

Comparison of the behaviour of *Brettanomyces bruxellensis* strain LAMAP L2480 growing in authentic and synthetic wines

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Abstract *Brettanomyces bruxellensis* is the main microorganism responsible for the production of off-flavours in wine. Studies have been carried out in synthetic cultures using *p*-coumaric acid for the production of vinyl and ethylphenols. The results obtained have been extrapolated to authentic wine, but there is no evidence that this correlation will be correct. We studied the behaviour of *B. bruxellensis* native strain LAMAP L2480 in authentic wine and in a synthetic medium with a chemical composition similar to the authentic wine used in this study (basal synthetic wine + pH, ethanol and hydroxycinnamic acid concentrations of commercial wine). In some assays, *B. bruxellensis* has been studied using media containing 100 mg L⁻¹ *p*-coumaric acid, so we also used the same concentration added to the authentic and synthetic wines. The microorganism showed better growth in authentic wine, regardless of the presence of *p*-coumaric acid. In the case of synthetic wine, the addition of *p*-coumaric acid caused a delay in

yeast growth and an increase in the production of volatile phenols. The coumarate decarboxylase activity did not show any difference regardless of the media and the presence of *p*-coumaric acid. Vinylphenol reductase showed higher activity when a higher concentration of *p*-coumaric acid was added in synthetic wine, but no change was observed in authentic wine.

Keywords *Brettanomyces bruxellensis* · Coumarate decarboxylase activity · Vinylphenol reductase activity · Spoilage yeast · Off-flavours

Introduction

Hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids) are weak acids authentically found in grape must. Due to their chemical nature, these compounds are capable of inhibiting microbial growth through a common mechanism for weak acids (Loureiro 2000; Stratford et al. 2007; Coulon et al. 2010). As hydroxycinnamic acids exhibit antimicrobial properties, microorganisms related to the fermentative process have developed different strategies in order to metabolise these compounds into less toxic ones (Coulon et al. 2010). In the case of yeasts, several species are able to transform hydroxycinnamic acids into volatile-vinyl derivatives through the activity of the cinnamate decarboxylase (CD) enzyme (Loureiro 2000). Only a few species are capable of reducing the vinyl compounds into ethyl derivatives, through a vinylphenol reductase (VR) activity (Chatonnet

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et al. 1992, 1995; Fleet 2003; Suárez et al. 2007; Wedral et al. 2010). Within this group, *Brettanomyces bruxellensis* has been described as the major wine spoilage yeast, due to its ability to produce vinyl and ethyl derivatives, particularly 4-vinylphenol (4VP), 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG) (Dias et al. 2003; Godoy et al. 2009; Harris et al. 2009; Benito et al. 2009). The presence of these compounds in wine, at concentrations above the sensorial threshold (230 and 47 $\mu\text{g L}^{-1}$ for 4EP and 4EG, respectively), is related to off-flavours described as ‘horse sweat’, ‘medicinal or ‘phenolic’ and it results in a decrease in the organoleptic quality of the product (Chatonnet et al. 1992; Suárez et al. 2007). Godoy et al. (2009) showed that when hydroxycinnamic acids are added to the culture media, the growth rate of *B. bruxellensis* is modified and the CD and VR activities increase. A similar observation about the growth of this microorganism has been described by other authors using model culture media (Dias et al. 2003; Benito et al. 2009; Harris et al. 2009, 2010). However, only a few studies have characterised the growth and off flavour production of *B. bruxellensis* in authentic wine (AW) (Grbin and Henschke 2000; Barata et al. 2008; Romano et al. 2008). Beltran et al. (2008) showed that in *Saccharomyces cerevisiae*, the cellular lipid composition and volatile content differed when grown in synthetic or AW. It is therefore important to determine whether the results obtained in synthetic wine (SW) can be extrapolated to AW in the case of *B. bruxellensis*.

In this work, our focus is to study the behaviour of *B. bruxellensis* LAMAP L2480, a native strain, in synthetic and authentic wines with similar chemical compositions, in order to determine whether the data obtained in synthetic medium can be extrapolated to an AW habitat.

