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Spoilage yeasts in Patagonian winemaking: molecular and physiological features of *Pichia guilliermondii* indigenous isolates

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ABSTRACT

Yeasts belonging to the genus *Dekkera*/*Brettanomyces*, especially the species *Dekkera bruxellensis*, have long been associated with the production of volatile phenols responsible for off-flavour in wines. According to recent reports, the species *Pichia guilliermondii* could also produce these compounds at the initial stages of fermentation. Based on the abundance of *P. guilliermondii* in Patagonian winemaking, we decided to study the relevance of indigenous isolates belonging to this species as wine spoilage yeast. Twenty-three indigenous isolates obtained from grape surfaces and red wine musts were analyzed in their capacity to produce volatile phenols on grape must. The relationship between molecular Random Amplified Polymorphic DNA (RAPD) and physiological (killer biotype) patterns detected in indigenous populations of *P. guilliermondii* and volatile phenol production was also evaluated. Different production levels of 4-ethylphenol, 4-vinylguaiacol and 4-ethylguaiacol were detected among the isolates; however, the values were always lower than those produced by the *D. bruxellensis* reference strain in the same conditions. High levels of 4-vinylphenol were detected among *P. guilliermondii* indigenous isolates. The combined use of RAPD and killer biotype allowed us to identify the isolates producing the highest volatile phenol levels.

Key words: *Pichia guilliermondii*, volatile phenols, spoilage yeasts, RAPD, killer biotype

RESUMEN

Levaduras contaminantes en vinos patagónicos: características moleculares y fisiológicas de los aislamientos indígenas de *Picchia guilliermondii*. Las levaduras del género *Dekkera*/*Brettanomyces*, sobre todo la especie *Dekkera bruxellensis*, siempre han sido asociadas con la producción de fenoles volátiles responsables de aromas desagradables en los vinos. Recientemente, se ha demostrado que la especie *Pichia guilliermondii* también es capaz de producir estos compuestos, particularmente durante las etapas iniciales de la fermentación. Dada la abundancia de *P. guilliermondii* en las bodegas de la Patagonia, se decidió evaluar la importancia de algunos aislamientos indígenas de esta especie como levaduras alterantes de vinos regionales. Se evaluó la capacidad de producir fenoles volátiles en ensayos sobre mosto de 23 aislamientos de *P. guilliermondii* provenientes de superficie de uvas y de mostos de fermentación de vinos tintos. Asimismo, se analizó la relación entre los patrones moleculares (RAPD) y fisiológicos (biotipo *killer*) de estos aislamientos y la producción de fenoles volátiles. Se detectaron diferentes niveles de producción de 4-etilfenol, 4-vinilguayacol y 4-etilguayacol entre los aislamientos de *P. guilliermondii* analizados; sin embargo, los valores obtenidos fueron en todos los casos inferiores a los producidos por *D. bruxellensis* cepa de referencia en las mismas condiciones. En general, se detectaron altos niveles de 4-vinilfenol en los mostos fermentados con los aislamientos indígenas de *P. guilliermondii.* El uso combinado de RAPD-PCR y el biotipo *killer* permitió identificar los aislamientos que producen los niveles más altos de fenoles volátiles.

Palabras clave: *Pichia guilliermondii*, fenoles volátiles, levaduras contaminantes, RAPD, biotipo *killer*

INTRODUCTION

The metabolism of the indigenous yeasts and bacterial biota is responsible for several changes in the organoleptic properties of wine during the process of fermentation, aging, and storage (12, 24). In particular, yeasts belonging to the genus *Dekkera*/*Brettanomyces* have been recognized as the sole agent capable of producing phenolic taint in wines associated with disagreeable aromas described as horse sweat, stable, leather, and others. Volatile phenols are originated from hydroxycinnamic acids (mainly *p*-coumaric, caffeic, and ferulic acids), natural constituents of the grape must and wine (25). These acids can be metabolized by different microorgan-

isms to form 4-vinyl derivatives, which can be reduced to 4-ethyl derivatives in wine by means of the sequential action of the enzymes hydroxycinnamate decarboxylase and vinylphenol reductase (11, 27). Hydroxycinnamate decarboxylase is present in a large number of yeasts and other microorganisms (5, 22, 23); however, vinylphenol reductase has only been associated with the species *Dekkera bruxellensis* and *Dekkera anomala* (6, 7). Recently, vinylphenol reductase activity has also been related to the species *Candida versatilis*, *Candida fermentati* and *Pichia guilliermondii* (26). Contrary to *D. bruxellensis*, the production of volatile phenols by these species in enological conditions has been poorly studied. Martorell *et al.* (17) have evidenced differential efficiencies of 4-ethylphenol production in synthetic media in *P. guilliermondii* isolates from enological origin. However, the possibility that high levels of 4-ethylphenol in wine are due to this species would be only related to its uncontrolled growth in grape juices before starter inoculation (2).

