International Journal of Food Microbiology 206 (2015) 51–55

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Relation between coumarate decarboxylase and vinylphenol reductase activity with regard to the production of volatile phenols by native *Dekkera bruxellensis* strains under 'wine-like' conditions

M.E. Sturm [a], M. Assof [a], M. Fanzone [a], C. Martinez [b], M.A. Ganga [b], V. Jofré [a], M.L. Ramirez [c,d], M. Combina [a,d,*]

[a] Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (EEA Mza INTA), San Martín 3853, 5507 Luján de Cuyo, Mendoza, Argentina
[b] Departamento de Ciencia y Tecnología de los Alimentos, Facultad Tecnológica, Universidad de Santiago de Chile, Alameda 3363, Santiago, Chile
[c] Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional 36, Km 601, Córdoba, Argentina
[d] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917 (C1033AAJ), Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 1 April 2015
Accepted 17 April 2015
Available online 23 April 2015

Keywords:
Dekkera bruxellensis
Volatile phenols
Enzymatic activities
Wine-like conditions

ABSTRACT

Dekkera/Brettanomyces bruxellensis is considered a major cause of wine spoilage, and 4-ethylphenol and 4-ethylguaiacol are the most abundant off-aromas produced by this species. They are produced by decarboxylation of the corresponding hydroxycinnamic acids (HCAs), followed by a reduction of the intermediate 4-vinylphenols. The aim of the present study was to examine coumarate decarboxylase (CD) and vinylphenol reductase (VR) enzyme activities in 5 native *D. bruxellensis* strains and determine their relation with the production of ethylphenols under 'wine-like' conditions. In addition, biomass, cell culturability, carbon source utilization and organic acids were monitored during 60 days. All strains assayed turned out to have both enzyme activities. No significant differences were found in CD activity, whilst VR activity was variable among the strains. Growth of *D. bruxellensis* under 'wine-like' conditions showed two growth phases. Sugars were completely consumed during the first growth phase. Transformation of HCAs into ethylphenols also occurred during active growth of the yeast. No statistical differences were observed in volatile phenol levels produced by the strains growing under 'wine-like' conditions, independently of the enzyme activity previously recorded. Furthermore, our results demonstrate a relationship between the physiological state of *D. bruxellensis* and its ability to produce ethylphenols. Inhibition of growth of *D. bruxellensis* in wine seems to be the most efficient way to avoid ethylphenol production and the consequent loss of wine quality.

1. Introduction

Wine spoilage is a serious problem for the wine industry because it renders the product unacceptable and can lead to huge economic losses (Oelofse et al., 2008). *Dekkera/Brettanomyces bruxellensis* has long been recognized as a common contaminant in wine (Chatonnet et al., 1992). Wine spoilage by *D. bruxellensis* has been associated with production of volatile acidity and phenolic off-flavours described as horse sweat, band aid, barnyard and burnt plastic (Loureiro and Malfeito-Ferreira, 2003; Oelofse et al., 2008). Formation of 4-ethylphenol and 4-ethylguaiacol occurs after metabolism of hydroxycinnamic acids (HCAs), *p*-coumaric and ferulic acid, respectively. Conversion of HCA precursors occurs in a two-step pathway. Initially, a decarboxylase enzyme yields a 4-vinyl derivative, which is then reduced to form the corresponding 4-ethyl derivative (Edlin et al., 1995). Coumarate decarboxylase and vinylphenol reductase were purified, characterised and partially sequenced in *D. bruxellensis* (Harris et al., 2009). Activities of these enzymes examined in culture media have shown to be strain-dependent within *D. bruxellensis* (Godoy et al., 2009; Sangorrín et al., 2013).

Various physicochemical factors have been shown to affect spoilage by *D. bruxellensis* in a given wine. Some of the wine factors/constituents (e.g. sulphur dioxide, ethanol, pH, sugar and oxygen concentration) have been reported to play a key role in cell growth and enzyme stability, and consequently, in the production of 4-ethylphenol (Dias et al., 2003; Godoy et al., 2008; Sturm et al., 2014).

