Inactivation of *Brettanomyces/ Dekkera* in wine barrels by high power ultrasound

Photographs used in this article have been taken at Tintara Winery, South Australia, courtesy of Shutterbug Studios.

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INTRODUCTION

While grape quality is recognised as the key component in the production of high quality wine, oak barrels are a critical ingredient in the production of red and certain white table wine styles. In modern winemaking, barrel stocks form a significant component of a winery's capital expenditure, representing the second greatest cost element in wine production (Anon. 2005).

Barrels require considerable care and attention, are difficult to clean and impossible to sterilise if they become contaminated with spoilage micro-organisms (Boulton *et al.* 1996). They are sources of *Brettanomyces/Dekkera* and other spoilage yeasts, lactic acid bacteria and acetic acid bacteria that can impart undesirable aroma and flavour compounds, such as 4-ethylphenol, 4-ethylguaiacol, 4-ethylcatechol, acetaldehyde, acetic acid, ethyl acetate, 2,4,6 trichloroanisole, tribromoanisole and 2-acetyltetrahydropyridine (Baldwin 1996; Fugelsang 1997; Hickin 1999; Leske 1999; Grbin and Henschke 2000; Fernandez *et al.* 2007; Anon. 2008), all of which can seriously compromise wine quality and lead to product loss.

In contrast to technological advances in the production of better quality wine, methods and technologies for barrel sanitation have changed little over the decades. Methods that are currently employed by the wine industry to remove tartrates and solid residues and to disinfect barrels have been reviewed by Yap *et al.* (2007a). Hot water and chemical treatments are ineffective as evidenced by the rampant spread of the spoilage yeast *Brettanomyces/Dekkera* in all wine-producing countries (Contero *et al.* 2006; Curtin *et al.* 2007). Pollnitz *et al.* (2000) found that wine stored in *Brettanomyces/Dekkera*-infected

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barrels that had been shaved and re-fired nonetheless contained 4-ethylphenol and 4-ethylguaiacol, confirming the presence of viable *Brettanomyces/Dekkera* cells in the wood. Given that *Brettanomyces/Dekkera* yeast can penetrate the wood to the same degree as the wine itself (Malfeito-Ferreira *et al.* 2004), there seems to be no treatment capable of effectively decontaminating infected barrels, as least not without markedly impacting on barrel properties or serviceable lifespan. At present, published research suggests that infected barrels cannot be effectively sterilised by hot water, steam, ozone, sulfited water, sulfur dioxide gas, and shaving and firing (Pollnitz *et al.* 2000; Arvik and Henick-Kling 2002; Malfeito-Ferreira *et al.* 2004). In effect, the presence of *Brettanomyces/Dekkera* within barrels can only be monitored, but not controlled (Arvik and Henick-Kling 2002).

Previous laboratory studies undertaken by the authors (Yap *et al.* 2007a) have shown that ultrasound inactivates viable *Brettanomyces/Dekkera* yeast cells. In a trial using a hand-held ultrasonic unit, 88.8% and 99.9% inactivation of cells was achieved using power outputs of 10 and 50W, respectively. At 50W power, 97-99.9% of viable cells were killed at 90 and 120 seconds, respectively. In a subsequent trial, a 1kW high power ultrasonic unit proved highly effective in inactivating *Brettanomyces/Dekkera bruxellensis* cells, where after five and seven minutes, treatments at 40°C, 99.58% and 99.97% of viable cells were inactivated, respectively.

This paper presents data on the efficacy of high power ultrasound (HPU) as a method for inactivating viable *Brettanomyces/Dekkera* yeast cells in barrels compared with high pressure hot water (HPHW) and mains pressure hot water (MPHW). This paper will also discuss the advantages HPU has over current technology and the benefits it will bring to the wine industry.

