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ORIGINAL PAPER

Characterization of glycolytic activities from non-*Saccharomyces* yeasts isolated from Bobal musts

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Abstract A large number of non-*Saccharomyces* yeasts were isolated from grapes of Bobal variety and identified according to their physiological and molecular characteristics. The yeasts were tested to determine the presence of β-glucosidase, β-xylosidase, α-arabinosidase, and α-rhamnosidase activities and five isolates were selected. All enzymatic activities were induced by the presence of glycosides as the only carbon source in the medium, which seems to be a characteristic of the yeast isolate, and were characterized according to different parameters of enological interest.

Keywords Glycosidases · Enzyme · Non-*Saccharomyces* · Yeasts · Wine

Introduction

It is well established that wine fermentations, as conducted by traditional methods (without inoculation), are not the result of the action of a single species or a single strain of yeast. Rather, the final products result from the combined actions of several yeast species which grow in succession throughout the fermentation process. Previous studies performed in various countries have described the isolation and identification of yeasts from grape surfaces, and quantitative data on the ecology of grape yeasts have concluded that the isolation process of the total yeast population from the grapes is complex and dependent on many factors [6, 8, 15, 29]. Fermentations are initiated by the growth of various species of *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, and *Torulaspora*. Their growth is generally limited to the first 2 or 3 days of fermentation, after which they die off. Subsequently, the most strongly fermenting and more ethanol-tolerant species of *Saccharomyces* take over the fermentation [8]. It is believed that during the first step of the fermentation low-fermentative yeasts produce some important reactions in must which improve the final flavor of wines.

The occurrence of glycosidically bound forms of monoterpenols in Muscat grapes and other cultivars has been established [34]. These forms, which are generally more abundant than free, flavor-active ones, occur mainly as monoglucosides or disaccharide glycosides: 6-*O*-α-L-arabinofuranosyl-β-D-glucopyranosides, 6-*O*-α-L-rhamnopyranosyl-β-D-glucopyranosides [35], or 6-*O*-β-D-apiofuranosyl-β-D-glucopyranosides [11]. Acid hydrolysis of grape glycosides has been studied as a method to release bound monoterpenes with a view to enhancing the aroma of grape juice [34]. However, acid hydrolysis promoted by heating causes rearrangement of the monoterpene aglycones [21].

As an alternative, enzymatic methods hold the potential for increasing the concentration of free flavoring substances in grape juice with minimal change in the natural monoterpene composition [32]. Recent studies demonstrated that enzymic hydrolysis of grape monoterpenyl diglycosides proceeds in two steps: first, the inter-sugar linkage is cleaved by either α-L-arabinofuranosidase, α-L-rhamnosidase, or β-D-apiosidase regardless of the structure of aglycone moiety and the corresponding monoterpenyl β-D-glucoside is released. The liberation of the aglycone moiety can only take place during the second

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step, which consists in the action of an β-D-glucosidase on the previous monoterpenyl β-D-glucosides [11].

Glycosidases such as β-glucosidase, β-xylosidase, β-apiosidase, α-rhamnosidase, and α-arabinofuranosidase have been described as being involved in flavor-releasing processes [36]. However, many studies have only focused on β-glucosidases because of their wide occurrence in plants, fungi, and yeasts [18]. The effect of β-glucosidases isolated from different yeast species on the hydrolysis of grape terpenyl-glycosides has been investigated. Grossmann et al. [10] studied the β-glucosidase from *Hansenula* species found in must. This enzyme, although able to liberate aroma substances in wine, seems to be less effective in must. According to Dubourdieu et al. [5], the liberation of terpenols during fermentation can be explained by yeast β-glucosidase activity. Studies developed by Vasserot et al. [33] were focused on the β-glucosidase activities of other yeast strains such as *Hanseniaspora vineae*, and Gunata et al. [12] studied *Candida* species. An extensive review of 317 strains from 20 wine yeast species indicates that yeasts of the *Candida*, *Debaryomyces*, *Hanseniaspora*, *Kloeckera*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Saccharomycodes*, *Schizosaccharomyces*, and *Zygosaccharomyces* genera carry out β-glucosidase activities [30]. Saha and Bothast [31] conducted a further screening of 48 yeast strains of the genera *Candida*, *Kluyveromyces*, *Debaryomyces*, and *Pichia* for production of extracellular glucose-tolerant β-glucosidase activity. All yeast strains tested produced extracellular β-glucosidase activity, but enzymes from only 15 yeasts showed very high glucose tolerance.

