Heat inactivation of wine spoilage yeast *Dekkera bruxellensis* by hot water treatment

V. Fabrizio¹, I. Vigentini², N. Parisi³, C. Picozzi², C. Compagno² and R. Foschino²

¹ Centro di Ricerca, Formazione e Servizi della Vite e del Vino, Riccagioia S.C.p.A., Torrazza Coste (PV), Italy
² Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Milan, Italy
³ Co.Pro.Vi. Società Cooperativa, Casteggio (PV), Italy

Significance and Impact of the Study: *Brettanomyces/Dekkera bruxellensis* is the main yeast involved in red wine spoilage that occurs during ageing in barrel, generating considerable economic losses. Current sanitization protocols, performed using different chemicals, are ineffective due to the porous nature of the wood. The thermal inactivation of *D. bruxellensis* cells by hot water treatment proves to be efficacious and easy to perform, provided that the holding time at the killing temperature takes into account the filling time of the vessel and the time for the heat penetration into the wood structure.

Keywords
*Brettanomyces*, decimal reduction time, *Dekkera*, thermal inactivation, wine, *z*-value.

Correspondence
Roberto Foschino, Department of Food, Environmental and Nutritional Sciences, Università degli Studi di Milano, Via G. Celoria, 2-20133 Milan, Italy.
E-mail: roberto.foschino@unimi.it

2015/0599: received 24 March 2015, revised 6 May 2015 and accepted 7 May 2015
doi:10.1111/lam.12444

Abstract
Cell suspensions of four *Dekkera bruxellensis* strains (CBS 2499, CBS 2797, CBS 4459 and CBS 4601) were subjected to heat treatment in deionized water at four different temperatures (55°C, 57°C, 60°C and 62.5°C) to investigate their thermal resistance. The decimal reduction times at a specific temperature were calculated from the resulting inactivation curves: the *D*-values at 55°C ranged from 63 to 79 s, at 57°C from 39 to 46 s, at 60°C from 19 to 20 s, and at 62.5°C from 13 to 17 s. The *z*-values were between 9.2 and 10.2°C, confirming that heat resistance is a strain-dependent character. A protocol for the sanitization of 225 l casks by immersion in hot water was set up and applied to contaminated 3-year-old barrels. The heat penetration through the staves was evaluated for each investigated temperature by positioning a thermal probe at 8 mm deep. A treatment at 60°C for an exposure time of 19 min allowed to eliminate the yeast populations up to a log count reduction of 8.

Introduction
The negative role of *Brettanomyces bruxellensis* (the anamorph of *Dekkera bruxellensis* species) has recently assumed increasing importance in winemaking since this spoilage yeast is considered the main hazard for red wines aged in wood (Suarez et al. 2006; Schifferdecker et al. 2014). The current incidence of the defect is not easy to be determined, partly because some oenologists are reluctant to admit the problem and partly because early contamination is difficult to detect. However, analytical data for Italian wines (Agnolucci et al. 2009; Campolongo et al. 2010) estimate that about 15% of the products is involved in the phenomenon, as the levels of volatile phenols in the investigated samples were higher than the thresholds of perception. Actually, the so-called ‘Brett’ defect in wine originates by the strain-specific capacity of *B. bruxellensis* (Vigentini et al. 2008) to produce volatile phenols through sequential enzymatic steps (Oelofse et al. 2008) in which the hydroxy-cinnamic acids, that are naturally present in the grape berries, are transformed in vinyl-phenols and ethyl-phenols. In particular, 4-ethyl-phenol and 4-ethyl-guaiacol give rise to disagreeable aromas, classified as ‘animal’ or ‘pharmaceutical’ notes, when their concentrations in wine are higher than 230 and 50 l g⁻¹ respectively (Suarez et al. 2006; Benito et al. 2009). Godoy et al. (2008) and Tchobanov et al. (2008) firstly and partially have purified a coumarate decarboxylase and a vinyl-phenol reductase; the role of these enzymes was then confirmed through the experiments of
Hixson et al. (2012). Recently, Granato et al. (2015) discovered that the vinyl phenol reductase is a moonlighting superoxide dismutase, with cofactor-binding structural features due to a specific change in the amino acid sequence.

