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ORIGINAL ARTICLE

Characterization of α-rhamnosidase activity from a Patagonian *Pichia guilliermondii* wine strain

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Abstract

Aims: The purpose of this study was to characterize the α-L-rhamnosidase of *Pichia guilliermondii* NPCC1053 indigenous wine strain from North-Patagonian region.

Methods and Results: The optimization of yeast culture conditions was carried out and the effects of oenological parameters on α-L-rhamnosidase activity were evaluated. Additionally, the effect of direct contact with must and wine on α-L-rhamnosidase activity was assayed. This strain showed an intracellular inducible α-L-rhamnosidase activity. This enzyme was active at pH, glucose and SO_2 concentrations usually found at the beginning of the fermentation as well as retained high levels of activity after 24 h of incubation in must. Furthermore, *P. guilliermondii* α-L-rhamnosidase was able to release monoterpenols and alcohols from grape glycosidic extracts.

Conclusions: The α-L-rhamnosidase belonging to *P. guilliermondii* indigenous wine yeast strain showed mainly an intracellular location and evidenced interesting oenological characteristics.

Significance and Impact of the Study: This study contributes to the knowledge of α-L-rhamnosidases from yeast origin because at present, there are few reports about this enzymatic activity in these micro-organisms. In addition, this work is relevant to the regional wine industry considering that this enzyme could be used in the production of more aromatic young wines.

Introduction

α-L-rhamnosidase (αRh) (E.C. 3.2.1.40) is an enzyme that hydrolyses the breakage of the glycosidic linkage of rhamnose with other compounds. This enzyme has been used in several industrial processes. In food industry, αRh has been successfully applied to the production of food additives (Giavasis *et al.* 2000) and beverage quality enhancement such as the debittering of grapefruit juices by hydrolysis of naringin (Puri *et al.* 1996, 2005; Prakash *et al.* 2002), the elimination of hesperidin crystals from orange (Terada *et al.* 1995) and the enhancement of fruit juice and wine aroma (Gunata *et al.* 1993; Gallego *et al.* 2001; Gunata 2002; Manzanares *et al.* 2003, 2007). In winemaking industry, it is well established that glucosides and disaccharide glycosides such as 6-O-α- L-arabinofuranosyl-β-D-glucopyranosides, 6-O-β-D-apiofuranosyl-β-D-glucopyranosides and 6-O-α-L-rhamnopyranosyl-β-D-glucopyranosides are a potential source of varietal aroma. Additionally, compounds as monoterpenols (e.g. linalool, geraniol, nerol), higher alcohols (e.g. 2-phenylethanol), norisoprenoids (e.g. damascenone) among others, have proved to be aglycons of such glycosides (Winterhalter and Skouroumounis 1997; Maicas and Mateo 2005; Swiegers *et al.* 2005). The release of these aroma precursor molecules (aglycons) by enzymatic hydrolysis requires two sequential reactions. During the first reaction, and depending on the conjugate, the glycosidic linkage is cleaved by either β-D-apiosidase, α-L-arabinofuranosidase or an α-L-rhamnosidase (αRh) releasing the β-D-glucoside. In a second reaction, the β-D-glucoside is hydrolysed by the action of a β-D-glucosidase causing the release of a

glucose and a volatile compound aromatically active (Gunata *et al.* 1988; Gunata 2002). Because grape and yeast glycosidases seem to be insufficient to process aromatic precursors completely during winemaking, the addition of exogenous glycosidases during or after the fermentation has become a common practice in wineries. αRh is a component of these commercial enzymatic preparations. The main sources of this enzyme are filamentous fungi, being *Aspergillus Niger* the most commonly used for its production. Other filamentous fungi such as *A. terreus*, *A. nidulans* and *A. aculeatus* have been reported as αRh producers with potential value in oenology and their enzymatic activities have been well characterized (Gallego *et al.* 1996; Orejas *et al.* 1999; Manzanares *et al.* 2003). However, there is a lack of information about the αRh activity in yeasts. So far, there have been only very few reports on the αRh production, low levels of this activity were found in yeast belonging to *Saccharomyces cerevisiae*, *Hansenula anomala*, *Debaryomyces ploymorphus*, *Candida guilliermondii* and *Aureobasidium pullulans* (Miklosy and Polos 1995; Rosi *et al.* 1995; Mcmahon *et al.* 1999) but, at present, there is only a αRh of yeast origin (*P. angusta* X349) that has been purified and characterized (Yanai and Sato 2000).

