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Screening of non-*Saccharomyces* wine yeasts for the production of β-D-xylosidase activity

Paloma Manzanares*, Daniel Ramón, Amparo Querol

Departamento de Biotecnologia de Alimentos, Instituto de Agroquímica y Tecnologia de Alimentos (CSIC), P. O. Box 73, 46100 Burjassot, Valencia, Spain

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Abstract

Fifty-four yeast strains belonging to the genera *Candida*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Zygosaccharomyces*, mainly isolated from grapes and wines, were screened for the production of β-D-xylosidase activity. β-D-xylosidase activity was only detected in eight yeast strains belonging to the genera *Hanseniaspora* (*H. osmophila* and *H. uvarum*) and *Pichia* (*P. anomala*). β-D-xylosidase preparations active against ρ-nitrophenyl-β-D-xyloside were characterised with respect to their optimal pH and temperature conditions. *H. uvarum* 11105 and 11107 and *P. anomala* 10320 β-D-xylosidase preparations were active at pH and temperature ranges and at concentrations of glucose and ethanol usually found during winemaking processes.

Keywords: Non-*Saccharomyces* wine yeasts; β-D-xylosidase activity; Wine

1. Introduction

The production of extracellular enzymes by yeasts has been reviewed by Fleet (1992). *Saccharomyces cerevisiae*, the main wine yeast, is not recognised as being a good producer of extracellular enzymes, whereas the so-called non-*Saccharomyces* wine yeasts (including genera such as *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces*) have been described as potential sources for the commercial production of enzymes such as proteases, esterases, pectinases, lipases and glycosidases for industrial use (Vaughn et al., 1969; Ravelomana et al., 1986; Lee et al., 1987; Rosi et al., 1994; Besançon et al., 1995; Saha and Bothast, 1996; Charoenchai et al., 1997).

Glycosidases, including β-D-apiosidase, α-L-arabinofuranosidase, β-D-glucosidase, α-L-rhamnosidase and β-D-xylosidase activities, have been described as being involved in flavour releasing processes (for a review see Winterhalter and Skouroumounis, 1997). However, many studies have only focused on β-D-glucosidase activities because of their widespread occurrence in plants, fungi and

*Corresponding author. Tel.: +34-96-3900022; fax: +34-96-3636301; e-mail: pmanz@iata.csic.es

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yeasts. Among glycosidases involved in flavour releasing processes, β-D-xylosidase is also a component of xylan degrading enzyme complexes which, together with β-1,4-xylanases, comprise the main enzymes required for the complete degradation of xylan, the major hemicellulolytic component of plant cell walls and the most abundant natural polysaccharide after cellulose (Biely, 1985). In addition to the application of xylanolytic systems for the conversion of xylan to xylose in the wastes of paper-pulp industry and agriculture, β-D-xylosidase can be used for the bioconversion of D-xylose to ethanol (Visser et al., 1992).

Although β-1,4-xylanases and β-D-xylosidase are widely distributed among mycelial fungi and bacteria, information on the production of these enzymes in yeast is very limited. Previous studies have focused mainly on the xylan degrading enzymes of the yeasts *Cryptococcus albidus* and *C. flavus* (Biely et al., 1980; Krátký and Biely, 1980; Biely and Petráková, 1984; Yasui et al., 1984; Morosoli et al., 1986). Induction of the xylan-degrading enzyme complex has been studied in *Trichosporon cutaneum* (Hrmová et al., 1984), and the purification of β-1,4-xylanase and β-D-xylosidase from *Pichia stipitis* has been reported (Özcan et al., 1991). Moreover, the conversion of D-xylose to ethanol by *P. stiptis* (Lee et al., 1986) and *Pachysolen tannophilus* (Slininger et al., 1982) has been described. However, there is a lack of information about β-D-xylosidase activity in yeasts of oenological origin.

In the present study we report the screening for β-D-xylosidase activity in 54 yeast strains belonging to eight genera and mainly isolated from grapes and wines. The production, cellular location and optimal conditions for activity of β-D-xylosidase preparations are also described. The possibility of using yeast β-D-xylosidase to improve the aroma and flavour properties of wine is also discussed.

2. Materials and methods

2.1. Yeast strains

A total of 54 yeast strains belonging to the genera *Candida*, *Dekkera*, *Hanseniaspora*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces* and *Zygosaccharomyces* were obtained from the Spanish Type Culture Collection (CECT) which were mainly isolated from grapes and wines (Table 1).

