Technical Brief

Filter Media Comparison for the Removal of *Brettanomyces bruxellensis* from Wine

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Abstract: *Brettanomyces bruxellensis* is a wine-spoilage yeast of significant concern to wine producers, and its removal by filtration is not fully understood. The aim of this work was to compare the efficacy of filters, having different compositions and micron ratings, to remove *B. bruxellensis* from wine. Wines inoculated with a *B. bruxellensis* strain were used, and the U.S. Food and Drug Administration guidelines for filter validation for aseptic processing were followed. Polypropylene filters of 0.6 and 1.0 µm ratings yielded wines with high *Brettanomyces* cell counts. No cells were detected in wines filtered through polyethersulfone filters of 0.45, 0.65, and 1.0 µm, or through X grade glass microfiber filter. This work showed that different filter compositions with similar micron ratings result in variable retention of *B. bruxellensis,* highlighting that filter media are relevant for removal mechanisms, and pointing to the importance of a complete characterization of filter media when addressing *Brettanomyces* removal from wine.

Key words: *Brettanomyces bruxellensis,* filter characteristics, filtration, removal, wine

The species *Brettanomyces bruxellensis* is a spoilage yeast found in wine production, which generates flavors such as animal, medicinal, sweet leather, barnyard, and clove-like that are detrimental to aroma profiles. The occurrence and distribution of *B. bruxellensis* during winemaking has been extensively described by several authors (Loureiro and Malfeito-Ferreira 2006, Suárez et al. 2007, Oelofse et al. 2008, Steensels et al. 2015). Different mechanisms and strategies to neutralize *Brettanomyces* have been studied and implemented, such as the addition of sulfur dioxide (SO_2) and dimethyl dicarbonate, as well as of weak acids, such as sorbic, benzoic, and fumaric acids. Other alternative methods include the addition of chitosan and the application of high pressure or high temperature (Oelofse et al. 2008). According to Curtin et al. (2015), a combination of chemical or macromolecular inhibitors along with physical removal represents a highly effective strategy to eliminate *Brettanomyces.*

Filtration has been considered a valuable process to reduce the number of contaminating microorganisms, namely yeasts. A filter can be described as a mechanical barrier, but one that acts far beyond a simple strainer. There are true depth filters and pleated depth filters, both of which are manufactured with random fibers that create a "depth" of media yielding a tortuous fluid path in which particles of many different sizes are retained. Another type of filter is the pleated membrane filter consisting of thin polymeric filter media characterized by limited depth, but far higher surface area, and which is assigned absolute ratings (Fugelsang and Edwards 2007). Several mechanisms are involved in microorganisms' removal by membranes that can be summarized as direct interception, inertial impaction, and charge effects (Starbard 2008). The issue of filtration has not yet been completely explored in the wine industry, unlike in other food-processing industries, mostly because of the unsupported belief that filtration with small-pore membranes removes essential compounds imparting wine-sensory characteristics. However, studies have shown that adequate filtration does not substantially affect red wine composition or sensory properties (Serrano and Paetzold 1994, Canas et al. 2011).

The removal of *Brettanomyces* cells from wine by filtration has been the subject of several studies, but is far from being fully understood (Renouf et al. 2007, Umiker et al. 2013). Currently, it is presumed that the variable and elongated cellular morphology of *B. bruxellensis* cells, influenced by factors like SO_2, must be taken into account before setting up the filtration (Curtin et al. 2015). In fact, Serpaggi et al. (2012) induced cells of *B. bruxellensis* to enter a viable but nonculturable state by SO_2 addition and observed that cell size decreases by 22%. However, Umiker et al. (2013) found that the exposure to molecular SO_2, although influencing culturability and cell morphology, did not affect cell removal by nylon membrane filtration.