Pichia guilliermondii NPCC1053 is an indigenous strain isolated from Negro River Upper Valley winegrowing region. This is a glycosidases-producing yeast that, in addition to *β*-glucosidase and *β*-xylosidase, which have been characterized in a previous work (Rodríguez *et al.* 2007), showed αRh activity (Rodríguez *et al.* 2004). The aims of this study were to characterize the *P. guilliermondii* αRh activity and to evaluate the effect of some relevant oenological features on this activity to consider its possible use in regional wine industry.

Materials and Methods

Yeast strain

Pichia guilliermondii NPCC1053 (NPCC, North Patagonian Culture Collection) is a α-L-rhamnosidase (αRh) producer Patagonian indigenous strain isolated from Malbec grapes from Comahue region and previously characterized by biochemical and molecular methods (Rodríguez *et al.* 2004).

Optimization of culture conditions

Several media containing 5 g l^{-1} of different carbon sources were assayed to evaluate the enzymatic activity levels. Media assayed were YNB-YEP (1·7 g yeast nitrogen base without amino acids and ammonium sulphate, 5 g yeast extract, 5 g peptone per litre, pH 5·5) and YNB (yeast nitrogen base 1·7 g and ammonium sulphate 5 g per litre, pH 5·5). Carbon sources were glucose, xylose, rhamnose and cellobiose. Liquid media were inoculated with 0·2 ml of an overnight GPY (5 g peptone, 5 g yeast extract and 40 g glucose per litre, pH 5·5) grown yeast culture and incubated at 28°C in an orbital shaker at 180 rev min^{-1}. After 20 h, the cultures were centrifuged (8000 ***g***, 10 min, 4°C), and both cells and culture supernatant were assayed for enzymatic activities. The dry weight was determined as described below.

The YNB-rhamnose medium (1·7 g YNB w/o amino acids and ammonium sulphate, 5 g rhamnose per litre, pH 5·5) supplemented with 0·15% (w/v) of total nitrogen (ammonium sulphate, phosphate or tartrate, peptone or urea) was used to study the effect of different nitrogen sources on αRh activity.

Kinetics of growth

Yeasts were grown in Erlenmeyer (1 l) filled with 250 ml of YNB-rhamnose and shaken at 180 rev min^{-1} in a Vicking shaker at 28°C. The liquid medium was inoculated with 0·2 ml of an overnight GPY grown yeast culture. Samples were taken at different times, and yeast growth and αRh activity were determined. The former was performed by both, measuring culture medium absorbance at 600 nm and determining dry weight, and enzymatic activity was measured as described below.

Enzymatic activity determination

αRh activity was measured using the appropriate *p*-nitrophenyl-α-L-rhamnoside (pNPR) as substrate. Fifty microliters of 0·2% (w/v) pNPR dissolved in water was mixed with 150 μl of citrate-phosphate buffer (100 mmol l^{-1}, pH 5·0) and 50 μl of an appropriate enzyme dilution. Incubation was realized at 30°C for 30 min, and the reaction was stopped by the addition of 250 μl of 0·25 mol l^{-1} sodium carbonate solution (pH 10·2). The release of *p*-nitrophenol (pNP) was measured spectrophotometrically at 405 nm in a Shimadzu UV-V spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The pNP molar extinction coefficient used was ε = 18·300 mol l^{-1} cm^{-1}. All assays were performed in duplicates. One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP per min under the above-mentioned experimental conditions. The capability of hydrolysing other artificial substrates as *p*-nitrophenyl-*β*-D-glucoside (pNPG) and *p*-nitrophenyl-*β*-D-xyloside (pNPX) by the cellular fractions was evaluated using the same enzymatic conditions previously described.