P. guilliermondii is a species frequently found in the Patagonian winemaking environment, and phenolic aroma detected in a young red wine has been recently associated with high-colony forming unit (CFU) numbers of this species in the initial stages of spontaneous fermentations (14). In order to analyze the potentiality of Patagonian *P. guilliermondii* indigenous isolates as relevant wine spoilage yeasts, 23 isolates obtained from different wine-related sources were studied in their capability to produce volatile phenols on grape must. The intra-specific variability of these isolates using molecular (RAPD) and physiological (killer biotype) characterization methods was also evaluated. Different production levels of volatile phenols were detected among the isolates and a particularly high 4-vinylphenol production was detected. The combined use of RAPD and killer biotype allowed us to identify the isolate capable of producing the highest volatile phenol levels.

MATERIALS AND METHODS

Yeasts

Twenty three isolates previously identified by physiological and morphological features as belonging to the species *P. guilliermondii* were used in this study. These isolates had been obtained from grape surfaces and grape musts in different Patagonian cellars (14, 18). All yeast cultures were deposited in the North Patagonian culture collection (NPCC).

Ten killer reference strains were employed: *Saccharomyces cerevisiae* YAT 679 (K1), *S. cerevisiae* NCYC 738 (K2), *S. cerevisiae* NCYC 671 (K3), *Candida glabrata* NCYC 388 (K4), *Wickerhamomyces anomala* (*ex-Pichia anomala)* NCYC 434 (K5), *Kluyveromyces marxianus* NCYC 587 (K6), *Candida valida* NCYC 327 (K7), *W. anomala* NCYC 435 (K8), *Williopsis saturnus* var. *mrakii* NCYC 500 (K9) and *Kluyveromyces lactis* var. *drosophilarum* NCYC 575 (K10).

Molecular analysis

Indigenous yeast identity was performed by RFLP (restriction fragment length polymorphism) analysis of ITS1-5.8S-ITS2 rDNA region amplified by PCR (polymerase chain reaction) using primers ITS1 and ITS4 (Table 1) as described by Esteve-Zarzoso et al. (9). Patterns obtained for each isolate were compared with those of reference strains available in the www.yeast-

Table 1. List of primers used in the present study

Primer	Sequence (5'- 3')	Target region
NL-1	GCATATCAATAAGCGGAGGAAAAG	D1/D2-26S rDNA
NL-4	GGTCCGTGTTTCAAGACGG	D1/D2-26S rDNA
ITS 1	TCCGTAGGTGAACCTGCGG	ITS1-5.8S-ITS2 rDNA
ITS 4	TCCTCCGCTTATTGATATGC	ITS1-5.8S-ITS2 rDNA
OPA 1	CAGGCCCTTC	Genomic DNA
OPA 2	TGCCGAGCTG	Genomic DNA
OPA 3	AGTCAGCCAC	Genomic DNA
OPA 7	GAAACGGGTG	Genomic DNA
OPA 8	GTGACGTAGG	Genomic DNA
OPA 9	GGGTAACGCC	Genomic DNA
OPA 10	GTGATCGCAG	Genomic DNA
OPA 11	CAATCGCCGT	Genomic DNA
OPA 15	TTCCGAACCC	Genomic DNA
OPA 16	AGCCAGCGAA	Genomic DNA

id.com database. The nucleotides sequences of the D1/D2-26S (using primers NL1 and NL4, Table 1) as well as ITS1-5.8S-ITS2 rRNA gene regions were analyzed for some randomly selected isolates.

RAPD analysis using ten different primers (Table 1) was carried out for intra-specific characterization according to the methodology described by Martorell *et al.* (17).

Killer biotype analysis

The killer sensitivity of the isolates against ten reference killer yeasts (killer biotype) was tested using the seeded agar-plate technique described by Sangorrín *et al.* (20). Each *P. guilliermondii* yeast isolate was suspended in sterile water ($1x10^6$ cells/ml) and 0.1 ml of this suspension was seeded as a lawn onto YEPD-MB agar plates (g/l: glucose 10, malt extract 3, peptone 5, yeast extract 3, agar 20, methylene blue 0.003, NaCl 1, buffered at pH 4.6 with 0.5 M phosphate-citrate). After this, the seeded plates were streaked with thick smears of 48 h killer cultures and incubated at 18 ± 2 °C for 48-72 h. The lawn *P. guilliermondii* yeast isolate was designated as sensitive when a clear zone of growth inhibition was observed surrounding the killer culture streaks. The experiments were performed in triplicates.

