

RESEARCH ARTICLE

The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine

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Dekkera/Brettanomyces bruxellensis;
4-ethylphenol; wine spoilage; phenolic taint.

Abstract

The wine spoilage yeast *Dekkera bruxellensis* was evaluated for the production of 4-ethylphenol under low concentrations (0.02–20 g L⁻¹) of glucose and fructose in synthetic media. Measurable amounts of 4-ethylphenol were produced over 0.2 g L⁻¹ of each sugar. The yeast growth rate and amount of biomass formed increased from 0.2 to 20 g L⁻¹ of glucose or fructose, being accompanied by increasing production of 4-ethylphenol. In red wines, the production of 4-ethylphenol was only observed in the presence of growing populations of indigenous or inoculated strains of *D. bruxellensis*. The production rate of 4-ethylphenol varied between 22 and 93 µg day⁻¹ either with inoculated strains or wild populations in bottled wines. The production rate of 4-ethylphenol as a function of the increase in the number of cells varied from 349 to 1882 µg L⁻¹ per one log CFU mL⁻¹. The effect of temperature on cellular viability and 4-ethylphenol production was tested in red wines with indigenous or inoculated strains of *D. bruxellensis*. Incubation temperatures of 15, 20 and 25 °C allowed cellular growth and volatile phenol production. Increasing incubation temperatures to 36 °C induced full viability loss of 10 strains of *D. bruxellensis* within < 12 h.

Introduction

Yeasts of species *Dekkera/Brettanomyces bruxellensis* are the most important microbiological threat to red wine stability (Loureiro & Malfeito-Ferreira, 2006). These yeasts produce volatile phenols that impart off-flavours described as 'phenolic', 'barnyard' or 'horse sweat', and cause serious economical losses (Suárez *et al.*, 2007). Spoilage occurs mainly during wine storage, particularly when oak barrels are used in the production of premium-quality red wines. However, young red wines in stainless-steel vessels or bottled wines are also prone to this type of spoilage (Rodrigues *et al.*, 2001; Renouf *et al.*, 2007).

Studies on the dissemination, monitoring and control of *D. bruxellensis* populations have shown that these yeasts are usually minor contaminants of wine-related environments (see the review of Loureiro & Malfeito-Ferreira, 2006). However, they are common contaminants of red wines particularly when wines are left unprotected by sulphur dioxide (Barata *et al.*, 2008). These authors have shown that

relatively high levels of this preservative are necessary to inactivate growing populations of *D. bruxellensis*. The option to increase sulphur dioxide usage is not in accordance with the consumer demand to decrease preservative levels in foods and beverages and so the effect of other factors on yeast activity must be studied.

We have characterized several factors determining the production of volatile phenols under oenological conditions, like the influence of carbon source or of ethanol content (Dias *et al.*, 2003). However, other aspects have not been elucidated, particularly those concerning the relation between cellular growth and rate of 4-ethylphenol production in red wines. The understanding of this relation would be particularly important because, at the winery level, the full eradication of these yeasts cannot be guaranteed. Therefore, the purpose of this work was to further characterize the factors affecting volatile phenol production by *D. bruxellensis* to better define preventative technological measures. In this paper, we describe the growth and production of

4-ethylphenol by *D. bruxellensis* as affected by sugar concentration and temperature under conditions mimicking wine production.

Materials and methods

Yeast strains and maintenance conditions

The strains of *D. bruxellensis* tested were either isolated from wines (strains ISA 1700, 1717, 1791, 2101, 2172 and 2173), barrique wood (ISA 2297) and insects in winery (ISA 2298) or obtained from culture collections (ISA 1649, type strain of *D. bruxellensis*). The strains were maintained in slants of GYP medium [20 g L⁻¹ glucose (Merck, Darmstadt, Germany), 5 g L⁻¹ yeast extract (Difco Laboratories, Detroit, MI), 10 g L⁻¹ peptone (Difco) and 20 g L⁻¹ agar pH 6.0] with addition of 5 g L⁻¹ of calcium carbonate (Merck), at 4 °C.