Cellular location

Cells were harvested at the end of exponential growth phase. Five millilitres of culture was centrifuged at 6000 ***g*** for 10 min, washed twice in citrate-phosphate buffer (100 mmol l^{-1}, pH 5·0), resuspended in this buffer (300 μl) and kept on ice. Glass beads (0·3-0·5 mm diameter) were added until the height of cellular suspension meniscus, and cells were disrupted by means of vortexing and cooling (30 and 30 s, respectively, for eight times). Cell debris was separated by centrifugation at 20 000 ***g*** for 20 min at 4°C. Cells debris and soluble cell extracts were assayed for parietal and intracellular activity, respectively. Additionally, parietal activity was determined in whole cells harvested from 1 ml of culture, washed twice in citrate-phosphate buffer (pH 5·0) and resuspended in 1 ml of this buffer. For the determination of extracellular activity, appropriate aliquots of culture supernatants of YNB grown cells were used.

Influence of oenological parameters on α-L-rhamnosidase activity

The effect of pH on yeast αRh activity was studied on whole cell under the standard enzymatic assay described above using citrate-phosphate buffer in the pH range from 3 to 7. The ethanol, glucose and sulphur dioxide effects on αRh activity were also determined on whole cell preparations by the addition of different concentrations of these compounds in the enzyme assay. Final concentrations from 0 to 20% (v/v) ethanol, from 0 to 1 mol l^{-1} glucose and from 0 to 150 ppm sulphur dioxide were assayed.

Must and wine effect on α-L-rhamnosidase activity

Pichia guilliermondii-αRh producer cells harvested from 1 ml of culture (YNB-rhamnose) were washed twice in distilled water and resuspended in 1 ml of young wine (pH: 3·5, 12·5% v/v ethanol and 38 mg l^{-1} free SO_2) and fresh must (21 Brix, pH: 3·7 and 43 mg l^{-1} free SO_2). Samples were incubated during 24 h at 25°C. At different times, cultures were centrifuged (8000 ***g***, 10 min, 4°C), washed twice in distilled water and resuspended in 1 ml of citrate-phosphate buffer, and the enzymatic activity was assayed as described above.

Zymograms

Native polyacrylamide gel electrophoresis was carried out using 6% (w/v) of acrylamide gel. Cellular extracts were used as enzymatic source. The enzymatic activity was revealed by overlaying the gel with 1 mmol l^{-1} 4-methylumbelliferyl-α-L-rhamnoside (MUR) during 15 min at room temperature and visualized by UV illumination. Additionally, 4-methylumbelliferyl-β-D-glucoside (MUG) and 4-methylumbelliferyl-β-D-xyloside (MUX) were also used as substrates.

Dry weight

Pellets from 10 ml of culture samples were obtained by centrifugation at 6000 ***g*** for 10 min, washed twice in 5 ml of cold sterile distilled water and resuspended in sterile distilled water. Then, pellets were placed on preweighed dishes and dried at 105°C until constant weight according with Rodríguez *et al.* (2004).

Liberation of aromatic compounds from glycoside precursors

Glycosidic precursors were obtained from Muscat grape juice using chromatography on Amberlite XAD-2 resin (Sigma, St Louis, MO). This method has been described by Gunata *et al.* (1985) and it was slightly modified. Two hundred millilitres of grape juice added with 20 μl of 0·1% (w/v) n-hetpyl-β-D-glucopyranoside (internal hydrolysis control) (Sigma) was eluted through the amberlite column, previously preconditioned with 50 ml methanol, 50 ml diethyl ether and 50 ml of water. After this, the column was washed with 200 ml of distilled water to eliminate sugars, acids and other grape juice water-soluble compounds, and free volatile compounds were eluted with 100 ml of dichloromethane: pentane (1 : 2, v/v). Glycoside precursors fraction was recupered with 100 ml of ethyl acetate:methanol (9 : 1, v/v); it was concentrated to dryness under reduced pressure (Büchi Rotavapor R-114 and Waterbath B-480; Büchi Labortechnik AG, Flawil, Switzerland) at 40°C and resuspended in 1 ml citrate-phosphate buffer (100 mmol l^{-1}, pH 5·0).