2.2. Plate screening

β-D-xylosidase screening was carried out on agar plates containing 1.7 g YNB (Yeast Nitrogen Base without amino acids and ammonium sulphate, Difco), 5 g ammonium sulphate, 5 g xylose and 20 g agar per litre. The pH was adjusted to 5.5. 4-Methylumbelliferyl-β-D-xyloside (MUX, Sigma) was spread onto the surface of the agar plates, yeasts were point-inoculated on the agar surface and the plates incubated at 25°C for 24 h. The hydrolysis of MUX by the action of β-D-xylosidase activity resulted in the release of 4-methylumbelliferone (MU) which can be visualised under UV illumination as fluorescent halos surrounding yeast growth.

Birchwood xylan (5 g/l, Sigma), glucose (5 g/l) and the combination of xylose and glucose (5 and 20 g/l, respectively) were also used as carbon sources for β-D-xylosidase induction experiments.

2.3. Liquid culture conditions for β-D-xylosidase production

Medium containing 1.7 g YNB, 5 g ammonium sulphate, 5 g yeast extract, 5 g peptone and 10 g xylose per litre was used for β-D-xylosidase production. The pH was adjusted to 5.5 and inoculated with 10^6 cells per ml from overnight cultures of GPY medium (contains 5 g yeast extract, 5 g peptone, 40 g glucose per litre, pH 5.5). Inoculated media were incubated at 25°C in an orbital shaker at 200 rpm for 3 days.

2.4. Enzyme activity assays

β-D-xylosidase activity was measured using ρ-nitrophenyl-β-D-xylopyranoside (pNPX, Sigma) as substrate. A 200 μl amount of 6.66 mM pNPX dissolved in McIlvaine buffer, pH 5.5, were mixed with 100 μl of an appropriate enzyme dilution. Incubation was done at 37°C and the reaction stopped by the addition of 300 μl of 0.25 M sodium carbonate solution. The release of ρ-nitrophenol was measured spectrophotometrically at 405 nm. All enzymatic measurements were done in duplicate.

Table 1
Yeast species screened for β-D-xylosidase activity

Species	Spanish Type Culture Collection (CECT) number	Isolation source
Candida cantarelli	11150	Grape must
Candida cantarelli	11170	Grape must
Candida dattila	10559	White wine
Candida dattila	10652	Unknown
Candida dattila	1962	Grapes
Candida dattila	10387	Grapes
Candida domerquiae	10650	Unknown
Candida intermedia var. *intermedia*	11154	Grapes
Candida stellata	11108	Wine grape
Candida stellata	11109	Wine
Candida vinaria	11177	Grape must
Candida vini	10053	Red wine
Dekkera bruxellensis	11045	Bordeaux wine
Hanseniaspora guilliermondii	11027	Grape must
Hanseniaspora guilliermondii	11104	Grape juice
Hanseniaspora osmophila	11206	Ripe Riesling grape
Hanseniaspora osmophila	11207	Grapes
Hanseniaspora uvarum	1444	Muscat grape
Hanseniaspora uvarum	10389	Grape juice
Hanseniaspora uvarum	11105	Grape must
Hanseniaspora uvarum	11106	Grape must
Hanseniaspora uvarum	11107	Grape must
Hanseniaspora uvarum	11156	Fruit must
Hanseniaspora uvarum	11026	Sugar cane
Metschnikowia pulcherrima	10388	Grapes
Metschnikowia pulcherrima	10408	Orange
Metschnikowia pulcherrima	10546	White wine
Metschnikowia pulcherrima	11202	Concord grapes
Pichia anomala	10320	White wine
Pichia anomala	10410	White wine
Pichia anomala	10590	Grape juice
Pichia anomala	10591	Grape juice
Pichia fermentans	10064	Red wine
Pichia membranaefaciens	10037	Red wine
Pichia membranaefaciens	10113	Grapes
Pichia membranaefaciens	10568	White grapes
Pichia membranaefaciens	10570	White wine
Rhodotorula acuta	11175	Grape must
Rhodotorula glutinis	10145	Unknown
Schizosaccharomyces pombe var. *pombe*	11197	Grape juice
Schizosaccharomyces pombe	1375	Unknown
Schizosaccharomyces pombe	1376	Unknown
Schizosaccharomyces pombe	1377	Unknown
Zygosaccharomyces bailii	11040	Wine
Zygosaccharomyces bailii	11041	Wine
Zygosaccharomyces bailii	11042	Grapes
Zygosaccharomyces mellis	11149	Wine grape
Zygosaccharomyces rouxii	1231	Syrup
Zygosaccharomyces rouxii	10137	Raisin
Zygosaccharomyces rouxii	11136	Wine grape
Zygosaccharomyces rouxii	11189	Portuguese white wine
Zygosaccharomyces rouxii	10381	Sugar cane molasses
Zygosaccharomyces rouxii	10425	Sugar cane molasses
Zygosaccharomyces rouxii	10445	Sugar cane molasses