Growth in synthetic media with reduced levels of sugars

The effect of low concentrations of sugars (glucose and fructose), mimicking the residual concentrations of sugars in wine at the end of fermentation, was studied using synthetic culture media because wines considered as dry have residual sugar levels < 2 g L⁻¹. A loopful of fresh culture (24–48 h) was used to inoculate 50 mL of 6.7 g L⁻¹ YNB broth (Difco) media containing 20 g L⁻¹ of glucose (Merck) or fructose (Merck) and subjected to orbital shaking at 26 ± 0.5 °C and 150 r.p.m. Growth was followed by turbidimetry at 640 nm (Bausch & Lomb, Spectronic 21). At an OD of about 0.5 U, cells were inoculated in the growth media as follows: the effect of different concentrations of glucose and fructose on the production of 4-ethylphenol was studied using a medium composed by 6.7 g L⁻¹ YNB (Difco), 100 mg L⁻¹ *p*-coumaric acid (Sigma Chemical Co, St. Louis, MO) with addition of 0.02, 0.2, 1 and 20 g L⁻¹ of each sugar separately. All media were adjusted to pH 3.50 ± 0.01 and sterilized by filtration through cellulose acetate membranes of 0.22-µm pore size and cultivation was carried out with orbital shaking at 26 ± 0.5 °C and 150 r.p.m. Specific growth rates (µ_c) were calculated from the slopes of the regression lines according to the equation $\ln x_t = x_0 + \mu_c t$, where x_t and x_0 represent the biomass in OD values in time t (h) and $t = 0$, respectively. Experiments were repeated at least twice with coefficients of variation < 10%.

Growth in wines and effect of temperature

A loopful of fresh culture (24–48 h) was used to inoculate 50 mL of culture broth [6.7 g L⁻¹ YNB, 20 g L⁻¹ glucose, 10% (v/v) ethanol, pH 3.50] previously filter sterilized through a membrane (0.22-µm pore size, 47 mm diameter). Incubation occurred at 25 °C with orbital shaking (120 r.p.m.).

When the OD was about 0.5 U, red wine blends were inoculated to give an initial population of about 10⁴ cells mL⁻¹.

Experimental red wines were obtained by blending several commercial red wines with < 2 g L⁻¹ of residual sugar. The free sulphur dioxide present in the blends was removed by adding acetaldehyde (Ribéreau-Gayon *et al.*, 2006). The ethanol content was adjusted to 12% (v/v) with a solution of 5 g L⁻¹ of tartaric acid (Merck) or 99% pure ethanol (Merck). The pH value was adjusted to 3.50 with concentrated NaOH (Merck) or HCl (Merck). The final wine blends were sterilized by filtration through cellulose acetate membranes (0.22-µm pore size, 47 mm of diameter, Millipore).

The effect of temperature was determined (1) using a gradient temperature incubator (Adzantech Mod TN-12, Japan) with 5 °C increments from 15 to 35 °C; (2) using Schott flasks filled with 1 L of red wine blend and incubated without agitation in refrigerated water baths (15, 20 °C) or in aerobic incubators (25, 36, 44 °C); and (3) using 0.375-L wine bottles closed with rubber stoppers and incubated as described in (2). During incubation, wine samples were decimally diluted and cellular viability was determined by surface plating 0.1 mL onto GYP medium, in duplicate. The experiments were conducted in duplicate, results not differing by more than 10%. The same experiment was repeated at least twice with different wine blends; the results shown correspond to representative trials.

The study of indigenous populations of *D. bruxellensis* was performed using naturally contaminated commercial red wines screened previously for the presence of viable cells by plating on *Dekkera/Brettanomyces* differential medium (DBDM) (Rodrigues *et al.*, 2001). The wines, with known ethanol content, total and volatile acidity, dry extract, pH and total and free sulphur dioxide concentrations, were poured into 1-L Schott flasks or into 0.375-L wine bottles and incubated as described before for inoculated wines. Viable *D. bruxellensis* counts (CFU mL⁻¹) were determined by plating known volumes of wine onto DBDM medium as described by Rodrigues *et al.* (2001).

