Thermal inactivation of the wine spoilage yeasts *Dekkera/Brettanomyces*

José António Couto [a,*], Filipe Neves [a,b], Francisco Campos [a], Tim Hogg [a]

[a] *Escola Superior de Biotecnologia, Universidade Católica Portuguesa, R. Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal*
[b] *Faculdade de Ciências da Universidade do Porto, Campus Agrário de Vairão, Rua Padre Armando Quintas, 4485-661 Vairão, Portugal*

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Abstract

The heat resistance of three strains of *Dekkera/Brettanomyces* (*Dekkera anomala* PYCC 5153, *Dekkera bruxellensis* PYCC 4801 and *Dekkera/Brettanomyces* 093) was evaluated at different temperatures between 32.5 and 55 °C. Thermal inactivation tests were performed in tartrate buffer solution (pH 4.0) and in wines. In the studies employing buffer as the heating menstruum, measurable thermal inactivation began only at temperatures of 50 °C. When heating was performed in wine, significant inactivation begins at 35 °C. Subsequent thermal inactivation tests were performed in buffer at various levels of pH, ethanol concentration, and various phenolic acids. Results from experiments in buffer with added ethanol suggest that the greater heat sensitivity shown in wines can be largely attributed to ethanol, although potentiation of this effect might be due to the phenolic content, particularly from ferulic acid. In the range of pH values tested (2.5–4.5), this factor had no influence in the heat inactivation kinetics. Relevant data, in the form of *D* and *Z* values calculated in the various environments, potentially useful for the establishment of regimes of thermal control of *Dekkera/Brettanomyces* yeasts in wine and contaminated equipment is presented.

1. Introduction

Yeasts of the genus *Dekkera/Brettanomyces* are well known to be involved in the production of volatile phenols (4-ethylphenol and 4-ethylguaiacol) in wines imparting aroma defects usually described as "horse sweat", "leather" and "animal" (Heresztyn, 1986; Chatonnet et al., 1995; Edlin et al., 1995; Chatonnet et al., 1997). In fact, the transformation of the hydroxycinnamic acids, p-coumaric and ferulic acids, into volatile phenols is predominantly associated with the activity of the *Brettanomyces* yeast genus and its ascosporogenous sexual form classified within *Dekkera* (Heresztyn, 1986; Chatonnet et al., 1995; Edlin et al., 1995). The mechanism of conversion involves a sequence of two enzymatic reactions. In the first, a carboxylase decarboxylates the hydroxy-

* Corresponding author. Fax: +351 225580088.
E-mail address: jagc@esb.ucp.pt (J.A. Couto).

cinnamic acids into the corresponding vinyl derivative (4-vinylphenol from p-coumaric acid or 4-vinylguaiacol from ferulic acid) and in the second reaction, a reductase converts the vinyl group into the corresponding ethyl compound (Heresztyn, 1986; Chatonnet et al., 1995).

The production of volatile phenols may occur at different stages of the winemaking process. However, *Dekkera/Brettanomyces* is often associated with spoilage of barrel-aged wines due to insufficient wood sanitation (Chatonnet et al., 1993). The unpleasant odour formed is the cause of important economic losses within the wine industry. Therefore, the monitoring and control of these organisms in the wine and wine contact surfaces is of major importance for wine producers. Aside from the rigorous application of effective cleaning and disinfectant regimes for contact surfaces, there are a number of winemaking practices which can limit the development of populations of *Dekkera/Brettanomyces* in wines. Low pH values (≤3.5), appropriate sulphur dioxide (SO_2) levels (0.8 ppm of the molecular form) and low aging temperatures (10–15 °C) are ordinary practices that can be used to limit *Dekkera/Brettanomyces* activity in wines. The judicious use of chemical preservatives, such as SO_2, during the winemaking process decreases the risk of microbial spoilage, but strains vary considerably in their SO_2 sensitivity (Du Toit and Pretorius, 2000). Hence, more drastic procedures or treatments may be required in these cases. Sterile filtration is effective at removing any type of microorganisms. This procedure is, however, regarded by some winemakers to be responsible for stripping wine fruit character, reducing body and viscosity and changing the mouth feel in a negative way. The economic losses and damage to reputation associated with *Dekkera/Brettanomyces* mean that the wine industry is constantly seeking to optimise current methods and find new approaches of monitoring and controlling this problem. The work presented in this study deals with the inactivation of *Dekkera/Brettanomyces* populations in wine by heat treatment. The heat resistance of three strains of this genus (*Dekkera anomala* PYCC 5153, *Dekkera bruxellensis* PYCC 4801 and *Dekkera/Brettanomyces* 093) was evaluated at temperatures between 32.5 and 55 °C. The aim of this study is to generate data useful for the conception of regimes for the inactivation of *Dekkera/Brettanomyces* in wines and contaminated equipment. Initial thermal inactivation tests were performed in buffer at various levels of pH, ethanol concentration, and various phenolic acids; subsequent tests were performed in wine.