COMMERCIAL APPLICATIONS OF HPU TECHNOLOGY

Uses of HPU in industrial processes, process parameters and the principal mechanism of high power ultrasound have been published (Clark 2008; Patist and Bates 2008; Mawson and Knoerzer 2007). Significant improvements in product quality, process enhancement and cost reduction are achievable on a commercial scale, with good returns on capital investment. The range of high power ultrasonic applications in the food industry include extraction, emulsification, homogenisation, crystallisation, filtration, screening, separation, viscosity alteration, de-foaming or foam reduction, enzyme and microbial inactivation, fermentation and heat transfer. The potential applications of HPU technology in the wine industry have been summarised by Yap *et al.* (2007b) and Jiranek *et al.* (2008). They include the management of micro-organisms at various stages of wine production, colour and flavour extraction, cold stabilisation, protein stabilisation, enhancement of yeast cell autolysis, de-foaming, degassing, plank cleaning and barrel and tank sanitisation. Yap *et al.* (2007a) have demonstrated that HPU is effective in removing tartrates and solid residues from oak barrels of various ages and planks. In comparative trials, HPHW cleaning (1000psi at 60°C) removed 50-90% of the tartrate deposit from surfaces of one-year-old barrels in 12 minutes, whereas HPU removed >99% of the tartrates after five minutes (Yap 2008). Studies on the effects of HPU by various authors on the growth and viability of pathogenic and food spoilage bacteria, *Saccharomyces cerevisiae*, fungi, algae and protozoa have been summarised by Jiranek *et al.* (2008).

METHODS FOR STUDYING THE INACTIVATION OF *BRETTANOMYCES/DEKKERA* CELLS IN BARRELS

The purpose of these studies were to evaluate the efficacy of HPU, HPHW and MPHW in inactivating viable *Brettanomyces/Dekkera* cells in barrels. As the distribution of *Brettanomyces/Dekkera* in naturally-infected barrels was thought to be too inconsistent to give reliable and reproducible results, their use was not considered. Instead, methods for the deliberate infection of oak wood with *Brettanomyces/Dekkera* and their subsequent recovery from the surface and subsurface, following treatment with HPU, HPHW and MPHW were developed for these studies. Using laboratory-infected oak blocks attached to the staves of barrels allowed testing to be performed under controlled conditions and enabled comparison of the treatments against controls. Blocks were cut from new American oak staves, as well as uninfected and tartrate-free staves of used one- and three-year old American oak barrels previously cleaned by high pressure hot water. The sterilised blocks were infected by suspending them in an actively growing liquid culture of *Dekkera bruxellensis* strain AWRI 1499.

A commercial standard static spray head was used to deliver HPHW (1000psi/60°C) or MPHW (70psi/60°C) through the bung-hole of the barrel. A water temperature of 60°C was chosen as the benchmark as it was the most commonly used temperature in the wine industry. The Cavitus Alpha prototype 4kW high power ultrasonic unit was used to introduce ultrasound into a 225L barrel filled with 60°C reverse osmosis water via the bung-hole or through an open head.

'Sliced block' method

A method was developed to enable studies to be carried out on the efficacy of HPU, HPHW and MPHW to inactivate *Brettanomyces/Dekkera* cells present on the surface of a stave, as well as at a depth of 2mm. Whole new American oak staves (27mm thick, medium + toast) were cut into blocks approximately 60mm in length, and a 4mm hole drilled in their centre to allow fixing of the 'sliced blocks' to the barrel during HPHW and MPHW treatment. Each block was then sawn in the same plane as the toasted surface to yield two pieces of wood – a 2mm thick slice containing the toasted surface and a 25mm thick slice. Each 2mm slice and its corresponding 25mm slice were labelled near the drilled holes using a marker pen, wrapped together tightly in aluminium foil and then sterilised by autoclaving. A second autoclaving occurred after the slices had been left overnight to allow germination of any spores surviving the initial autoclaving.

The sterile 2mm slices were then threaded in groups of 12 onto surface-sterilised (70% v/v ethanol-dipped) lengths of nylon fishing line and immersed into the vigorously growing *Brettanomyces/Dekkera bruxellensis* broth culture for 12 days.

Sterilised stainless steel washers were fixed to each group of 2mm slices to ensure that they remained evenly submerged in the culture. Following removal from the infection culture, the 2mm slices were gently jiggled in 2 x 10L vessels of sterile saline to remove 'unbound' cells. The 2mm slices were then re-assembled with their pre-sterilised corresponding 25mm slices using a single sterile staple along the wood grain on one side. A sterilised 30mm-wide rubber band was wrapped around each assembled unit to prevent penetration of the HPU and hot water from the cut sides of the block during treatment. Finally, a piece of surface sterilised parafilm was wrapped around the sides of the assembled sliced blocks to hold everything in place. Each assembled sliced block was stored in sterile 500mL bags until required.