Yeast cells were harvested from 1 ml of rhamnose cultured broth by centrifugation and resuspended in 730 μl of citrate-phosphate buffer (pH 5·0) containing 250 μl of glycosidic precursors and 20 μl of 0·1% (w/v) 2-octanol (Fluka Chemie AG, Buchs, Switzerland) as internal standard. The mixture was incubated for 48 h at 40°C. The liberated volatile compounds were extracted five times with 200 μl of dichloromethane:pentane (1 : 2, v/v). After this, the solvent was evaporated by a N_2 stream to a residual volume of 50 μl. The samples were stored at −20°C until gas chromatography (GC) analyses were carried out. All assays were performed in triplicate. Glycoside extracts without cell preparations and the converse of this one were included as negative control. The free compounds released from glycosidic precursor were identified comparing their retention times with those of standard

compounds (Sigma). GC was carried out using an HP5890 series II GC (Hewlett Packard, Waldbronn, Germany) equipped with a HP-Innowax capillary column (60 m × 0·25 mm i.d. × 0·25 μm film thickness, Hewlett Packard) and a flame ionization detector. The GC operating conditions were according to those described by Rodríguez *et al.* (2007).

Statistical analysis

ANOVA and Tukey honest significant difference test with $\alpha = 0{\cdot}05$ were performed by mean comparison. The data normality and variance homogeneity in the residuals were verified by Lilliefors and Bartlet tests, respectively.

Chemicals

The carbon and nitrogen sources, pNPR, pNPG, pNPX, MUR were supplied by Sigma, and culture medium constituents by Difco.

Results

Optimization of culture conditions

Pichia guilliermondii NPCC1053 was grown on various culture media. The αRh activity was only detected when rhamnose was used as carbon source and the production was higher in YNB medium (32 U g^{-1} dry weight) than in YNB-YEP medium (4 U g^{-1} dry weight). Once the culture medium to be used for αRh production was defined, the effect of nitrogen source on enzymatic activity was assayed. The highest activity levels were detected in media containing the three ammonium salts assayed (Table 1). Under assayed condition, αRh activity levels were not detected when urea and peptone were used as nitrogen source (Table 1). Because no significant differences among the three ammonium salts were observed, sulphate ammonium was chosen for the subsequent experiments.

Table 1 Effects of different nitrogen sources on *Pichia guilliermondii* α-L-rhamnosidase activity

Nitrogen source	αRH activity*
Ammonium sulphate	100·0 ± 30·3[b]
Ammonium phosphate	91·1 ± 1·1[b]
Ammonium tartrate	124·2 ± 1·3[b]
Peptone	1·2 ± 0·1[a]
Urea	nd

*Percentage of αRH activity estimated respect of YNB-rhamnose-ammonium sulphate (32 U g^{-1} dry weight).
Different letters indicate significant differences among values (Tukey test, $P < 0{\cdot}05$, $n = 2$).