Materials and methods

Microorganisms

Brettanomyces bruxellensis LAMAP L2480 was obtained from the Laboratorio de Biotecnología y Microbiología Aplicada (LAMAP) of Universidad de Santiago de Chile, which was isolated from Cabernet Sauvignon wine. The strain was maintained on YPD medium (20 g L^{-1} glucose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 20 g L^{-1} agar and 5 g L^{-1} calcium carbonate) until use.

Culture conditions

Prior to performing SW and AW assays, an adaptation step was carried out (Sturm et al. 2010). *B. bruxellensis* LAMAP L2480 was grown in YPD medium for 10 days at 28 °C. Later, each culture was grown in 10 mL of YPD1 (40 g L^{-1} glucose, 5 g L^{-1} peptone and 5 g L^{-1} yeast extract) supplemented with 6 % (v/v) of ethanol and pH 6 at 28 °C for 48 h. Subsequently, the total volume of the inoculum was added to fresh medium prepared in a 1:1 proportion of SW:YPD2 (20 g L^{-1} glucose, 5 g L^{-1} peptone and 5 g L^{-1} yeast extract, equilibrated to pH 4 with acetic acid) or AW:YPD2, and the culture was incubated for 3 days at 28 °C. Finally, 9 volumes of SW or AW were added to 1 volume of culture, and yeasts were grown until a concentration of 10^8 cells mL^{-1} . A volume from the adapted culture obtained was used to inoculate SW or AW to a final concentration of 10^6 cells mL^{-1} . SW (0.6 g L^{-1} glucose, 1.2 g L^{-1} fructose, 0.3 g L^{-1} trehalose, 2 g L^{-1} yeast extract, 1 g L^{-1} $(\text{NH}_4)_2\text{SO}_4$, 0.4 g L^{-1} MgSO_4 , 2 g L^{-1} KH_2PO_4 , 0.25 mg L^{-1} biotine, 0.0045 mg L^{-1} thiamine and 10 % alcohol, pH 3.7) media were prepared as described by Sturm et al. (2010). In the case of AW, the strain was grown in Cabernet Sauvignon (Central Valley, Chile, 2011) wine. This wine was used because it is the most produced wine in Chile (Wines of Chile 2013), and up to a 5 % of domestic production is lost every year due to this fungus (Saavedra et al. 2005). SW and AW were sterilised by filtration (filter of 0.45 μm in diameter; Pall Corporation, Port Washington, USA), and both wines had a final alcohol concentration of 10 % p/v (Sturm et al. 2010). In the case of SW, ethanol was added, and on the other hand, AW was de-alcoholated by osmotic distillation (Valdes et al. 2009) until 10 % ethanol. Hydroxycinnamic acids were added to both media until the concentration described in the text. The cultures were maintained at 28 °C for 10 d.

Growth kinetics

Growth of *B. bruxellensis* LAMAP L2480 in each culture medium was determined in a Neubauer chamber (Precicolor, HBG, Germany) each 24 h. The specific growth rates (μ) and generation time (t_g) were calculated by least-squared fitting of the linear part of the authentic logarithmic growth plot.

Chemical composition of commercial wine and volatile phenol production

Ethanol, *p*-coumaric acid and ferulic acid concentrations were obtained by the method described by Ross et al. (2009). The pH was determined using a pH metre (HI 2221 Calibration Check Ph/ORP Meter, Hanna Instruments, USA). The transformation of *p*-coumaric acid into 4VP and 4EP was determined using the method described by Ross et al. (2009) and adapted by Godoy et al. (2009). Quantification was performed by comparison against an external standard of *p*-coumaric acid (range 0–112 mg L⁻¹), 4VP (range 0–100 mg L⁻¹) and 4EP (range 0–112 mg L⁻¹) at 200 nm.