Treatment of infected sliced blocks with HPU and HPHW

For HPU treatment each assembled sliced block was aseptically transferred onto a surface-sterilised steel bracket with the 2mm slice facing outwards and then submersed to the depth of the bilge in a water-filled barrel (Figure 1). For HPHW treatment the assembled sliced blocks were aseptically affixed to the bilge region of the barrel with sterilised stainless steel screws after removing a headstave. After replacing the headstave, HPHW was applied with a standard commercial static spray head. Following treatment, all assembled sliced blocks were aseptically transferred to separate sterile 500mL bags.

The sliced blocks were treated at 60°C with HPU for five, eight or 12 minutes or with HPHW for three, five or eight minutes. Following treatment, the 2mm slice was separated from its corresponding 25mm slice, and the front (top surface) and back (representing a subsurface depth of 2mm) swabbed (Quick Swabs, 3M™). Swab areas (area 3.46cm^2) were defined by the random placement of two sterilised stainless steel washers (21mm ID) on the surface of the slice. Dilutions of each swab in sterile saline were plated onto Wallerstein's Laboratory Nutrient Agar, supplemented with 2mg/L cycloheximide. All swab plates were incubated at 25°C for 12 days prior to counting.

Initial cell numbers on the surfaces of the 2mm slices yielded an average of 7000 ± 4000 colony-forming units (cfu) per mL per cm^2 oak wood surface. The standard deviations were consistent with all the sliced blocks examined – we believe this could be attributed to the porous, variable nature of the wood which hide or expose the viable cells differently in each block tested.

This study found that 100% of the cells on the surface and at 2mm were inactivated following HPU and HPHW treatments at all time points.

Treatment of infected sliced blocks with HPHW and MPHW

This study was carried out to determine if HPHW and MPHW would have the same effect on *Brettanomyces/Dekkera* cells present in different parts of the barrel. The sliced blocks were aseptically affixed to the inside of the barrel with sterilised stainless steel screws in four positions (A, B, C and D) as shown in Figure 1. Sliced block A was affixed to the headstave and D to a stave directly opposite the bung-hole. After replacing the headstave, HPHW or MPHW was applied with a standard commercial static spray head.

The sliced blocks were treated for three, five and eight minutes with HPHW and MPHW. Following treatment, only the surface (top) of the 2mm slice was swabbed using 3M Quick Swabs. Initial cell numbers on the surfaces of the 2mm slices yielded an average of 2700 ± 400 colony forming units (cfu) per mL per cm^2.

The reduction of viable cells on the surface of infected new oak staves compared with the control sample is shown in Figure 2. Greatest reduction in cell numbers was achieved in positions A and D, although after three minutes' treatment with MPHW and HPHW, the percent inactivation was only 11.5% and 48.8%, respectively. With longer treatment times, fewer viable *Brettanomyces/Dekkera* cells were detected in positions A and D. In the earlier study (see above) 100% of *Brettanomyces/Dekkera* cells on the surface and at 2mm of sliced blocks in position D were inactivated by HPHW treatments. However, in this study, only 99.8% were killed after eight minutes. HPHW and MPHW treatment of sliced blocks located in positions B and C (see Figure 1) in the arc between Block D opposite the bung-hole and Block A on the barrel head showed extremely variable results. Percent inactivation in position B ranged from 82-100% and in position C, 0-99%. This study showed that the ability of HPHW and MPHW to kill viable *Brettanomyces/Dekkera* cells in a barrel is highly dependant on their location. Viable

Figure 1. *Brettanomyces/Dekkera* infected sliced blocks clipped onto surface-sterilised metal holders (A), which in turn were clipped onto the side of the barrel (B) for treatment with HPU applied through the open head of a 225L barrel of water. Positions of slice blocks in the barrel (C) for HPHW and MPHW treatment (Block D is affixed to a stave directly opposite the bung-hole).

cells present on the barrel head and bilge region appear most vulnerable whereas those present in other regions of the barrel have greater chances of survival.