The overall information about the growth physiology of *D. bruxellensis* and metabolite production appears to be sometimes contradictory. Some authors concluded that production of ethylphenols was intrinsically related to *D. bruxellensis* growth (Barata et al., 2008b; Dias et al., 2003; Vigentini et al., 2008), whereas other studies suggest the existence of a sulphite-induced viable but non-culturable subpopulation, which is able to produce vinylphenols and ethylphenols (Agnolucci et al., 2010; Laforgue and Lonvaud-Funel, 2012; Serpaggi et al., 2012). Previous studies arrived at conclusions using different assay conditions, which are often far from vinification conditions (e.g. complex media

* Corresponding author at: Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (EEA Mza INTA), San Martín 3853, 5507 Luján de Cuyo, Mendoza, Argentina. Tel.: +54 261 4963020x295.
E-mail address: combina.mariana@inta.gob.ar (M. Combina).

http://dx.doi.org/10.1016/j.ijfoodmicro.2015.04.023
0168-1605/

without ethanol, high glucose content, high concentration of HCA precursors, and elevated pH). This latter establishes a controversy regarding previous publications on the field, and evidences the need of further studies designed with controlled and reproducible 'wine-like' conditions that can shed light on the topic.

The aim of the present study was to establish a relationship between the coumarate decarboxylase and vinylphenol reductase activity in 5 native *D. bruxellensis* strains and their potential to produce ethylphenol under 'wine-like' conditions. Moreover, in order to determine at what stage ethylphenols are synthesised during *D. bruxellensis* growth; biomass, cell culturability, carbon source utilization and production of vinyl/ethylphenols and organic acids were monitored during 60 days in 'wine-like' conditions.

2. Materials and methods

2.1. Yeast strains

The 5 yeast strains used in the present study (VC20, B11, PM14, LA17 and CH29) were isolated from contaminated red wines from different Argentine wineries. They were identified as *D. bruxellensis* by sequencing of the D1/D2 domain of the 26S ribosomal gene and deposited in the Culture Collection of the INTA Oenological Research Centre, Mendoza, Argentina (GenBank accession numbers: KM236195, KM236196, KM236197, KM236198, KF002710).

2.2. Enzymatic assays

D. bruxellensis strains were grown at a concentration of 10^8 cells/mL in liquid medium (YNB 6.7 g/L, glucose 20 g/L, ethanol 5% v/v and *p*-coumaric acid 0.6 mM). Detection of coumarate decarboxylase (CD) activity was carried out according to Godoy et al. (2008). One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 μmol of *p*-coumaric acid per min. Detection of vinylphenol reductase (VR) activity was carried out according to Godoy et al. (2009). One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 μmol of NADPH per min. After the reaction had finished, HCAs and volatile phenols in the media (YNB) were analysed by HPLC and GC, respectively, as described below.

2.3. Growth media and 'wine-like' conditions

2.3.1. Pre-culture growth media

Strains were aerobically grown during 2 days at 28 °C in 20 mL of YPD medium (glucose 40 g/L, yeast extract 5 g/L, peptone 5 g/L) supplemented with 6% ethanol and adjusted to pH 6.0 with 1 M HCl. In order to obtain a better adaptation to the test conditions, the strains were transferred to 40 mL of YPD medium with glucose concentration halved (glucose 20 g/L) adjusted to pH 4.0 with 1 M HCl and supplemented with 50% of red wine (pH 4.0, ethanol content 14.4% v/v and without sulphur dioxide) (Sturm et al., 2010). Yeasts were incubated for 3 days at 28 °C without shaking. Growth was monitored by measuring the optical density (OD) at 640 nm until the population reached the highest density ($5 \pm 1 \times 10^7$ CFU/mL), which was just at the end of the exponential growth phase.