Determination of 4-ethylphenol

Production of 4-ethylphenol in synthetic media or red wines was measured according to a protocol described by Rodrigues *et al.* (2001). Briefly, the volatile phenol was extracted using ether–hexane from a 10-mL sample with pH adjusted to 8 with NaOH. The quantitation was achieved by adding 10 mg L⁻¹ 3,4-dimethylphenol (Fluka, Buchs, Switzerland) as an internal standard, followed by GC (Fisons Instruments, model 8130, Rodano, Italy) with a DB-Wax column (30 m, 0.53 mm ID, 0.25-µm film thickness, J&W Scientific, Folsom). Program conditions were as follows: initial temperature 50 °C, increase in temperature 5 °C min⁻¹, second temperature 215 °C, second increase in temperature 20 °C min⁻¹, and final temperature 250 °C for

Fig. 1. Growth (filled symbols) and 4-ethylphenol production (open symbols) by *Dekkera bruxellensis* ISA 1791 as a function of glucose (a) and fructose (b) concentration in YNB with 100 mg L⁻¹ *p*-coumaric acid, at initial pH 3.50, 26 °C and 150 r.p.m. Symbols: (▼, ▽) 0.02, (▲, △) 0.2, (●, ○) 1 and (■, □) 20 g L⁻¹.

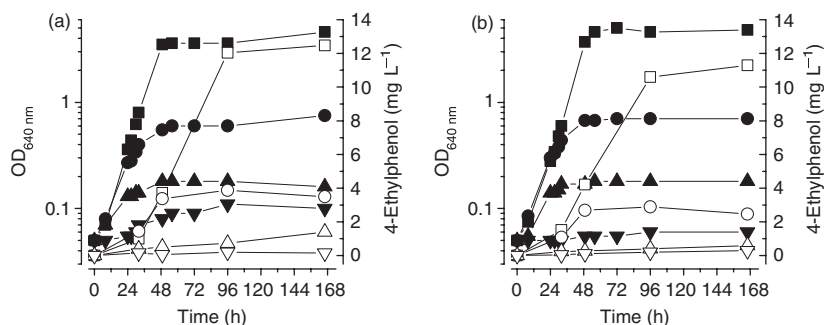
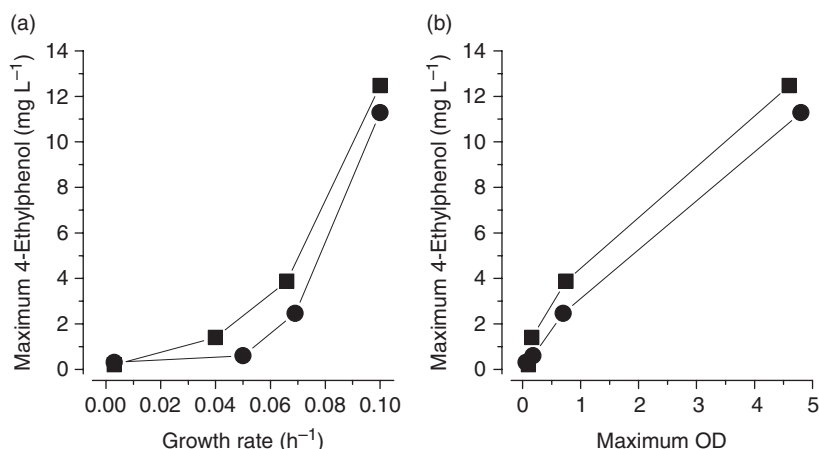


Fig. 2. Maximum production of 4-ethylphenol as a function of growth rate (a) and maximum OD (b) attained by *Dekkera bruxellensis* ISA 1791 growing in YNB with glucose (■) or fructose (●), 100 mg L⁻¹ *p*-coumaric acid, at initial pH 3.50, 26 °C and 150 r.p.m.



15 min. Injector and detector temperatures were 260 °C. Hydrogen was used as a carrier gas at 80 kPa pressure (2.0 mL min⁻¹). The sample volume injected was 1 µL. The coefficients of variation of the chromatographic determination (six injections of the same extract) and of the extraction procedure (six extractions of the same wine sample) were < 5.1%.