Treatment of infected one- and three-year-old staves with HPU and HPHW (1000psi/60°C)

Stave pieces (10 x 5cm) were cut from tartrate-free one- and three-year-old staves (American oak, medium toast), sterilised by autoclaving and then immersed in YPD medium (300mL) containing 0.01% (w/v) cycloheximide. *Dekkera bruxellensis* (5 x 10^7 cells/mL) was directly inoculated into this medium and incubated at 30°C for five days. The stave pieces were then removed from the medium and immediately used for the respective trials. After treatment, the samples were refrigerated overnight (4°C) and processed the following day.

Triplicate core samples were taken from each treated and control stave, and 2mm slices to a depth of 4mm were removed. The slices were milled in 50mL of 0.9% saline (IKA A11 grinder, Crown Scientific) using a method previously shown not to affect cell viability (data not shown). The suspensions were centrifuged, the supernatant removed and the pellet re-suspended in 0.9% saline (1mL). Aliquots of 10µL were plated onto YPD agar and incubated to determine cell counts.

In this study, the number of viable *D. bruxellensis* cells present on the surface (2mm slice) and sub-surface (4mm slice) of infected staves after five, eight, 12 minutes' exposure to HPU in a barrique containing water at 60°C was determined and compared with the effect of HPHW treatment for three, five and eight minutes on one-year-old infected staves. The infected

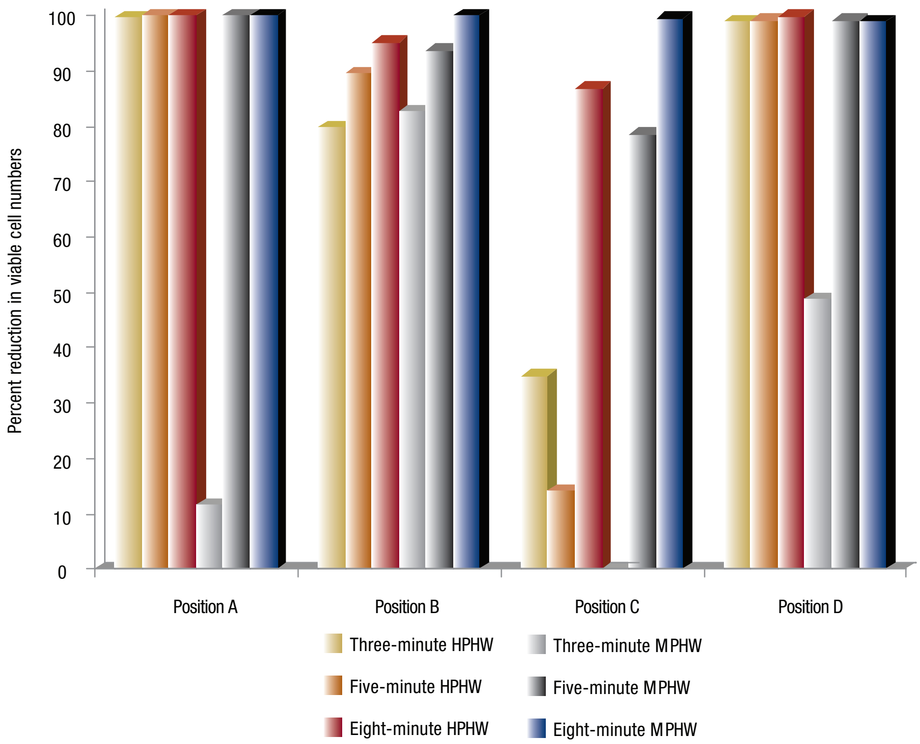

Figure 2. Reduction of viable *Dekkera bruxellensis* cells (AWRI 1499) on the surface of infected new oak staves, compared with the untreated control sample, using HPHW (1000psi/60°C) and MPHW (70psi/60°C).

stave pieces for HPU treatment were attached to the barrel staves in the region of the bilge. Cell counts were expressed as colony forming units per the volume of the 2mm core sample slice (approximately 142mm^3).

