

ORIGINAL ARTICLE

Study of the coumarate decarboxylase and vinylphenol reductase activities of *Dekkera bruxellensis* (anamorph *Brettanomyces bruxellensis*) isolates

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Abstract

Aim: To evaluate the coumarate decarboxylase (CD) and vinylphenol reductase (VR) activities in *Dekkera bruxellensis* isolates and study their relationship to the growth rate, protein profile and random amplified polymorphic DNA (RAPD) molecular pattern.

Methods and Results: CD and VR activities were quantified, as well, the growth rate, intracellular protein profile and molecular analysis (RAPD) were determined in 12 isolates of *D. bruxellensis*. All the isolates studied showed CD activity, but only some showed VR activity. Those isolates with the greatest growth rate did not present a different protein profile from the others. The FASC showed a relationship between RAPD molecular patterns and VR activity.

Conclusion: CD activity is common to all of the *D. bruxellensis* isolates. This was not the case with VR activity, which was detected at a low percentage in the analysed micro-organisms. A correlation was observed between VR activity and the RAPD patterns.

Significance and Impact of the Study: This is the first study that quantifies the CD and VR enzyme activities in *D. bruxellensis*, demonstrating that these activities are not present in all isolates of this yeast.

Introduction

Dekkera bruxellensis has been described as the main spoilage micro-organism in red wines (Loureiro and Malfeito-Ferreira 2003; Suárez *et al.* 2007). Its presence in wines is associated with the detection of phenolic flavours that have a negative impact on the organoleptic characteristics of the wine (Chatonnet *et al.* 1992). As a result, much of the research on *D. bruxellensis* has been focused on the development of detection methods (Rodrigues *et al.* 2001; Cocolin *et al.* 2004) and few works have been carried out on its physiological characterization (Dias *et al.* 2003; Silva *et al.* 2004; Romano *et al.* 2008). Only in recent years have genetic studies been published (Conterno *et al.* 2006; Martorell *et al.* 2006; Curtin *et al.* 2007; Woolfit *et al.* 2007). The pro-

duction of these undesired phenolic compounds by *D. bruxellensis* may be through the metabolization of cinnamic acids found in plant cell walls (Barthelmebs *et al.* 2001). These acids are associated with an antimicrobial function (Stead 1995), such that micro-organisms able to ferment plant products show enzymatic activities that allow metabolizing less toxic compounds. For this, the cinnamic acids are decarboxylated into vinylphenol derivatives by the action of a coumarate decarboxylase (CD) activity and then reduced to an ethyl derivative through a vinylphenol reductase (VR) activity (Chatonnet *et al.* 1992). Some studies have shown differences between *D. bruxellensis* isolates in the production of these phenolic aromas (Conterno *et al.* 2006). However, these differences remain unexplained making it necessary to perform studies to find a possible explanation.

The objective of this study was to evaluate CD and VR activities from a group of isolates of *D. bruxellensis* and analyse a possible relationship between growth rates, intracellular protein profiles and molecular patterns obtained by random amplification of polymorphic DNA (RAPD) analysis.

Materials and Methods

Micro-organisms and growth conditions

Strains of *D. bruxellensis* and *Saccharomyces cerevisiae* were obtained from the collection of the Laboratorio de Biotecnología y Microbiología Aplicada of the Universidad de Santiago de Chile. The origin *D. bruxellensis* strains is: L987, L988, L1359 isolate from Mendoza-Argentina (32·8°S and 68·8°W), CECT-1450 obtained from Spanish Type Culture Collection. The remaining strains were obtained from different regions of Chile: *D. bruxellensis* L1630 from Rengo (34·24°S and 70·51°W), L1687 and L1700 from Pirque (33·38°S and 70·34°W), L1730 and L1750 from Isla de Maipo (33·44°S and 70·53°W), L2099, L2107 and L2108 from Peumo (34·23°S and 71·10°W) and *Saccharomyces cerevisiae* L16 from Cauquenes (35·57°S and 72·19°W). Micro-organisms were maintained in YPD agar (0·5% peptone, 0·5% yeast extract, 4% glucose and 20 g l⁻¹ of agar, pH 6·0) with 5 g l⁻¹ of CaCO₃.