Results

Effect of glucose and fructose concentration on the production of 4-ethylphenol in synthetic medium

The growth and production of 4-ethylphenol by *D. bruxellensis* ISA 1791 in synthetic media with low glucose or fructose concentrations is shown in Fig. 1. The behaviour was similar for both sugars. The production of 4-ethylphenol was detected at sugar concentrations over 0.2 g L⁻¹ and increased under higher sugar concentrations. The process was correlated with the growth rate and with the amount of biomass produced as shown in Fig. 2.

Growth and 4-ethylphenol production in naturally contaminated red wines

Two commercial red wines naturally contaminated with *D. bruxellensis* and other microbial flora were incubated under

different temperatures. One wine (I) showed an initial 2×10^3 CFU mL⁻¹ of *D. bruxellensis* and an initial high 4-ethylphenol concentration of 1500 µg L⁻¹. The other wine (II) showed a higher contamination load of 3×10^4 CFU mL⁻¹ and a lower 4-ethylphenol content of 350 µg L⁻¹. Both wines were incubated statically at 15 and 25 °C and maintained in Schott flasks in contact with air. Fig. 3 shows the evolution in the plate counts of viable wild populations of *D. bruxellensis* (Fig. 3a) and respective production of 4-ethylphenol (Fig. 3b) during the incubation period. The experiment was ended when a film appeared on the surface level of the wine accompanied by acetic acid production. This event was due to the development of a natural population of acetic acid bacteria stimulated by the contact of the wine with air, leading to *D. bruxellensis* death (results not shown). This outcome occurred after 30 days in the wine II incubated at 25 °C (viability was reduced from 10^5 to 10^4 CFU mL⁻¹ and 4-ethylphenol production was arrested) and so results were not presented for this trial. In wine I, this event occurred after 45 days, being possible that the lower increment in viable counts between 30 and 45 days, under 25 °C, was the result of the onset of bacterial growth.

The results shown in Fig. 3 demonstrate that the production of 4-ethylphenol began with the onset of *D. bruxellensis* growth, in both wines and under both temperatures. During the initial lag phase of about 15 days, even under high contamination loads, there was no measurable increase in the production of 4-ethylphenol. The growth phase was

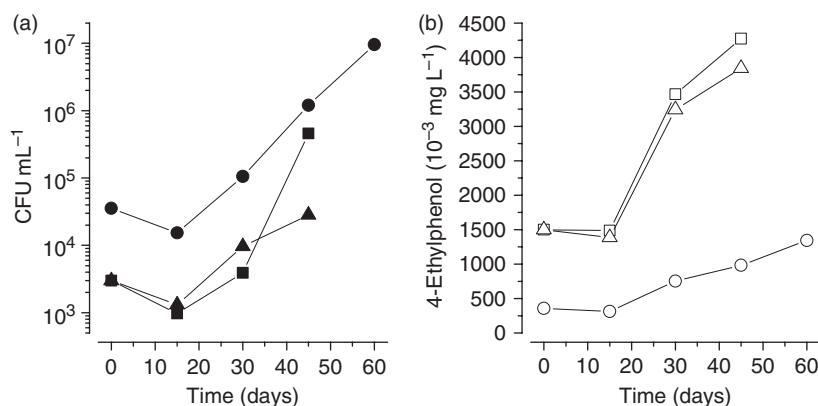


Fig. 3. Growth (a) and 4-ethylphenol production (b) in commercial red wines (wine I, ■, □, ▲, △; wine II, ●, ○) contaminated by wild populations of *Dekkera bruxellensis* incubated statically at 15 (●, ○, ■, □) and 25 °C (▲, △).