***P. guilliermondii* monoculture fermentations**

Fermentations were carried out using Syrah red grape juice (238.7 g/l of total reducing sugars, 4.75 g/l of total acidity expressed as tartaric acid, pH 3.82) from the North-Patagonian region, filter-sterilized and supplemented with 100 mg/l of *p*-coumaric acid (Sigma-Aldrich, Argentina). The concentration of precursor molecules *p*-coumaric and ferulic acids in the must before the addition of *p*-coumaric acid were 4.8 mg/l and 2.6 mg/l, respectively. Fermentations were carried out in 15 ml screw cap tubes containing 10 ml of must prepared as described above. After inoculation with an initial population of 10^4 cells/ml of each *P. guilliermondii* yeast culture, tubes were incubated at 26 °C during 30 days without agitation. *D. bruxellensis* and *Candida boidinii* monoculture fermentations were carried out as control. Yeast growth at the end of the monoculture fermentations was evaluated by viable yeast enumeration on GPY-agar plates (g/l: glucose 40, peptone 5, yeast extract 5, agar 20). The experiments were performed in duplicates.

Volatile phenol detection

Concentrations of 4-vinylphenol (4-VP), 4-ethylphenol (4-EP), 4-vinylguaiacol (4-VG) and 4-ethylguaiacol (4-EG) were analyzed by headspace solid-phase microextraction (HS-SPME) with polyacrylate fibers (PA, Varian, Argentina) and gas chromatography/mass spectrometry (GC/MS) using a Varian CP-3800 gas chromatograph with an ion trap mass detector Saturn 2200. Separation was performed using a Factor Four VF-5MS (30 m x 0.25 mm x 0.25 mm), and the carrier gas was helium with a flow-rate of 1 ml/min. The oven temperature was programmed as follows: 50 °C (3 min), 15 °C/min to 80 °C (1 min), 2.5 °C/min to 120 °C (1 min), 30 °C/min to 250 °C (5 min), and the detector temperature was set at 250 °C.

For sample preparation, 10 ml of sample (musts) were placed into a 20-ml vial, with 3 g of NaCl and 200 µl of 13 mg/l anisole in ethanol (final internal standard concentration of 260 mg/l) and a magnetic stirrer. Samples were equilibrated for 30 min at 40 °C and magnetically stirred at 1000 rpm before extraction. Polyacrylate fiber was exposed to the sample headspace during 60 min, under the same conditions of temperature and agitation. The fiber was inserted into the injection port of the gas chromatograph for thermal desorption at 280 °C during 5 min. Standards were supplied by Sigma–Aldrich (Argentina).

Statistical analysis

Analysis of variance (ANOVA) and Tukey's honestly significant difference tests (HSD) with α=0.05 were performed for mean comparison. The data normality and variance homogeneity in the residuals were verified by Lilliefors and Bartlet tests respectively. Principal Component Analysis (PCA) on the centered and standardized quantitative variables (4-ethylphenol, 4-vinylguaiacol and 4-ethylguaiacol levels) was performed using the NTSYS program (19).

RESULTS AND DISCUSSION

Molecular characterization of Patagonian *P. guilliermondii* indigenous isolates

All the indigenous isolates showed the same ITS/RFLP pattern characterized by an amplified product of 650 bp and restriction fragments with *Cfo*I (300+270 bp), *Hae*III (400+120+80 bp) and *Hin*fI (320+300 bp), already reported for *P. guilliermondii* (Yeast-id database). This result as well as the ITS1-5.8S-ITS2 and D1/D2 26S rDNA gene sequence analysis confirmed that our isolates belonged to the species *P. guilliermondii* (data not shown).