Materials and methods

Microorganisms, culture maintenance and growth media

The strains used in this work were *D. anomala* PYCC 5153 and *D. bruxellensis* PYCC 4801, obtained from the Portuguese Yeast Culture Collection (Institute Gulbenkian de Ciência, Oeiras, Portugal) and *Dekkera/Brettanomyces* 093 isolated in our laboratory from a contaminated wine. The cultures were maintained on slants prepared with YM broth (Difco, Detroit, USA) with 2% agar (Lab M, Bury, UK), pH adjusted to 5 with H_2SO_4 6M, at 4 °C, with monthly transfers to maintain strain viability.

Identification of wine isolates

Wine samples were used to inoculate WL medium (Difco) supplemented with 100 mg/l of cycloheximide, 10 mg/l of p-coumaric acid and 50 mg/l of chloramphenicol. Inoculated media were incubated at 25 °C for 12 days. Representative colony forms were isolated and maintained on YM agar (Difco) slants at 4 °C prior to identification. The methodology adopted for the identification of the yeast isolates was based on discriminatory tests described in Barnett et al. (1994) using morphological, physiological and biochemical tests. Sugar assimilation tests were carried out using API ID 32C galleries (Biomérieux, Marcy l'Étoile, France) with Yeast Nitrogen Base (Difco) as suspending diluent. The commercially available computer program of Barnett et al. (1994) was used for identification.

Inoculum development for thermal inactivation experiments

Growth curves were obtained for the three strains in YM broth (pH 5.0) at 30 °C in static cultures.

Growth was monitored by measuring the optical density at 650 nm on a UNICAM 8620 UV/VIS (Cambridge, UK) spectrophotometer. On knowing the growth kinetics, cultures were grown in YM broth at 30 °C to the late exponential (A_{650}=6–7) or stationary (A_{650}=8–9) growth phases, harvested by centrifugation at 3000 g for 5 min at room temperature, and washed twice with sterile buffer solution. The cell pellet was suspended in sterile tartrate buffer solution (3 g/l tartaric acid) and this suspension was used as the inoculum in the thermal inactivation experiments.

Wine parameters

The pH of the wines was determined by potentiometry using a Crison micro pH 2002 (Crison Instruments SA, Barcelona, Spain) pH meter. The total and free sulphur dioxide (SO_2) concentrations were determined by the standard iodine titration method (the recommended procedure by OIV) using starch for the detection of the end-point. For the determination of the total polyphenol index, wines were diluted (1:100) in distilled water and the UV absorption, at 280 nm, was measured using 1 cm optical path cuvettes on a UNICAM 8620 UV/VIS (Cambridge, UK) spectrophotometer.

Thermal inactivation experiments

5 ml samples of the above described inoculum was added to 95 ml of tartrate buffer at pH 4.0 (adjusted with a solution of NaOH 3M) or sterile-filtered (0.45 μm filter pore size) red wines, as the heating menstruum, giving an initial inoculum of approximately 1×10^7 CFU/ml. The heating menstruum was previously stabilised at the desired temperature in a Tectron 3473100 thermostatted water bath (JP-Selecta, Barcelona, Spain). A stirring drive unit (Variomag, H+P Labortechnik, Oberschleissheim, Germany) and submerged magnetic stirrers were used for the agitation of cultures. Heating time and sampling frequency was based on the culture growth conditions and previous survival experiments. After being heated, the samples were removed at regular intervals, serially diluted (decimal dilutions in sterile 9 ml Ringer solution [Lab M, Bury, UK]) and plated in duplicate on YM agar using the drop count technique (Miles and Misra, 1938) for the determination of viability. Counts were made after incubation at 25 °C for 72 h and cell densities were reported as CFU/ml of sample. The results presented are the average of three separate experiments.