The aim of this study was to investigate the presence of various glycolytic activities in enological indigenous non-*Saccharomyces* yeasts from the Utiel-Requena region (Spain) and characterize them by different physicochemical parameters of enological interest in order to determine the possibility of using these yeasts as a source of enzymes for winemaking.

Materials and methods

Yeast strains

Musts were collected from nine cellars from the D.O. Utiel Requena (eastern Spain). A total of 15 must samples were taken before fermentation. Each sample was spread onto malt agar (20 g/L glucose, 20 g/L malt extract, 2 g/L peptone, 20 g/L agar) or lysine agar [13] and 49 colonies were finally isolated. Isolates were identified according to their morphological, physiological, and molecular characteristics as proposed by Kreger van Rij [14] and Barnett [2].

Preliminary enzymatic assay

Screening for activities was carried in triplicate out on agar plates containing 1.7 g/L yeast nitrogen base (Difco), 5/L g ammonium sulfate, 5 g/L glucose, and 20 g/L agar. Glycosidase activities were determined by using the appropriate 4-methylumbelliferyl glycoside (Sigma) as substrate, as described by Manzanares et al. [7, 19]. The presence of enzymatic activity was visualized under UV light as a fluorescent halo surrounding yeast growth on the plate.

Induction media

Isolated strains were grown in YEPD medium (1% yeast extract, 2% mycological peptone, 2% glucose) at 28°C for 24 h. Cells were centrifuged at 3,500 *g* for 10 min, the supernatant was discarded and resuspended in saline solution (0.9% NaCl in purified water). Cells were counted in a Thoma chamber and inoculated in induction medium at an initial concentration of 1×10^6 cfu/mL. Basal induction medium composition was 1 g/L yeast extract and 10 g/L mycological peptone. This medium was supplemented with 50 g/L of naringin, xylan, salicin, or glucose. Cultures were maintained at 28°C for 48 h.

Enzymatic assay

After incubation in induction medium, 2-ml aliquots of yeast cultures were centrifugated and the pellets were resuspended in 750 µl 0.1 M pH 5.0 citrate phosphate buffer. Then, 250 µl of a 1 mg/ml 4-nitrophenyl-β-D-glucopyranoside (pNPG), 4-nitrophenyl-β-D-xylopyranoside (pNPX), 4-nitrophenyl-α-L-arabinopyranoside (pNPA), or 4-nitrophenyl-α-L-rhamnopyranoside pNPR) solution was added and the mixture was incubated at 40°C for 90 min. Enzyme substrates were obtained from Sigma. To stop the reaction, 1.0 ml of Na_2CO_3 0.2 M was added. Yellow color released from the substrate was measured at 404 nm and activity was expressed as nanokatals (1 nkat = 1 nmol of pNP liberated in 1 min by 10^6 yeast cells) [4]. Assays were performed in triplicate.

Effect of pH and temperature on glycosidase activities

For pH assays, washed cells were resuspended into 0.1 M citrate–phosphate buffer spanning the pH values from 2.2 to 9.2; determination of activities were made as described in the previous section. Assays were performed in triplicate.

The influence of temperature on glycosidase activities was determined by measuring activity at different temperatures in the range 20 to 60°C. Assays were performed in triplicate.

Influence of some compounds on glycosidase activities

The effect of xylan on glycosidase activities was determined by varying the concentration of this compound in the induction medium from 0 to 5% (w/v). The effect of various levels of glucose, ethanol, and EDTA on glycosidase activities was determined by alteration of these parameters in the assay mixture, prepared as described in the previous section and supplemented with 1–20% (w/v) of glucose, 1–20% (v/v) of ethanol, or 0.01–0.2 M of EDTA, respectively. Blanks were prepared similarly, but without addition of any compund. Assays were performed in triplicate.

Results and discussion

A total of 49 non-*Saccharomyces* yeasts belonging to 11 different species were isolated from Bobal musts obtained from the Utiel-Requena region by using lysine agar as differential medium [13] (Table 1). As can be seen, main genera detected are *Hanseniaspora* and *Pichia* (35% each one) and, to a lesser extent, *Torulaspora* (14% of isolates). These data are similar to those previously published regarding this and other Spanish regions [6, 17, 28], indicating the quantitative importance of the aforementioned genera in the grapes. Some authors have assigned a great importance to the presence of these yeasts as a way to improve some wine characteristics, mainly aromatic ones [9, 15, 20].