The reduction of viable *Dekkera bruxellensis* cells (AWRI strain 1499) in the surface slice (0-2mm) and sub-surface slice (2-4mm) of infected one- and three-year-old oak staves, compared with the control sample, using HPU and HPHW are summarised in Figures 3 and 4. Initial cell populations in the surface slice for treatment by HPU were 5974 and 4512cfu/mm^3 for the one- and three-year old staves, respectively. No viable cells were detected at any time at 60°C, suggesting that HPU treatment was effective in deactivating all viable cells in one- and three-year old infected wood. The number of cells detected at 2-4mm below the surface of the control stave for the one- and three-year old infected staves was 18.5 and 84.0cfu/mm^3, respectively. HPU at 60°C destroyed all the cells.

Surface and sub-surface slices of one-year infected staves were exposed to HPHW for three, five and eight minutes. The surface and sub-surface control staves contained 8129 and 20cfu/mm^3, respectively. Although significant reduction in cell numbers occurred in the surface slices after all treatment times, at no time was total elimination of cells achieved, unlike that seen to occur in the HPU trials at 60°C. Further, there was no consistent trend in the reduction of numbers of viable cells with increasing time of HPHW exposure. Although some reduction in cell numbers was achieved in the subsurface (2-4mm depth), total elimination was not achieved, again, unlike the case for the HPU treatments. The data does, however, suggest a decrease in the number of viable cells with increased time of exposure to hot water.

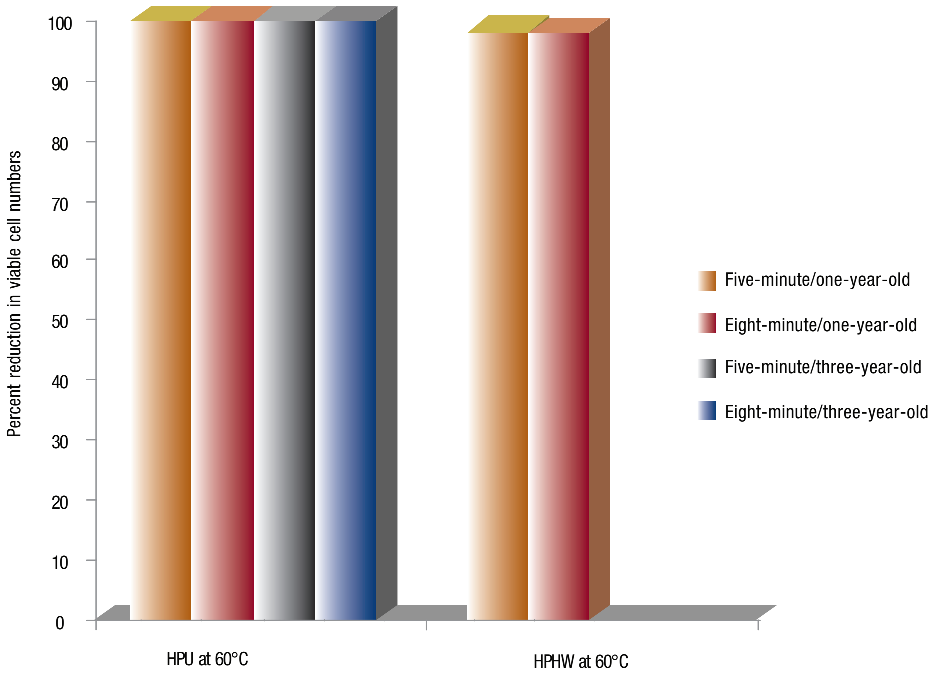

Figure 3. Reduction of viable *Dekkera bruxellensis* cells (AWRI 1499) on the surface (0-2mm) of infected one- and three-year-old oak staves by HPU at 60°C or HPHW (1000psi at 60°C). Exposure times used for each treatment were three, five and eight minutes.

DISCUSSION AND CONCLUSION

Cooperage remains an expensive consumable in the production of wine. In-barrel wine spoilage and short barrel life are problems currently facing the wine industry. The infection of the barrel with spoilage micro-organisms, especially *Brettanomyces/Dekkera* yeast, can have a marked influence on whether a barrel is reused, and hence the cost effectiveness of its purchase. To overcome these problems, it is desirable for the wine industry to employ technologies that will eliminate solid residues and inactivate *Brettanomyces/ Dekkera* and other spoilage micro-organisms from the interior of barrels.