Enzymatic assays

CD activity and protein extracts were prepared and quantified as indicated by Godoy et al. (2009). VR activity was quantified as follows: in SW, a mixture with 100 µL protein extract, 50 mM phosphate buffer pH 6.0, 1.6 mM NADPH and 12.5 mM 4VP was incubated at 25 °C for 25 min. The reaction was stopped adding 25 mM Tris–HCl and 0.3 % SDS. VR activity was monitored by the decrease in absorbance of NADPH at 340 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 µmol of NADPH per minute. In AW, VR activity was quantified through the production of 4EP determined by HPLC, as described above. In this case, the unit (U) of enzyme activity was defined as 4EP (mg/L) produced per minute.

Statistical analysis

All experiments were performed in triplicate, and the results were analysed using Statgraphics Plus v.5.0 (Manugistics, Inc.). Differences between treatment means were compared using the LSD test at $p < 0.05$.

Results

Growth of *B. bruxellensis* in synthetic wine and authentic wine media

In general, the physiological characterization of the yeast *B. bruxellensis* has been performed in laboratory

medium or wine-like synthetic medium (Dias et al. 2003; Harris et al. 2009; 2010; Benito et al. 2009; Godoy et al. 2009; Ganga et al. 2011). However, Beltran et al. (2008) showed that *S. cerevisiae* growth is different when it is grown in synthetic or authentic wine. Based on this, we did a comparative study on *B. bruxellensis* growth in SW and AW. For this, we first characterized the AW used: Cabernet Sauvignon variety (2009 year) with pH 3.7 and concentration of *p*-coumaric acid, ferulic acid and caffeic acid of 0.3, 0.6 and 4.5 mg L⁻¹, respectively.

A similar composition was established in SW, with 10 % ethanol concentration. In this case, the wines were named AW0 (authentic wine) and SW0 (synthetic wine). On the other hand, to allow a comparison of our results with other authors (Dias et al. 2003; Barata et al. 2008) who used synthetic media with 100 mg L⁻¹ of *p*-coumaric acid, we added this concentration to both wines (parallel assays) named AW1 and SW1. *P*-coumaric acid delayed the growth in *Brettanomyces anomalus*, while in different isolates of *B. bruxellensis* studied, an increase of velocity was detected (Barata et al. 2008; Godoy et al. 2009). In our case, the growth of *B. bruxellensis* was different in SW and AW independent of the presence of high concentrations of *p*-coumaric acid, showing a better growth in AW (Fig. 1). The yeast presented a lag phase of 24 h in authentic wine (AW0 and AW1), while in synthetic wine (SW0 and SW1), a longer lag phase was observed (48 h) (Table 1). The addition of

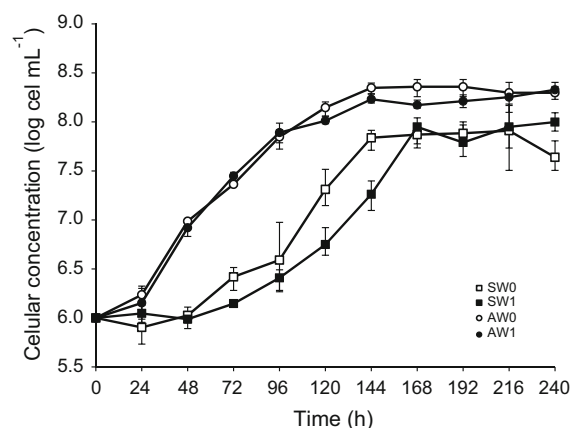


Fig. 1 Growth of *B. bruxellensis* LAMAP L2480 in synthetic wine (squares) and authentic wine (circles). Basal (empty symbols) or supplemented (filled symbols) with 100 mg L⁻¹ *p*-coumaric acid. Data are mean \pm SD of three independent experiments

Table 1 Growth rates of *B. bruxellensis* LAMAP L2480 in synthetic (SW) and authentic wines (AW); basal media (0) and supplemented (1) with 100 mg L⁻¹ *p*-coumaric acid

Condition	Lag phase (h)	Log phase (h)	Specific growth rate (μ h ⁻¹)	Generational time (h)
SW0	48 ^a	144 ^b	0.043 \pm 0.003 ^b	16.05 \pm 1.10 ^a
SW1	48 ^a	168 ^a	0.037 \pm 0.004 ^a	18.85 \pm 1.77 ^b
AW0	24 ^b	144 ^b	0.040 \pm 0.001 ^{ab}	17.27 \pm 0.45 ^{abc}
AW1	24 ^b	144 ^b	0.039 \pm 0.001 ^{ab}	17.79 \pm 0.58 ^{bc}

Means of the same column with the same superscript letters are not significantly different ($p < 0.05$)

p-coumaric acid delayed the stationary phase in SW1 until 168 h, concomitant with a decreased growth rate (0.037 h⁻¹) and a higher generational time (18.85 h) in comparison with SW0 (Table 1).