Table 1. Parameters related with the rate of 4-ethylphenol (4-EP) production by wild populations of *Dekkera bruxellensis*

Sample	Temperature (°C)	4-EP production rate (µg day ⁻¹)	Days to produce 620 µg L ⁻¹ 4-EP	Growth rate (day ⁻¹)	4-EP/log CFU (µg L ⁻¹)
Wine I	15	93	6.7	0.20	874 ($r=0.86$)
Wine I	25	82	7.6	0.10	1882 ($r=0.99$)
Wine II	15	22	28.2	0.14	349 ($r=0.987$)

accompanied by an increase in 4-ethylphenol production, being possible to determine the rate of 4-ethylphenol production for the three different situations (Table 1). The wine II, with a lower 4-ethylphenol concentration and higher counts of *D. bruxellensis*, showed a lower rate of 4-ethylphenol production of 22 µg day⁻¹, when incubated at 15 °C. At this temperature, the wine I with higher initial counts and a lower 4-ethylphenol content showed a higher production rate of 93 µg day⁻¹. This wine at 25 °C yielded a slightly lower rate (82 µg day⁻¹), which was negatively influenced by wine acetification in the last determination of the experiment. Using these rates of 4-ethylphenol production, it is possible to calculate the number of days to reach a concentration similar to the preference threshold of 620 µg L⁻¹, as determined by Chatonnet *et al.* (1990). The results presented in Table 1 show that, when *D. bruxellensis* is actively growing, this threshold may be achieved within a week.

The previous data were obtained in wines incubated in Schott flasks with air contact at the wine surface. Knowing that oxygen stimulates *D. bruxellensis* growth and volatile phenol production (Malfeito-Ferreira *et al.*, 2001) we checked whether these 4-ethylphenol production rates could be reached under conditions similar to bottled wine. A group of eight red wines, initially contaminated by 500–5 × 10³ CFU mL⁻¹ of *D. bruxellensis* and with 440–2440 µg L⁻¹ of 4-ethylphenol, were incubated for 57 days in 0.375-L wine bottles closed with rubber stoppers. The 4-ethylphenol production rates ranged from 24 to 90 µg day⁻¹, which agrees with the values shown in Table 1. We

found no relation between those rates and the chemical composition of wines (results not shown).

The production rate of 4-ethylphenol can be calculated as a function of the increment in cellular counts, thus obtaining a parameter independent from the microbial growth rate. This parameter was estimated from the linear slopes of the log viable counts vs. 4-ethylphenol for the two wines maintained at 15 and 25 °C (results not shown). Then, it is possible to estimate the increase in 4-ethylphenol production when *D. bruxellensis* counts increase by one log cycle by calculating the slopes of the regression lines. This parameter illustrates the production rate of 4-ethylphenol independently from the growth rate, and the results are given in Table 1. The range of values varied between 349 µg L⁻¹ for wine I (at 15 °C) and 1882 µg L⁻¹ for wine II (at 25 °C), implying that the production rate is dependent, at least, on the yeast strain, incubation conditions and on the characteristics of the wine.

Effect of temperature on *D. bruxellensis* viability and 4-ethylphenol production

The previous experiments have shown that the testing of naturally contaminated wines in contact with air was not appropriate for long periods because bacterial wine contaminations also developed, leading to *D. bruxellensis* death. Consequently, another set of experiments was performed with filter sterile red wines inoculated with pure cultures of *D. bruxellensis* strains.

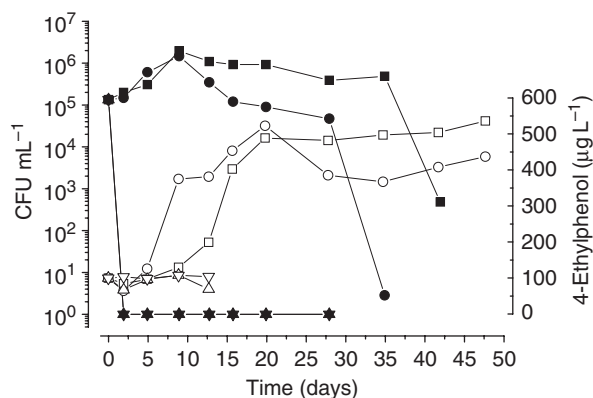


Fig. 4. Effect of temperature on the viability (filled symbols) and 4-ethylphenol production (open symbols) of *Dekkera bruxellensis* ISA 1791 inoculated in red wine with 12% (v/v), pH 3.50, without free sulphur dioxide (\square , \blacksquare , 15 °C; \circ , \bullet , 25 °C; \triangle , \blacktriangle , 36 °C; ∇ , \blacktriangledown , 44 °C).