2.3.2. Chemically defined wine medium

The chemically defined wine medium was formulated so as to reproduce important characteristics of red wine: glucose 0.6 g/L, fructose 1.2 g/L, trehalose 0.3 g/L, yeast extract 2 g/L, $(NH4)_2SO_4$ 1 g/L, $MgSO_4 \cdot 7\ H_2O$ 0.4 g/L, KH_2PO_3 2 g/L, biotin 0.45 mg/L, thiamine 25 mg/L, ethanol 10% v/v, *p*-coumaric acid 5.28 mg/L, ferulic acid 0.66 mg/L. The pH was adjusted to 3.8 with tartaric acid (85% w/v). Ethanol, vitamins and filter-sterilised (0.2 μm pore size cellulose filter) HCA solutions were added to the previously autoclaved base medium in order to make sure that all these components were available in their natural forms (Salameh et al., 2008). This medium includes *p*-coumaric and ferulic acid at a ratio of 8:1 with a final HCA concentration of 6 mg/L. The proportion and concentration of these HCAs represent the average ratio found in red wines (Chatonnet et al., 1992; Hixson et al., 2012).

The assay was carried out in triplicate in 700 mL experimental units during 60 days at 28 °C. Oxygen-limiting conditions were obtained using bottles hermetically sealed with a rubber cap. Experimental units were manually shaken daily; followed by flushing pure nitrogen (Linde S.A., Mendoza, Argentina) in order to avoid oxidative reactions during sampling. Temperature and oxygen conditions used were selected to favour synthesis of volatile phenols under oenological conditions according to Dias et al. (2003) and Curtin et al. (2013). Cultures were inoculated at a concentration of $5.6 \pm 1.7 \times 10^5$ CFU/mL. The chemically defined wine medium without inoculation was used as control.

2.4. Determination of biomass and culturability

D. bruxellensis growth samples were taken every 3–8 days. Biomass was calculated by determination of the OD at 640 nm. Cellular culturability was assayed on WL Nutrient agar (Oxoid Co.) plates and weekly confirmed on *Dekkera* selective culture medium (glucose 10 g/L, yeast extract 10 g/L, agar 15 g/L, chloramphenicol 0.5 g/L, cycloheximide 0.05 g/L, *p*-coumaric acid 0.4 g/L, pH 4.7 adjusted with 1 M HCl, ethanol 6%). Plates were incubated during 7–10 days at 28 °C.

2.5. Analysis of carbon sources, organic acids and hydroxycinnamic acids

Fructose, glucose, trehalose, ethanol, glycerol, acetic acid, succinic acid, citric acid, malic acid and lactic acid concentrations were determined by HPLC using a Shimadzu Prominence HPLC equipment (Shimadzu, USA) equipped with HPX-87H Aminex ion-exclusion column according to Nissen et al. (1997). Hydroxycinnamic acids were analysed using a Perkin-Elmer Series 200 HPLC system equipped with a diode array detector (PerkinElmer, Shelton, CT, USA) according to Fanzone et al. (2012). All individual phenol compounds were confirmed by HPLC-DAD/ESI-MS as described by Monagas et al. (2005). Quantitative determinations were made by using the external standard method with commercial standards of *p*-coumaric acid (501-98-4) and ferulic acid (537-98-4) (Sigma-Aldrich, St. Louis, MO, USA). Calibration curves were obtained by injection of standard solutions under the same assay conditions, and within the range of concentrations observed ($R^2 \geq 0.94$).

2.6. Analysis of vinyl/ethylphenols

Free volatile phenols were determined using headspace solid-phase microextraction (HS-SPME) and gas chromatography/mass spectrometry (GC/MS) according to Sangorrín et al. (2008). Chromatographic experiments were performed using a Varian CP-3800 gas chromatograph with an ion trap mass detector (MS; Saturn 2200). Compounds were detected using selective ion storage (SIS). Standards of 4-ethylphenol (123-07-9), 4-vinylphenol (2628-17-3) (10% w/v solution in propylene glycol) and anisole (100-66-3) (internal standard, I.S.) were supplied by Sigma-Aldrich (Argentina). The efficiency of the conversion of HCAs into ethylphenols was calculated as the ratio of the maximum production of 4-ethylphenol and 4-ethylguaiacol observed in the assays and the theoretical maximum concentration estimated for total conversion of *p*-coumaric and ferulic acid quantified in the medium on day 0 (Dias et al., 2003).