In the present work, several filters commercially available as pleated cartridges for the wine industry were compared for their efficacy in removing *B. bruxellensis* from wine. The filters differed in composition and micron rating, namely depth

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filters made of polypropylene (PP, 0.6 and 1.0 µm), borosilicate glass microfiber (GF, X, and V), and polyethersulfone membrane filters (PES, 0.45, 0.65, and 1.0 µm). The U.S. Food and Drug Administration (FDA) guidelines for filter validation for aseptic processing, aimed at the pharmaceutical industry, were followed, thus providing a margin of safety well beyond that expected in wine production (FDA 2004).

Materials and Methods

Yeast strain and culture conditions. A strain of *B. bruxellensis* ISA 1791 from Instituto Superior de Agronomia, Lisbon University, Portugal, isolated from Portuguese red wine was used. Strain cultures were maintained on YPD agar (5 g/L yeast extract, 10 g/L bacto-peptone, 20 g/L glucose, and 20 g/L agar).

A YPD agar culture grown for 72 hrs was used to inoculate YPD broth, which was then incubated at 25°C with orbital shaking (150 rpm) for 38 hrs. Cells were harvested by centrifugation at 5000*g* for 30 min, at 4°C (Heraeus) prior to wine transfer.

Red wine preparation. A red wine (14.4% [v/v] ethanol content, 2.8 g/L residual sugars, 5.98 g/L total acidity, 0.46 g/L volatile acidity, 12 mg/L free SO_2, 112 mg/L total SO_2, and pH 3.4) from a 2009 harvest was utilized. Equimolar hydrogen peroxide was added to promote SO_2 oxidation to sulfate. By adding a tartaric acid (5 g/L) solution to the previously SO_2-neutralized wine, two wines with different ethanol content were prepared: 6% (v/v) and 12% (v/v). A sodium hydroxide solution was used to adjust the pH to 3.5. The two wines were filter-sterilized through 0.45 µm mixed-cellulose ester (ME) membranes (Whatman). The 12% (v/v) ethanol wine was the initial wine, hereafter referred as V_i. The wine sterility was checked as described below.

The cells obtained as described above were transferred to the 6% (v/v) ethanol wine with a cell density of ~4×10^7 cells/mL and incubated at 25°C for 24 hrs, for wine adaptation (Barata et al. 2008). Cells were again harvested by centrifugation as described above, resuspended in V_i, and incubated at 25°C for 24 hrs, as recommended for validation tests for microorganism adaptation to the liquid to be filtrated (PDA 1998). After this period, the inoculated wine, hereafter referred to as V_B, was used in the filters tests. A target of 1.6×10^6 cells/mL or higher was established, so that 100 mL of inoculated wine would represent a challenge of 10^7 cells/cm^2 of filter surface (47 mm diam disks, corresponding to ~16 cm^2 effective filtration area) (FDA 2004).

Filter description and tests. The filters used in this study, recommended for the wine industry, are described in Table 1. To perform the tests, we used 47 mm diam disks from each filter medium. For PES 0.45 and 0.65, two lots were assayed. The disks were sterilized in the autoclave at 121°C for 15 min. A sterilized Analytical Stainless-Steel Filter Holder (ASSFH) (Millipore) positioned over a 500 mL Kitasato flask connected to a vacuum/pressure pump (Millipore) was used. After addition of 100 mL of the V_B wine, a vacuum of 600 mmHg was established, and the filtrated wine was aseptically collected. Each filter lot was tested in duplicate, resulting in two or four filter disks analyzed per each filter media.

Microbiological analysis. V_i and V_B were analyzed by plate counting after membrane filtration through 0.20 µm ME (Whatman), with a Microfil Filtration System with three filter supports (Millipore). Two sample volumes, 1 and 50 mL, of V_i were filtered, and the membrane was placed over YPD agar added with chloramphenicol (0.01% [w/v]) and biphenyl (0.015% [w/v]), bacteria and filamentous fungi inhibitors, respectively, according to International Organization of Vine and Wine (OIV) recommendations for yeast counting, and plates were incubated at 28°C for five days. V_B was analyzed after serial dilutions with a solution of 8.5 g/L NaCl and 1.0 g/L tryptone (autoclaved for 15 min at 121°C) to obtain a countable number of colonies.

