Application of Heated Water to Reduce Populations of *Brettanomyces bruxellensis* Present in Oak Barrel Staves

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**INTRODUCTION**

Contaminated oak barrels are well-known to contribute to the spread of the yeast *Brettanomyces bruxellensis* within wineries (Chatonnet et al., 1992; Fugelsang & Edwards 2007). To minimise the potential for spoilage, various sanitation methods for barrels have been evaluated, including microwaves (González-Arenzana et al., 2013), ozone (Guzzon et al., 2011; 2013) and high power ultrasonic waves (Yap et al., 2008; Schmid et al., 2011). However, culturable yeasts have been recovered from 8 mm deep within staves (Malfeito-Ferreira et al., 2004; Barata et al., 2013; Cartwright et al., 2018), a depth beyond penetration by these methods.

As such, alternative approaches to these methods, such as using heat (e.g. steam or hot water) have been more widely adopted by the wine industry. In fact, *B. bruxellensis* can be inactivated even at mildly elevated temperatures, beginning at approximately 50°C (Couto et al., 2005; Fabrizio et al., 2015). More recently, Cartwright et al. (2018) reported that steaming staves for 9 to 12 minutes eliminated yeast populations, depending on oak species and toasting level. Even though evidence does not currently exist, application of commercial steaming units could result in uneven heating or “cold spots” within barrels, thereby not completely inactivating viable cells.

Compared to steam, filling and/or submersion with heated water may, in fact, more reliably reduce microbial populations by attaining uniform temperatures throughout the barrel. Of the few studies available, Fabrizio et al. (2015) noted that immersing barrels for extended periods of time in heated water significantly reduced resident *B. bruxellensis* populations. However, this study analysed rinse water from barrels to evaluate efficacy of heated water treatments. Even though *B. bruxellensis* can deeply penetrate staves, there are areas where cells will not be removed through rinsing. In fact, the yeast can survive application of steam to the surface of wood if present at depths of 5 to 9 mm (Cartwright et al., 2018).

The objective of this research was to determine the impact of heated water on the recovery of *B. bruxellensis* from oak staves.

**MATERIAL AND METHODS**

**Cultures and barrels**

Strains of *B. bruxellensis* I1a and E1 (Jensen et al., 2009), media used for starter culture preparation and recovery (WRM and EBB), as well as sources of barrels were described by Cartwright et al. (2018). Briefly, a commercially prepared Cabernet Sauvignon wine (pH 3.39, 6.09 g/L titratable acidity as tartaric acid, 13.5% v/v alcohol, and 18 mg/L free SO$_2$) was added to 16 L new American or French oak barrels with light or heavy toast levels prior to inoculation, with *B. bruxellensis* strain 11a or E1, with an incubation period of several weeks. Additionally, four 225 L three-year-old commercial barrels infected with an unidentified *B. bruxellensis* strain(s) were obtained from a regional winery.
Heated water or wine treatments

Center portions of side staves from disassembled barrels were sawn into 3 x 3 cm cubes. A stainless steel plate (0.3 cm x 28 cm x 43 cm), fitted with bottomless circular chambers (4 cm diameter x 2 cm depth), was manufactured locally and used to hold 24 individual cubes immersed approximately 2 mm into heated water. The loaded plate was placed into 4 L of circulating distilled water held at 50°C, 60°C, 70°C, or 80°C. During heat application, a steel cover was overlaid to prevent movement of the stave cubes. Selected cubes were fitted with thermocouples (Omega Engineering, Stamford, CT) installed into 1.0 mm diameter holes and were centrally located at varying depths 1.5 cm into each block. Each experiment was conducted twice, with three cubes removed at each designated time and the water changed each time. After heating, cubes were sawn into 4 mm thick horizontal cross-sections and individually placed into sterile 100 mL sampling bags (Nasco, Salida, CA). Sterilized wine recovery medium (WRM, 40 mL) was added to all bags, which were then incubated at 27°C for at least 60 days.

Decimal reduction times at a given temperature to kill 1 log of yeast (DT) were estimated by heating 3 x 3 cubes obtained from heavy toasted 16 L barrels (French oak) in a water bath. The heated cubes were immediately placed in sterile bags and allowed to cool to room temperature for at least 24 hours. Wood shavings were then prepared using a sterilized 2.5 cm Forstner drill bit, which bored 2 mm holes in increments. The shavings were collected and transferred into 25 mL EBB medium, to incubation at 20°C with shaking (100 rpm) for 12 hours to remove cells from oak before determination of yeast culturability.

All equipment used (table and band-saws, Forstner drill bits, heating plate, etc.) were regularly sanitized using 70% v/v ethanol.

Analyses

Culturability was determined using both manual and spiral plate methods (Autoplate 4000 spiral plater, Spiral Biotech, Bethesda, MD), and Wallerstein Differential medium (Difco, Detroit, MI). After incubation at 27°C for six days, colonies were counted and tentatively identified based on physical appearance as well as cellular morphologies viewed with phase-contrast microscopy. Identification of randomly selected colonies (three per barrel) was later confirmed using genetic ITS sequencing (MIDI Labs, Newark, DE).