Experiments were performed in tartrate buffer at various pH values, in the range 2.5–4.5, and different ethanol concentrations (10–13% v/v). The effect of several phenolic acid compounds was also tested. Fresh concentrated solutions (10 g/l) of ferulic, vanillic, caffeic and gallic acids, all from Sigma-Aldrich (Steinheim, Germany), were prepared in pure (99.5% v/v) ethanol and added to the tartrate buffer (pH 4.0) at a concentration of 500 mg/l.

D and Z values

D values (the time needed to inactivate 90% of the population) were calculated as the negative reciprocal slope of the linear regression of survivor curves obtained by plotting logarithms of survival counts versus their corresponding heating times. *Z* values (the rise in temperature necessary to reduce the *D* value by 10) were calculated as the negative reciprocal slope of the linear regressions obtained by plotting *D* values versus their corresponding temperatures.

Results and discussion

D. bruxellensis and *D. anomala* were chosen to carry out this study because *D. bruxellensis* is the most prevalent species of this genus in wine (Chatonnet et al., 1997; Sponholz, 1993) and is, together with *D. anomala*, regarded as the most efficient wine microorganism in the conversion of p-coumaric acid into 4-ethylphenol (Heresztyn, 1986; Chatonnet et al., 1997; Fugelsang and Zoecklein, 2003). Initial thermal inactivation tests were performed in tartrate buffer at pH 4.0. Fig. 1 shows the thermal inactivation of populations of *D. bruxellensis* PYCC 4801 harvested at the late exponential growth phase. No significant effect on cell survival was found at 45 °C over the 12-min period of exposure. Measurable thermal inactivation began only at 50 °C, the log numbers apparently decreasing in a linear manner with time. Reproducible semilogarithmic curves

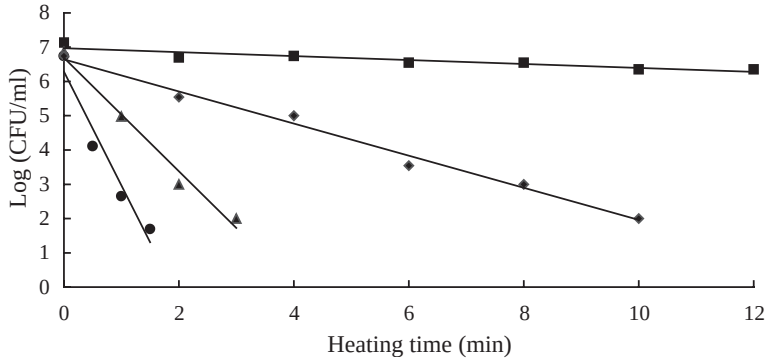

Fig. 1. Thermal inactivation of *D. bruxellensis* PYCC 4801 in tartrate buffer (pH 4.0) at 45 (■), 50 (♦), 52.5 (▲) and 55 °C (●). Relative standard deviation of log CFU never varied more than 12% of the mean value in each time point.

were obtained without any apparent deviations associated with cell clumping and variable heat resistance within the cell population. Similar results were obtained for *D. anomala* PYCC 5153 and for *Dekkera/Brettanomyces* 093. The *D* and *Z* values determined from the experiments employing tartrate buffer as the heating menstruum are shown in Table 1. As expected, the *D*-values decreased with increasing temperature. It is possible to observe that, for the 3 strains studied, cells harvested at the stationary phase exhibit a higher heat resistance than cells harvested at the late exponential growth phase. This is specially noted in the D_{45} values for *D. bruxellensis* PYCC 4801 and *D. anomala* PYCC 5153 and in the D_{50} values for strain 093. It can also be noted that the two strains, representing different species of *Dekkera*, show similar heat resistances. The strain isolated in our laboratory in the course of this work (*Dekkera/Brettanomyces* 093) seems to be less resistant than the culture collection strains when cells are harvested at the stationary phase.

