainless steel (around 106 cells/cm²) after 14 days of incubation in a YPD medium. Currently, the lactic acid solution is not a harsh enough treatment to eradicate *B. bruxellensis*, but it is a promising approach, which could be improved by increasing contact time or the concentration of the solution. The winery method demonstrated its effectiveness, but two strains exhibited VBNC cells after treatment. These results show that as for SO_2[2, 3, 4], sanitation represents a stress for yeast with a strain-dependent response. The development of cheap and reliable detection methods which are reproducible at strain level could allow *B. bruxellensis* contamination to be predicted and controlled. ■

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