3. Results

Five native *D. bruxellensis* strains were grown in a culture medium supplemented with *p*-coumaric acid or 4-vinylphenol depending on the enzymatic activity to be assayed. The enzymatic activities of CD and VR, and volatile phenol production are shown in Table 1. It can be

Table 1
Quantification of enzymatic activities (CD and VR) and volatile phenol production for *Dekkera bruxellensis* native strains in culture medium.

Strain	CD activity (U/mg)	Final 4-VP (mg/L)	VR activity (U/mg)	Final 4-EP (mg/L)
VC20	25.57 ± 7.17^{a}	47.95 ± 5.09^{c}	12.5 ± 1.2^{a}	38.03 ± 0.14^{a}
B11	29.72 ± 4.63^{a}	11.83 ± 3.04^{a}	7.6 ± 1.7^{a}	63.03 ± 1.14^{c}
PM14	26.94 ± 9.27^{a}	31.63 ± 9.89^{b}	9.6 ± 0.3^{a}	54.71 ± 5.21^{b}
LA17	12.43 ± 0.31^{a}	0^{a}	25.0 ± 0.3^{b}	68.32 ± 0.16^{c}
CH29	19.55 ± 11.28^{a}	8.69 ± 2.48^{a}	21.4 ± 3.0^{b}	63.96 ± 2.38^{c}

CD: coumarate decarboxylase; VR: vinylphenol reductase; 4-VP: 4-vinylphenol; 4-EP: 4-ethylphenol.
Values within a column followed by the same letter are not significantly different according to Duncan's Test ($p < 0.05$).

observed that all five strains showed CD and VR activity in the presence of their respective precursor compound. No statistical difference ($p < 0.05$) was observed for CD activity among the strains studied. In contrast, statistical differences ($p < 0.05$) were detected regarding VR activity, arranging the strains within two groups. The first group contained LA17 and CH29 strains and showed the highest VR activity, whereas the second group contained VC20, B11 and PM14 strains, and showed the lowest VR activity. However, there was no relation between enzymatic activity and the ethylphenol concentrations produced (Table 1).

In order to determine the relation between the VR activity and the potential production of volatile phenols in wine, the same strains were grown under 'wine-like' conditions. All five native *D. bruxellensis* strains were able to grow in the chemically defined wine medium, increasing their populations up to $5.3 \pm 2.5 \times 10^{6}$ CFU/mL during the first 5–10 days before entering the stationary phase. Culturability curves showed two growth phases. The first one was observed within the first 20–25 days followed by a gradual decrease in growth. The strains experienced a secondary growth cycle between 27 and 39 days of the assay. After that, population culturability gradually decreased at different rates depending on the strain. Fig. 1a shows growth curves of 3 native *D. bruxellensis* strains (VC20, B11 and LA17) which are included as representative examples of all the strains assayed.

Meanwhile, the majority of the sugars present in the medium were used in relatively early stages of growth (Fig. 1b). Strains completely consumed glucose in 3–5 days, fructose in 10–12 days and trehalose in 19–20 days, except for strain CH29, which degraded trehalose more slowly than the other strains (data not shown). No change was observed in ethanol, glycerol, acetic acid, citric acid, malic acid and lactic acid concentrations during the 60 days (data not shown), whilst a gradual increase in succinic acid concentrations was evidenced (Fig. 1b).

Under 'wine-like' conditions, all *D. bruxellensis* strains assayed showed a similar behaviour with regard to HCA metabolism. All strains were able to metabolise HCAs producing their respective volatile phenols (Fig. 1c). The strains metabolised between 64 and 83% of the initial concentration of *p*-coumaric acid during the first 10 days, which was strain dependent. As a result, an increase in volatile phenols was observed in the same period, representing 63–77% of the final concentration of 4-ethylphenol. Simultaneously, between 23 and 53% of the initial ferulic acid concentration was metabolised, and as a result 4-ethylguaiacol production was between 48 and 87%. Ferulic acid was used at a lower rate than *p*-coumaric acid (Fig. 1c). The greatest accumulation of ethylphenols was observed during the late exponential and early stationary growth phase, although 4-ethylphenol production started as early as yeast growth began. Synthesis of ethylphenols continued until day 30, but demonstrated a slower accumulation rate. The maximum level of 4-ethylphenol coincided with the period of high yeast culturability (Fig. 1a/c). Reaction intermediates (vinylphenols) were not detected during the assay, which suggests that in these high yeast populations, transformation of HCAs into ethylphenols occurred immediately (Fig. 1c).