The efficacy of HPU treatment in reducing numbers of *Dekkera bruxellensis* cells on the surface and sub-surface of

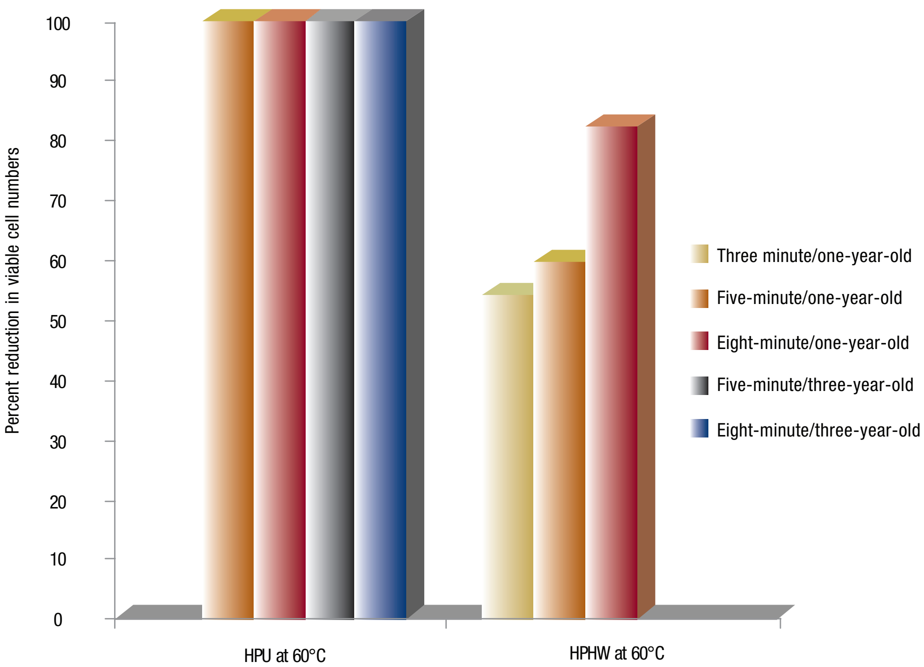

Figure 4. Reduction of viable *Dekkera bruxellensis* cells (AWRI 1499) on the subsurface (2-4mm) of infected one- and three-year-old oak staves by HPU at 60°C or HPHW (1000psi at 60°C). Exposure times used for each treatment were three, five and eight minutes.

barrel wood has been demonstrated in the present studies. Infected new, one- and three-year-old staves were used to compare barrel sanitising techniques currently applied in wineries (hot water washes at high and mains pressures). Viable cells were dramatically reduced (>1000 x reduction) on the surface of wood of all ages studied with total inactivation occurring most successfully at 60°C with five-minute HPU exposure. Although sub-surface infection numbers were much lower in the control staves, HPU exposure on these samples also showed reduction in cell numbers for all ages of wood. Again, the most appropriate combination of HPU and temperature was 60°C for five minutes, which yielded a greater than 1000-fold reduction.

These studies have also clearly established that the present and most widely adopted cleaning technique of applying high pressure or mains pressure hot water sprays to the interior of barrels does not completely inactivate *Brettanomyces/Dekkera* cells. Further, the location of viable cells within the barrel environment determines their chances of survival, with populations within the arc of the barrel between the headstave and bilge having the greatest opportunity to survive and proliferate.

In summary, these findings indicate that the application of high power ultrasound for barrel disinfection has the potential to eliminate or at least drastically reduce wine spoilage by *Brettanomyces/Dekkera* or possibly other spoilage yeasts and bacteria, and provide considerable cost savings through:

- offering an effective, consistent or uniform means (i.e. no 'hit or miss') of disinfecting barrels
- reducing sulfur dioxide consumption
- avoidance of hazardous or taint-causing chemicals
- lowering labour and energy requirements
- extending barrel life (by possibly one to two years) by effectively removing tartrates, solid residues, micro-organisms, biofilms and taint compounds from surfaces and pores
- removing anthocyanin pigments that can be broken down by *Brettanomyces/Dekkera* (glucose released through this can be used as a carbon source for growth)
- using a clean technology, resulting in no chemical wastes from the cleaning process.

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