Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Molecular and physiological comparison of spoilage wine yeasts

M.P. Sangorrín[1], V. García[2], C.A. Lopes[1,3], J.S. Sáez[1,3], C. Martínez[2,4] and M.A. Ganga[2]

1 Grupo de Biodiversidad y Biotecnología de Levaduras, Instituto Multidisciplinario de Investigación y Desarrollo de la Patagonia Norte (IDEPA CONICET-UNCo), Dpto. de Química, Facultad de Ingeniería, Universidad Nacional del Comahue, Buenos Aires, Argentina
2 Laboratorio de Biotecnología y Microbiología Aplicada, Departamento en Ciencia y Tecnología de los Alimentos, Universidad de Santiago de Chile (USACH), Santiago, Chile
3 Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, Río Negro, Argentina
4 Centro de Estudios en Ciencia y Tecnología de los Alimentos (CECTA), Universidad de Santiago de Chile (USACH), Santiago, Chile

Keywords
Dekkera bruxellensis, off-flavour, phenols, *Pichia guilliermondii*, wine.

Correspondence
M. Angelica Ganga, Laboratorio de Biotecnología y Microbiología Aplicada, Departamento en Ciencia y Tecnología de los Alimentos, Universidad de Santiago de Chile (USACH), Obispo Manuel Umaña 050, Estación Central, Santiago, Chile. E-mail: angelica.ganga@usach.cl

2013/1506: received 22 August 2012, revised 21 November 2012 and accepted 11 December 2012

doi:10.1111/jam.12134

Abstract

Aims: *Dekkera bruxellensis* and *Pichia guilliermondii* are contaminating yeasts in wine due to the production of phenolic aromas. Although the degradation pathway of cinnamic acids, precursors of these phenolic compounds has been described in *D. bruxellensis*, no such pathway has been described in *P. guilliermondii*.

Methods and Results: A molecular and physiological characterization of 14 *D. bruxellensis* and 15 *P. guilliermondii* phenol-producing strains was carried out. Both *p*-coumarate decarboxylase (CD) and vinyl reductase (VR) activities, responsible for the production of volatile phenols, were quantified and the production of 4-vinylphenol and 4-ethylphenol were measured. All *D. bruxellensis* and some *P. guilliermondii* strains showed the two enzymatic activities, whilst 11 of the 15 strains of this latter species showed only CD activity and did not produce 4-EP in the assay conditions. Furthermore, PCR products obtained with degenerated primers showed a low homology with the sequence of the gene for a phenyl acrylic acid decarboxylase activity described in *Saccharomyces cerevisiae*.

Conclusions: *D. bruxellensis* and *P. guilliermondii* may share a similar metabolic pathway for the degradation of cinnamic acids.

Significance and Impact of the Study: This is the first work that analyses the CD and VR activities in *P. guilliermondii*, and the results suggest that within this species, there are differences in the metabolization of cinnamic acids.

Introduction

The presence of phenolic aromas is associated to microbiological contamination by *Dekkera bruxellensis* or its anamorph *Brettanomyces bruxellensis* (Chatonnet *et al.* 1992; Curtin *et al.* 2007; Suárez *et al.* 2007). This yeast produces aromas described as 'phenolic', 'animal', 'horse sweat' and 'stable' which are collectively known as 'Brett character' (Chatonnet *et al.* 1992; Suárez *et al.* 2007). Pizarro *et al.* (2007) chemically characterized this defect describing it as being composed of a series of volatile phenols, including 4-ethylguaiacol (4-EG), 4-ethylphenol (4-EP), 4-vinylguaiacol (4-VG) and 4-vinylphenol (4-VP). A concentration >425 $\mu g\ l^{-1}$ of a mixture of 1 : 10 of 4-EG/4-EP produces an animal aroma, whilst a concentration >770 $\mu g\ l^{-1}$ of a 1 : 1 mixture of 4-VG/4-VP produces a strong pharmaceutical aroma (Chatonnet *et al.* 1995).

Although at a global scale, *D. bruxellensis* would be the main producer of these volatile phenols in wine, other species have also been described (Dias *et al.* 2003a; Martorell *et al.* 2006; Lopes *et al.* 2009a). Dias *et al.* (2003a) showed that *Candida winckerhamii*, *Candida cantarelli*, *Lactococcus lactis*, *Debaryomyces hansenii*

and *Pichia guilliermondii* are also able to produce volatile phenols with a similar efficiency to *D. bruxellensis*. This observation was confirmed by Barata *et al.* (2006) who showed the capacity of *P. guilliermondii* to produce volatile phenols in wine must. Similar results were obtained by Lopes *et al.* (2009a) who detected the production of phenolic compounds, particularly 4-VP in must inoculated with different *P. guilliermondii* isolates. Likewise, Martorell *et al.* (2006) showed that different isolates of this species produce different concentrations of phenolic compounds in synthetic culture media. Additionally, mixed cultures of the spoilage yeasts *D. bruxellensis* and *P. guilliermondii* produced an increase in the total volatile phenols concentration regarding as compared with pure cultures of the same species (Sáez *et al.* 2010).

Chatonnet *et al.* (1992) proposed a biosynthetic pathway for the synthesis of volatile phenols from cinnamic acids in *D. bruxellensis*. This yeast transforms these organic acids, naturally present in the must, into vinyl and ethyl derivatives through a cinnamate decarboxylase (CD) and a vinylphenol reductase (VR). These enzymes have been purified and characterized in *D. bruxellensis* (Godoy *et al.* 2008; Tchobanov *et al.* 2008). Until now, a similar biosynthetic pathway has not been described for *P. guilliermondii*.