No significant differences were observed regarding residual HCAs and final volatile phenol levels among the strains assayed in 'wine-like' conditions, although certain strains had previously shown statistical differences in the VR activities (Table 1). All five strains were able to produce ethylphenol at a concentration exceeding the threshold commonly established for wine off-aromas (Chatonnet et al., 1992). The conversion efficiency of *p*-coumaric acid into 4-ethylphenol ranged from 63 to 67% and was strain-dependent. In contrast, the efficiency of the conversion of ferulic acid into 4-ethylguaiacol was lower than that of *p*-coumaric acid: 28 to 40%.

4. Discussion

Our results showed that none of the five *D. bruxellensis* strains exhibited significant differences in CD activity, whilst VR activity was variable among the strains. This variability in the VR activities was not evidenced under 'wine-like' conditions, in which no statistical differences were found regarding volatile phenol levels produced among the different strains assayed.

Growth of *D. bruxellensis* has been examined under different assay conditions, with varying results (Barata et al., 2008a; Curtin et al., 2013; Dias et al., 2003; Vigentini et al., 2008). As it has been suggested by Vigentini et al. (2008), appropriate experimental conditions are essential to study the metabolism of *D. bruxellensis* during the winemaking process, and 'wine-like' media may be suitable for this purpose. These types of media allow a realistic analysis of the substrates used and the products formed under 'wine-like' conditions. In that sense, the results obtained in our work, could reflect a more realistic scenario, as all conditions have been carefully selected in order to be the closest possible to winemaking environment.

The biphasic growth curve of viable populations of *D. bruxellensis* in 'wine-like' conditions recorded in our study has been previously described by Fugelsang and Zoecklein (2003) and Coulon et al. (2010) in wines. The ability to resume growth after a growth decline detected after plating seems to be typical of *D. bruxellensis* (Barata et al., 2008a). Nevertheless, this behaviour has only been reported after growth of the yeast in wine or 'wine-like' conditions and it also requires a higher incubation time to be observed (more than 30 days).

Some authors analysed the role of sugars (glucose, fructose, galactose and trehalose) on growth and ethylphenol production by *D. bruxellensis* and they concluded that 200 to 275 mg/L of residual sugars contained enough carbon to support the development of this yeast and consequently the production of volatile phenols (Barata et al., 2008b; Chatonnet et al., 1995). In line with this, our results showed that 210 mg/L of sugars (glucose, fructose and trehalose) was enough to allow *D. bruxellensis* growth. Sugars were completely consumed during the first growth phase, whilst consumption of glucose and fructose was faster than trehalose. Only few studies have reported on the kinetics of fructose and trehalose utilization by *D. bruxellensis* in the presence of ethanol and under other stress conditions with varying results. Dias et al. (2003) found that the trehalose intake rate by *D. bruxellensis* was much lower than glucose under oenological conditions. Moreover, Vigentini et al. (2008) found that *D. bruxellensis* used fructose as a preferred carbon source in a synthetic medium with a high concentration of ethanol.

In the current study, all five *D. bruxellensis* strains were able to simultaneously metabolise *p*-coumaric and ferulic acid with production of their respective volatile phenols, being the conversion rate of ferulic acid lower than *p*-coumaric acid. In contrast, Oelofse et al. (2009) suggested that the conversion pathway of ferulic acid as precursor was preferred to *p*-coumaric acid by different *D. bruxellensis* strains grown in wine spiked with similar amounts of both compounds (100 mg/L). This tendency was not observed in our study, mainly because the 'wine-like' media included a lower concentration of HCAs (6 mg/L) with a *p*-coumaric:ferulic acid ratio of 8:1. Moreover, it has been proven that ferulic acid is slightly more toxic to the yeast than *p*-coumaric acid