In order to evaluate the intra-specific genetic variability, all the isolates were subjected to RAPD analysis using ten different primers (Table 1). RAPD analysis has been previously used for intra-specific characterization of different species (1, 3, 16); however, no information

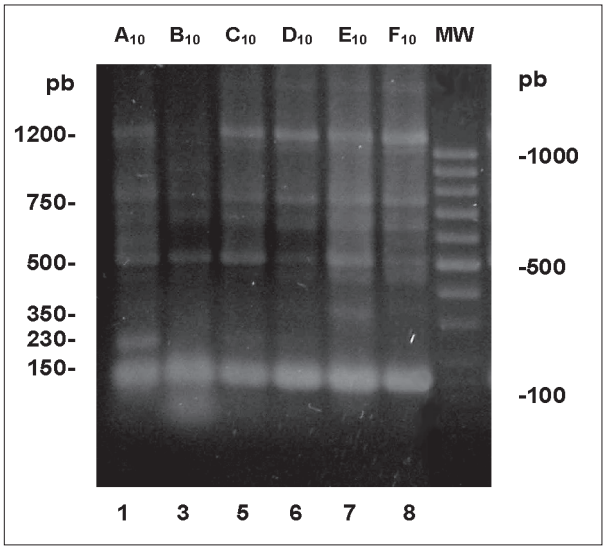

Figure 1. Molecular patterns detected among *P. guilliermondii* isolates using RAPD analysis with primer OPA10. Capital letters at the top of the Figure indicate the corresponding RAPD pattern of the isolates. MW: 100 pb molecular weight marker.

Table 2. Origin, RAPD pattern and killer biotype of the 23 *P. guilliermondii* indigenous isolates

P. guilliermondii isolates			OPA 10 RAPD pattern	Killer biotype[1]	Combined profile[2]
Nº	Code	Origin			
1	NPCC1051	Red grape surface	A_{10}	K5,K6,K7,K8,K10	I
2	NPCC1052	Red grape surface	A_{10}	K5,K8,K9,K10	II
3	NPCC1053	Red grape surface	B_{10}	K8,K10	III
4	NPCC1054	Red grape surface	A_{10}	K5,K8,K9,K10	II
5	NPCC1055	Fresh red must	C_{10}	K5,K8,K9,K10	IV
6	NPCC1056	Fresh red must	D_{10}	K5,K8,K9,K10	V
7	NPCC1057	Fermenting red must	E_{10}	K10	VI
8	NPCC1058	Fermenting red must	F_{10}	K10	VII
9	NPCC1059	Fermenting red must	C_{10}	K10	VIII
10	NPCC1060	Fermenting red must	C_{10}	K10	VIII
11	NPCC1061	Fermenting red must	D_{10}	K10	IX
12	NPCC1062	Fresh red must	A_{10}	K4,K5,K6,K10	X
13	NPCC1063	Fresh red must	D_{10}	K5,K10	XI
14	NPCC1064	Fermenting red must	A_{10}	K10	XII
15	NPCC1065	Fermenting red must	C_{10}	K10	VIII
16	NPCC1066	Fermenting red must	C_{10}	K10	VIII
17	NPCC1067	Fermenting red must	C_{10}	K5,K10	XIII
18	NPCC1068	Fermenting red must	C_{10}	K10	VIII
19	NPCC1069	Fermenting red must	C_{10}	K10	VIII
20	NPCC1070	Fermenting red must	C_{10}	K10	VIII
21	NPCC1071	Fermenting red must	C_{10}	K10	VIII
22	NPCC1072	Fermenting red must	C_{10}	K10	VIII
23	NPCC1073	Fermenting red must	C_{10}	K10	VIII
Total patterns			6	6	13

[1] Letter K followed by arabic numbers indicate the reference killer strains (identity in Materials and Methods) with lethal action on the respective *P guilliermondii* isolate.
[2] Profiles obtained using combined RAPD and killer biotype patterns.
NPCC: North Patagonian *culture collection,* Neuquén, Argentina.

was found on the use of this molecular analysis in *P. guilliermondii* diversity studies.

Four out of ten primers analyzed (OPA 3, OPA 9, OPA 10 and OPA 16) generated satisfactory and reproducible amplifications; however, three of them (OPA 3 and OPA 9 and OPA 16) rendered identical patterns for all the *P. guilliermondii* isolates. Therefore, only primer OPA10 showed capability to detect some degree of intra-specific genetic variability, rendering six different patterns (Figure 1, Table 2). A main pattern (pattern C_{10}) was detected in 52% of the isolates and was mainly associated with yeast cultures isolated from fermenting red musts (Table 2).