In the wine industry, Brettanomyces contamination is hard to manage as this yeast is able to survive and grow in conditions that are prohibitive for many other microorganisms (low pH, anaerobic conditions, high ethanol concentration and very low amount of fermentable sugar). The currently applied control actions, such as the addition of sulphur dioxide and/or membrane filtration, are not effective because of the presence of resistant strains (Barata et al. 2008; Agnolucci et al. 2010; Curtin et al. 2012; Vigentini et al. 2013) or the occurrence of cross contaminations in cellar operations. Moreover, some strains of B. bruxellensis were able to form biofilms under different growth conditions (Joseph et al. 2007; Galafassi et al. 2015), which allows them to resist sanitation treatments. The origin of this species in the supply chain of wine has not yet been defined, but the prevailing opinion is that it is due to improper or insufficient hygiene practices (Boulton et al. 1996; Fugelsang and Edwards 2007). Actually, the genus Brettanomyces belongs to the minority native microflora of grapes (Renouf and Lonvaud-Funel 2007; Barata et al. 2012); when inside the cellar, yeast cells colonize the porous surfaces, especially the wood of barrels, and the equipment parts that are difficult to clean, such as pumps, pipes, grids and filters (Coulter et al. 2004; Guzzon et al. 2011). The economic loss and the damage to brand reputation associated with the ‘Brett’ spoilage lead wine industries to seek new approaches for preventing and controlling the problem and to optimize the sanitation techniques.

**Results and discussion**

**Kinetics of heat inactivation of Dekkera bruxellensis strains in water**

To investigate the resistance of yeast species at high temperatures, controlled cell suspensions of four different strains were placed in capillary tubes and subjected to heat treatment by immersion in a thermostatic water bath. The attainment of the desired temperature inside the capillary tube occurred after 15 s; thus, the holding time started after this period. The choice of testing the heat resistance of the cells in deionized water was determined by the supposition that a heating operation with hot water could be easy to implement in a cellar situation for the sanitization of the barrels. The thermal inactivation curves at different temperatures (55°C, 57°C, 60°C and 62°C) for the investigated D. bruxellensis strains are shown in Fig. 1. The reported values are the mean of two independent trials. As expected, a logarithmic decrease in the concentration of viable cells was observed with time of exposure, for all the considered temperatures. Comparable results were obtained for CBS 2499, CBS 2797 and CBS 4601 strains, whereas CBS 4459 strain proved to be less resistant than the others. The D-values, at a specific constant temperature and for each strain, were deduced from regression lines, which showed correlation coefficients ($R^2$) equal or higher than 0.87 (Table 1). Then z-values were calculated for each strain by linear

![Figure 1](https://example.com/figure1.png)
regression among the log D-values and the temperature values (Table 1). Thermal sensitivity was confirmed to be a strain-specific character. The particular heat resistance of CBS 2797 might be attributed to the formation of cell clumping observed at the microscope for the strain in these experimental conditions.

The D-values and z-values calculated in this study were higher than those reported by Couto et al. (2005) for the same yeast species. However, even if one of the tested temperature was the same (55°C) for both works, the strains, heating protocols and media used for the cell suspension were different and these factors are known to heavily affect the thermal resistance of micro-organisms (Jay et al. 2005). Despite the interest in the wine and beer industry for B./D. bruxellensis, there are no data in the literature on the thermal parameters regarding the inactivation of this species in water and our findings fill this gap.

On the basis of the results obtained for the most resistant strain (CBS 2797), we calculated the time needed to reduce the cell concentration of eight logarithmic cycles of D. bruxellensis, which could potentially contaminate the internal surface of a cask. These values for water treatment were calculated as 9.7 min at 55°C, 5.4 min at 57.5°C, 3.0 min at 60.0°C and 1.7 min at 62.5°C.

Trials on contaminated barrels

Barata et al. (2013) observed that Brettanomyces is able to penetrate the inner layers of wood staves – to 8 mm – corresponding to the maximum level of wine penetration. The profiles of heat penetration through the oak wood were preliminarily evaluated and recorded at the investigated temperatures by placing a probe at 8 mm in depth (Fig. 2). The heat propagation was very slow, since, as expected, this material has a low thermal conductivity, ranging between 0.16 and 0.46 W m⁻¹ K⁻¹ (Lagüela et al. 2015). So, given the time required to achieve the selected temperature in the inner layers where Brettanomyces/Dekkera can develop and taking into account a magnitude of 8 D-value of the most resistant strain, a protocol of thermal sanitation of the barrel by water treatment was designed. Even if a measurable inactivation began as early as 55°C, we decided to apply the treatment at 60°C for 19 min, including the time needed for the filling of the cask (approximately 6 min) and that for the heat propagation into the wood (10 min).