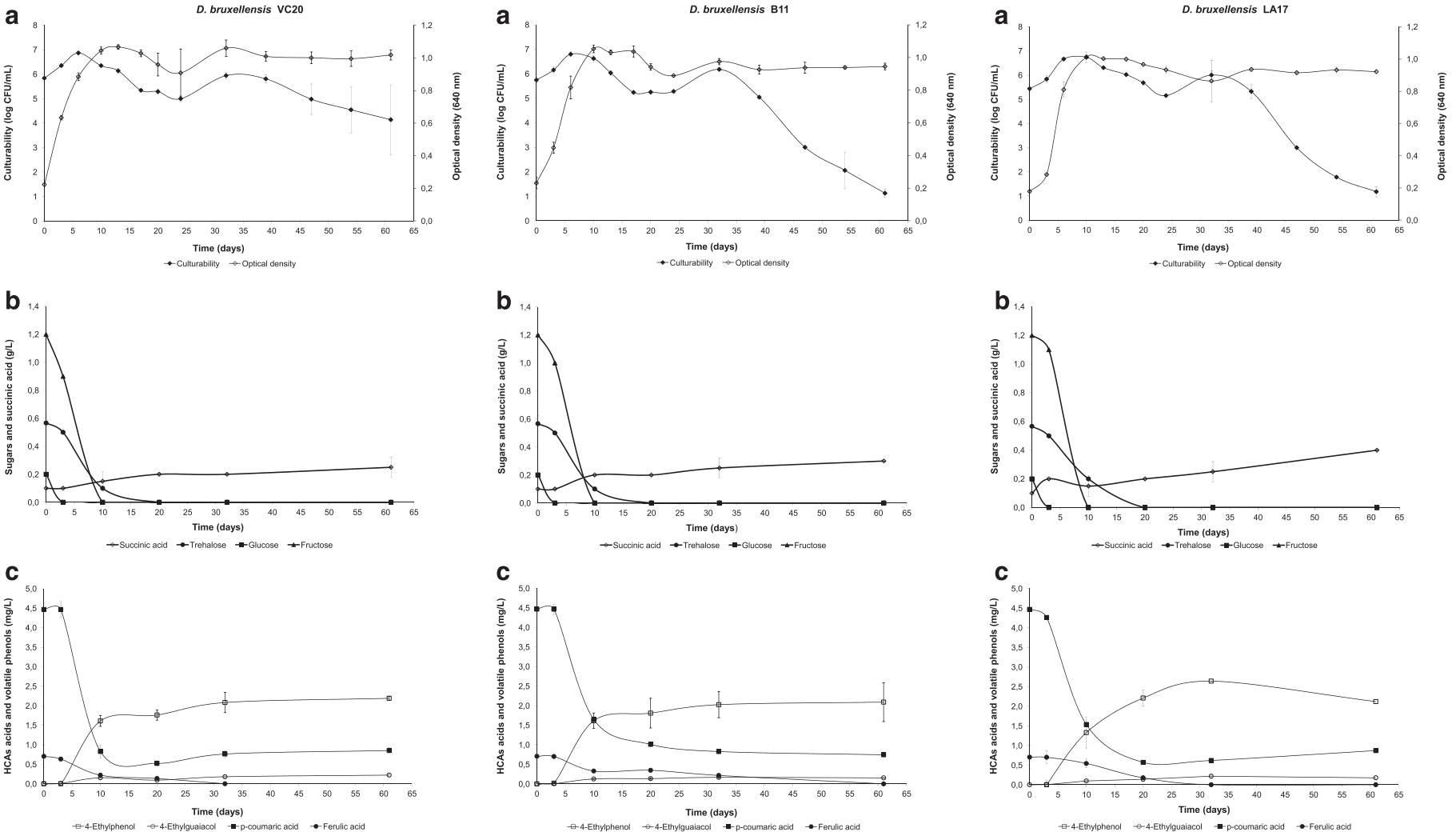

Fig. 1. Three native *D. bruxellensis* strains (VC20, B11 and LA17) monitored during 60 days under 'wine-like' conditions. a) Growth curves through measuring OD_{640} and after cell counts (CFU/mL). b) Sugar consumption and succinic acid production. c) Hydroxycinnamic acid consumption and volatile phenol production.