In order to find a second additional tool for *P. guilliermondii* isolate characterization, we evaluated its killer sensitivity patterns (killer biotype) against a panel of ten well-known killer yeasts. This physiological method has been reported to be a good diversity index when used in combination with molecular markers as mtDNA-RFLP (mitochondrial DNA restriction analysis) or RAPD analyses (4, 13). We observed a similar discriminatory capacity of killer biotype (six different patterns) regarding RAPD analyses (Table 2). Moreover, a relationship between the killer sensitivity and the origin of the isolates was detected: *P. guilliermondii* isolates recovered from grapes and fresh musts (musts without an evident beginning of fermentation) exhibited a higher killer sensitivity spectrum than that presented by the isolates recovered from active fermenting musts (Table 2). Ninety three percent of the isolates from active fermenting musts showed killer sensitivity

Table 3. Production of volatile phenols by *P. guilliermondii* indigenous isolates and reference strains

P. guilliermondii isolates		Volatile phenol production (µg/l)[2]			
Combined profile[1]	Nº	4-VG	4- EG	4-VP	4-EP
I	1	637.74 ± 126.94 ^{ab}	0.47 ± 0.04^{a}	>3000	34.87 ± 2.77^{ab}
II	2	661.31 ± 84.91 ^{ab}	0.32 ± 0.09^{a}	>3000	63.04 ± 3.38^{ab}
II	4	628.19 ± 174.14 ^{ab}	0.30 ± 0.03^{a}	>3000	51.27 ± 7.62^{b}
III	3	771.29 ± 241.87 ^{ab}	0.80 ± 0.75^{ab}	>3000	57.76 ± 6.29^{b}
IV	5	874.99 ± 25.74^{a}	1.56 ± 0.42^{bc}	>3000	196.95 ± 36.94^{a}
V	6	759.96 ± 122.44 ^{ab}	1.59 ± 0.21^{bc}	>3000	128.25 ± 39.73^{ab}
VI	7	671.28 ± 23.00 ^{ab}	2.18 ± 0.32^{c}	>3000	61.79 ± 26.27^{ab}
VII	8	601.30 ± 40.41 ^{ab}	0.74 ± 0.27^{ab}	>3000	96.76 ± 14.65^{ab}
VIII	9	374.87 ± 129.50 ^{b}	0.17 ± 0.09^{a}	>3000	57.15 ± 37.96^{b}
VIII	10	502.69 ± 9.36 ^{ab}	0.45 ± 0.13^{a}	>3000	64.49 ± 7.60^{ab}
VIII	15	528.72 ± 169.35 ^{ab}	0.30 ± 0.16 ^{a}	>3000	35.29 ± 21.41^{b}
VIII	16	688.64 ± 11.34 ^{ab}	0.70 ± 0.21^{ab}	>3000	65.14 ± 8.53^{ab}
VIII	18	637.93 ± 61.36 ^{ab}	0.65 ± 0.16 ^{ab}	>3000	61.95 ± 8.50^{ab}
VIII	19	556.79 ± 69.38 ^{ab}	0.27 ± 0.06 ^{a}	>3000	105.01 ± 87.17^{ab}
VIII	20	535.46 ± 54.09 ^{ab}	0.33 ± 0.11 ^{a}	>3000	62.93 ± 6.83^{ab}
VIII	21	685.30 ± 43.76 ^{ab}	0.50 ± 0.19^{a}	>3000	135.50 ± 13.34^{ab}
VIII	22	699.78 ± 7.19 ^{ab}	0.42 ± 0.11^{a}	>3000	143.56 ± 4.92^{ab}
VIII	23	710.71 ± 19.22 ^{ab}	0.41 ± 0.19^{a}	>3000	82.36 ± 5.61^{ab}
IX	11	585.40 ± 77.28 ^{ab}	0.62 ± 0.20^{a}	>3000	146.59 ± 95.70^{ab}
X	12	706.79 ± 220.20 ^{ab}	0.48 ± 0.00^{a}	>3000	25.66 ± 2.55^{b}
XI	13	481.49 ± 19.60 ^{ab}	0.46 ± 0.05 ^{a}	>3000	68.78 ± 34.90^{ab}
XII	14	568.68 ± 33.68 ^{ab}	0.45 ± 0.05 ^{a}	>3000	44.07 ± 2.99^{b}
XIII	17	558.43 ± 6.89 ^{ab}	0.39 ± 0.01 ^{a}	>3000	73.48 ± 59.36^{ab}
D. bruxellensis		<10	534.08 ± 110.43	591.88 ± 176.25	722.40 ± 388.29
C. boidinii		243.33 ± 42.91	0.19 ± 0.09	468.81 ± 4.08	<10

[1] Profiles obtained using combined RAPD and killer biotype patterns (Table 1).
[2] 4-VG: 4-vinylguaiacol; 4-EG: 4-ethylguaiacol; 4-VP: 4-vinylphenol; 4-EP: 4-ethylphenol values are expressed as media ± standard deviation. Values not sharing the same superscript letter (a, b, c) within the vertical line are significantly different (ANOVA and Tukey's HSD test, α=0.05, n= 2). Only the values obtained for the *P. guilliermondii* isolates were taken into consideration in order to evaluate the statistical significances.

pattern K10, i.e. they were only sensitive against *K. lactis* var. *drosophilarum* NCYC 575 killer strain. Therefore, the sensitivity toxin profiles could be revealing yeast isolates with particular physiological characteristics associated with the specific substrate of origin. The same differential origin-related behaviour was observed when these *P. guilliermondii* isolates were exposed to different physical and chemical stress conditions as well as against different regional killer yeasts (15).