One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 μmol of ρ-nitrophenol per hour at 37°C in McIlvaine buffer, pH 5.5.

2.5. Cellular location

The cellular location of β-D-xylosidase was investigated following the protocol described previously (González-Candelas et al., 1995), except for the use of McIlvaine buffer, pH 5.5.

For the determination of extracellular activity the culture supernatants of xylose grown cells were used. Cell wall bound activity was determined in liquid culture media. Cell extracts were assayed for intracellular β-D-xylosidase activity.

2.6. pH and temperature optima

The pH optima for the β-D-xylosidase preparations were determined by incubating the enzyme solutions with pNPX in McIlvaine buffers in the pH range 3 to 8. The optimum temperature was determined at the optimum pH by assaying the enzyme preparations at different temperatures (30–60°C).

2.7. Influence of oenological parameters on β-D-xylosidase activity

The effect of the oenological parameters glucose and ethanol at concentrations ranging from 0 to 1 M and from 0 to 15% (v/v), respectively, was investigated.

3. Results

3.1. Plate screening for β-D-xylosidase activity

The 54 yeast strains were examined for their production of β-D-xylosidase activity using D-xylose as inducer. β-D-xylosidase activity was detected after 24 h of incubation at 25°C (Fig. 1) as UV fluorescent halos surrounding yeast colonies. Of all the yeast strains tested, only the genera *Hanseniaspora* and *Pichia* produced β-D-xylosidase activity. Of the genus *Hanseniaspora*, only yeasts belonging to the species *H. osmophila* and *H. uvarum* showed β-D-xylosidase activity. Of the seven strains of *H. uvarum* tested, four strains produced the enzyme (10389, 11105, 11106, and 11107), whereas both strains of *H. osmophila* produced the activity. None of the two strains of *H. guilliermondii* produced β-D-xylosidase. In the genus *Pichia*, strains of the species *P. anomala*, *P. fermentans* and *P. membranaefaciens* were tested and only two strains belonging to *P. anomala* (10320 and 10590) showed β-D-xylosidase activity. These eight β-D-xylosidase-producing strains were used for further work.

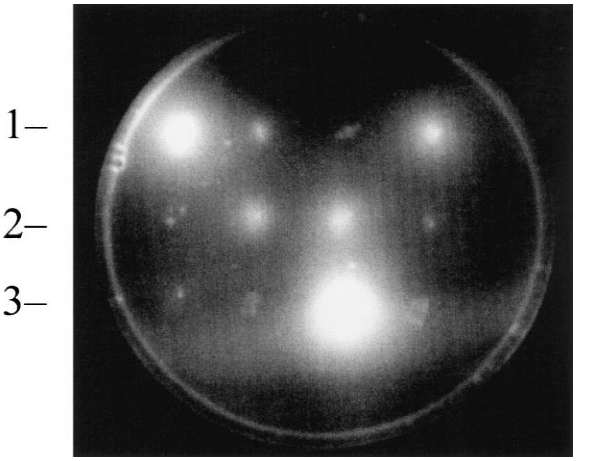

Fig. 1. Plate assay for the detection of β-D-xylosidase activity using D-xylose as inducer and MUX as substrate. β-D-xylosidase activity was visualised as fluorescent halos surrounding yeast growth. From left to right, row 1: *Pichia anomala* 10320, *Hanseniaspora uvarum* 10389, *Candida cantarelli* 11150, *Hanseniaspora uvarum* 11107; row 2: *Zygosaccharomyces rouxii* 10445, *Hanseniaspora uvarum* 11105, *Hanseniaspora uvarum* 11106, *Hanseniaspora uvarum* 11156; row 3: *Hanseniaspora guilliermondii* 11027, *Metschnikowia pulcherrima* 10546, *Pichia anomala* 10590, *Pichia membranaefaciens* 10570.