The effect of temperature on the viability of *D. bruxellensis* ISA 1791 was first determined in a gradient temperature incubator. At 15 and 20 °C, growth proceeded after inoculation without any decrease in viability. At 25 °C, an initial decrease in viability was observed but growth resumed after 75 h of incubation. Temperatures of 30 and 35 °C induced full viability loss within < 12 h (results not shown).

According to the view that relatively mild temperatures induced rapid viability loss, another set of experiments was conducted in higher volumes of wine in contact with air in Schott flasks without shaking, as depicted in Fig. 4. The results of cellular viability and 4-ethylphenol production showed that at 36 and 44 °C, death occurred in < 24 h, without variation in the 4-ethylphenol content. At 15 and 25 °C, cellular death was not observed and 4-ethylphenol increased from 100 to about 500 $\mu\text{g L}^{-1}$ in 15 days. The respective production rates were about 27 $\mu\text{g day}^{-1}$ of 4-ethylphenol, and 400 μg of 4-ethylphenol was produced during the increase of one log of viable counts. These values are within the range presented in Table 1 for other wines. The production kinetics was similar to that presented in synthetic culture media (see Fig. 1), the onset of 4-ethylphenol production was at about the mid-exponential phase and the maximum amount was achieved during the stationary phase (Fig. 4).

These results, showing the lethal effect under moderate temperatures, if valid for other strains, could provide stronger evidence that mild heat treatments are suitable to control these yeasts. Thus, a total of 10 *D. bruxellensis* strains (ISA 1600, ISA 1649, ISA 1700, ISA 1700, ISA 1791, ISA 2101, ISA 2172, ISA 2173, ISA 2297, ISA 2298) were subjected to similar incubation conditions, in Schott flasks, under an inhibitory temperature range. At 36 °C, all strains were killed within < 24 h of incubation, while at 33 °C only one strain (ISA 2172) recovered after an initial death phase

(results not shown). These results were further validated with red wine naturally contaminated by wild *D. bruxellensis* strains in situations closer to winery practice. Incubation of red wine, in rubber-stopped 0.375 L⁻¹ bottles, with initial *D. bruxellensis* counts of about 10⁵ CFU mL⁻¹, at 36 and 44 °C, resulted in cellular death within < 12 h (results not shown), demonstrating that relatively low temperatures are effective in killing high contamination loads of *D. bruxellensis*.

Discussion

The production of 4-ethylphenol by *D. bruxellensis* depends on several factors such as the carbon source, ethanol content and temperature (Dias *et al.*, 2003). In this work, we further demonstrated that the concentration of carbon source (glucose or fructose) also influences the production of 4-ethylphenol. In addition, the production rate is linked to the growth rate and the highest levels of 4-ethylphenol were observed under the highest levels of biomass attained. These results, derived from cultures in synthetic media, suggested that 4-ethylphenol production depends on the activity of growing yeast populations and on the amount of biomass accumulated. Fugelsang & Zoecklein (2003) also found a correlation with 4-ethylphenol production and the cumulative cell exposure, but the highest increase in 4-ethylphenol production was observed during the cellular decline phase, which does not correspond to our observations.

Dias *et al.* (2003) showed that in the absence of carbon sources there is no production of 4-ethylphenol. However, these results were obtained in synthetic media and were not validated in wines. In dry wines, residual sugars are below 2 g L⁻¹, but this small amount is enough to support volatile phenol production. However, even in the presence of a carbon and energy source, we were able to demonstrate that the levels of volatile phenols do not increase in commercial red wines when *D. bruxellensis* is not growing. This conclusion has direct implications in the management of the phenolic taint, because it shows that the primary objective should be the prevention of actively growing populations and not the reduction of *D. bruxellensis* to the lowest possible level, as suggested by Renouf *et al.* (2007). In fact, complete absence of viable cells of *D. bruxellensis* is not easy to achieve under winery conditions, especially when oak ageing is used due to the porous nature of the wood (as discussed by Loureiro & Malfeito-Ferreira, 2006). Another practical implication is related to the methodologies required for an appropriate microbiological control. Besides yeast detection using selective culture media, it is advisable to check regularly the concentration of 4-ethylphenol as a chemical indicator of microbial activity during storage. In our experience, we managed to achieve stable levels of 4-ethylphenol (*c.* 100 $\mu\text{g L}^{-1}$) with viable but nongrowing *D. bruxellensis* populations of 2000 CFU mL⁻¹ during

barrique storage at low temperatures (6–8 °C). Removal and inactivation of *D. bruxellensis* by strict process operations (e.g. heat treatment, sterile filtration) would only be advisable when there is an increase in the 4-ethylphenol levels.