The fact that different strains belonging to a same species can produce significantly different amounts of volatile phenols or other metabolic products makes the use of molecular techniques necessary to allow the intraspecific differentiation of the yeasts. This characterization could be also very helpful in establishing the origin of wine spoilage yeasts, their routes of contamination and the association with different metabolic capacities (Suárez *et al.* 2007; Lopes *et al.* 2009a,b). Different techniques like RAPD-PCR, RFLP's of mtDNA restriction analysis, AFLP and fluorescence *in situ* hybridization were applied for strain discrimination in this species (Mitrakul *et al.* 1999; Dias *et al.* 2003a; Cocolin *et al.* 2004; Agnolucci *et al.* 2009; Godoy *et al.* 2009). On the other hand, the characterization at intraspecific level in *P. guilliermondii* has been less studied; mtDNA restriction analysis, RAPD-PCR and physiological (killer biotype) characterization methods have been already evaluated (Martorell *et al.* 2006; Lopes *et al.* 2009a).

In this work, both the production of volatile phenols and the associated CD and VR activities (described in *D. bruxellensis*) were evaluated *in vitro*in *D. bruxellensis* and *P. guilliermondii* strains collected in different wine-producing areas. Molecular relationships among strains based on RAPD patterns, and its association with the capacity to produce volatile phenols was also analysed.

Materials and methods

Yeast strains and culture conditions

Yeast strains used in this study included 14 isolates of *D. bruxellensis* and 15 isolates of *Pichia guilliermondii* obtained from the collection of the Biotechnology and Applied Microbiology Laboratory of the Universidad de Santiago de Chile (LAMAP) and North Patagonia Culture Collection (NPCC), Neuquén, Argentina (Table 1).

The yeast were grown in YPD medium (yeast extract 5 g l^{-1}, peptone 5 g l^{-1} and glucose 20 g l^{-1}) for 4 days. These cultures were used to inoculate 100 ml of YNB medium (yeast nitrogen base 6·7 g l^{-1} and glucose 20 g l^{-1}) supplemented with ethanol 5% and *p*-coumaric acid 0·6 mmol l^{-1} as previously described by Godoy *et al.* (2008).

All cultures were incubated at 28°C in an orbital shaker (150 rev min^{-1}) for 5 days. The cultures were controlled by cell count using a Neubauer chamber until a concentration of 1×10^8 cells ml^{-1} was obtained.

RAPD-PCR analysis

Intraspecific characterization was carried out by RAPD analysis according to the methodology described by Martorell *et al.* (2006). For the preparation of DNA samples, the yeasts were grown for 72 h in YPD medium. DNA was obtained by the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). RAPD-PCR analysis was carried out using seven primers from the series OPA (Operon Technologies Inc., Alameda, CA, USA); OPAE-09, OPAE-12, OPAE-15, OPAR-08, OPAR-14, OPAS-05 and OPAS-11. DNA amplification was carried out in a PTC-100 Peltier Thermal Cycler, MJ Research (Galenica, Santiago, Chile) in a final volume of 25 μl. The reaction mixture contained 2·5 μl Taq Buffer 10× with KCl (Fermentas, Hanover, MD, USA), 0·1 mmol l^{-1} dNTPs, 2 mmol l^{-1} $MgCl_2$, 3 μmol l^{-1} primer, 500 ng de DNA molde y 1 U Taq polimerase. The RAPD-PCR products were visualized on a 2% agarose gel. The band patterns were analysed using the Quantity One software (BioRad, Hercules, CA, USA).

Determination of protein and enzymatic assays

The protein extracts were prepared as indicated by Godoy *et al.* (2008), and the proteins were quantified according to the method described by Bradford (1976) using bovine serum albumin as standard. The determination of CD activity was based on the methodology described by Godoy *et al.* (2008) with some modifications. The reaction mixture contained 200 μl of protein

Table 1 Geographical and molecular pattern of *Dekkera bruxellensis* and *Pichia guilliermondii*

Strain	Designation	Source	Pattern types obtained by RAPD-PCR composite patterns by OPA primer							Haplotype (RAPDs)
			E09	E12	E15	R08	R14	S05	S11	
D1	CECT 1451^T	Collection	C	D	D	F	I	F	K	1
D2	L2480	RW_1 C	A	A	A	A	B	A	B	2
D3	L2652	RW_2 C	J	K	D	H	D	B	A	3
D4	L2676	RW_3 C	E	E	F	D	H	E	C	4
D5	L2686	RW_4 C	C	D	E	C	E	G	H	5
D6	400	RW_6 P	B	C	E	B	F	H	G	6
D7	419	RW_6 P	I	H	G	G	M	D	I	7
D8	1327	RW_7 P	H	J	C	I	A	C	O	8
D9	1600	RW_7 P	D	F	F	D	H	G	D	9
D10	1700	RW_8 P	E	G	F	D	J	G	F	10
D11	1701	RW_5 P	C	I	D	D	C	H	E	11
D12	1791	RW_9 P	C	D	D	D	L	I	N	12
D13	2101	RW_6 P	G	B	E	E	G	H	M	13
D14	2113	RW_{11} P	F	B	B	D	J	H	L	14
P1	2105	G_6 P	R	R	T	U	R	R	S	15
P2	2136	G_6 P	R	R	T	U	R	R	T	16
P3	2141	Dr_{10} P	R	R	T	U	R	R	U	17
P4	2131	RW_6 P	R	R	T	U	R	R	S	15
P5	NPCC1051	G_1 A	P	P	P	P	P	P	P	18
P6	NPCC1052	G_2 A	P	P	P	P	P	P	P	18
P7	NPCC1053	G_3 A	S	P	Q	Q	P	P	P	19
P8	NPCC1055	M_4 A	P	P	P	P	P	P	P	18
P9	NPCC1056	M_4 A	T	P	R	R	P	P	Q	20
P10	NPCC1057	FM_5 A	P	P	P	P	P	P	P	18
P11	NPCC1058	FM_5 A	P	P	P	P	P	P	P	18
P12	NPCC1061	FM_5 A	Q	Q	S	T	Q	Q	R	21
P13	NPCC1063	M_7 A	P	P	P	P	P	P	P	18
P14	NPCC1067	M_8 A	P	P	P	P	P	P	P	18
P15	NPCC1071	M_8 A	P	S	P	S	P	P	P	22