(Harris et al., 2008), and consequently high ferulic acid levels could lead to different results of those obtained in 'wine-like' conditions. This finding again highlights the importance of carrying out assays using test conditions the closest possible to real conditions in order to obtain results that can be extrapolated, in this particular case, to yeast behaviour in wine.

Our work evidences a relationship between the physiological state of *D. bruxellensis* and its ability to produce ethylphenols. In fact, culturable populations were able to synthesise large quantities of ethylphenols in 'wine-like' conditions. In conclusion, potential spoilage of wine by *D. bruxellensis* was more related to the ability of the strains to develop in the wine environment than the CD and VR enzymatic activity recorded in laboratory conditions. Therefore, the most efficient way to prevent wine spoilage by *D. bruxellensis* should be the control of its development.

Acknowledgements

This research is part of the National PNAIyAV N° 1130032 Project and it is financially supported by the Instituto Nacional de Tecnología Agropecuaria (INTA). M.E. Sturm wants to thank INTA for funds to support her doctoral studies.

References

Agnolucci, M., Rea, F., Sbrana, C., Cristani, C., Fracassetti, D., Tirelli, A., Nuti, M., 2010. Sulphur dioxide affects culturability and volatile phenol production by *Brettanomyces/Dekkera bruxellensis*. Int. J. Food Microbiol. 143, 76–80.

Barata, A., Caldeira, J., Botelheiro, R., Pagliara, D., Malfeito-Ferreira, M., Loureiro, V., 2008a. Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. Int. J. Food Microbiol. 121, 201–207.

Barata, A., Pagliara, D., Piccininno, T., Tarantino, F., Ciardulli, W., Malfeito-Ferreira, M., Loureiro, V., 2008b. The effect of sugar concentration and temperature on growth and volatile phenol production by *Dekkera bruxellensis* in wine. FEMS Yeast Res. 8, 1097–1102.

Chatonnet, P., Dubourdieu, D., Boidron, J., Pons, M., 1992. The origin of ethylphenols in wines. J. Sci. Food Agric. 60, 165–178.

Chatonnet, P., Dubourdieu, D., Boidron, J.N., 1995. The influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. Am. J. Enol. Vitic. 46, 463–468.

Coulon, J., Perello, M.C., Lonvaud-Funel, A., de Revel, G., Renouf, V., 2010. *Brettanomyces bruxellensis* evolution and volatile phenols production in red wines during storage in bottles. J. Appl. Microbiol. 108, 1450–1458.

Curtin, C.D., Langhans, G., Henschke, P.A., Grbin, P.R., 2013. Impact of Australian *Dekkera bruxellensis* strains grown under oxygen-limited conditions on model wine composition and aroma. Food Microbiol. 36, 241–247.

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.

Edlin, D.A.N., Narbad, A., Dickinson, J.R., Lloyd, D., 1995. The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. FEMS Microbiol. Lett. 125, 311–316.

Fanzone, M., Zamora, F., Jofré, V., Assof, M., Gómez-Cordovés, C., Peña-Neira, A., 2012. Phenolic characterisation of red wines from different grape varieties cultivated in Mendoza province (Argentina). J. Sci. Food Agric. 92 (3), 704–718.

Fugelsang, K.C., Zoecklein, B.W., 2003. Population dynamics and effects of *Brettanomyces bruxellensis* strains on Pinot Noir (*Vitis vinifera* L.) wines. Am. J. Enol. Vitic. 54, 294–300.

Godoy, L., Martínez, C., Carrasco, N., Ganga, M.A., 2008. Purification and characterization of a p-coumarate decarboxylase and a vinylphenol reductase from *Brettanomyces bruxellensis*. Int. J. Food Microbiol. 127 (1/2), 6–11.

Godoy, L., Garrido, D., Martínez, C., Saavedra, J., Combina, M., Ganga, M.A., 2009. Study of the coumarate decarboxylase and vinylphenol reductase activities of *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) isolates. Lett. Appl. Microbiol. 48, 452–457.