Selection of isolates

Following the identification of the isolates, a preliminary assay was performed to quantify their glycolytic activities [7]. On the basis of the results obtained in the preliminary assay, the five most interesting isolates were selected for subsequent experiments; two of these isolates belonged to *Hanseniaspora uvarum* species and the others to the species *H. vineae*, *Pichia anomala*, and *Torulaspora delbrueckii*.

Table 1 Non-*Saccharomyces* yeast isolates from Bobal must

Yeast species	Number of isolates	Percent
Hanseniaspora uvarum	10	20.4
Hanseniaspora vineae	3	6.1
Hanseniaspora guilliermondii	4	8.2
Issatchenkia occidentalis	1	2.0
Issatchenkia terricola	3	6.1
Kluyveromyces thermotolerans	2	4.1
Metschnikowia pulcherrima	2	4.1
Pichia anomala	8	16.3
Pichia fermentans	2	4.1
Pichia membranifaciens	7	14.3
Torulaspora delbrueckii	7	14.3
Total	49	100

Determination of glycolytic activities

Following the procedure described in the "Induction media" section in the "Materials and methods", no activity was detected when basal medium (BM) was used. Taking into account previous results [20], we have considered the possibility that such activities were inducible. Consequently, experiments were repeated by supplementing basal medium with 5% of each of the following compounds: glucose, salicin (2-(hydroxymethyl)-phenyl-β-D-glucopyranoside), xylan (poly(β-D-xylopyranose [1 → 4])), and naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside). Only the presence of xylan or naringin stimulated the enzymatic activities under investigation (Table 2). Therefore, it seems that the activities under study in this research are not attributed to the pool of constitutive enzymes of the different yeasts studied but they are induced as a response to unfavorable conditions in the medium, mainly the absence of an easily assimilable carbon source and/or effect of catabolite repression.

H. vineae isolate shows low values for different activities, clearly lower than the other isolates. These data do not agree with results obtained in preliminary enzymatic assay. The presence in the yeast of different enzymes capable of hydrolyzing 4-methylumbelliferyl glycosides, probably a glucanase [20], is possible; as a consequence, data obtained by hydrolysis of such glycosides are not completely guaranteed and they should be confirmed by other assays.

Regarding the other isolates, there were large differences between the two isolates of *H. uvarum*. One of them showed detectable activities only when the medium was supplemented with xylan (0.2–0.3 nkat), while the other one shows higher activities when naringin was added to the medium. *P. anomala* shows the highest activities, mainly for β-glucosidase. Finally, *T. delbrueckii* showed the highest values when medium was supplemented with narigin; nevertheless, there were no differences among the four activities assayed.

Effect of xylan concentration on activity

As naringin is not capable of inducing the four activities studied in all five isolates, this work is limited to studying the influence of xylan concentration (Fig. 1). Both isolates of *H. uvarum* show similar data; maximum activities are obtained when medium is supplemented with 1–2% (w/v)

Table 2 Glycolytic activity (nkat) assays in several induction media

Induction medium	Substrate	*H. uvarum (I)*		*H. uvarum (II)*		*H. vineae*		*P. anomala*		*T. delbrueckii*	
		Activity	SD (%)	Activity	SD (%)	Activity	SD (%)	Activity	SD (%)	Activity	SD (%)
BM + xilane	pNPG	0.216	7.9	0.605	6.5	0.076	3.5	1.430	8.6	0.124	5.1
	pNPA	0.267	6.8	0.308	8.2	0.033	4.8	0.115	4.8	0.191	6.2
	pNPX	0.289	7.3	0.303	7.4	0.037	5.2	0.360	6.3	0.130	4.9
	pNPR	0.307	6.1	0.292	6.6	0.048	4.9	0.185	5.1	ND	
BM + naringin	pNPG	ND		0.930	8.6	ND		1.568	9.2	0.676	7.1
	pNPA	ND		0.233	5.1	ND		0.529	6.8	ND	
	pNPX	0.249	8.1	0.780	6.9	ND		0.975	7.1	0.519	6.2
	pNPR	ND		0.459	6.8	ND		0.656	6.5	0.742	6.9