In code: D, *D. bruxellensis*; P, *P. guilliermondii*; *Designation*: Only number, Martorell *et al.* (2006); CECT, Collection Spain Culture Type; ^{T}, Type strain; L, Laboratory of Biotechnology and Applied Microbiology of the Universidad de Santiago de Chile (LAMAP); NPCC, Noth Patagonia Collection Culture; *In source:* G, grape; Dr, *Drosophila*; M, fresh must; FM, fermenting must; RW, red wine; A, Argentina; C, Chile; P, Portugal. In number subindice vineyard or winery source. OPA primers, E09, E12, E15, R08, R14, S05, S11.

extract, 50 mmol l^{-1} phosphate buffer pH 6·0 and 12·2 mmol l^{-1} of *p*-coumaric acid, and incubated at 40° C for 40 min. Subsequently, the mixture was diluted 50 times with bidistilled water to avoid interference with the proteins. CD activity was monitored by the decrease in absorbance at 350 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 μmol of *p*-coumaric acid per minute. The VR activity was performed as described by Godoy *et al.* (2009).The reaction mixture contained 200 μl protein extract, 50 mmol l^{-1} phosphate buffer pH 6·0, 0·15 mmol l^{-1} NADPH and 2 mmol l^{-1} 4-vinylphenol. It was incubated at 20°C for 60 min, and the reaction was stopped with 25 mmol l^{-1} Tris–HCl, 0·3% SDS. The VR activity was monitored by the decrease in absorbance at 340 nm of NADPH, an oxidizable cofactor present in the reaction. One unit (U) of enzymatic activity was defined as the amount of enzyme that consumes 1 μmol of NADPH per minute.

Volatile phenol production

The transformation of *p*-coumaric acid into 4-VP and 4-EP was monitored using the method described by Ross *et al.* (2009). Chromatographic separation was performed with a waters HPLC equipped (Waters Corporation, Milford, MA, USA) with a 600 pumps, an UV visible detector. Millenium software (Waters, Oshawa, Canada) was employed for chromatographic control. The separations were performed on a reversed phase column C18 (150 × 4·6 mm). Chromatographic separation was carried out using a gradient elution of (A) H_2O: acetic acid

90 : 10 and (B) Methanol as follows: 0–10 min A 100%, 10–15 min A 70%, B 30%, 15–30 min A 30%, B 70%, 30 –35 min A 100%. The flow rate was 1·0 ml min^{-1}, and the temperature was set at 45°C. Quantification was performed by comparison against an external standard of *p*-coumaric acid (range, 0–112 mg l^{-1}), 4-VP (range, 0–100 mg l^{-1}) and 4-EP (range, 0–112 mg l^{-1}).

Amplification of the putative CD gene from spoilage yeast

The *Saccharomyces cerevisiae PAD1* gene (access number NM_001180846) encodes for a protein with phenyl acrylic acid decarboxylase activity (Clausen *et al.* 1994), which metabolizes the cinnamic acids to vinyl derivatives. We used the sequence of putative *PAD* gen of *Debaromyces hansenii* CBS767, *Candida albicans* WO-1 and *Giberella zeae* PH-1 (www.ncbi.nlm.nih.gov). The resulting alignment showed highly conserved areas from which the degenerate primers 1F (5′-ATTACTGGT GCNACAGGT-3′) and 3R (5′-CATGGAACAGGGCAC RACAATC-3′) were designed. The PCR reaction was carried out in a thermal cycler (PTC-100 Peltier Thermal Cycler; MJ Research) using the following programme: one initial step of 95°C for 5 min and 35 cycles consisting of 95°C for 1 min, an annealing of 50°C for 1 min and a final extension of 72°C for 1 min. The resulting amplicons were analysed by electrophoresis in 1% agarose gels. Those products with the highest intensity were extracted and purified using the FavorPrep GEL/pCR Purification Mini Kit (Biotech Corp., Kuala Lumpur, Malaysia). Subsequently, the eluted DNA fragments were cloned in pGEM ®-T and sequenced in Macrogen (Seul, Korea).

Statistical analysis

All experiments were performed in duplicate, and the results were analysed using Statgraphics Plus 5.0 (Manugistics, Inc., Rockville, MD, USA). Differences between treatment means were compared using the Tukey's test at $P < 0{\cdot}05$.

The genetic relationship among strains was evaluated using Principal Coordinates Analysis (PCoA) and depicted in a 2D scatter plot by the Numerical Taxonomy System program (NTSYS) (Rohlf 2005). A matrix value of 1 or 0 was assigned to denote the presence or absence of a particular band according to the RAPD-PCR profiles for each strain (qualitative variables). A minimum-length spanning tree was calculated from the simple matching coefficient matrices and superimposed on PCo plots to help detect local distortions. Principal Component Analysis (PCA) on the centred and standardized quantitative variables (4-VP, 4-EP, CD and VR activities) was also performed using the same software.

Results

Molecular characterization of the isolates

The intraspecific characterization of the isolates was carried out by RAPD-PCR analysis with OPA series primers. In the case of *D. bruxellensis* 14 different haplotypes were identified, whilst eight haplotypes were obtained for *Pichia guilliermondii* (Table 1, Fig. 1). To simplify the profile analysis and detect potential relationships among isolates, RAPD-PCR patterns were converted into binary data matrices and subjected to PCoA (Fig. 2). The first two axes of the plot obtained after PCoA explained 50 and 83% of the total variability detected among *D. bruxellensis* and *P. guilliermondii*, respectively. Scatter-plots resulting from these two axes demonstrated different strain clusters for each species (Fig. 2a,b). For *D. bruxellensis,* one cluster groups the majority of the studied strains and only strains D2 and D3 do not fall within this cluster (Fig. 2a). On the other hand, the *P. guilliermondii* strains formed two clusters: cluster I contains strains P1–P4 and cluster II contains the rest of the strains. Additionally, cluster II is not as homogenous as cluster I since isolates P7, P9 and P12 are separated from the rest of the strains.