Chemical analyses (pH, titratable acidity and SO₂) were performed by standardized methods (Edwards & Watson, 2013); 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) by gas chromatography–mass spectroscopy (Jensen et al., 2009); ethanol using an ebulliometer (Alla, Chemillé, France).

Analysis of variance (ANOVA) and Tukey’s HSD for mean separation was determined at p≤0.05 using XLSTAT software (Addinsoft, New York, NY).

RESULTS AND DISCUSSION

Heating staves

Increases in temperature of the 3 x 3 cubes at different layers during water heating depended primarily on stave depth, not on oak species or the toasting level. In fact, since American and French oak from 16 L barrels heated almost identically (p≤0.05), data were pooled to further assess heating profiles within staves (Fig. 1). Typically, innermost surfaces directly in contact with water reached the treatment temperature within ≤5 minutes, while furthermost surfaces (≥20 mm) remained below 30°C, similar to the observations regarding 225 L barrels (data not shown). At 9.5 mm, a stave depth slightly beyond where B. bruxellensis has been recovered (Malfeito-Ferreira et al., 2004; Barata et al., 2013; Cartwright et al., 2018), heated treatments of 50°C (Fig. 1A) or 60°C (Fig. 1B)
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resulted in maximum temperatures of 35° or 47°C after 45 minutes, respectively. However, shorter times of 25 minutes or 15 minutes were required at 70° (Fig. 1C) or 80°C (Fig. 1D), respectively, in order to reach ≥50°C. Cubes never attained ≥60°C at 9.5 mm depths within 45 minutes. Temperatures higher than those have been reported by Cartwright et al. (2018) for the same oak staves, but were heated by steam.

**Yeast recoveries from heated staves**

*B. bruxellensis* strain I1a was found in the 5 mm to 9 mm layer of heavy toasted 16 L barrels made from French oak, but not in the same layer within the light or heavy toasted American oak (Fig. 2 to 5). These analyses were in agreement with observations of 16 L barrels as reported by Cartwright et al. (2018), where the two types of oak had different permeabilities to *B. bruxellensis*, yet similar thermal conductivities.

In general, as the temperature of heated water increased, shorter treatment periods were required to eliminate culturable cells. Application of 50°C (Fig. 2) resulted in elimination of the yeast after 60 minutes from the 0 mm to 4 mm layer of American oak, but required 120 minutes for the French oak due to the presence of *B. bruxellensis* in the 5 mm to 9 mm layer. After 30 minutes of heating American oak, 15 days of incubation in WRM was required to detect *Brettanomyces* populations. An incubation period of 15 days implied that the populations were low but still culturable after water heating. In comparison, 30 days of incubation was needed to observe viable cells from staves treated for 60 minutes for French oak. For French oak treated with water at 60°C (Fig. 3), 70°C (Fig. 4) or 80°C (Fig. 5), the times needed to eradicate the yeast decreased from 45, 30, to 20 minutes, respectively. At these same temperatures, yeast recovery was not observed from the American oak after 30, 20, or 15 minutes, respectively. While these data were based on heavy toasted staves, similar trends were observed for the light toasted French and American oak (data not shown).

**D**ₜ values for *B. bruxellensis* strains I1a (Fig. 6A) or E1 (Fig. 6B) were determined using cubes prepared from 16 L heavy toasted French oak staves. From initial populations of approximately 6 x 10³ cfu/g, culturabilities were monitored over time within the 0 mm to 2 mm layer immersed in the heated water. Based on slopes of best fit lines (r² ≥0.88), Dₜ₅₀°C, Dₜ₆₀°C, Dₜ₇₀°C, and Dₜ₈₀°C were estimated to be 223 sec, 50 sec, 20 sec, and 11 sec for strain I1a (Fig. 6A), values similar to that of strain E1 (Fig. 6B).

To date, the limited studies available regarding thermal inactivation of *B. bruxellensis* relied upon liquid cultures, not on oak staves as described in the current research. Couto et al. (2005) noted Dₜ₅₀°C to be 204 to 228 sec for strain PYCC 4801, in agreement with strains I1a and E1 (Fig. 6). Using liquid cultures, Couto et al. (2005) observed Dₜ₅₀°C of a single strain of *B. bruxellensis* to vary between 18 to 24 sec whereas Fabrizio et al. (2015) found Dₜ₅₀°C values to range from 19.5 to 20.7 sec. Furthermore, Fabrizio et al. (2015) noted that three strains behaved similarly, while the fourth exhibited a slightly greater heat resistance. In general, the Dₜ values reported by Couto et al. (2005) and Fabrizio et al. (2015) at temperatures >50°C were lower than those obtained with staves, possibly due to matrix effects, as oak exhibits a lower thermal conductivity (Lagüela et al., 2015) compared to

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**FIGURE 2**

Culturabilities of *B. bruxellensis* strain I1a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 50°C water treatments. Data represent means ± standard error (n=6).
Figure 3: Culturabilities of *B. bruxellensis* strain IIa recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 60°C water treatments. Data represent means ± standard error (n=6).