It is interesting to note that only the reference strain *K. lactis* var. *drosophilarum* NCYC 575 (K10), was effective against all *P. guilliermondii* isolates (Table 2). To a lesser extent, both *W. anomala* killer reference strains (K5 and K8, exhibiting different killer activities) were capable of killing a high percentage of spoilage isolates (Table 2).

Finally, the combined use of killer biotype and RAPD patterns allowed us to increase the discriminatory capacity exhibited by the molecular methods themselves to differentiate indigenous *P. guilliermondii* isolates (Table 2).

Volatile phenol production by *P. guilliermondii* strains in single cultures

The capacity of indigenous *P. guilliermondii* isolates to produce volatile phenols during winemaking was tested

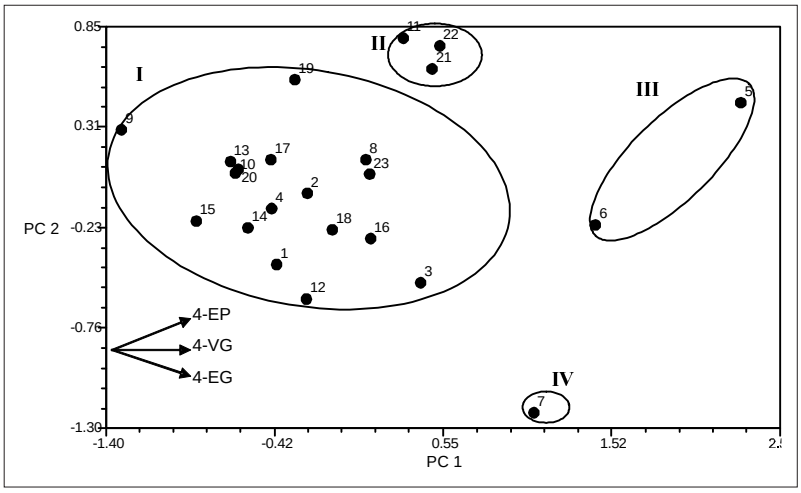

Figure 2. Principal Component Analysis (PCA) of 4-ethylphenol (4-EP), 4-vinylguaiacol (4-VG) and 4-ethylguaiacol (4-EG) levels obtained after must fermentation with the 23 *P. guilliermondii* indigenous isolates. Symbols indicate each isolate. Arrows inside the Figure indicate the eigenvectors obtained in PCA.

in microfermentations. Fermentations inoculated with spoilage yeasts *D. bruxellensis* and *C. boidinii* spoilage species commonly found in Patagonian cellars (21), were carried out as positive and negative controls for ethylphenol-production respectively.

All three species evaluated were capable of growing in must, reaching similar cell counts before 30 days of fermentation as well as of converting the *p*-coumaric acid added and the ferulic acid naturally present in the must into the respective volatile phenols. However, significant differences in the levels of these compounds were detected among species (ANOVA and Tukey's HSD test, $\alpha = 0.05$, n = 2), being *D. bruxellensis* the species showing the highest levels of both 4-EG and 4-EP final products. Several works showed the different ability of *P. guilliermondii* and *D. bruxellensis* to produce phenol volatiles (2, 8, 17). Our results confirmed those observations and identified for the first time 4-VG and 4-VP highly productive strains isolated from Patagonian wines.

According to our results, *D. bruxellensis* was able to consume ferulic acid naturally present in the must, yielding high levels of 4-EG (534.08 µg/l) and non detectable levels of 4-VG (Table 3). On the contrary, *P. guilliermondii* isolates consumed the ferulic acid, producing high levels of 4-VG (Table 3), and low levels of 4-EG (< 2 µg/l) (Table 3). Suezawa and Suzuki (26) showed that 4-VG is converted to 4-EG by *C. versatilis* and *C. fermentati;* however, as it was observed in our work, they did not detect this activity in *Candida guilliermondii* (anamorph of *P. guilliermondii).* Regarding volatile phenols derived from *p*-coumaric acid, *P. guilliermondii* produced high levels of the intermediary compound 4-VP (more than 3000 µg/l) and medium levels of 4-EP (lower than 200 µg/l), when compared with the values obtained with *D. bruxellensis.* These differences could be related to different metabolic rates for both enzymes involved in the two species analyzed. However, more studies are being carried out in our laboratory in order to elucidate these differences.