Harris, V., Ford, C., Jiranek, V., Grbin, P., 2008. *Dekkera* and *Brettanomyces* growth and utilisation of hydroxycinnamic acids in synthetic media. Appl. Microbiol. Biotechnol. 78, 997–1006.

Harris, V., Ford, C., Jiranek, V., Grbin, P., 2009. Survey of enzyme activity responsible for phenolic off-flavour production by *Dekkera* and *Brettanomyces* yeast. Appl. Microbiol. Biotechnol. 81 (6), 1117–1127.

Hixson, J.L., Sleep, N.R., Capone, D.L., Elsey, G.M., Curtin, C.D., Sefton, M.A., Taylor, D.K., 2012. Hydroxycinnamic acid ethyl esters as precursors to ethylphenols in wine. J. Agric. Food Chem. 60, 2293–2298.

Laforgue, R., Lonvaud-Funel, A., 2012. Hydroxycinnamic acid decarboxylase activity of *Brettanomyces bruxellensis* involved in volatile phenol production: relationship with cell viability. Food Microbiol. 32, 230–234.

Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. Review. Int. J. Food Microbiol. 86, 23–50.

Monagas, M., Suarez, R., Gómez-Cordovés, C., Bartolomé, B., 2005. Simultaneous determination of nonanthocyanin phenolic compounds in red wines by HPLC-DAD/ESI-MS. Am. J. Enol. Vitic. 56, 139–147.

Nissen, T., Schulze, U., Nielsen, J., Villadsen, J., 1997. Flux distribution in anaerobic, glucose-limited continuous cultures of *Saccharomyces cerevisiae*. Microbiology 143, 203–218.

Oelofse, A., Pretorius, I.S., du Toit, M., 2008. Significance of *Brettanomyces* and *Dekkera* during winemaking: a synoptic review. S. Afr. J. Enol. Vitic. 29 (2), 128–144.

Oelofse, A., Lonvaud-Funel, A., du Toit, M., 2009. Molecular identification of *Brettanomyces bruxellensis* strains isolated from red wines and volatile phenol production. Food Microbiol. 26 (4), 377–385.

Salameh, D., Brandam, C., Medawar, W., Lteif, R., Strehaiano, P., 2008. Highlight on the problems generated by *p*-coumaric acid analysis in wine fermentations. Food Chem. 107, 1661–1667.

Sangorrín, M.P., Lopes, C.A., Jofré, V., Querol, A., Caballero, A.C., 2008. Spoilage yeasts from Patagonian cellars: characterization and potential biocontrol based on killer interactions. World J. Microbiol. Biotechnol. 24, 945–953.

Sangorrín, M.P., García, V., Lopes, C.A., Sáez, J.S., Martínez, C., Ganga, M.A., 2013. Molecular and physiological comparison of spoilage wine yeasts. J. Appl. Microbiol. 114, 1066–1074.

Serpaggi, V., Remize, F., Recorbet, G., Gaudot-Dumas, E., Sequeira-Le Grand, A., Alexandre, H., 2012. Characterization of the "viable but non culturable" (VBNC) state in the wine spoilage yeast *Brettanomyces*. Food Microbiol. 30, 438–447.

Sturm, M.E., Rojo, M.C., Ciklic, I., Ramirez, M.L., Combina, M., 2010. Development of a model-wine media to evaluate *Dekkera bruxellensis* growth in mimic wine conditions. Biocell 34, 48 (Supplement).

Sturm, M.E., Arroyo-López, F.N., Garrido-Fernández, A., Querol, A., Mercado, L.A., Ramirez, M.L., Combina, M., 2014. Probabilistic model for the spoilage wine yeast *Dekkera bruxellensis* as a function of pH, ethanol and free SO_2 using time as a dummy variable. Int. J. Food Microbiol. 170, 83–90.

Vigentini, I., Romano, A., Compagno, C., Merico, A., Molinari, F., Tirelli, A., 2008. Physiological and oenological traits of different *Dekkera/Brettanomyces bruxellensis* strains under wine-model conditions. FEMS Yeast Res. 8 (7), 1087–1096.