Quantification of volatile phenols and enzymatic activity

D. bruxellensis and *P. guilliermondii* strains were grown in medium supplemented with *p*-coumaric acid. The production of volatile phenols, non-metabolized *p*-coumaric acid and enzymatic activities of CD and VR were determined (Table 2). All the *D. bruxellensis* strains produced more than 0·44 mg l^{-1} of 4-EP, which is considered the sensory threshold for this compound (Ferreira *et al.* 2002). The majority of the *P. guilliermondii* strains produced high levels of 4-VP except strains P1–P4. Likewise, all the strains studied produced 4-VP at levels which exceeded the sensory threshold of 0·18 mg l^{-1}(Ferreira *et al.* 2002).

As shown in Table 2, there are significant differences in the production of 4-EP among the strains studied. Strains D3, D4, D7, D10, D12 and D14 produced concentrations >60 mg l^{-1}, whilst for strains D1, D2, D8 and P2 <25 mg l^{-1} of 4-EP were detected in the culture media. The rest of the strains produced intermediate values of this compound. In the conditions assayed, strains P5–P15 of *P. guilliermondii* (Table 2) did not produce 4-EP, whilst P1–P4 did. However, these strains produced

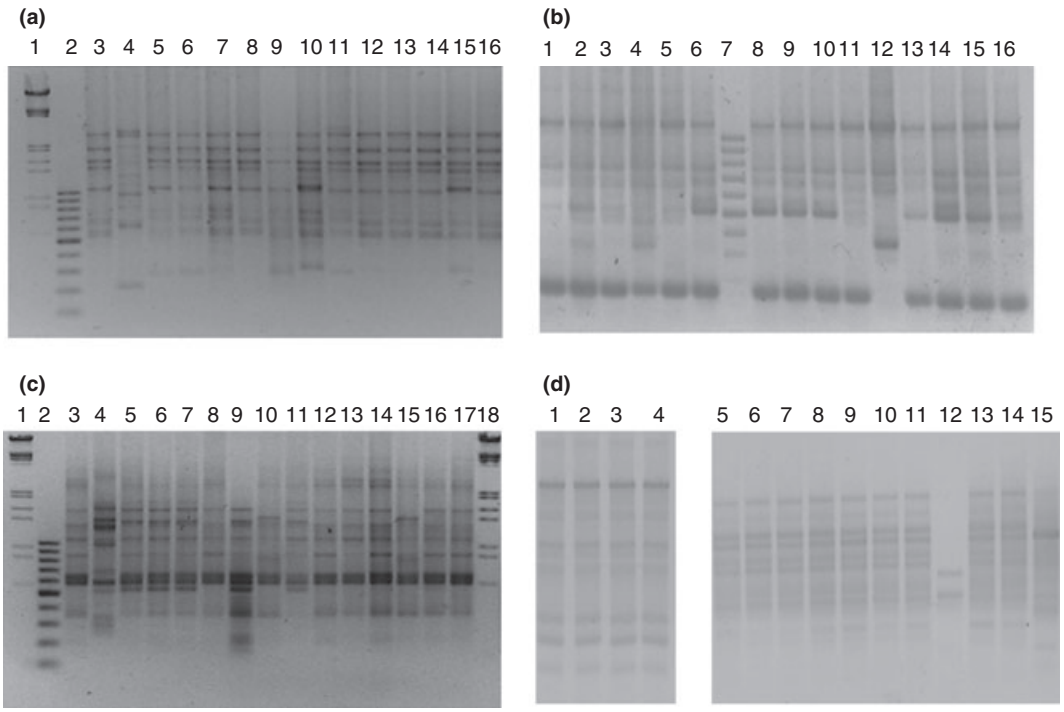

Figure 1 Molecular patterns detected among the *Dekkera bruxellensis* and *Pichia guilliermondii* strain using RAPD analysis. (a) Analysis of *D. bruxellensis* strains with primer OPA-E09. Lane 1: λ*Eco*RI/*Hin*dIII molecular weight standard, Lane 2: 100 bp molecular weight standard, Lanes 3–16: D9, D7, D13, D6, D1, D2, D11, D10, D12, D8, D14, D4, D5 and D3, respectively. (b) *P. guilliermondii* strain with primer OPA-E09. Lanes 1–6: strains P5, P7, P1, P9, P2, P5, Lane 7: 100 bp molecular weight standard, Lanes 8–16: strains P6, P7, P8, P3, P12, P10, P11, P13 and P4, respectively. (c) *D. bruxellensis* strains with primer OPA-E12. Lanes 1 and 18: λ*Eco*RI/*Hin*dIII molecular weight standard, Lane 2: 100 bp molecular weight standard, Lanes 3–17: strains D9, D3, D7, D13, D6, D1, D2, D11, D10, D12, D8, D14, D10, D4 and D5, respectively. (d) *P. guilliermondii* strains with primer OPA-E12. Lanes 1–15: Strains P1–P15.

on average a lower concentration of 4-EP than *D. bruxellensis* (Table 2). We can observe that all the *D. bruxellensis* strains and strains P1–P4 of *P. guilliermondii* showed CD and VR activities. In the case of CD activity, except for isolate D4 (which showed the greatest specific activity), there were no significant differences among the strains studied (Table 2). When analysing strains D2 and P3, we observed that on average, D2 had the greatest VR activity, whilst P3 on average had the lowest out of the strains studied. However, it was not possible to correlate the VR activity and the production of 4-EP.