Three different recycled 3-year-old oak barrels (225 l barriques) supposed to be contaminated by Brettanomyces/Dekkera populations, the wines derived from them being affected by ’Brett’ spoilage, were used for the experiment. The inside surface of the cask was subjected to microbiological analysis before and after heating in hot water. After the thermic exposure for the designated time, the barrel was emptied and allowed to cool to room temperature. In Table 2, the results of plate counts before and after the heat treatment are shown. The yeast concentrations in the rinsing suspensions before the trials varied from 2.5 × 10² to 2.3 × 10⁶ CFU ml⁻¹, whereas no colony in 100 ml was detected afterwards. Therefore, the immersion of the casks in hot water has allowed to eliminate the yeast populations until an estimated log count <8, with a log count reduction in presumptive Brettanomyces/Dekkera of 7-54 in the case of barrel 3. Despite the stink coming from the used barrels, the fraction of the viable population attributable to Brettanomyces/Dekkera genus was a minority or lower than the detection limit. According to Rodrigues et al. (2001), the

![Figure 2](image_url)
Table 2 Yeast cell concentrations found in different samples of 3-year-old barrels collected before and after the heat treatment in water at 60°C for 19 min

<table>
<thead>
<tr>
<th>Barrel</th>
<th>Plate counts before the heat treatment (CFU/100 ml)</th>
<th>Per cent of presumptive Brettanomyces colonies</th>
<th>Plate counts after the heat treatment (CFU/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.5 × 10⁴</td>
<td>&lt;10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>2.1 × 10⁶</td>
<td>10</td>
<td>&lt;1</td>
</tr>
<tr>
<td>3</td>
<td>2.3 × 10⁸</td>
<td>15</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Recognition of Brettanomyces/Dekkera colonies grown onto Dekkera/Brettanomyces Differential Medium (DBDM) has been done through the observation of colony morphology, colour change in medium due to acidification, detection of phenolic taint by smelling and observation of cell morphology by optical microscope.

The management of Brettanomyces/Dekkera contamination is a complex activity because of the capability of this yeast to settle in points of the cellar equipment difficult to sanitize. As regards chemical or physical agents used in the disinfection of the cask, the ability of B./D. bruxellensis species to penetrate in the inner layers of the staves can ensure its survival, explaining the failures observed in some practices (Boulton et al. 1996; Coulter et al. 2004; Barata et al. 2013). Guzzon et al. (2011) have compared the effects of different sanitization treatments of yeast populations inhabiting the barriques: the use of steam proved to be more effective than UV irradiation or ozone treatment, but it was insufficient to eliminate the microbial contaminants totally. The porous nature of wood has probably protected the cells from direct UV irradiation, while the presence of abundant organic matter inside the barrels may have reacted with the ozone making this treatment less effective. Schmid et al. (2011) tested the effectiveness of ultrasound HPU high power (17 W l⁻¹) of oak staves contaminated with B. bruxellensis under controlled conditions. Satisfactory results were obtained after an application time longer than 8 min in combination with water at 60°C; this treatment resulted in complete removal of the deposits of tartrate as well as a reduction in the inoculated yeast cells of three orders of magnitude. Recently, an innovative application based on a 3 min treatment with high-frequency microwaves (González-Arenzana et al. 2013) was not completely effective in destroying yeast cells, showing a reduction from 35 to 67% for Brettanomyces populations. Under our conditions, provided that the holding time at the killing temperature takes into consideration either the filling time of the barrel and the time for heat penetration into the wood structure, the thermal inactivation of D. bruxellensis cells by hot water immersion proves to be a valid technique. In conclusion, intervention in a cellar situation seems feasible because it is easy to manage and eco-friendly, does not involve a significant increase in costs and avoids steam production and excessive aerosol dissemination.

Materials and methods

Yeast strains, growth conditions and culture maintenance

The strains used in this work were D. bruxellensis CBS 2499, CBS 2797, CBS 4459 and CBS 4601, which were originally isolated from spoiled wines. Fresh cultures were grown in YPD broth (10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ glucose, pH 6.5) at 25°C in static conditions. Concentrated cultures of each strain were maintained at −80°C in YPD broth with the addition of 20% glycerol. To assess the cell concentration in liquid suspension, regression curves were drawn comparing plate counts (CFU ml⁻¹), obtained in YPD medium added with agar 15 g l⁻¹, with cellular biomass (OD at 650 nm) determined by spectrophotometer measurements (Perkin Elmer, Waltham, MA).