αRh production, cellular location and hydrolysis of other artificial substrates

The α-rhamnosidase activity evolution during the *P. guilliermondii* growth under optimal conditions (YNB-rhamnose supplemented with ammonium sulphate) was evaluated. Additionally, enzyme cellular locations were determined at culture time of highest activity. Figure 1 showed that the αRh activity of *P. guilliermondii* was associated with its cellular growth and the maximum value of total enzymatic activity (33 U g^{-1} dry weight) was at the end of exponential growth phase (sixth day). With regard to αRh cellular location, 84% of total enzymatic activity was determined in the supernatant fraction (intracellular activity), while the rest of the activity was associated to parietal fraction (Table 2). No differences were observed in the parietal activity values obtained from whole cell and cell debris (also called parietal cellular fraction) indicating that the cellular rupture methods not affected the enzymatic activity. No αRh activity was detected in culture medium (extracellular activity). On the other hand, the capability of hydrolysing other substrates such as pNP-β-D-glucoside (βGl activity) and pNP-β-D-xyloside (βXy activity) by the cellular fractions was assayed. Both parietal and intracellular cellular fractions of *P. guilliermondii* were also able to hydrolyse in descending order pNPG and pNPX to a much lesser extent than pNPR (Table 2). Moreover, the parietal fraction showed increased capability for hydrolysing pNPG (86·8%) and pNPX (91·9%) compared to the intracellular fraction (9·8 and 6·8%, respectively) (Table 2).

Zymograms

Three identical zymograms were performed using cellular extracts of *P. guilliermondii* previously grown in YNB-rhamnose. An intense activity band showing a scarce electrophoretic mobility (Rf 0·12 *c.*) was observed in the

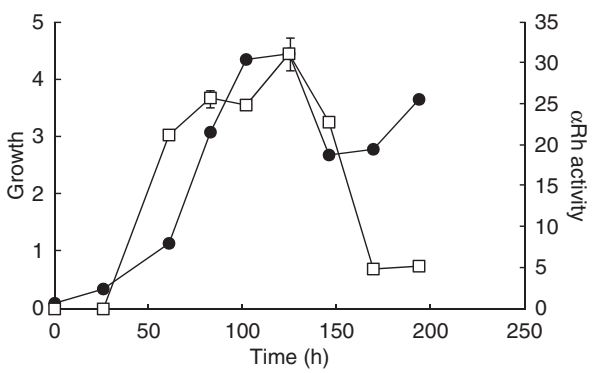

Figure 1 *Pichia guilliermondii* NPCC1053 growth (●) expressed as DO 600 and α-rhamnosidase (αRh) activity (□) as U g^{-1} dry weight.

Table 2 Cellular location of *Pichia guilliermondii* αRh activity and capability of the different cellular fractions to hydrolyse other substrates

	Enzymatic activity*			
			Cellular fractions	
Substrate	Whole cell (parietal)	Crude extracts (total)	Parietal (%)†	Intracellular (%)†
*p*NP α-L-rhamnoside	3·31 ± 0·20	33·17 ± 5·62	3·57 ± 0·10 (10·7)	27·61 ± 2·59 (83·2)
*p*NP-β-D-glucoside	1·66 ± 0·06	1·74 ± 0·10	1·51 ± 0·11 (86·8)	0·17 ± 0·00 (9·8)
*p*NP-β-D-xyloside	0·96 ± 0·09	0·74 ± 0·09	0·68 ± 0·11 (91·9)	0·05 ± 0·00 (6·8)

*Enzymatic activity is expressed as U g^{-1} dry weight.
†In parenthesis percentage of enzymatic activity using crude extracts activity as 100%.
*p*NP, *p*-nitrophenol.

zymogram revealed with MUR (Fig. 2). The intensity of this band was proportional to the amounts of cellular extracts placed (Fig. 2). In addition, the native gel revealed using MUG as substrate showed a single band with very slight βGl activity (data not shown) and it was associated with the same protein band that showed intense αRh activity. No band was observed in the zymogram when MUX was used as substrate.

Influence of oenological parameters on α-rhamnosidase activity

The influence of pH, glucose, ethanol and SO_2 concentrations on αRh activity was evaluated. The *P. guilliermondii* αRh showed the maximum activity at pH 6, while at pH typically found in wine and must (3·5–3·8), the αRh only retained the 50% of its activity (Fig. 3). The different concentrations of glucose and SO_2 assayed did not affect the αRh activity. Nevertheless, this activity was affected by the increase in ethanol concentration, showing a reduction of 60% of its activity at ethanol concentration commonly encountered in wines (12%) (Fig. 3).