Principal component analysis was used to cluster the strains showing similar production values of 4-VP and 4-EP as well as enzymatic activities (Fig. 3). The PCA plot explained 75% of the total variability in the data in the first two dimensions and four clusters were identified: cluster I contained *P. guilliermondii* P5–P15 strains and was characterized by a high production of 4-VP and a low concentration of residual *p*-coumaric acid; cluster II grouped the *P. guilliermondii* P1–P4 strains and *D. bruxellensis* D2, which showed intermediate levels of this enzymatic activity; cluster III grouped those *D. bruxellensis* strains that showed the highest values of 4-EP and enzymatic activities. These left a low concentration of residual *p*-coumaric acid in the culture medium, similarly to cluster I. On the other hand, cluster IV contained those *D. bruxellensis* strains, which produced the highest amount of 4-EP and enzymatic activities and left a low concentration of *p*-coumaric acid. The *D. bruxellensis* strains D3 and D4 are the greatest producers of 4-EP, with a high CD and VR activity.

Identification of the putative *CD* gene in *Pichia guilliermondii*

In *S. cerevisiae* the phenyl acrylic acid decarboxylase is encoded by the *PAD1* gene (Clausen *et al.* 1994). This enzymatic activity is responsible for transforming cinnamic acids in vinylderivatives, similarly to what the CD activity carries out in *D. bruxellensis*. To identify the possible *CD* gene of *D. bruxellensis* and *P. guilliermondii*, the *PAD* gene sequences of *S. cerevisiae YJM789*, *Debaryomyces hansenii* CBS767, *Candida albicans* WO-1 and *Gibberellazeae* PH-1 were aligned (www.ncbi.nlm.nih.gov), and

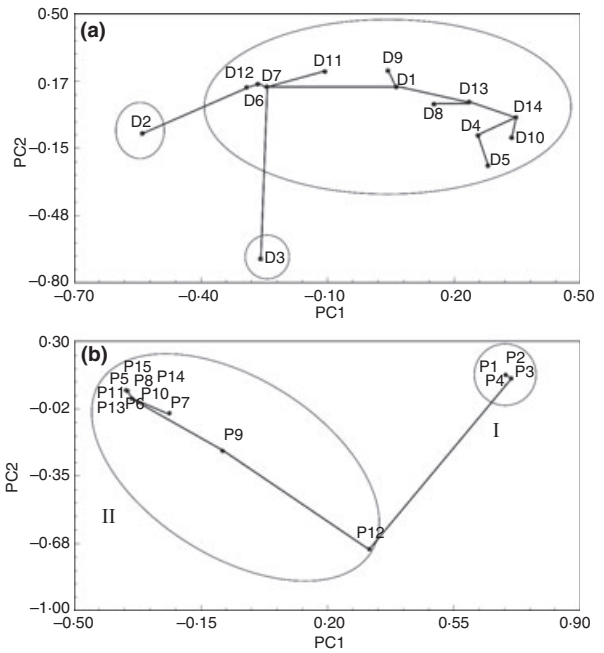

Figure 2 Principal coordinates analysis of the individual RAPD-PCR profiles for 14 strains of *Dekkera bruxellensis* (a) and 15 strains of *Pichia guilliermondii* (b) *D. bruxellensis* (D); *P. guilliermondii* (P).

primers 1F and 3R were designed (see Materials and Methods). Subsequently, the DNA of *D. bruxellensis* D2 and *P. guilliermondii* P1 and P14 strains was amplified using these primers. In *D. bruxellensis*, a 200 bp fragment was obtained, which had scarce homology with the *PAD1* gene of *S. cerevisiae*. In the case of *P. guilliermondii* isolates P1 and P14, the amplicons were of 900 and 1100 bp, respectively. The homology detected between these two sequences obtained from different *P. guilliermondii* strains studied was very low (25%).

Discussion

In the present study, we evidenced a high degree of molecular polymorphism in *Dekkera bruxellensis* and *Pichia guilliermondii.* Using seven OPA primers, we differentiated 14 isolates of *D. bruxellensis* by the combined haplotype of the RAPD-PCR composite patterns (Table 1, Fig. 1). Using three different OPA primers (OPA-2, OPA-3 and OPA-9) Martorell *et al.* (2006) did not show genetic differences among the D6–D14 *D. bruxellensis* strains tested in this work; however, by increasing the number of primers, we obtained a greater differentiation at the genetic level. In fact, we were able to differentiate strains D7, D9 and D12, which in the study by Martorell *et al.* (2006) showed a different mtDNA restriction pattern but a similar RAPD-PCR pattern. Agnolucci *et al.* (2009), using three different OPA primers only, obtained six different profiles when analysing 84 *D. bruxellensis* isolates. By sequencing the 26S rDNA segment, Conterno *et al.* (2006) grouped 47 *D. bruxellensis* strains into six clusters. Curtin *et al.* (2007) analysed 244 Australian isolates and found only eight different genotypes. On the other hand, Oelofse *et al.* (2009) used different molecular methods (PCR-DGG; ISS-PCR; REA-PFGE) to analyse *D. bruxellensis* strains and concluded that the species has a low genetic variability.

The PCoA carried out with the RAPD-PCR data for the *D. bruxellensis* strains showed a large cluster that grouped most of the strains analysed, whilst strains D2 and D3 were grouped apart (Fig. 2a).

The production of volatile phenols (4-VP and 4-EP) and the enzymatic activities (CD and VR) showed very heterogeneous values (Table 2, Fig. 3). A similar observation was made by Conterno *et al.* (2006) and Oelofse *et al.* (2009) who stated that isolates from the same or different geographical zones show differences in the production of volatile phenols even though they are genetically close.