Significant differences were detected in 4-EP, 4-VG and 4-EG production levels among *P. guilliermondii* isolates, revealing the existence of strains with different metabolic capacities (Table 3). Principal Component Analysis (PCA) was used in order to cluster these isolates according to the production of volatile phenols (Figure 2). PCA analysis explained the 85% of total variability in the data in the first two dimensions. Four clusters of isolates could be distinguished in the PCA chart: i) cluster I is composed by the majority of the isolates bearing similar medium capacities for volatile phenol production; ii) cluster II comprises three isolates with very similar metabolic capacities mainly characterized by the elevated production of 4-EP; iii) cluster III segregated according to the ability of the isolates to produce the highest levels of the three volatile phenols including the highest levels of 4-EP and 4-VG; and iv) finally, cluster IV is composed only by isolate 7 showing the highest of 4-EG levels (Figure 2).

Finally, although the ethylphenol levels (4-EG and 4-EP) produced by the *P. guilliermondii* isolates do not seem to be dangerous for winemaking (7, 11), it could be interesting to study the effect of the high vinylphenol levels (4-

VG and 4-VP) produced by these isolates. According to previous publications, cumulative perception thresholds of 770 and 426 µg/l have been reported for vinyl- and ethylphenols respectively (7, 10); therefore, the vinylphenol levels produced by *P. guilliermondii* isolates could be related to the detection of unpleasant aromas in wines.

Regarding to the use of the intra-specific characterization methods applied in this work, we observed that neither the RAPD nor the killer biotype were able to differentiate the isolates capable of producing the highest levels of volatile phenols (Table 2). However, unique combined profiles for these isolates (profiles IV, V and VI corresponding to isolates 5, 6 and 7, respectively) were observed when the combined use of both molecular and physiological characterization methods were taken into account (Figure 2).

In conclusion, the spoilage yeast *P. guilliermondii* was confirmed to be present in grapes and wine fermentations in Patagonia. All 23 isolates tested were able to produce volatile phenols, and they were particularly able to synthesize high amounts of the intermediary compounds 4-VG and 4-VP in comparison with the levels produced by the *D. bruxellensis* reference strain in the same conditions. We also demonstrated that the combined use of the RAPD and killer biotype analyses proved to be an interesting tool in the fingerprinting of particularly dangerous *P. guilliermondii* spoilage strains.