When heating was performed in wine, a slight effect on cell survival is observed at 32.5 °C and significant inactivation begins at 35 °C (Table 2). As expected, the heat sensitivity of the yeast strains studied is much higher in wine than in simple buffer solutions. In fact, it is shown that relatively

Table 1
Decimal reduction times (D_T) and *Z* values determined in tartrate buffer (pH 4.0)

Strain	Temperature (°C)	Exponential phase		Stationary phase	
		D_T (min)	*Z* (°C)	D_T (min)	*Z* (°C)
D. bruxellensis PYCC 4801	45.0	17.4 (r^2=0.85)	5.8 (r^2=0.98)	56.0 (r^2=0.80)	4.4 (r^2=1.00)
	50.0	3.8 (r^2=0.99)		3.4 (r^2=0.98)	
	52.5	1.0 (r^2=0.94)		1.1 (r^2=1.00)	
	55.0	0.3 (r^2=0.95)		0.4 (r^2=1.00)	
D. anomala PYCC 5153	45.0	33.3 (r^2=0.81)	4.5 (r^2=1.00)	48.5 (r^2=0.87)	4.3 (r^2=0.99)
	50.0	2.0 (r^2=0.97)		2.4 (r^2=0.94)	
	52.5	0.6 (r^2=0.95)		0.9 (r^2=0.97)	
	55.0	0.2 (r^2=1.00)		_[a]	
093	45.0	17.6 (r^2=0.82)	5.7 (r^2=0.99)	17.5 (r^2=0.82)	5.5(r^2=0.95)
	50.0	2.7 (r^2=0.96)		4.8 (r^2=0.95)	
	52.5	1.0 (r^2=0.91)		0.7 (r^2=0.99)	
	55.0	0.3 (r^2=1.00)		0.3 (r^2=1.00)	

Values represent the mean of at least two experiments; relative standard deviation never varied more than 12% of the mean value.
[a] Not determined.

Table 2
Decimal reduction times (D_T) and Z values determined in wine

Strain	Temperature (°C)	Wine A		Wine B	
		D_T (min)	Z (°C)	D_T (min)	Z (°C)
D. bruxellensis PYCC 4801	32.5	23.3 (r^2=0.75)	3.3 (r^2=0.96)	14.8 (r^2=0.63)	4.3 (r^2=0.94)
	35.0	2.3 (r^2=0.75)		2.1 (r^2=0.94)	
	37.5	0.7 (r^2=0.96)		1.0 (r^2=0.96)	
D. anomala PYCC 5153	32.5	8.9 (r^2=0.80)	3.7 (r^2=0.97)	7.9 (r^2=0.75)	4.5 (r^2=0.93)
	35.0	1.4 (r^2=0.98)		4.0 (r^2=0.84)	
	37.5	0.4 (r^2=1.00)		0.6 (r^2=1.00)	
093	32.5	16.6 (r^2=0.93)	3.8 (r^2=0.99)	14.4 (r^2=0.80)	4.2 (r^2=0.96)
	35.0	4.6 (r^2=0.86)		2.2 (r^2=0.97)	
	37.5	0.8 (r^2=0.92)		0.9 (r^2=0.96)	

Values represent the mean of at least two experiments; relative standard deviation never varied more than 14% of the mean value.

low temperatures are capable of inactivating *Dekkera/Brettanomyces* in wine. It is very well known that the microbial heat resistance is profoundly influenced by environmental physicochemical factors (Farkas, 1997). The influence of selected wine parameters (pH, ethanol and phenolic acids) on the thermal inactivation of the strains used was assayed in buffer solution. At the range of pH values tested (2.5–4.5), this factor showed no significant influence in the heat inactivation kinetics of *D. bruxellensis* PYCC 4801 (data not shown) and *Dekkera/Brettanomyces* 093 (Fig. 2). In the experiments done with different phenolic acids (ferulic, vanillic, caffeic and gallic acids at 500 mg/l), all compounds caused a decrease in the D values, ferulic acid having the strongest effect (Table 3). This effect can be observed in both strains studied and for both inactivation temperatures tested. The effect of several wine associated phenolic acids on the growth and viability of wine organisms has been determined by a number of authors. The effect of common hydroxycinnamic acids, at concentrations of 100 and 500 mg/l, on the growth of 11 strains of wine yeasts, other than *Dekkera/Brettanomyces*, was studied by Stead (1995). Ferulic acid was found to be the most generally inhibitory hydroxycinnamic acid, appreciably inhibiting *Pichia anomala*, *Debaryomyces hansenii* and *Saccharomyces cerevisiae* when added at the concentration of 500 mg/l. Although not comparable in absolute terms, these results are in agreement with the results of the thermal inactivation experiments obtained in the