Volatile phenol production

B. bruxellensis is able to metabolise *p*-coumaric acid to 4VP and 4EP (Chatonnet et al. 1997). To study the effect on 4VP and 4EP production in the culture medium, 100 mg L⁻¹ *p*-coumaric acid has been used (Dias et al. 2003; Godoy et al. 2009). In order to establish if differences exist in the production of phenolic derivatives by *B. bruxellensis* LAMAP L2480 in SW and AW, a similar concentration of *p*-coumaric acid was used. The concentration of this acid and 4EP was determined in different growth phases in SW1 and AW1 (Fig. 2). In both media, *B. bruxellensis* showed a similar consumption of *p*-coumaric acid in lag and log phases; however, the major uptake of *p*-coumaric acid observed was at the stationary phase (Fig. 2). Total consumption of *p*-coumaric acid in SW1 and AW1 was 97 and 49 % of the initial concentration, respectively (Fig. 2a, b). A trace amount of 4VP was detected for a short time during the stationary phase in AW1 with a maximum content of 9.4 mg L⁻¹ (data not shown). In the case of 4EP, the maximum production was detected in the stationary phase in both media but the final content was 64.1 mg L⁻¹ in SW1, 5.3 times higher than in AW1 (12.1 mg L⁻¹) (Fig. 2). Interestingly, the major accumulation of 4EP was observed in SW (Fig. 2).

CD and VR enzymatic activities

Some authors have described an increase in CD and VR activities in the presence of hydroxycinnamic acids (Harris et al. 2009, 2010; Dias et al. 2003; Benito

et al. 2009; Godoy et al. 2009). To compare our results and those obtained by these authors, an analysis of the CD and VR activities of the yeast growth in synthetic and authentic wines was performed, using media with high and low concentrations of *p*-coumaric acid (SW0, SW1, AW0, AW1). This analysis was done in lag, log

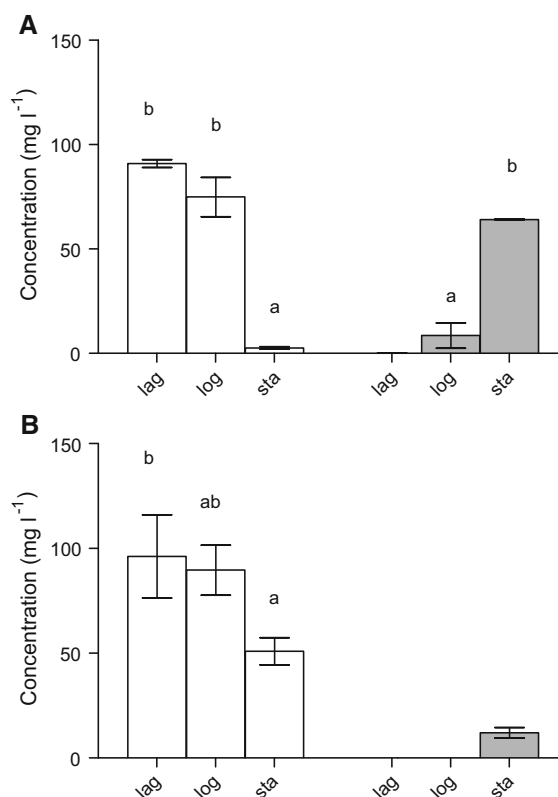
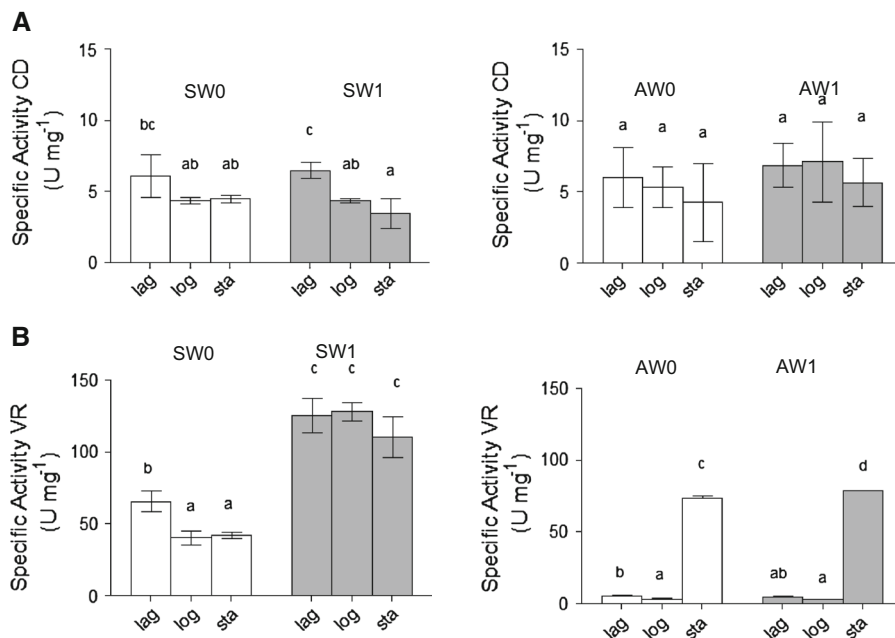