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REFERENCES

1. Baleiras Couto B, van der Vossen M, Hofstra H, Huis in't Veld JH. RAPD analysis: a rapid technique for differentiation of spoilage yeasts. Int J Food Microbiol 1994; 24: 249-60.
2. Barata A, Nobre A, Correia P, Malfeito-Ferreira M, Loureiro V. Growth and 4-ethylphenol production by the yeast *Pichia guilliermondii* in grape juices. Am J Enol Vitic 2006; 57: 133-8.
3. Budjoso G, Egli CM, Henick-Kling T. Inter-and intraspecific differentiation of natural wine strains of *Hanseniaspora* (*Kloeckera*) by physiological and molecular methods. Food Technol Biotechnol 2001; 39: 19-28.
4. Buzzini P, Turchetti B, Vaughan-Martini AE. The use of killer sensitivity patterns for biotyping yeast strains: the state of the art, potentialities and limitations. FEMS Microbiol Lett 2007; 7: 749-60.
5. Cavin JF, Barthelmebs L, Guzzo J, Van Beeumen J, Samyn B, Travers JF, *et al.* Purification and characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum.* FEMS Microbiol Lett 1997; 147: 291-5.
6. Chatonnet P, Dubordeau D, Boidron JN. The Influence of *Brettanomyces/Dekkera* sp. yeasts and lactic acid bacteria on the ethylphenol content of red wines. Am J Enol Vitic 1995; 46: 463-8.
7. Chatonnet P, Viala C, Dubourdieu D. Influence of polyphenolic components of red wines on the microbial synthesis of volatile phenols. Am J Enol Vitic 1997; 48: 443-8.
8. Dias L, Pereira-da-Silva S, Tavares M, Malfeito-Ferreira M, Loureiro V. Factors affecting the production of 4-ethylphenol by the yeast *Dekkera bruxellensis* in enological conditions. Food Microbiol 2003; 20: 377-84.
9. Esteve-Zarzoso B, Belloch C, Uruburu F, Querol A. Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and two ribosomal internal transcribed spacers. Int J Syst Bacteriol 1999; 49: 329-37.
10. Etiévant PX, Issanchou SN, Marie S, Ducruet V, Flanzy C. Sensory impact of volatile phenols on wine aroma: influence of carbonic maceration and time of storage. Sci Aliments 1989; 9: 19-33.
11. Godoy L, Martínez C, Carrasco N, Ganga MA. Purification and characterization of a p-coumarate decarboxylase and a vinylphenol reductase from *Brettanomyces bruxellensis*. Int J Food Microbiol 2008; 127: 6-11.
12. Lambrechts MG. Pretorius IS. Yeast and its importance to wine aroma: a review. S Afr J Enol Vitic 2000; 21: 97-129.
13. Lopes CA, Lavalle TL, Querol A, Caballero AC. Combined use of killer biotype and mtDNA-RFLP patterns in a Patagonian wine *Saccharomyces cerevisiae* diversity study. Antonie van Leeuwenhoek 2006; 89: 147-56.
14. Lopes CA, Rodríguez ME, Sangorrín M, Querol A, Caballero AC. Patagonian wines: implantation of an indigenous strain of *Saccharomyces cerevisiae* in fermentations conducted in traditional and modern cellars. J Ind Microbiol Biotechnol 2007; 34: 139-49.
15. Lopes CA, Sáez JS, Sangorrín MP. Differential response of *Pichia guilliermondii* spoilage isolates to biological and physical-chemical factors prevailing in Patagonian wine fermentations. Can J Microbiol 2009; doi: 55: 801-809.
16. Martorell P, Fernández-Espinar MT, Querol A. Molecular monitoring of spoilage yeasts during the production of candied fruit nougats to determine food contamination sources. Int J Food Microbiol 2005; 101: 293-302.
17. Martorell P, Barata A, Malfeito-Ferreira M, Fernández-Espinar MT, Loureiro V, Querol A. Molecular typing of the yeast species *Dekkera bruxellensis* and *Pichia guilliermondii* recovered from wine related sources. Int J Food Microbiol 2006; 106: 79-84.
18. Rodríguez ME, Lopes CA, van Broock M, Vallés S, Ramón D, Caballero AC. Screening and typing of Patagonian wine yeasts for glycosidase activity. J App Microbiol 2004; 96: 84-95.
19. Rohlf FJ. NTSYSpc: Numerical taxonomy and multivariate analysis system, version 22 Exeter software: Setauket, New York, 2005.
20. Sangorrín MP, Zajonskovsky I, Lopes CA, Rodríguez ME, van Broock MR, Caballero AC. Killer behaviour in wild wine yeasts associated with Merlot and Malbec type musts spontaneously fermented from Northwestern Patagonia (Argentina). J Basic Microbiol 2001; 41: 105-13.
21. Sangorrín MP, Lopes C A, Jofré V, Querol A, Caballero, A C. Spoilage yeasts associated with Patagonian cellars: characterization and potential biocontrol based on killer interactions. World J Microbiol Biotechnol 2008; 24: 945-53.

22. Shinohara T, Kubodera S, Yanagida F. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. J Biosci Bioeng 2000; 90: 90-7.
23. Smit AL, Cordero Otero RR, Lambrechts MG, Pretorius IS, Van Rensburg P. Enhancing volatile phenol concentrations in wine by expressing various phenolic acid decarboxylase genes in *Saccharomyces cerevisiae.* J Agric Food Chem 2003; 51: 4909-15.
24. Stratford, M. Food and beverage spoilage yeast. In: Querol, A & Feet, G (Eds) Yeasts in food and beverages. Springer-Velarg Berlin Heidelberg, Berlin, 2006; p. 336-79.
25. Suárez R, Suárez-Lepe JA, Morata A, Calderón F. The production of ethylphenols in wine by yeasts of the genera *Brettanomyces* and *Dekkera*: A review. Food Chem 2007; 102: 10-21.
26. Suezawa Y, Suzuki M. Bioconversion of ferulic acid to 4-vinylguaiacol and 4-ethylguaiacol and of 4-vinylguaiacol to 4-ethylguaiacol by halotolerant yeasts belonging to the genus *Candida.* Biosci Biotechnol Biochem 2007; 71: 1058-62.
27. Tchobanov I, Gal L, Guilloux-Benatier M, Remize F, Nardi T, Guzzo J, *et al*. Partial vinylphenol reductase purification and characterization from *Brettanomyces bruxellensis*. FEMS Microbiol Lett 2008; 284: 213-7.

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