The use of RAPD-PCR with eight primers resulted in six different haplotypes within the 15 *P. guilliermondii* isolates (Table 1). This technique showed a lower capacity to differentiate strains within this species than that obtained in *D. bruxellensis*. The *P. guilliermondii* strains were grouped in two clusters: cluster I grouped strains that were very homogenous, whilst the strains of cluster II showed differences between them. Martorell *et al.* (2006) analysed 32 isolates of this species from Alentejo (Portugal) using mtDNA-RFLP and found a great genetic variability, whereby seven different profiles were detected. Four of these isolates showing a greater production of 4-EP were selected and included in our study. Although we differentiated these isolates using RAPD-PCR, the PCoA confirmed a high genetic relationship between them (Fig. 2b). This genetic relationship was also evident in their physiological evaluation where they were found in cluster II in the PCA as a result of their production of volatile phenols as well as the enzymatic activities analysed (Fig. 3). On the other hand, the *P. guilliermondii* P5–P15 strains used in this study have been previously identified as different strains by molecular and physiological techniques (Lopes *et al.* 2009a,b). In the present study, we confirmed the genetic differentiation between these strains using eight OPA primers, which grouped the strains in cluster II. Within this cluster isolates P7, P9 and P12, which came from different samples, were clearly distinguished.

With regard to the physiological characteristics of the *P. guilliermondii* P1–P4 strains, Martorell *et al.* (2006) found that this species was capable of producing levels of

Table 2 Quantification of precursor acid, volatile phenols and enzymatic activities (CD and VR) for the micro-organisms of this study

Strain	*p*-coumaric acid (mg l^{-1})	CD activity (U mg^{-1})	4-VP (mg l^{-1})	VR activity (mU mg^{-1})	4-EP (mg l^{-1})
D1	$6{\cdot}37^{ab}$	$13{\cdot}37^{a}$	$90{\cdot}23^{de}$	$21{\cdot}50^{e}$	$22{\cdot}13^{abc}$
D2	$52{\cdot}44^{e}$	$8{\cdot}06^{a}$	$21{\cdot}83a^{b}$	$31{\cdot}80^{e}$	$22{\cdot}11^{abc}$
D3	nd	$15{\cdot}03^{a}$	$1{\cdot}50^{a}$	$27{\cdot}50^{e}$	$69{\cdot}28^{hi}$
D4	nd	$39{\cdot}96^{b}$	$1{\cdot}08^{a}$	$24{\cdot}20^{e}$	$73{\cdot}08^{hi}$
D5	$11{\cdot}64^{abcd}$	$9{\cdot}33^{a}$	$49{\cdot}79^{bc}$	$24{\cdot}10^{e}$	$48{\cdot}33^{defg}$
D6	$7{\cdot}34^{abc}$	$9{\cdot}30^{a}$	$13{\cdot}23^{a}$	$21{\cdot}29^{de}$	$54{\cdot}73^{efgh}$
D7	nd	$9{\cdot}65^{a}$	nd	$21{\cdot}60^{e}$	$67{\cdot}88^{ghi}$
D8	nd	$11{\cdot}54^{a}$	$95{\cdot}13^{e}$	$20{\cdot}70^{cde}$	$12{\cdot}88^{ab}$
D9	$0{\cdot}34^{a}$	$8{\cdot}66^{a}$	$93{\cdot}85^{e}$	$25{\cdot}80^{e}$	$29{\cdot}72^{bcd}$
D10	nd	$10{\cdot}16^{a}$	$6{\cdot}25^{a}$	$17{\cdot}60^{abcde}$	$61{\cdot}71^{fghi}$
D11	$0{\cdot}42^{a}$	$7{\cdot}80^{a}$	$62{\cdot}41^{cd}$	$20{\cdot}80^{cde}$	$39{\cdot}45^{cde}$
D12	$0{\cdot}07^{a}$	$8{\cdot}92^{a}$	$3{\cdot}25^{a}$	$20{\cdot}30^{bcde}$	$76{\cdot}43^{i}$
D13	$8{\cdot}39^{abc}$	$6{\cdot}51^{a}$	$13{\cdot}66^{a}$	$26{\cdot}20^{e}$	$58{\cdot}29^{efghi}$
D14	$0{\cdot}05^{a}$	$15{\cdot}71^{a}$	nd	$11{\cdot}50^{a}$	$72{\cdot}12^{hi}$
P1	$46{\cdot}67^{e}$	$6{\cdot}95^{a}$	$2{\cdot}20^{a}$	$2{\cdot}15^{ab}$	$28{\cdot}30^{bcd}$
P2	$74{\cdot}89^{f}$	$9{\cdot}54^{a}$	nd	$2{\cdot}82^{abc}$	$7{\cdot}80^{a}$
P3	$24{\cdot}12^{cd}$	$9{\cdot}60^{a}$	$1{\cdot}70^{a}$	$0{\cdot}18^{a}$	$38{\cdot}20^{cde}$
P4	$15{\cdot}38^{abcd}$	$4{\cdot}95^{a}$	$1{\cdot}72^{a}$	$2{\cdot}88^{abc}$	$44{\cdot}50^{def}$
P5	$2{\cdot}01^{ab}$	$10{\cdot}42^{a}$	$105{\cdot}70^{e}$	nd	nd
P6	0·79a	$3{\cdot}82^{a}$	$107{\cdot}41^{e}$	nd	nd
P7	$1{\cdot}57^{ab}$	$1{\cdot}74^{a}$	$112{\cdot}00^{ce}$	nd	nd
P8	$2{\cdot}03^{ab}$	$2{\cdot}18^{a}$	$107{\cdot}30^{e}$	nd	nd
P9	$5{\cdot}33^{ab}$	$4{\cdot}75^{a}$	$104{\cdot}70^{e}$	nd	nd
P10	$0{\cdot}09^{a}$	$11{\cdot}26^{a}$	$99{\cdot}66^{e}$	nd	nd
P11	$0{\cdot}37^{a}$	$8{\cdot}29^{a}$	$109{\cdot}42^{e}$	nd	nd
P12	nd	$16{\cdot}46^{a}$	$95{\cdot}61^{e}$	nd	nd
P13	$18{\cdot}12^{bcd}$	$2{\cdot}92^{a}$	$89{\cdot}86^{bc}$	nd	nd
P14	$16{\cdot}17^{abcd}$	$5{\cdot}31^{a}$	$85{\cdot}01^{de}$	nd	nd
P15	$28{\cdot}29^{d}$	$15{\cdot}09^{a}$	$85{\cdot}07^{de}$	nd	nd

4-VP, 4-vinylphenol; 4-EP, 4 ethylphenol; nd, not detected; CD, cinnamate decarboxylase; VR, vinyl reductase.
Values within a column followed by the same letter are not significantly different according to Tukey's tests ($P > 0{\cdot}05$).