To evaluate the filters' efficacy for removing the *Brettanomyces* cells from the wine, two volumes, 1 and 50 mL, of the filtrated wine were also analyzed by plate counting after membrane filtration. To reinforce the results, six randomly filtrated wine samples were additionally checked with differential media for *Brettanomyces,* namely *Dekkera*/*Brettanomyces* differential medium (DBDM) agar and DBDM broth (STAB VIDA), using 1 mL for DBDM broth and 25 mL for DBDM agar. Plates were incubated at 28°C for 20 days, and results were evaluated as recommended by the manufacturer.

Validation of experimental conditions. To verify the eventual interference of the apparatus in the retention of microorganisms, filtration with the ASSFH without any test filter was performed. A volume of 100 mL V_B was passed through the ASSFH, and the collected wine was serially diluted and analyzed as described above. To verify whether wine constituents retained by 0.20 µm ME membranes interfere with *Brettanomyces* colony development in the filtrated wine analysis, 0 (for control), 1, or 50 mL of V_i was filtered concurrently with V_B wine, using the aforementioned procedures.

Results and Discussion

Putative interferences of the ASSFH apparatus and of the wine constituents with *Brettanomyces* cell counts were evaluated to validate the experimental conditions used. ME

Table 1 Characteristics of the filters tested (Amazon Filters).

Commercial designation	Composition[a]	Micron rating	Lot number	Filter code
03PP001	PP	1.0	W97334/1-001	PP 1.0
16PPG006	PP	0.6	W14472	PP 0.6
16FPW00V	GF	V[b]	W13729	GFV
16FPW00X	GF	X[b]	W13730	GFX
16VPW010	PES	1.0	W106653/3-001	PES 1.0
16VPW006	PES	0.65	W97334/8-001	PES 0.65-V
			W106653/2-001	PES 0.65-N
16VPW004	PES	0.45	W97334/7-001	PES 0.45-V
			W106653/1-001	PES 0.45-N

[a]PP: polypropylene; GF: borosilicate glass microfiber with epoxy resin binder/polypropylene; PES: polyethersulfone.
[b]V was equivalent to 0.8 µm, and X was equivalent to 0.5 µm (Amazon Filters, personal communication, 2016).

membranes (0.20 µm) used to analyze the filtrated wine retain many wine constituents, namely polyphenol compounds, which could interfere with *Brettanomyces* colony development, as their concentrations on the membrane are higher than in the wine (Scalbert 1991). Thus, the method needed to be validated when 1 or 50 mL of the filtrated wine were analyzed. The results obtained are presented in Table 2. A culturable yeast population of 3.4×10^6 cfu/mL was obtained for V_B, which functioned as a test suspension for these controls, fulfilling the proposed target of at least 1.6×10^6 cells/mL. For V_i, no yeast colonies were detected. Colony counts for the ASSFH apparatus control were slightly lower than those of the test suspension, and *Brettanomyces* counts in the presence of the wine constituents retained by the membrane were similar to those from the test suspension. Therefore, as method validation requires controls with cell counts of at least half the value of the test suspension, the procedures used in the present work proved to be adequate.

The filter tests were carried out on more than one day, corresponding to a V_B culturable yeast population between 1.6×10^6 and 3.4×10^6 cells/mL. This challenge concentration of 10^7 organisms per cm^2 of effective filtration area was established and in agreement with the recommendation of the FDA for manufacturing sterile drugs and biological products with aseptic processing regarding filtration efficacy evaluation (FDA 2004). This would represent a worst-case challenge to the filter and should result in no passing of the challenge microorganism. Even though this recommendation is intended for the pharmaceutical industry in which controls are much more restricted because of health considerations or even life-or-death consequences, its application to wine-filtration procedures would guarantee a margin of safety toward the spoilage yeast *B. bruxellensis*.