Birchwood xylan, glucose, and glucose in combination with xylose were tested as inducers or repressors of β-D-xylosidase activity. Xylan was able to induce β-D-xylosidase activity in the eight yeast strains. After 24 h of plate growth, clear halos were visible around the two *P. anomala* and two *H. osmophila* strains. After 72 h, β-D-xylosidase activity could also be detected in the *H. uvarum* strains. Similar behaviour was observed when glucose at 0.5% (w/v) was used. Although yeast growth on plates was better in the presence of glucose compared to xylose or xylan, fainter halos were observed. Using combinations of 2% (w/v) glucose

Table 2
Effect of different carbon sources on the induction of β-D-xylosidase activity in the eight selected yeast strains

Strain	Carbon source[a]						
	Xylose	Xylan		Glucose		Xylose/glucose	
	24 h	24 h	48 h	24 h	48 h	24 h	48 h
Pichia anomala 10320	+ +	+	+	+	+	+	+
Pichia anomala 10590	+ +	+	+	+	+	+	+
Hanseniaspora uvarum 10389	+	−	+	−	+	−	−
Hanseniaspora uvarum 11105	+	−	+	−	+	−	−
Hanseniaspora uvarum 11106	+	−	+	−	+	−	−
Hanseniaspora uvarum 11107	+	−	+	−	+	−	−
Hanseniaspora osmophila 11206	+	+	+	+	+	+	+
Hanseniaspora osmophila 11207	+	+	+	+	+	+	+

[a] Xylose, xylan and glucose at 5 g/l. When combined, xylose and glucose were used at 5 and 20 g/l, respectively.
Positive result: +, moderate fluorescent halo surrounding yeast growth; + +, strong fluorescent halo surrounding yeast growth.
Negative result: −.

with 0.5% (w/v) xylose resulted in a lack of enzyme production at 24 h; after 72 h only *H. osmophila* and *P. anomala* strains showed β-D-xylosidase activity.

A summary of the β-D-xylosidase activity plate screening data is presented in Table 2.

3.2. Production and cellular location

The eight strains noted above were used to study β-D-xylosidase production and also its cellular location. After 72 h of incubation at 25°C, β-D-xylosidase activity was measured in culture supernatants (extracellular activity), whole culture aliquots (extracellular plus cell wall bound activities) and cell free extracts (intracellular activity). In Table 3 the β-D-xylosidase activities measured as well as the percentages of extracellular, cell wall bound and intracellular activities with respect to the total are shown.

P. anomala 10590 yielded the highest level of β-D-xylosidase production (total activity), followed by *P. anomala* 10320, the two *H. osmophila* strains 11206 and 11207, and finally *H. uvarum* 11107. The remaining strains produced significantly lower amounts of enzyme. *P. anomala* 10590 also showed the highest percentage of extracellular β-D-xylosidase activity whereas *H. uvarum* 10389 only produced cell wall bound β-D-xylosidase. Whilst neither of the *H. osmophila* strains produced any extracellular activity, more than 70% of the total activity produced by each was located intracellularly.

Table 3
β-D-xylosidase production and cellular location in the eight selected yeast strains after 72 h incubation at 25°C

Strain	% activity			
	Total activity (mU/ml)[a]	Extracellular	Cell wall	Intracellular
Pichia anomala 10320	118.6	20.7	72.3	7.1
Pichia anomala 10590	242.1	84.3	7.6	8.1
Hanseniaspora uvarum 10389	16.5	0	100	0
Hanseniaspora uvarum 11105	17.0	54.7	45.3	0
Hanseniaspora uvarum 11106	32.1	28.0	67.0	5
Hanseniaspora uvarum 11107	92.3	12.1	81.1	6.7
Hanseniaspora osmophila 11206	99.8	0	27.7	72.3
Hanseniaspora osmophila 11207	102.8	0	26.6	73.4

[a] U = the amount of enzyme releasing 1 μmol of ρ-nitrophenol per hour at 37°C.