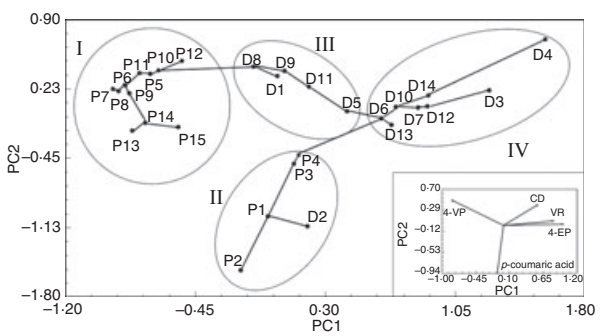

Figure 3 Principal component analysis of 4-vinylphenol, 4-ethylphenol cinnamate decarboxylase and vinyl reductase activity levels obtained from 14 strains of *D. bruxellensis* (D) and 15 strains of *Pichia guilliermondii* (P).

4-EP close to 65 mg l^{-1} in synthetic medium without ethanol. On the other hand, in a study by Barata *et al.* (2006), the same strains produced approximately 4–12 mg l^{-1} 4-EP in grape juice prior to the onset of fermentation by *S. cerevisiae*. In our study, the production of 4-EP was carried out in synthetic medium with 5% ethanol and intermediate levels of 4-EP were obtained when compared with those reported by these authors (8–44 mg l^{-1}). This inhibition of 4-EP production in wine spoilage yeasts by increasing concentrations of ethanol has been previously demonstrated (Dias *et al.* 2003b; Silva *et al.* 2004). It is important to note that in the conditions assayed in this work only strains P1–P4 were able to produce 4-EP and had VR activity indicating a large phenotypic diversity between the *P. guilliermondii* strains isolated in different wine-producing areas. Strains P5–P15 used in this study have been previously described as producers of low levels of 4-EP (0·1 mg l^{-1}) in must (Lopes *et al.* 2009a), this metabolite was not detected in the conditions assayed and with the method of detection used. This difference could be related to the presence of ethanol in the culture media used in this work, the use of different detection methods: gas chromatography in Lopes *et al.* (2009a) and HPLC in this work or the presence of

certain compounds in the must that could induce the enzymatic activities responsible for the production of these volatile phenols. The low production of this compound and the lack of VR activity observed in the P5–P15 strains suggest that the yeast growth conditions for the production of 4-EP is different to that required by the other strains. These differences could be explained by the low sequence similarity of the putative *CD* gene, only 25% between strains P1 and P14 from cluster I and II, respectively (Fig. 2b).

Based on the proposed stoichiometry for the production of 4-VP and 4-EP by *D. bruxellensis* (Suárez *et al.* 2007) and from the analysis of Table 2, it is possible to observe that in strains D3, D4, D6, D7, D10, D12–14 and P1–P4, the sum of 4-VP + 4-EP + residual *p*-coumaric acid is less than the initial concentration of the cinnamic acid added to the culture medium (100 mg l^{-1}). The production of ethoxyphenols through the interaction of ethanol with *p*-coumaric acid and phenols may explain the lack of stoichiometry of the enzymatic reaction (Dugelay *et al.* 1995). An alternative explanation is that the *p*-coumaric acid may be absorbed by the yeast wall (Salameh *et al.* 2008) and therefore not be completely available for its metabolization by the yeast.

On the other hand, the comparison of the putative segment for the *D. bruxellensis CD* gene shows a low homology with the *PAD1* gene of *S. cerevisiae*. This could be due to the phylogenetic distance between both species. Woolfit *et al.* (2007) carried out a partial sequence of the *D. bruxellesis* genome and calculated the identity between amino acids with orthologous proteins from both microorganisms and showed that there is only a 49·9% identity between *S. cerevisiae* and *D. bruxellensis*. Likewise, the homology detected between the possible *CD* gene of *P. guilliermondii* and the *PAD1* gene was very low. Similar percentages were obtained in our study between the 1100 and 900 pb amplicons of *P. guilliermondii* and the *PAD1* gene of *S. cerevisiae* (42·6 and 30%, respectively). In a recent study, Huang *et al.* (2012) identified the *CgPAD* gene of *Candida guilliermondii (*anamorph of *P. guilliermondii)*; the sequence of this gene also exhibited very low sequence similarity to our amplicons (37%). Since degenerate primers were employed in our study based on other known *PAD* genes that encode for the decarboxylase activity domain of the yeast, it is possible that we amplified the gene of another enzyme responsible for the decarboxylation of *p*-coumaric acid.

The *P. guilliermondii* P1–P4 strains do not consume all the *p*-coumaric acid and produce 4-EP, whilst the other strains consume the precursor in greater quantity and produce high levels of 4-VP, which could indicate that the VR activity is limiting for the metabolization of *p*-coumaric acid. This is the first study that detects and evaluates the CD and VR enzymatic activities in *P. guilliermondii*. These enzymes could be part of an equivalent two step biosynthesis pathway for the production of volatile phenols in *D. bruxellensis*.

Acknowledgements

This work was supported by FONDECYT grant number 1080376, ProyectoApoyo de VisitasExtranjero USACH and Programa de Cooperación Científico-Tecnológica between MINCyT and CONICYT CH/09/12.

References

Agnolucci, M., Vigentini, I., Capurso, G., Merico, A., Tirello, A., Compagno, C., Foschino, R. and Nuti, M. (2009) Genetic diversity and physiological traits of *Brettanomces bruxellensis* strain isolated from Tuscan Sangiovese wine. *Int J Food Microbiol* **130**, 238–244.