As *Brettanomyces* culturability is affected by free SO_2, its neutralization was performed in the wine to enable the use of plate count techniques to evaluate the number of cells passing through the tested filters. Although other methods such as real time (RT)-PCR can quantify even nonculturable cells, plate counts with membrane filtration enable the analysis of larger sample volumes and detection of lower cell numbers (Portugal and Ruiz-Larrea 2013). Furthermore, Umiker et al. (2013) reported that the exposure to molecular SO_2, although influencing culturability and cell morphology, does not affect *Brettanomyces* cell removal by nylon membrane filtration.

Filters of different compositions and micron ratings were evaluated in duplicate and, if available, from different production lots. The results obtained are presented in Table 3 and correspond to the analysis by plate counting of the wines obtained after filtration through the filters in the test. In the present work, 47 mm diam filter disks (Table 1) were evaluated, corresponding to pleated filters in cartridges commercialized for the wine industry. It must be emphasized that the other components of the cartridge increase filter resistance and retention capacity, so an even better performance is expected than with the disks alone.

The PP filters, which are depth filters indicated as membrane prefilters and as final polishing filters for liquid processing applications, had low retention of *B. bruxellensis* cells. For the PP 1.0 filter, it was not possible to count the colonies in any of the filtrated volume analyzed because the colony numbers largely exceeded 400 cfu. Higher retention of *Brettanomyces* cells was observed for the PP 0.6 filters. We observed that one of the duplicates gave a higher retention, yielding an estimated value of 4.9×10^2 cfu/mL (with a logarithmic reduction level or LRV, of 3.5), while the other duplicate gave a cfu value that was roughly estimated to be double (LRV of 3.2) of that value (results not shown). Therefore, PP filters showed poor efficacy, as a high number of cells could pass through these filters. These results were reinforced by plate count on DBDM and growth in DBDM broth, as shown in Table 4. Although 0.6 and even 1.0 µm are values

Table 2 Validation of experimental conditions.

Experimental controls	V_B volume[a] (mL)	cfu	N[b]
Test suspension	10^{-4}	337, 335	3.4×10^6
	10^{-5}	29, 44	
ASSFH[c] apparatus	10^{-4}	249, 344	3.0×10^6
	10^{-5}	40	
Wine constituents			
0 mL V_i	10^{-4}	354, 336	3.4×10^6
	10^{-5}	40	
1 mL V_i	10^{-4}	302, 357	3.3×10^6
	10^{-5}	37	
50 mL V_i	10^{-4}	335, 325	3.3×10^6
	10^{-5}	36	

[a] V_B analysis using described procedures. V_B, 24 hrs inoculated wine.
[b]N, number of cfu/mL.
[c]Analytical stainless-steel filter holder.

Table 3 Results obtained by plate counting of the filtrated wine for duplicate tests performed with each filter.

Filter code[a]	Yeast population
PP 1.0	$>4.0 \times 10^2$ cfu/mL
	$>4.0 \times 10^2$ cfu/mL
PP 0.6	$>4.0 \times 10^2$ cfu/mL
	$>4.0 \times 10^2$ cfu/mL
GF V	2.7×10^1 cfu/mL
	$>4.0 \times 10^2$ cfu/mL
GF X	<1.0 cfu/50 mL
	<1.0 cfu/50 mL
PES 1.0	<1.0 cfu/50 mL
	<1.0 cfu/50 mL
PES 0.65-V	<1.0 cfu/50 mL
	<1.0 cfu/50 mL
PES 0.65-N	<1.0 cfu/50 mL
	<1.0 cfu/50 mL
PES 0.45-V	<1.0 cfu/50 mL
	<1.0 cfu/50 mL
PES 0.45-N	<1.0 cfu/50 mL
	<1.0 cfu/50 mL

[a]PP: polypropylene with 0.6 and 1.0 µm micron ratings; GF: borosilicate glass microfiber with X and V grades; PES: polyethersulfone membrane with 0.45, 0.65, and 1.0 µm micron ratings.

of micron ratings often considered adequate for *Brettanomyces* removal from wine, as observed by Umiker et al. (2013) for nylon membranes and Renouf et al. (2007) for cellulose filters, filter performance is complex and depends not only on the micron rating but also on the composition.