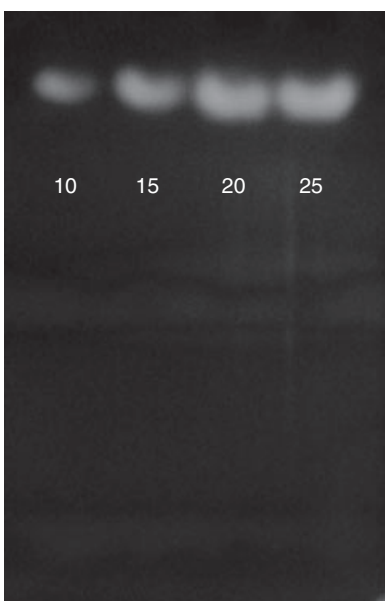

Figure 2 Zymogram revealed with 4-methylumbelliferyl-α-L-rhamnoside. *Pichia guilliermondii* NPCC1053 cellular extracts. The numbers under the bands indicate the cellular extracts microliters (μl).

Must and wine direct effect on α-rhamnosidase activity

Cells of *P. guilliermondii* were inoculated in both fresh must and young wine and conserved under anaerobic condition during 24 h to evaluate the direct effect of these substrates on αRh. The results evidenced that αRh was slightly affected by the must preserving 80% of its activity at the assay end (Fig. 4). On the other hand, *P. guilliermondii* αRh lost 50% of its activity after 24 h in contact with wine (Fig. 4).

Liberation of aromatic compounds from glycoside precursors

The hydrolysis of glycosidic precursors extracted from Muscatel grape juice from cell preparations was evaluated. The *P. guilliermondii*-αRh producer strain was able to significantly increase the content of all aromatic compounds assayed except 2-ethyl-1-hexanol and benzyl alcohol (Table 3).

Discussion

Unlike α-L-rhamnosidases from bacteria and filamentous fungi, characterization of these enzymes in yeasts is very limited today (Miklosy and Polos 1995; Mcmahon *et al.* 1999; Yanai and Sato 2000; Rodríguez *et al.* 2004). In this work, the results obtained from optimization assays suggested that αRh was not produced constitutively in *P. guilliermondii* but only when the yeast was grown in rhamnose broth media (Fig. 1 and Table 1), confirming the inducible nature proposed for this αRh in a previous work from agar plate assays (Rodríguez *et al.* 2004). Similar nature has been reported in many α-rhamnosidases of bacteria (Miake *et al.* 2000; Orillo *et al.* 2007) and fungi origin. (Orejas

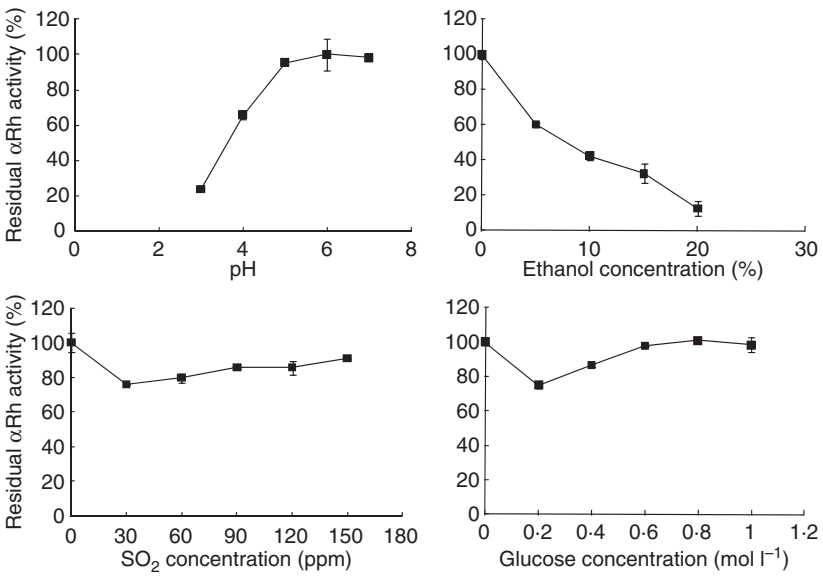

Figure 3 Effects of pH, ethanol, glucose and SO_2 concentrations on αRh activity of *Pichia guilliermondii* NPCC1053.