Barata, A., Nobre, A., Correia, P., Malfeito-Ferreira, M. and Loureiro, V. (2006) Growth and 4-ethylphenol production by 1 the yeast *Pichia guilliermondii* in grape juices. *Am J Enol Vitic* **57**, 133–138.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.

Chatonnet, P., Dubourdie, D., Boidron, J.N. and Pons, M. (1992) The origin of ethylphenols in wines. *J Agric Food Chem* **60**, 165–178.

Chatonnet, P., Dubourdieu, D. and 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.

Clausen, M., Lamb, C.J., Megnet, R. and Doerner, P.W. (1994) *Pad*1 encodes phenylacrylic acid decarboxylase which confers resistance to cinnamic acid in *Saccharomyces cerevisiae*. *Gene* **142**, 107–112.

Cocolin, L., Rantsiou, K., Iacumin, L., Zironi, R. and Comi, G. (2004) Molecular detection and identification of *Brettanomyces/Dekkera bruxellensis* and *Brettanomyces/Dekkeraanomalus* in spoiled wines. *Appl Environ Microbiol* **70**, 1347–1355.

Conterno, L., Joseph, C.M.L., Arvik, T.J., Henick-Kling, T. and Bisson, L.F. (2006) Genetic and physiological characterization of *Brettanomycesbruxellensis* strains isolated from wines. *Am J Enol Vitic* **57**, 139–147.

Curtin, C.D., Bellon, J.R., Henschke, P.A., Godden, P.W. and De Barros Lopes, M.A. (2007) Genetic diversity of *Dekkera bruxellensis* yeasts isolated from Australian wineries. *FEMS Yeast Res* **7**, 471–481.

Dias, L., Dias, S., Sancho, T., Stender, H., Querol, A., Malfeito-Ferreira, M. and Loureiro, V. (2003a) Identification of yeasts isolated from wine-related

environments and capable of producing 4-ethylphenol. *Food Microbiol* **20**, 567–574.

Dias, L., Pereira-da-Silva, S., Tavares, M., Malfeito-Ferreira, M. and Loureiro, V. (2003b) Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis*in enological conditions. *Food Microbiol* **20**, 377–384.

Dugelay, L., Beaumes, R., Gunata, Z., Razungles, A. and Bayonove, C. (1995) Evolution de l'arome au cours de la conservation du vin: formation de 4-(l-étoxyethyl) pheno et de 4-(étoxyethyl) guaiacol. *Sci Aliments* **15**, 423–433.

Ferreira, V., Ortin, N., Escudero, A., López, R. and Cacho, J. (2002) Chemical characterization of the aroma of Grenache Rosé wines: aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *J Agric Food Chem* **50**, 4048–4054.

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

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

Huang, H., Chen, L., Tokashiniki, M., Ozawa, T., Taira, T. and Ito, S. (2012) An endogenous factor enhances ferulic acid decarboxylation catalyzed by phenolic acid decarboxylase from *Candida guilliermondii*. *AMB Express* **4**, 2–10.

Lopes, C.A., Jofré, V. and Sangorrín, M.P. (2009a) Spoilage yeasts in Patagonian winemaking: molecular and physiological features of *Pichia guilliermondii* indigenous isolates. *Rev Argent Microbiol* **41**, 177–184.

Lopes, C.A., Sáez, J.S. and Sangorrín, M.P. (2009b) Differential response of *Pichia guilliermondii* spoilage isolates to biological and physicochemical factors prevailing in Patagonian wine fermentation. *Can J Microbiol* **55**, 801–809.

Martorell, P., Barata, A., Malfeito-Ferreira, M., Fernández-Espinar, M.T., Loureiro, V. and Querol, A. (2006) Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources. *Int J Food Microbiol* **106**, 79–84.

Mitrakul, C., Henick-Kling, T. and Egli, C. (1999) Discrimination of *Brettanomyces/Dekkera*yeast isolates from wine using various DNA fingerprinting. *Food Microbiol* **16**, 3–14.

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

Pizarro, C., Pérez del Notario, N. and González Sáiz, J.M. (2007) Determination of Brett character responsible compounds in wines by using multiple headspace solid-phase microextraction. *J Chromatogr A* **A1143**, 176–181.

Rohlf, F.J. (2005) *NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System [Computer Program]. Version 2.2 Exeter software*. Setauket, NY: Applied Biostatistics Incorporation.

Ross, K.A., Beta, T. and Arntfield, S.D. (2009) A comparative study on the phenolic acids identified and quantified in dry beans using HPLC as affected by different extraction and hydrolysis methods. *Food Chem* **113**, 336–344.

Sáez, J.S., Lopes, C.A., Kirs, V.C. and Sangorrín, M.P. (2010) Enhanced volatile phenols in wine fermented with *Saccharomyces cerevisiae* and spoiled with *Pichia guilliermondii* and *Dekkera bruxellensis*. *Lett Appl Microbiol* **51**, 170–176.

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

Silva, P., Cardoso, H. and Gerós, H. (2004) Studies on the wine spoilage capacity of *Brettanomyces/Dekkera* spp. *Am J Enol Vitic* **55**, 65–72.

Suárez, R., Suárez-Lepe, J.A., Morata, A. and Calderón, F. (2007) The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*. A review. *Food Chem* **102**, 10–21.

Tchobanov, I., Gal, L., Guilloux-Benatier, M., Remize, F., Nardi, T., Guzzo, J., Serpaggi, V. and Alexandre, H. (2008) Partial vinylphenolreductase purification and characterization from *Brettanomyces bruxellensis*. *FEMS Microbiol Lett* **284**, 213–217.

Woolfit, M., Rozpedowska, E.L., Piskur, J. and Wolfe, K. (2007) Genome survey sequencing of the wine spoilage yeast *Dekkera (Brettanomyces) bruxellensis*. *Eukaryot Cell* **6**, 721–733.