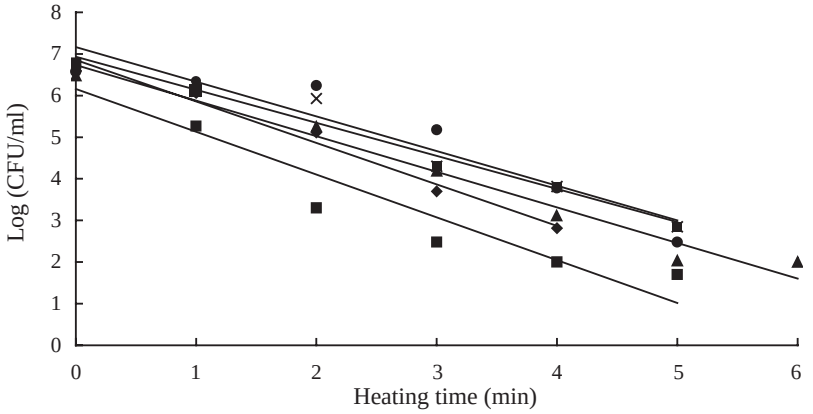

Fig. 2. Thermal inactivation of *Dekkera/Brettanomyces* 093 at 52.5 °C in tartrate buffer at pH 2.5 (●), 3.0 (♦), 3.5 (▲), 4.0 (■) and 4.5 (×). Relative standard deviation of log CFU never varied more than 11% of the mean value in each time point.

Table 3
Decimal reduction times (D_T) determined in tartrate buffer (pH 4.0) with added phenolic acids (500 mg/l) or with ethanol (12% v/v)

	D. bruxellensis PYCC 4801		*Dekkera/Brettanomyces* 093	
	D_{45}	D_{50}	D_{45}	D_{50}
Control	17.4 (r^2=0.85)	3.0 (r^2=0.98)	17.5 (r^2=0.82)	3.9 (r^2=0.96)
Ferulic acid	2.4 (r^2=1.00)	0.2 (r^2=1.00)	3.4 (r^2=0.93)	–[a]
Caffeic acid	10.9 (r^2=0.75)	1.1 (r^2=0.99)	12.3 (r^2=0.74)	0.9 (r^2=0.96)
Vanilic acid	13.3 (r^2=0.70)	0.6 (r^2=1.00)	14.6 (r^2=0.85)	1.1 (r^2=0.96)
Gallic acid	12.0 (r^2=0.65)	1.2 (r^2=0.91)	36.6 (r^2=0.72)	1.5 (r^2=0.92)
Ethanol (12% v/v)	0.7 (r^2=0.99)	–[a]	0.3 (r^2=1.00)	–[a]

Values represent the mean of at least two experiments; relative standard deviation never varied more than 12% of the mean value.
[a] Not determined.

present study at least in terms of the relative responses of the different species. Heat inactivation experiments were also done with 12% (v/v) ethanol in tartrate buffer (pH 4.0). Table 3 presents data that show the influence of ethanol on the yeast heat resistance at 45 °C. These experiments strongly suggest that the greater heat sensitivity exhibited in wines, in comparison to buffer without ethanol as heating menstruum, can be largely attributed to ethanol. The increased heat lethality in the presence of ethanol has been noted previously. In studies with *S. cerevisiae*, Leão and Van Uden (1982) observed that alkanols enhanced yeast thermal death and that the enhanced potency of alcohols at elevated temperatures may result not only from increased plasmatic membrane disorder, but also from a higher concentration of alcohol within the membrane. A similar synergistic ethanol/temperature effect was also found in bacteria (Adams et al., 1989; Jones, 1989). The combined effects of high temperature and ethanol are, however, regarded as quite complex. This effect might, however, be potentiated by the phenolic content of wines, as suggested, particularly, from the assay with ferulic acid. Fig. 3 shows, in the same graphic, the effect of the addition of ethanol (12% v/v) and ferulic acid (500 mg/l) to the heating menstruum in the inactivation of *D. bruxellensis* PYCC 4801 at 45 °C. Lower concentrations of ferulic acid, for example 100 mg/l (a level closer to those normally encountered in wines), also led to a reduction of the measured yeast thermal resistance (data not shown). Besides the strain variability, the behaviour of yeasts in terms of heat sensitivity, will depend on the wine composition. In respect to SO_2, the levels found in the wines employed in this study (Table 4), which can be considered low in winemaking terms, are not expected to significantly affect the yeast sensitivity to heat.

