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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

**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

Yeast

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. makii NCYC 500 (K9) and Kluyveromyces lactis var. drosophilairum 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>
<thead>
<tr>
<th>Table 1. List of primers used in the present study</th>
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<td>Primer</td>
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<tr>
<td>NL-1</td>
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<td>NL-4</td>
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<tr>
<td>ITS 1</td>
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<td>ITS 4</td>
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<td>OPA 1</td>
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<td>OPA 2</td>
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<td>OPA 3</td>
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<tr>
<td>OPA 7</td>
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<tr>
<td>OPA 8</td>
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<tr>
<td>OPA 9</td>
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<tr>
<td>OPA 10</td>
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<tr>
<td>OPA 11</td>
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<tr>
<td>OPA 15</td>
</tr>
<tr>
<td>OPA 16</td>
</tr>
</tbody>
</table>
id.com database. The nucleotide sequences of the D1/D2-26S 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 (1x10⁶ 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 10⁶ cells/ml of each *P. guilliermondii* yeast culture, tubes were incubated at 26 °C during 30 days without agitation. *D. bruxellensis* and *Candida boidini* 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).

**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 *Cotl* (300+270 bp), *Haelli* (400+120+80 bp) and *Hinf* (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 has been previously used for intra-specific characterization of this species.

**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).

![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.](image-url)
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 C10) 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 2. Origin, RAPD pattern and killer biotype of the 23 *P. guilliermondii* indigenous isolates

<table>
<thead>
<tr>
<th><em>P. guilliermondii</em> isolates</th>
<th>OPA 10 Killer biotype(1) Combined profile(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nº Code Origin pattern</td>
<td>Combined profile pattern</td>
</tr>
<tr>
<td>1 NPCC1051 Red grape surface A10 K5,K6,K7,K8,K10 I</td>
<td></td>
</tr>
<tr>
<td>2 NPCC1052 Red grape surface A10 K5,K8,K9,K10 II</td>
<td></td>
</tr>
<tr>
<td>3 NPCC1053 Red grape surface B10 K8,K10 III</td>
<td></td>
</tr>
<tr>
<td>4 NPCC1054 Red grape surface A10 K5,K8,K9,K10 II</td>
<td></td>
</tr>
<tr>
<td>5 NPCC1055 Fresh red must C10 K5,K8,K9,K10 IV</td>
<td></td>
</tr>
<tr>
<td>6 NPCC1056 Fresh red must D10 K5,K8,K9,K10 V</td>
<td></td>
</tr>
<tr>
<td>7 NPCC1057 Fermenting red must E10 K10 VI</td>
<td></td>
</tr>
<tr>
<td>8 NPCC1058 Fermenting red must F10 K10 VII</td>
<td></td>
</tr>
<tr>
<td>9 NPCC1059 Fermenting red must C10 K8,K10 VIII</td>
<td></td>
</tr>
<tr>
<td>10 NPCC1060 Fermenting red must C10 K10 VIII</td>
<td></td>
</tr>
<tr>
<td>11 NPCC1061 Fermenting red must D10 K10 IX</td>
<td></td>
</tr>
<tr>
<td>12 NPCC1062 Fresh red must A10 K4,K5,K6,K10 X</td>
<td></td>
</tr>
<tr>
<td>13 NPCC1063 Fresh red must D10 K5,K10 XI</td>
<td></td>
</tr>
<tr>
<td>14 NPCC1064 Fermenting red must A10 K10 XII</td>
<td></td>
</tr>
<tr>
<td>15 NPCC1065 Fermenting red must C10 K10 VIII</td>
<td></td>
</tr>
<tr>
<td>16 NPCC1066 Fermenting red must C10 K10 VIII</td>
<td></td>
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<tr>
<td>17 NPCC1067 Fermenting red must C10 K5,K10 XIII</td>
<td></td>
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<tr>
<td>18 NPCC1068 Fermenting red must C10 K10 VIII</td>
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<tr>
<td>19 NPCC1069 Fermenting red must C10 K10 VIII</td>
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</tr>
<tr>
<td>20 NPCC1070 Fermenting red must C10 K10 VIII</td>
<td></td>
</tr>
<tr>
<td>21 NPCC1071 Fermenting red must C10 K10 VIII</td>
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</tr>
<tr>
<td>22 NPCC1072 Fermenting red must C10 K10 VIII</td>
<td></td>
</tr>
<tr>
<td>23 NPCC1073 Fermenting red must C10 K10 VIII</td>
<td></td>
</tr>
<tr>
<td>Total patterns</td>
<td>6 6 13</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th><em>P. guilliermondii</em> isolates</th>
<th>Volatile phenol production (µg/l)(2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Combined Nº</td>
</tr>
<tr>
<td>I 1</td>
<td>637.74 ± 126.94</td>
</tr>
<tr>
<td>II 2</td>
<td>661.31 ± 84.91</td>
</tr>
<tr>
<td>II 4</td>
<td>628.19 ± 174.14</td>
</tr>
<tr>
<td>III 3</td>
<td>771.29 ± 241.87</td>
</tr>
<tr>
<td>IV 5</td>
<td>874.99 ± 25.74</td>
</tr>
<tr>
<td>V 6</td>
<td>759.96 ± 122.44</td>
</tr>
<tr>
<td>VI 7</td>
<td>671.28 ± 23.00</td>
</tr>
<tr>
<td>VII 8</td>
<td>601.30 ± 40.41</td>
</tr>
<tr>
<td>VIII 9</td>
<td>374.87 ± 129.50</td>
</tr>
<tr>
<td>VIII 10</td>
<td>502.69 ± 9.36</td>
</tr>
<tr>
<td>VIII 15</td>
<td>528.72 ± 169.35</td>
</tr>
<tr>
<td>VIII 16</td>
<td>688.64 ± 11.34</td>
</tr>
<tr>
<td>VIII 18</td>
<td>637.93 ± 61.36</td>
</tr>
<tr>
<td>VIII 19</td>
<td>556.79 ± 69.38</td>
</tr>
<tr>
<td>VIII 20</td>
<td>535.46 ± 54.09</td>
</tr>
<tr>
<td>VIII 21</td>
<td>685.30 ± 43.76</td>
</tr>
<tr>
<td>VIII 22</td>
<td>699.78 ± 7.19</td>
</tr>
<tr>
<td>VIII 23</td>
<td>710.71 ± 19.22</td>
</tr>
<tr>
<td>IX 11</td>
<td>585.40 ± 77.28</td>
</tr>
<tr>
<td>X 12</td>
<td>706.79 ± 220.20</td>
</tr>
<tr>
<td>XI 13</td>
<td>481.49 ± 19.60</td>
</tr>
<tr>
<td>XII 14</td>
<td>568.68 ± 33.68</td>
</tr>
<tr>
<td>XIII 17</td>
<td>558.43 ± 6.89</td>
</tr>
<tr>
<td><em>D. bruxellensis</em></td>
<td>&lt;10</td>
</tr>
<tr>
<td><em>C. boidinii</em></td>
<td>243.33 ± 42.91</td>
</tr>
</tbody>
</table>

(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...
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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