Enzymatic assays

CD activity

CD activity was detected with the method described by Edlin *et al.* (1998). One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 µmol of *p*-coumaric acid min⁻¹ at 40°C.

VR activity

VR activity was detected with the method described by Kato *et al.* (1991). One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 µmol of NADPH min⁻¹ at 25°C.

In both the cases the intracellular protein extract of *S. cerevisiae* L16, obtained as described next, was used as the control.

Intracellular protein extract and SDS-PAGE

Dekkera bruxellensis was grown in yeast nitrogen base medium with amino acids (YNB) (Difco), 20 g l⁻¹ of glucose, 50 ml l⁻¹ of ethanol and 0·6 mmol l⁻¹ of *p*-coumaric acid, and was maintained at 28°C for 72 h. Subsequently, it was centrifuged at 12 000 g for 10 min at 4°C and the pellet was subjected to enzymatic lysis (Sambrook *et al.*

1989). The proteins were quantified using the method described by Bradford (1976) using a calibration curve with bovine serum albumin (BSA; Winkler, Chile) as standard. The protein profile was performed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 5% stacking gel and 12% resolving gel; Sambrook *et al.* 1989). The protein gels were stained with silver stain (Switer *et al.* 1979).

Growth kinetics

The *D. bruxellensis* isolates were inoculated in YPD medium at 28°C for 24 h, and then 1 × 10⁶ cells were inoculated in 200 ml of YNB with amino acids, 20 g l⁻¹ of glucose and 50 ml l⁻¹ of ethanol. The culture conditions were 28°C for 96 h and agitation at 140 rev min⁻¹ (Multitron, Infors AG, Switzerland). Growth was determined by measuring absorbance at 640 nm. Parallel to this, other cell cultures were grown in the aforementioned conditions with the addition of 0·6 mmol l⁻¹ of *p*-coumaric acid. The specific growth rates (µ) were calculated by least-squared fitting to the linear part of the natural logarithmic growth plot.

DNA extraction

Genomic DNA extraction was performed using the Wizard[®] Genomic DNA Purification Kit (Promega, USA).

RAPD-PCR

RAPD analyses were performed using 11 primers from the OPA series (OPAR-14, OPAE-12, OPAS-11, OPAE-15, OPAS-04, OPAR-20, OPAD-08, OPAE-09, OPAS-05, OPAR-01, OPAR-08; Operon Technologies, CA, USA). The polymerase chain reaction (PCR) was performed using the Light Cycler Fast Start DNA Master SYBR Green I Kit (Roche), in a final volume of 20 µl, containing 3 mmol l⁻¹ of MgCl₂, 0·5 µmol l⁻¹ of each primer and 20 ng of DNA, in accordance with the instructions of the supplier. The PCR programme was denaturation at 95°C for 10 min, 40 cycles of amplification with denaturation of 0 s at 95°C, annealing at 35°C or 40°C for 5 s, depending on the melting temperature of the primer and an extension of 72°C for 80 s. PCR products were visualized by electrophoresis using a 1·4% agarose gel. The size of the bands was detected using Quantity One software (Bio-Rad, USA).

Statistical analyses

All experiments were performed in duplicate. Differences between treatment means were compared using the

Duncan's test at 95% confidence level. For the RAPD-PCR data analysis, the cluster analysis was performed by Dice Coefficient and UPGMA using FreeTree and Tree-View softwares. Re-sampling was performed using bootstrap with 100 iterations.

Factorial analysis of simple correspondence (FASC)

Results from the molecular methods were summarized in three two-way tables: matrix 1 consists of 24 rows (2×12 isolates of *D. bruxellensis*) and 237 columns (CD and VR activities and for each DNA fragment obtained by the RAPD technique); matrix 2 consists of 24 rows (2×12 isolates of *D. bruxellensis*) and 68 columns (one for each protein band from the protein profile detected by SDS-PAGE); and matrix 3 consists of matrixes 1 and 2 (for the total variance explanation improvement). Subsequently, a FASC was performed on matrixes 1, 2 and 3 using symmetrical and row principal normalization criteria. FASC plot is basically a multidimensional map indicating relative distance between isolates and CD-VR activities. This plot shows how the CD-VR activities (variables) influence the clustering of isolates around particular groups. The analysis conforms homogenous groups from eventual similarities (or differences) of the isolates/CD-VR activities (Greenacre 1984). All these analyses were performed using the SPSS 10.0 (SPSS, Inc, Illinois, USA) and Statgraphics Plus 5.0 (Manugistic, Inc.) software.