Protocol for the heat treatment

For each strain, cells grown in YPD broth at the late exponential phase were collected by centrifugation at 5000 g for 15 min, washed twice and resuspended in deionized water (pH 7.0), to obtain a concentration of approximately 10⁶ CFU ml⁻¹. Cell suspensions were then distributed in sterile glass capillary tubes (3 mm, Norell NMR tubes; Sigma-Aldrich, Germany) that allowed a quick distribution of heat. The capillary tubes were set into a rack to permit simultaneous dipping in a thermostatic water bath with an immersion circulator (Thermo Fisher Scientific, Waltham, MA). Tests were performed at 55.0, 57.5, 60.0 and 62.5°C (±0.5°C). A thermal probe (Lacor Menaje Profesional, Bergara, Spain) was inserted into a tube containing only water to monitor the internal temperature, and the holding time started when the desired temperature had been attained. After immersing in hot water, the tubes were removed at specific times of exposure and cooled on ice before retrieving the content for the plate counts. The recovered samples were then serially diluted in sterile peptone water (peptone, 10 g l⁻¹, pH 6.5) and plated on WL agar (Merck, Germany). Counts were carried out after incubation at 25°C for 7 days.

Data analysis

The thermal resistance of D. bruxellensis strains was evaluated by calculating D-values and z-value, according to Bigelow’s model (Stumbo 1973). The decimal reduction
time at a specific temperature, D-value, is given by the reciprocal of the slope of the inactivation curve, found through linear regression, obtained by plotting the log counts of the surviving yeast cells vs the corresponding time of exposure. The z-value is given by the reciprocal of the slope of the correlated line, obtained through linear regression, between the log D-values vs the temperature values; this corresponds to the temperature increase in a heat treatment that is required to obtain a 10-fold reduction in the D-value. Regression coefficients (R²) were calculated to assess the correlation of the experimental data with the regression lines.

Determination of the thermal profile in oak wood

Heat penetration through the staves of barrels was evaluated for each investigated temperature. The measurement was carried out by positioning a thermal probe (Lacor Menaje Profesional) at 8 mm (±1 mm) inside a oak wood piece that was previously removed from the bottom of a barrel (Fig. 3). After immersion in the thermostatic water bath, the temperature values were recorded continuously, until the required temperature was achieved. The experiment was replicated three times at different points.

Heat treatment of the barrels

The treatment of the barrel was done in a thermostatic water bath (±1°C) endowed with a system for water circulation and a mechanical crane for the transfer of the cask, which was accommodated into the tub full of preheated potable water at 61°C (Appiano Romano, Costruzioni Meccaniche, Guarene, Italy). An electronic control unit and related software allowed the control of the physical parameters and the movement of the mechanical parts (Appiano Romano, Costruzioni Meccaniche). The filling time of the barrel was measured in 6 min ± 10 s. The temperature and the heating time were selected on the basis of prior results obtained for the most resistant strain.

The contaminated barrels were sampled before and after the heat treatment to verify the rate of inactivation of the spoilage micro-organisms. To recover the yeast cells, the barrel was rinsed with 3 l of sterile peptoned water, thoroughly agitated for 15 min and subsequently this suspension was recovered in a sterile container. The sample was then serially diluted in sterile peptoned water or filtered by a 0.45 μm membrane, depending on the expected colony count, and plated on DBDM (6.7 g l⁻¹ YNB, ethanol 6%, 10 mg l⁻¹ cycloheximide, 100 mg l⁻¹ p-coumaric acid, 22 mg l⁻¹ bromocresol green and 20 g l⁻¹ agar, pH 5.4) plus 100 mg l⁻¹ chloramphenicol. Colonies, presumptively recognized as Brettanomyces/Dekkera, were counted after incubation at 25°C for 12 days.

Acknowledgements

The authors thank Francesca Fiorini and Tatiana Macca-rini for excellent technical assistance, and the YeSVitE consortium (EU project, 7FP-IRSES-GA no. 612441) for helpful discussion and precious collaboration.

Conflict of Interest

No conflict of interest declared.

References

barriques after different sanitation treatments. *J Food Res* 2, 140–149.