Figure 4: Culturabilities of *B. bruxellensis* strain IIa recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 70°C water treatments. Data represent means ± standard error (n=6).
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Heating liquid cultures. In addition, heat resistance may vary between different strains of B. bruxellensis as suggested by Fabrizio et al. (2015).

Given differences in dimensions (i.e., stave thickness) and age compared to the 16 L barrels, cubes were sawn from 225 L barrel staves and exposed to heated water using previously described protocols (Fig. 7 to 10). Unlike the 16 L barrels, culturable cells were recovered from 5 mm to 9 mm layers from American oak barrels, most likely because these had been commercially used for three years, allowing slow but deeper penetration of the yeast (Cartwright et al., 2018). Like the 16 L barrels, however, yeast recovery was largely affected by water temperature and time of heat exposure. For example, culturable cells were recovered from the 5 mm to 9 mm layer, but not the 0 to 4 mm for both American and French oaks after 60 minutes of heating at 50°C (Fig. 7). Elimination of B. bruxellensis from these staves was noted after 120 minutes of heating. As the temperature of water was increased, the heating time, which resulted in no recoverable cells from the 5 mm to 9 mm stave layer, also decreased from 45 minutes (60°C; Fig. 8) to 30 minutes (70°C; Fig. 9), and then to 20 minutes (80°C; Fig. 10). Overall, inactivation of the unidentified strain(s) that was present in the 225 L barrels followed the same trends as those observed in the 16 L barrels infected with another strain of B. bruxellensis.

Even though some studies concluded that heat treatments did not adequately remove B. bruxellensis from staves, others disagreed. For instance, Barata et al. (2013) concluded that steam treatments of 10 minutes for French

FIGURE 5
Culturabilities of B. bruxellensis strain I1a recovered from heavy toasted American (A) or French (B) 16 L oak barrels following 80°C water treatments. Data represent means ± standard error (n=6).

FIGURE 6
D-values for B. bruxellensis strain I1a (A) or E1 (B) recovered from the first 2 mm depths of heavy toasted 16 L French oak barrels following heated water treatments. Data represent means ± standard error (n=3).
FIGURE 7
Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatment with 50°C water. Data represent means ± standard error (n=6).

FIGURE 8
Culturabilities of an unidentified strain(s) of *B. bruxellensis* recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatment with 60°C water. Data represent means ± standard error (n=6).
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FIGURE 9
Culturabilities of an unidentified strain(s) of \textit{B. bruxellensis} recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatments with 70°C water. Data represent means ± standard error (n=6).

FIGURE 10
Culturabilities of an unidentified strain(s) of \textit{B. bruxellensis} recovered from medium-heavy toasted American (A) or medium-light toasted French (B) 225 L oak barrels following treatments with 80°C water. Data represent means ± standard error (n=6).
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populations present in... Even though Fabrizio... in wine barrels by high power ultrasound. Wine... populations from oak barrel staves using steam. -/ (2015) reported... and (2011) concluded that this approach... in (2018) noted that... for strains I1a and E1, where populations exceeded 10... continued to produce 4-EP and 4-EG... B. bruxellensis... of wine ageing barriques after different sanitation treatments. J. Food Res. 2, 140-149.

Volatile phenol production

Upon incubation of stave layers or shavings in WRM, recovered B. bruxellensis continued to produce 4-EP and 4-EG (data not shown). Based on analysis of staves from 16 L barrels, concentrations surpassed 2000 µg/L 4-EP and 300 µg/L 4-EG for strains I1a and E1, where populations exceeded 10⁶ cfu/mL after 60 days. The concentrations produced by these strains were similar to those reported by Oswald & Edwards (2017) using a different wine. Even though similar populations were reached, the unidentified strain(s) originating from 225 L barrels produced lower concentrations of volatile phenols; ≤1300 4-EP and 180 µg/L 4-EG. Neither 4-EP or 4-EG were detected in WRM, where populations remained below the limit of detection; <30 cfu/mL.

CONCLUSIONS

This study evaluated the efficacy of heated water and wine treatments towards the removal of B. bruxellensis from a wide range of infected barrels. Water heated to 70°C or 80°C eradicated the yeast if present at depths of 0 mm to 4 mm (20 or 15 minutes, respectively) or 5 mm to 9 mm (30 or 20 minutes, respectively), evidenced by a lack of culturable cells – even after incubation of stave cross-sections in a wine recovery medium for 60 days. Future research should focus on application of heated water on entire barrels, including any potential changes to the sensory impacts of treated barrels.

LITERATURE CITED