BM basal medium, *ND* not detected, *SD* standard deviation

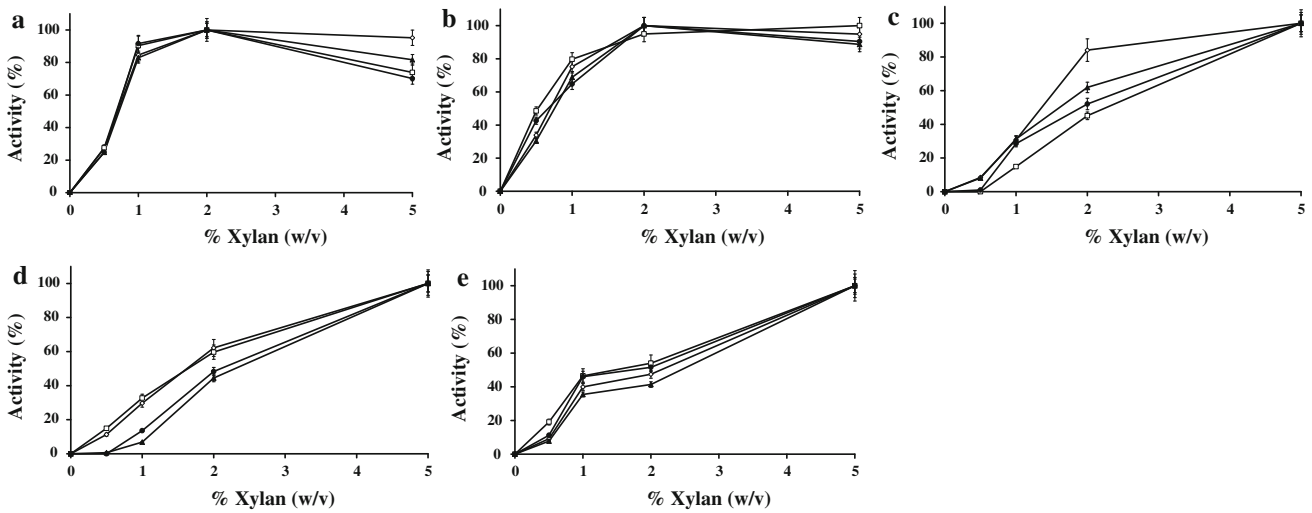

Fig. 1 Effect of xylan concentration (% w/v) on the glycolytic activities from **a** *H. uvarum* (I), **b** *H. uvarum* (II), **c** *H. vineae*, **d** *P. anomala*, **e** *T. delbrueckii*. α-Arabinosidase (*squares*), β-glucosidase (*diamonds*), α-rhamnosidase (*triangles*), β-xylosidase (*circles*). Maximum values are those reported in Table 2. Assays were performed in triplicate

of xylan with only slight changes at higher concentrations. The other three isolates show the highest values with 4–5% (w/v) in the medium. Low activity can be detected at low xylan concentrations, lower than 0.5% (w/v).

Effect of pH on activity

All isolates show a very low activity at pH 2.2 and a rapid increase when pH arises to 3.2; at this pH value, *H. uvarum* II and *P. anomala* β-glucosidase activity reaches the maximum value. Except for *H. uvarum* II, no decreases in the different activities were observed for higher pH values; these results could suggest a difference in the amino acid sequence in the active site of the enzymes, such that *H. uvarum* II contains a protonatable amino acid at high pH value in this catalytic part of the enzyme. This fact could also explain the differences in activity observed between the two isolates of *H. uvarum* species. The optimum pH values for the different activities in the five isolates are shown in Table 3.

Effect of glucose on activity

Activities drop at 5% glucose for all isolates, but 25–30% of activity could be detected at 20% glucose, except for *P. anomala*, which remained almost 40% for the four activities assayed at this glucose concentration (Fig. 2). At low glucose concentration, α-rhamnosidase and β-glucosidase in *H. vineae* showed a high degree of activity (85 and 100%). *H. uvarum* (II) and *T. delbrueckii* activities were highly affected by glucose content, with a higher slope in the *Torulaspora* isolate.