Fig. 2 Quantification of *p*-coumaric (white square) and 4EP (shaded square) during *B. bruxellensis* LAMAP L2480 growth in the lag, exponential (log) and stationary (sta) phases SW1 (a) and AW1 (b). Data are mean \pm SD of three independent measures. The same superscript letters are not significantly different ($p < 0.05$)

Fig. 3 CD activity (a) and VR activity (b) during *B. bruxellensis* LAMAP L2480 growth in synthetic wine (SW) or authentic wine (AW) in the lag, exponential (log) and stationary (sta) phases. Basal (white square) or supplemented (shaded square) wine with 100 mg L⁻¹ of *p*-coumaric. Data are mean ± SD of three independent determinations. Each graphic was analysed by separated. The same superscript letters are not significantly different (*p* < 0.05)



and stationary phases (Fig. 3a). In the case of SW0 and SW1, the higher average activity was observed in the lag phase (6 U mg⁻¹, approximately), decreasing in the log and stationary phases. This may result from a basal concentration of hydroxycinnamic acids (0.3 mg L⁻¹) present in SW0, which would be sufficient to induce the expression of this activity in the yeast. In the case of authentic wine, CD activity did not show any differences between the phases in AW0 and AW1 (Fig. 3a). It is possible that the complex composition of AW affects the expression of CD activity.

In the case of the initial VR activity, this activity was determined by different VR methods: in SW by quantification of NADPH (Godoy et al. 2009) while in AW by quantification of 4EP (Ross et al. 2009). This was mainly due to the amount of proteins obtained from yeast extract, those grown in SW was higher than that obtained in AW. While the concentration of yeasts recovered at the end of the process was similar in both wines used, the extraction of cell extract protein was lower in those grown in AW, which may be due to the composition of the cell wall of yeast changes according the medium used. Therefore, the VR activity was compared within the same culture medium (synthetic or authentic) (Fig. 3b). In SW0, the VR activity detected was 65.7 U mg⁻¹ during Lag phase, while in SW1, the activity was promoted up to 125, 128 and

110 U mg⁻¹ in the lag, log and stationary phases, respectively (Fig. 3b). In the case of AW, there was a mild increase in VR activity during stationary phase in the presence of *p*-coumaric acid (74 and 79 U mg⁻¹ in AW0 and AW1, respectively) (Fig. 3b).