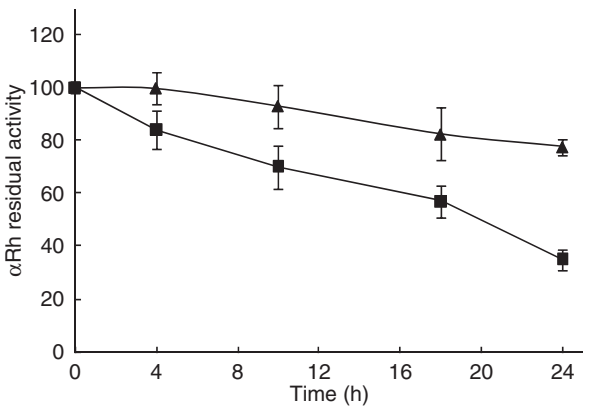

Figure 4 Effect of must (▲) and wine (■) direct contact on αRh activity of *Pichia guilliermondii* NPCC1053.

Table 3 Volatile compound released from grape glycoside extracts in *in vitro* assays using yeast whole cell preparations

Volatile compounds	RA* (x ± SD) Control†	*Pichia guilliermondii*‡	*P* value
Monoterpenols			
Geraniol	3·31 ± 1·05[a]	44·96 ± 10·72[b]	0·002558
Linalool	1·89 ± 0·25[a]	45·12 ± 2·10[b]	0·000004
Nerol	0·90 ± 0·21[a]	16·91 ± 3·70[b]	0·001753
α-Terpineol	2·16 ± 0·55[a]	7·39 ± 1·11[b]	0·002087
Alcohols			
2-Ethyl-1-hexanol	47·12 ± 13·94[a]	52·23 ± 17·01[a]	0·708317
1-Hexanol	1·43 ± 0·64[a]	5·58 ± 0·15[b]	0·000342
Benzyl alcohol	3·81 ± 0·60[a]	6·88 ± 2·21[a]	0·82156
2-Phenylethanol	3·26 ± 0·74[a]	29·49 ± 7·67[b]	0·004184
Acid			
Geranic acid	0·62 ± 0·11[a]	4·85 ± 1·80[b]	0·015757

*RA: values expressed in percentages of relative areas.
†Glycosidic extracts without yeast contact.
‡Glycosidic extracts treated with the yeast.
Different letters indicate significant differences among values of the same line (ANOVA and Tukey test, n = 3).

et al. 1999; Manzanares *et al.* 2000; Yanai and Sato 2000; Koseki *et al.* 2008). As regards cellular location, the αRh activity was mainly intracellular (Table 2). This location was also described for the α-rhamnosidase of *P. angusta* yeast (Yanai and Sato 2000) and of several bacteria (Miake *et al.* 2000; Zverlov *et al.* 2000; Hashimoto *et al.* 2003; Orillo *et al.* 2007), while in filamentous fungi this activity has been found mainly in extracellular location (Gallego *et al.* 2001; Manzanares *et al.* 2001, 2003; Scaroni *et al.* 2002; Yu *et al.* 2004; Koseki *et al.* 2008). On the other hand, the hydrolysis relative rates of p-nitrophenyl-glycosides (Table 2) evidenced that *P. guilliermondii* αRh intracellular and parietal fractions were highly specific to the α-L-rhamnopyranoside configuration. Both fractions also showed hydrolytic activities against pNP-β-D-glucose and pNP-β-D-xylose. However, these βGl and βXy activities were much weaker than αRh activity, and they were mainly associated to the parietal fraction (Table 2). This fact together with that obtained from zymograms, where an intense αRh (Fig. 2) and other very slight βGl activities were associated with protein bands with identical electrophoretic mobility, may suggest that the parietal and intracellular α-rhamnosidases could be isoenzymes although more exhaustive studies must be performed to verify this hypothesis.