3.3. pH and temperature optima of β-D-xylosidase preparations

3.3.1. Effect of pH on activity

The influence of pH on the β-D-xylosidase activities of the eight selected strains was studied in the pH interval 3 to 8. The pH optima of the enzymatic preparations of *P. anomala* strains 10320 and 10590 were found to be 6.5–7 and 7–7.5, respectively, for *H. uvarum* strains 10389 and 11106 and *H. osmophila* strains 11206 and 11207, an optimal pH of 7–7.5 was found, whereas a lower optimum, 4.5–5, was found for β-D-xylosidase preparations from *H. uvarum* 11105 and 11107 (results not shown). At pH 3–3.8, the usual pH range found in winemaking, only three strains showed β-D-xylosidase activity. *P. anomala* 10320 maintained 30% of its maximum activity whereas *H. uvarum* 11105 and 11107 maintained approximately 70 and 80% of their maximum activities, respectively. For this reason, these strains were selected to study the influence of oenological parameters on β-D-xylosidase activity.

3.3.2. Effect of temperature on activity

The optimal temperature for the eight enzymatic preparations was determined in the range 30 to 60°C. The optimal value in the case of *P. anomala* strains 10320 and 10590 was found to be 40–45°C, a similar optimal temperature of 45°C was found for the enzymatic preparations from *H. uvarum* 10389 and 11106, whereas a lower temperature range, 35–37°C, was found for *H. uvarum* 11105 and 11107 preparations and finally, 50°C was the optimal temperature found for the β-D-xylosidase activities of both *H. osmophila* strains (results not shown).

The activity of the three strains active at pH 3–3.8 was also tested at 25°C, a temperature value within the range (20–30°C) usually encountered in winemaking. *P. anomala* 10320 β-D-xylosidase yielded approximately 5% of its maximum activity whereas *H. uvarum* 11105 and 11107 strains maintained 85 and 59% of their maximum activities, respectively (results not shown).

3.4. Effects of oenological parameters

In view of the potential application of β-D-xylosidase activity for wine flavour enhancement, the influence of glucose and ethanol at concentrations that normally occur in musts during winemaking was investigated.

Glucose at concentrations up to 1 M, a level usually found in unfermented grape juice, reduced the activity of *H. uvarum* 11105 β-D-xylosidase by 10%, whereas reductions of 16 and 19% were found for the *P. anomala* 10320 and *H. uvarum* 11107 activities, respectively (results not shown). Ethanol concentrations up to 10% (v/v) did not affect β-D-xylosidase activity in any of the three strains and ethanol at 15% (v/v) was found to be an activator of *P. anomala* 10320 β-D-xylosidase activity, yielding an increase of 55% with respect to the original activity (results not shown). For *H. uvarum* 11105 and 11107, inhibitions of 41 and 26%, respectively were found.

4. Discussion

Although β-D-xylosidases have been described as being involved in flavour releasing processes there are no data available about the potential of these enzymes to improve the aroma and flavour properties of wine. Despite it being well known that non-*Saccharomyces* yeasts can grow during the early stages of juice preparation and wine fermentation in conditions of high levels of glucose and low pH values and that they are good producers of extracellular enzymes, there is a lack of information about β-D-xylosidase activity in yeasts of oenological origin. In our work, eight yeast strains belonging to the genera *Hanseniaspora* and *Pichia* have been selected from a general screening of 54 oenological yeast strains to determine whether they may be a useful source of β-D-xylosidase activity with the potential for beneficial use during winemaking processes. Results from the screening for β-D-xylosidase production suggest that this activity is quite rare in yeasts of oenological origin and is generally restricted to the genera *Hanseniaspora* and *Pichia*. Within these two genera, only species corresponding to *H. osmophila*, *H. uvarum* and *P. anomala* showed β-D-xylosidase activity. The genera *Cryptococcus* and *Pichia* have been described previously as being able to hydrolyse xylan (Lee et al., 1986) but there are no data concerning the genus *Hanseniaspora*. As

suggested by Lee et al. (1986) for the ability of yeasts to hydrolyse xylan, the occurrence of β-D-xylosidase activity may be of value for taxonomic purposes.