Regarding the GF filters, we observed that GF V filter disks presented differences in the duplicate results of the retention tests performed (Table 3). In fact, one duplicate presented an LRV of 5.1, while the LRV for the other could not be quantified because too many colonies had formed. For GF X filtration disks, no culturable yeast cells were detected in any of the volumes analyzed. DBDM results confirmed these observations (Table 4). These depth filters are recommended to be used prior to membrane filtration, with GF V for membranes ranging from 0.6 to 1.0 µm and GF X for membranes ranging from 0.2 to 0.45 µm, while also promoting bioburden reduction. Of note, we observed that the GF X filters had good efficacy in *Brettanomyces* removal with a very high retention capacity (LRV higher than 8.5).

The results obtained with the filters PES 1.0, 0.65, and 0.45 indicated no detection of culturable *Brettanomyces* cells in the filtrated volumes analyzed, regardless of the batch and duplicates (LRV higher than 8.2). DBDM incubations confirmed the absence of yeast growth. PES 1.0, despite having a similar or even higher micron rating, was more effective at removing *Brettanomyces* from wine than PP 1.0, PP 0.6, and GF V. PES filters yielded a very effective retention level, which underlines the importance of the mechanisms involved in microorganism removal by membranes, such as direct interception, inertial impaction, and charge effects (Starbard 2008). Nevertheless, the flow speed was slower than with the other filters, probably because of PES membrane adsorption of polyphenols and polysaccharides, as reported by Ulbricht et al. (2009). In practice, these membranes are used after prefilters, usually depth filters with higher dirt-holding capacity, leaving PES membranes to ensure microbiological stabilization. These results emphasize the importance of the filter media, including filter composition and micron rating, on *Brettanomyces* removal efficacy.

PES and X grade GF filters were considered quite promising for *Brettanomyces* removal from wine. Umiker et al. (2013) found that the efficacy of nylon membrane filters was strain-dependent, with the required pore size varying from 0.8 to 1.2 µm, depending on the strain. Therefore, to extend the knowledge about these filters' efficacy for *Brettanomyces* removal from wine, additional tests are planned with different yeast strains and types of wine.

Table 4 Results of filtered wine analysis by plate count on *Dekkera/Brettanomyces* differential medium (DBDM) agar and in DBDM broth.

Filter code[a]	*Brettanomyces* population (cfu/25 mL)	DBDM broth alteration
PP 1.0	>4.0 × 10^2	+
PP 0.6	>4.0 × 10^2	ND[b]
GF V	ND	+
GF X	<1.0	–
PES 1.0	<1.0	–
PES 0.65-N	<1.0	–
PES 0.45-N	<1.0	–

[a]PP: polypropylene with 0.6 and 1.0 µm micron ratings; GF: borosilicate glass microfiber with X and V grades; PES: polyethersulfone membrane with 0.45, 0.65, and 1.0 µm micron ratings.
[b]ND: not determined.

Conclusion

The PES filters showed high *B. bruxellensis* removal efficacy for all micron ratings tested. Similar efficacy was achieved for the X grade GF filter. In contrast, low retention was obtained with PP, even with 0.6 micron rating, and with V-grade GF filters. A major observation from this work is that different filter compositions with similar micron ratings gave different retention of *B. bruxellensis,* highlighting the relevance of the filter media in the removal mechanisms. Thus, this work points toward the importance of a complete characterization of the filtration media, and not just the pore size, by enologists prior to wine filtration, and by researchers in scientific studies concerning *Brettanomyces* removal by filtration.

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