Pichia guilliermondii NPCC1053 was isolated from grape surface and selected through a protocol intended to

find non-*Saccharomyces* indigenous yeasts with interesting properties for a potential use in more aromatic wine production (Rodríguez *et al.* 2004). In this context, it became necessary to assess the effect of some environmental conditions usually encountered in must and wine on αRh activity as well as to assay the ability of this enzyme to hydrolyse natural glycoside precursors. The former could be useful to determine the most appropriate time to use it, the whole yeasts or their enzymatic products, during a winemaking process (initial, middle or end stages of fermentation) and the latter is a decisive criterion for the technological purpose of this work. Under assay conditions, two oenological parameters, pH and ethanol concentrations, showed a significant effect on αRh activity (Fig. 3). While the strong and negative effect of high ethanol concentration (12%) on *P. guilliermondii* αRh activity (Fig. 3) seems to be a common effect to all αRh, independently of their bacterial (Orillo *et al.* 2007) or fungic origin (Orejas *et al.* 1999; Spagna *et al.* 2000; Yanai and Sato 2000), the pH effect shows significant differences within the fungi group. Filamentous fungi αRhs show optimal activities at acidic pH (pH 4–5), whereas the optimum pHs of *P. angusta* and *P. guilliermondii* yeast αRhs were near to neutrality (pH 6). This could to be a disadvantage for the *P. guilliermondii* yeast αRh oenological purpose when compared with the filamentous fungi αRhs. However, and in contrast with that observed for *P. angusta* αRh which lost practically all of its activity at acidic pH (Yanai and Sato 2000), *P. guilliermondii* αRh maintained significant levels of residual activity (50%) at acidic pH (3·5–3·8). On the other hand, the results obtained from the assays of *P. guilliermondii* αRh exposition to must and wine, where the oenological parameter effects were simultaneously evaluated, seem to indicate a better response system than that expected from individual assays under similar conditions (Figs 3 and 4), and it was particularly certain for the must assay where after 24 h of exposition the *P. guilliermondii* αRh maintained 80% of its optimum activity. These results pointed out the importance of using an experimental approach closer to the real technological conditions in the evaluation of micro organisms, or their parts, with industrial purposes.

Finally, the capability of *P. guilliermondii* αRh to hydrolyse natural substrates as glycosidic precursors extracted from Muscatel grape juice was also confirmed. This αRh-producing yeast strain was able to increase the amounts of almost all assayed compounds and interestingly, significantly increased the amounts of aromatically relevant compounds such as monoterpenols and 2-phenylethanol (Table 3). These aromatic compounds were mainly related to the floral aroma of wine (Swiegers *et al.* 2005).

As a conclusion, we want to highlight two important aspects of this work, (i) the characterization of an α-rhamnosidase activity from yeast origin—specifically from a *P. guilliermondii* NPCC1053 indigenous strain and (ii) the technological relevance of the *P. guilliermondii* αRh for winemaking industry. We think that *P. guilliermondii* αRh could contribute positively to the wine sensorial quality bringing out its varietal aromatic notes and preserving the wine distinctive characteristics typical of our wine region and, that its application would be desirable during the early stages of fermentation, using either the enzymatic preparation of αRh or the αRh-producing yeast itself. The latter could be considered as the most adequate alternative as *P. guilliermondii* has been found associated with musts at initial fermentation stages in Patagonian winemaking (Lopes *et al.* 2007). However, subsequent studies will be required for this purpose.

Acknowledgements

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