Results

Quantification of the CD and VR activities

Dekkera bruxellensis produces 4-ethylphenol by the action of CD and VR activities (Chatonnet *et al.* 1992). To determine if both enzymatic activities would be affected by the presence of *p*-coumaric acid in the growth media, the isolates were grown in the presence or absence of this cinnamic acid (Table 1). We observed that all the isolates presented CD activity whether they were grown in the presence or absence of *p*-coumaric acid. VR activity was only detected in 4 of the 12 isolates analysed. Both enzymatic activities increased when the protein extracts were obtained from cultures grown in the presence of *p*-coumaric acid. When the quantification of the CD activity in the protein extracts obtained from microorganisms grown with *p*-coumaric acid were compared, six groups ($P < 0.05$) were obtained. In the order of increasing levels of activity were L2108-L1630, CECT 1451-L1700, L2107-L1750-L1687-L987, L1730, L988 and L1359-L2099. No statistical difference was observed for CD activity in the *S. cerevisiae* yeast, used as control, when the micro-organ-

Table 1 Coumarate decarboxylase (CD) and vinylphenol reductase (VR) activities of *Dekkera bruxellensis* isolates grown in presence or absence of *p*-coumaric acid

Isolated	Culture media			
	Without <i>p</i> -coumaric acid		With <i>p</i> -coumaric acid	
	CD activity (U mg ⁻¹)	VR activity (U mg ⁻¹)	CD activity (U mg ⁻¹)	VR activity (U mg ⁻¹)
<i>Saccharomyces cerevisiae</i>				
L16	16.5 ^c	n.d.	18.8 ^{ft}	n.d.
<i>D. bruxellensis</i>				
L2108	0.1 ^a	n.d.	2.5 ^a	n.d.
L1630	0.3 ^a	n.d.	3.3 ^a	n.d.
CECT 1451	0.3 ^a	14.7 ^b	3.1 ^b	139.9 ^b
L1700	1.2 ^{ab}	n.d.	7.1 ^b	n.d.
L2107	1.5 ^{ab}	15.1 ^b	10.7 ^c	152.6 ^c
L1750	0.8 ^a	23.9 ^c	11.1 ^c	275.4 ^d
L1687	1.1 ^{ab}	n.d.	11.6 ^c	n.d.
L987	1.2 ^{ab}	n.d.	12.3 ^{cd}	n.d.
L1730	4.0 ^b	n.d.	13.9 ^d	n.d.
L988	1.6 ^{ab}	0.4 ^a	15.7 ^e	3.7 ^a
L1359	1.1 ^{ab}	n.d.	18.9 ^f	n.d.
L2099	2.1 ^{ab}	n.d.	19.6 ^f	n.d.

n.d., not detected

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

*Means of the same enzymatic activity are not significantly different ($P < 0.05$).

ism was grown in the presence or absence of *p*-coumaric acid. VR activity was not detected in this yeast.

Specific growth rate and SDS-PAGE

The isolates L987, L988, L1359, L1687, L1700, L1730, L1750, L2099 and CECT1451 show a statistically significant increase ($P < 0.05$) on their specific growth rates when this value is compared with those obtained in the absence of *p*-coumaric acid (Table 2). The growth rate of these isolates increased by between 108% and 170%, with the isolate L1730 being the one showing the greatest positive effect. The isolates L2107 and L1630 showed a decrease in their growth in the presence of cinnamic acid. The isolate L2108 showed no statistical difference. Figure 1 shows that of the 12 isolates studied, 9 have a similar protein profile. In the last four, dominant bands of 37, 34, 23 and 21 kDa approximately are observed.