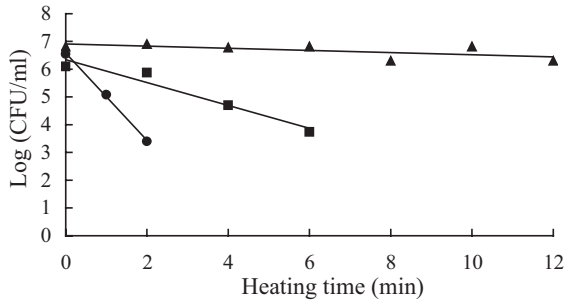

Fig. 3. Thermal inactivation of *D. bruxellensis* PYCC 4801 at 45 °C in tartrate buffer (pH 4.0) (▲) and in tartrate buffer with 500 mg/l ferulic acid (■) and 12% v/v ethanol (●). Relative standard deviation of log CFU never varied more than 12% of the mean value in each time point.

Those yeasts which are of the genus *Dekkera* can, in certain situations, sporulate and, although no specific reference can be found concerning the heat

Table 4
Chemical characterisation of wines

Parameter	Wine	
	Wine A	Wine B
pH	3.51	3.60
Ethanol (%v/v)[a]	12.8	12.0
SO_2 total (mg L^{-1})	32.0	41.6
SO_2 free (mg L^{-1})	9.6	19.2
Total polyphenols index	46.8	43.4

[a] As indicated by the wine producer.

Table 5
Heat treatment regimes for a 6D reduction of yeast populations in wine

Wine A						Wine B					
D. bruxellensis PYCC 4801		*D. anomala* PYCC 5153		Strain 093		*D. bruxellensis* PYCC 4801		*D. anomala* PYCC 5153		Strain 093	
T (°C)	Time (min)	*T* (°C)	Time (min)	*T* (°C)	Time (min)	*T* (°C)	Time (min)	*T* (°C)	Time (min)	*T* (°C)	Time (min)
37.5	4.23	37.5	2.40	37.5	4.8	37.5	6.00	37.5	3.60	37.5	5.40
40.8	0.42	41.2	0.24	41.3	0.48	41.0	0.60	41.8	0.36	41.7	0.54
44.1	0.04	44.9	0.02	45.1	0.05	44.5	0.06	46.1	0.04	45.9	0.05

resistance of these ascospores, these are likely to be more heat resistant than the vegetative forms. It is not certain that this is of relevance in this case as it is generally recognised that the specific conditions which would permit *Dekkera* to sporulate are highly unlikely to occur in the wine environment. It is also important to say that the physiological state of the yeast populations used in this study will probably be different from those naturally occurring in wines and this might influence the relative heat resistances of such populations. However, the approach employed here, of preparing high density cultures in rich medium prior to heat challenge experiments, is standard in microbiology and the *D* and *Z* values published in the literature are largely derived from such experiments.

The release of metabolites (such as volatile phenols) into the wine upon cell death and autolysis when inactivation regimes are applied can be hypothesised; preliminary experiments in our laboratory, however, indicate that the thermal treatments used did not augment the concentration of ethylphenols in wines. Another important practical concern is the impact of the heat treatments on the aromatic and flavour characteristics of wines. It is our opinion that the sensorial effects of such regimes would be more appropriately established on a case by case basis as the expectations of wine quality vary so greatly.

As an illustration, 6D inactivation (99.9%) regimes for *D. bruxellensis* PYCC 4801, *D. anomala* PYCC 5153 and *Dekkera/Brettanomyces* 093 in wines A and B, were determined and are shown in Table 5. These data mean that, taking *D. bruxellensis* PYCC 4801 in wine B as an example, a population of 1 million cells/ml can be inactivated in 6 min at 37.5 °C or in 0.6 min at 41 °C. In conclusion, the results presented here provide useful quantitative data for the conception of effective regimes for the thermal destruction of *Dekkera/Brettanomyces* populations in contaminated wines.

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