Low levels of β-D-xylosidase activity were found in our strains, ranging from 16.5 to 242.1 mU/ml (total β-D-xylosidase activity). The production of reduced amounts of β-D-xylosidase is a common feature already described for xylan degrading yeasts (Biely et al., 1980; Hrmová et al., 1984). For *Pichia stipitis*, β-D-xylosidase activities found in the culture supernatants of 3-day xylose- and xylan-grown cells were 25 and 29 mU/ml/h, respectively (Özcan et al., 1991), in good agreement with our data for extracellular β-D-xylosidase.

In plate assays the β-D-xylosidase activity of the eight selected strains was found to be induced by xylose and xylan, whereas lower levels of β-D-xylosidase activity were found in the presence of glucose. When glucose was present at 2% (w/v), no activity could be detected in the four strains of *H. uvarum* even after 72 h of incubation. These results suggest that β-D-xylosidase is not produced constitutively in these strains and that its synthesis is under carbon catabolite repression. Similar results were described for both the β-xylanase and β-D-xylosidase of *Cryptococcus albidus* (Biely et al., 1980). By contrast, *P. stipitis* β-D-xylosidase is synthesised constitutively to a considerable degree (Özcan et al., 1991).

With respect to the location of the enzyme activity, it was found to be different between the genera and also within the genus *Hanseniaspora*. Both *P. anomala* strains have extracellular, cell wall bound and intracellular β-D-xylosidase activities. *P. anomala* 10320 showed mainly cell wall bound activity whereas in *P. anomala* 10590 the enzyme was mainly extracellular. *H. uvarum* strains showed the lowest levels of β-D-xylosidase production, the majority of which was cell wall bound. In the case of *H. uvarum* 10389 the only activity produced was cell wall associated. High intracellular β-D-xylosidase activity ($>70\%$ of the total) was found in the two strains of *H. osmophila* and no extracellular activity could be detected. In *Trichosporom cutaneum*, β-D-xylosidase activity could be detected extracellularly and cell wall associated but most was located in the cell interior (Hrmová et al., 1984). In *P. stipitis*, 60% of total activity was found in the culture supernatant, 22% in supernatant plus intact cells and 18% in crude cellular extracts (Özcan et al., 1991).

No data about optimal temperature values for yeast β-D-xylosidases could be found in the literature. In this study, similar optimum temperature ranges were observed for *P. anomala* strains (40–45°C), *H. uvarum* 10389 and 11106 (45°C), and a slightly higher optimum (50°C) for *H. osmophila*. *H. uvarum* strains 11105 and 11107 exhibited a lower optimal temperature range (35–37°C).

Except for *H. uvarum* strains 11105 and 11107 which showed an acidic optimum pH range (4.5–5), neutral pH optima in the range 6.5–7.5 were found for the other six yeast β-D-xylosidases. Only in the case of *C. albidus* β-D-xylosidase have pH studies been described, the protein exhibiting maximum hydrolytic activity at pH 5.4 (Biely et al., 1980). Nevertheless in the pH range (3–3.8) usually found in winemaking, *P. anomala* 10320 β-D-xylosidase activity was 30% of its maximum value, and the *H. uvarum* 11105 and 11107 activities maintained 70 and 80% maximum activity, respectively. With respect to the usual range of temperature (20–30°C) in winemaking, both *H. uvarum* strains 11105 and 11107 maintained their activity (85 and 59%, respectively) whereas *P. anomala* 10320 β-D-xylosidase only showed 5% of its maximum activity. Moreover, in the usual ranges of ethanol and glucose concentrations during winemaking β-D-xylosidase activity could be detected in the three β-D-xylosidase preparations. All maintained more than 80% of their maximum activity in 1 M glucose, whereas 15% ethanol (v/v) increased *P. anomala* 10320 β-D-xylosidase activity and decreased the *H. uvarum* 11105 and 11107 activities by 40 and 25%, respectively.

This study has revealed the potential of certain oenological yeasts to produce β-D-xylosidase activity. Moreover β-D-xylosidase preparations from the *P. anomala* 10320 and *H. uvarum* 11105 and 11107 strains maintained their activities at pH and temperature values and at concentrations of glucose and ethanol typically found during winemaking processes, thus potentially allowing their application for the improvement of the aroma and flavour properties of wine. Considering the effects of the oenological parameters on the β-D-xylosidase activities, our

research is now focused on improving β-D-xylosidase production as well as the purification of the *H. uvarum* 11105 and 11107 enzymes.

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