Molecular characterization

The UPGMA cluster analysis shows that there is no relationship between the isolates that had both enzymatic activities and their RAPD patterns. A similar observation was performed when comparing the growth rate with

Table 2 Growth rates of *Dekkera bruxellensis* isolates in the presence or absence of *p*-coumaric acid

Isolate	Specific growth rate μ (h^{-1})	
	Culture media without <i>p</i> -coumaric acid	Culture media with <i>p</i> -coumaric acid
L2107	0.032 ^{ab}	0.028 ^{a*}
L2108	0.032 ^{ab}	0.033 ^b
L1630	0.034 ^{ab}	0.026 ^{a*}
L1730	0.033 ^{ab}	0.056 ^{e*}
L2099	0.034 ^b	0.038 ^{bc*}
CECT 1451	0.035 ^b	0.039 ^{c*}
L1700	0.031 ^a	0.050 ^{d*}
L988	0.046 ^c	0.054 ^{de*}
L987	0.056 ^e	0.075 ^{g*}
L1750	0.062 ^f	0.066 ^{f*}
L1359	0.052 ^d	0.068 ^{f*}
L1687	0.071 ^g	0.085 ^{h*}

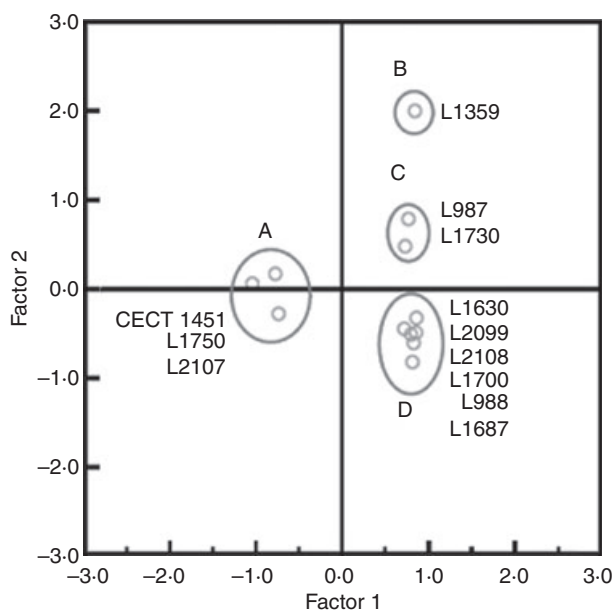
Means of the same column with the same superscript letters are not significantly different ($P < 0.05$).

*Means of the same row are significantly different ($P < 0.05$).

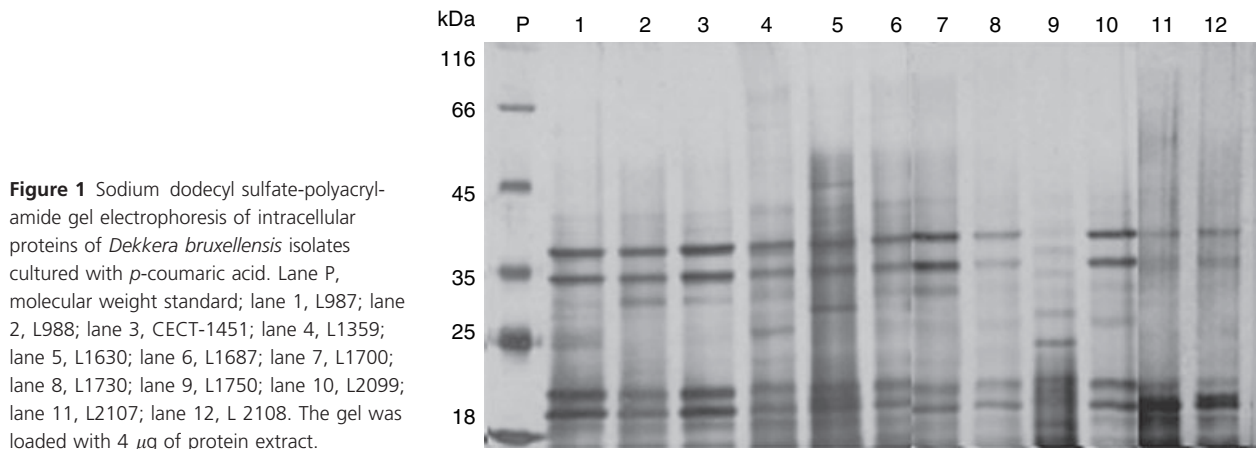
molecular patterns in the presence or absence of *p*-coumaric acid. The FASC was performed on the three matrices, selecting two principal factors according to the maximum percentages of inertia, composed of each of the first successive factors: 86.5% in matrix 1, 64.2% in matrix 2 and 67.9% in matrix 3. By the maximum inertia explanation, only matrix 1 was considered in the following analyses. These results suggest that RAPD-PCR provides a better explanation for the clusters conformed by the 12 isolates. Figure 2 shows the distribution of the tendency of the points, obtaining four characteristic groups: A, B, C and D.