Discussion

The metabolism of hydroxycinnamic acids in *B. bruxellensis* is very important in winemaking. In general, the volatile phenols’ production is associated with yeast tolerance to toxic phenolic compounds, relating the antimicrobial effects of *p*-coumaric acid to an increase of the lag phase and a decrease of growth rate (Baranowski et al. 1980; Edlin et al. 1995; Chambel et al. 1999; Harris et al. 2010). In this work, we observed that the lag phase was longer in SW than authentic wine (SW0 and AW0) (Table 1). *B. bruxellensis* LAMAP L2480 was obtained from wine; thus, its growth would be favoured in this environment (Deak et al. 2007), and this was shown by a better adaptation in AW (Fig. 1). However, *p*-coumaric acid did not affect the growth kinetics of *B. bruxellensis* in AW0 and AW1. An average growth velocity of 0.039 (μ h⁻¹) was maintained with a stationary phase of 144 h and an average generation time of 17.53 h. Deprotonated hydroxycinnamic acids in the cells have two effects, a decrease in

the internal pH (Stratford et al. 2007; Mira et al. 2010; Godoy et al. 2013) resulting in an increased H⁺-ATPase activity liberating H⁺ to the exterior of the cell, and the need to decarboxylate the anions produced through a metabolic pathway (Chambel et al. 1999; Coulon et al. 2010). Therefore, the profile of volatile phenols and CD and VR activity was investigated. The decrease of *p*-coumaric observed was related with the accumulation of 4EP (Fig. 2). The maximum content of 4EP was noticed in the stationary phase (64 mg L⁻¹) in SW1 (Fig. 2a), and similar results were found in previous works reaching 40–70 from 100 mg L⁻¹ of *p*-coumaric acid (Dias et al. 2003). Otherwise, Barata et al. (2008) determined that in AW, the production of 4EP starts with the growth of *B. bruxellensis* and increases during the exponential phase. However, in this work, 4EP was detected in traces in both the *lag* and *log* phases, reaching the higher concentration in the stationary phase (Fig. 2). Interestingly, the major accumulation of 4EP was observed in SW (Fig. 2a). Petrozziello et al. (2014) showed the presence of polyphenols in the medium produced a significant decrease in the volatilities of 4EF and 4EG. In our case, there were no polyphenols in the SW, but there are in AW, which could be the cause of our observation. Moreover, it is known that different factors exist which affect the reduction of *p*-coumaric acid and the formation of 4EP (Salameh et al. 2008; Benito et al. 2009). An example is the condensation between vinylphenol and anthocyanins, improving wine properties and preventing the formation of ethylphenol (Morata et al. 2005). Enzyme activities may act differently depending on strain varieties and genetic differences (Godoy et al. 2009).

It is known that enzymatic activity increases with *p*-coumaric in *B. bruxellensis* (Godoy et al. 2008; Harris et al. 2009), the same as it was reported in other microorganisms such as *Bacillus pumilus*, *Lactobacillus plantarum* or *Dekkera anomala* (Degrossi et al. 1995; Edlin et al. 1998). In this study, CD activity did not show differences independent of the media and the concentration of *p*-coumaric (Fig. 3a). It could be due to the basal concentration of hydroxycinnamic acids (0.3 mg L⁻¹) present in SW0 and AW0. However, the profile of VR activity was different in SW and AW (Fig. 3b). Moreover, the addition of *p*-coumaric acid into the media only promoted the activity in SW1 (50 %), and any change was detected in AW1 (Fig. 3b). We observed that the increase of VR activity in SW1 could be well correlated with the

high content of 4EP, while in AW1, the 4EP accumulation was lower compared with VR activity (Figs. 2, 3b). At present, any data of VR activity have been reported in protein extracts from AW, and some authors have described that VR activity of *B. bruxellensis* is induced by 4VP in culture media (Godoy et al. 2009; Harris et al. 2009). Moreover, as mentioned above, the volatile 4VP is able to react with other components such as anthocyanin present in wine (Benito et al. 2009). Taken together, it suggests that it could be an important loss of 4VP in AW1, leading to less VR activity and therefore less accumulation of 4EP.

In conclusion, it is necessary to carry out more studies of wine yeasts in authentic grape must in order to understand the real fermentation process.

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