The results presented in this work concerning the effect of temperature on the inactivation of viable populations of *D. bruxellensis* demonstrate that this environmental factor may be regarded as another hurdle in the prevention of the phenolic taint. Couto *et al.* (2005) already presented lethal heat-treatment parameters (*D* and *z*) for *D. bruxellensis*, showing that significant inactivation of *D. bruxellensis* in wine began at 35 °C, stimulated by the ethanol content of wine. In addition, our data show that relatively mild temperatures (about 36 °C) overnight are a reasonable technological option when wines are found contaminated by viable *D. bruxellensis* and 4-ethylphenol tends to increase. According to our empirical experience, this mild temperature has no obvious detrimental effects on wine quality and may be achieved using electric devices to heat wine in stainless-steel vessels or simply by heating bottled wine, without disgorging, if contamination could not have been avoided during bottling. Any occasional loss in wine quality due to heating must be weighed with the certain loss of quality if viable cells grow in the bottled product.

In conclusion, our work showed that the production of 4-ethylphenol in red wines is related to the presence of growing populations of *D. bruxellensis*, demonstrating that the primary management objective should not be their complete elimination but their maintenance at constant levels. If the numbers of *D. bruxellensis* increase, removal or inactivation procedures must be performed, among which wine thermal treatment is a feasible practice.

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References

- Barata A, Caldeira J, Botellheiro R, Pagliara D, Malfeito-Ferreira M & Loureiro V (2008) Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. *Int J Food Microbiol* **121**: 201–207.
- Chatonnet P, Boidron JN & Bons M (1990) Élevage des vins rouges en fûts de chêne: évolution de certains composés volatils et de leur impact aromatique. *Sci Alim* **10**: 565–587.
- Couto JA, Neves F, Campos F & Hogg T (2005) Thermal inactivation of the wine spoilage yeasts *Dekkera/Brettanomyces*. *Int J Food Microbiol* **104**: 337–344.
- Dias L, Pereira-da-Silva S, Tavares M, Malfeito-Ferreira M & Loureiro V (2003) Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. *Food Microbiol* **20**: 377–384.
- Fugelsang K & Zoecklein B (2003) Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot Noir (*Vitis vinifera* L.) wines. *Am J Enol Vitic* **54**: 294–300.
- Loureiro V & Malfeito-Ferreira M (2006) Spoilage activities of *Dekkera/Brettanomyces* spp. *Food Spoilage Microorganisms* (Blackburn C, ed), pp. 354–398. Woodhead Publishers, Cambridge.
- Malfeito-Ferreira M, Rodrigues N & Loureiro V (2001) The influence of oxygen on the “horse sweat taint” in red wines. *Ital Food Beverage Technol* **24**: 34–38.
- Renouf V, Lonvaud-Funel A & Coulon J (2007) The origin of *Brettanomyces bruxellensis* in wines: a review. *J Int Sci Vigne Vin* **41**: 161–173.
- Ribéreau-Gayon P, Dubourdieu D, Donèche B & Lonvaud A (2006) *Handbook of Enology (Vols. 1 and 2). The Microbiology of Wine and Vinification*. John Wiley & Sons Ltd, Chichester.
- Rodrigues N, Gonçalves G, Pereira-da-Silva S, Malfeito-Ferreira M & Loureiro V (2001) Development and use of a new medium to detect yeasts of the genera *Dekkera/Brettanomyces* spp. *J Appl Microbiol* **90**: 588–599.
- Suárez R, Suárez-Lepe JA, Morata A & Calderón F (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: a review. *Food Chem* **102**: 10–21.