Discussion

In this work, CD and VR activities were quantified in the intracellular protein extracts of 12 isolates of *D. bruxellen-*

**Figure 2** Factorial analysis of simple correspondence performed on data from matrix 1.

sis. The results showed that all the isolates possessed CD activity, while VR activity does not seem to be common among them. Both enzymatic activities increased when the micro-organism was grown with *p*-coumaric acid. The isolates that presented VR activity had variable CD activity, suggesting that they are not closely related, i.e. those isolates that have high CD activity also have high VR activity and vice versa. In the case of the *S. cerevisiae* isolate studied, we only detected CD activity and did not detect significant differences of this activity when it was grown with or without *p*-coumaric acid. Chatonnet *et al.* (1992) described that *S. cerevisiae* is only capable of producing 4-vinylphenol from *p*-coumaric acid. We believe that those isolates that had both enzymatic activities

**Figure 1** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of intracellular proteins of *Dekkera bruxellensis* isolates cultured with *p*-coumaric acid. Lane P, molecular weight standard; lane 1, L987; lane 2, L988; lane 3, CECT-1451; lane 4, L1359; lane 5, L1630; lane 6, L1687; lane 7, L1700; lane 8, L1730; lane 9, L1750; lane 10, L2099; lane 11, L2107; lane 12, L2108. The gel was loaded with 4 μg of protein extract.

would rapidly metabolize *p*-coumaric acid, transforming it into a less toxic compound. Thus, this isolate might grow more rapidly when cultivated with cinnamic acid. However, this relationship was not observed. This result suggests that there may be other enzymes involved in the metabolization of cinnamic acids or that this adaptability depends on possible changes in the plasmatic membrane that could regulate the permeability to these cinnamic acids (Piper *et al.* 1998).

Edlin *et al.* (1995) shown that in the case of *Dekkera anomalous*, the presence of *p*-coumaric in the culture media resulted in a decrease in the growth rate of 30%. In our case, the presence of this cinnamic acid in the culture media generated a positive effect on the growth rate of some isolates. The difference in the results could be because of the yeast species studied and the concentration of *p*-coumaric acid used. Likewise, we observed that the increase in the growth rate in the culture media in the presence of *p*-coumaric acid is not a common characteristic among the isolates.

We did not observe a relationship between protein profiles and the micro-organisms with the greatest growth rate when grown in the presence of *p*-coumaric acid. Similar results were obtained when relating the protein profile and the enzymatic activities studied. When the protein profiles of the isolates with VR activity were analysed, it was observed that they shared a common protein profile, except isolate L1750. A similar observation was carried out when the protein profiles of the isolates with VR activity (CECT-1451, L2107, L1750 and L988) were compared with those without this activity.

The detection of 4-ethylphenol in wine has been used as a method of identification of the presence of *Dekkera* sp. (Pollnitz *et al.* 2000). Conterno *et al.* (2006) analysed several isolates from different geographic areas worldwide and showed that some isolates can produce large amounts of this compound, while others have little or no production. Based on our results, not all *D. bruxellensis* isolates present both enzymatic activities that have been proposed in the production of 4-ethylphenol. As we did not find any apparent relationship between the RAPD analysis and the growth rate or enzymatic activities, an FASC was performed (Fig. 2). The apparent associations among different strains, relating CD and VR activities with the molecular profiles were evident. The relationship of both factors (F1 and F2 being orthogonal to each other) allowed us to affirm in a practical way that every factor provides a different meaning of the phenomenological nature of the isolates (Kerouanton *et al.* 1998). In Fig. 2, factor 1 clearly sorts the isolates of *D. bruxellensis* from left to right in function of its major to minor VR and CD activities, with the isolates from group A being associated with greater VR activity.

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