Table 3 Values of pH (±0.1) for highest glycolytic activities in the five isolates studied

Activity	*H. uvarum (I)*	*H. uvarum (II)*	*H. vineae*	*P. anomala*	*T. delbrueckii*
β-Glucosidase	6.2	4.2	4.2	3.2	5.2
α-Arabinosidase	6.2	4.2	4.2	4.2	5.2
β-Xylosidase	5.2	5.2	4.2	4.2	5.2
α-Rhamnosidase	6.2	4.2	4.2	4.2	5.2

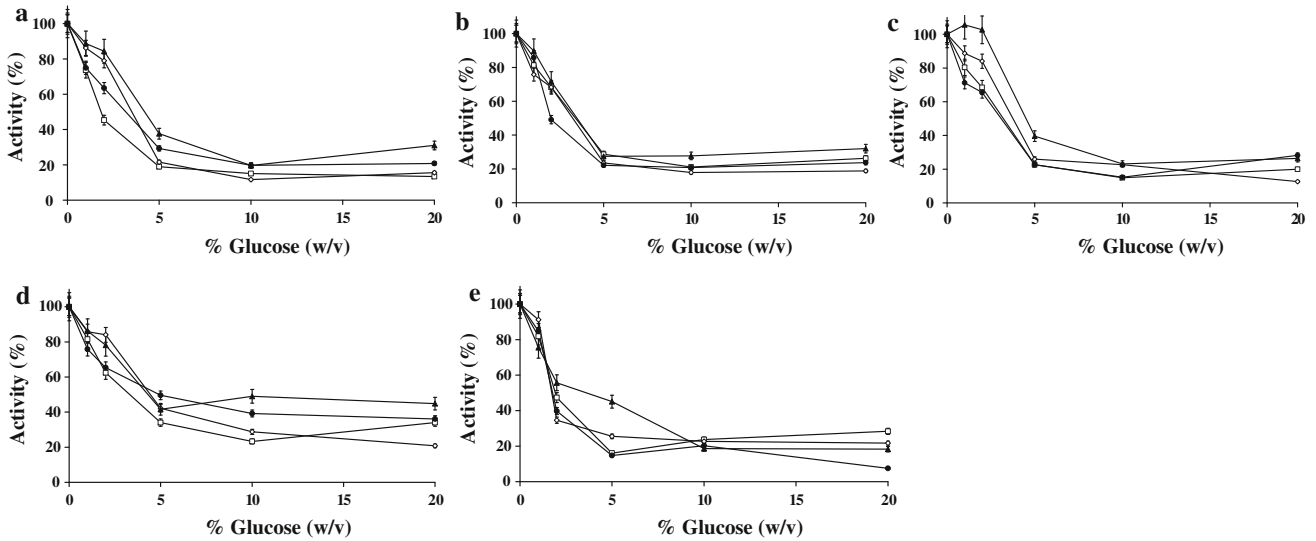

Fig. 2 Effect of glucose concentration (% w/v) on the glycolytic activities from **a** *H. uvarum* (I), **b** *H. uvarum* (II), **c** *H. vineae*, **d** *P. anomala*, **e** *T. delbrueckii*. α-Arabinosidase (*squares*), β-glucosidase (*diamonds*), α-rhamnosidase (*triangles*), β-xylosidase (*circles*). Maximum values are those reported in Table 2. Assays were performed in triplicate

Effect of ethanol on activity

Figure 3 shows the influence of ethanol added to the reaction mixture on the different activities. *H. uvarum* (I), *P. anomal*a, and *T. delbrueckii* isolates showed a similar behavior, so that the four activities had a slight decrease at the highest ethanol content, remaining at 80% activity at 20% ethanol. On the other hand, *H. uvarum* (II) (except for α-rhamnosidase) and *H. vineae* activities were more affected by ethanol; only 50–60% of activity was maintained at 20% ethanol. The ethanol had a low inhibitory effect on glycolytic activities and even showed a stimulatory effect. The change in polarity of the medium induced by ethanol could alter enzyme conformation and, consequently, its active site, thus reducing its activity. Nevertheless, the enzymatic activities were unaffected by such phenomenon; this may be due to both the intrinsic structural characteristics of the enzyme and/or active site and a probable protective mechanism that reduces the unfolding rate because of immobilization of the enzyme on the yeast cell membranes [1]. Alternatively, higher ethanol concentrations may have altered membrane permeability thereby allowing easier access between the intracellular enzyme and substrate [26].

Effect of temperature on activity

The optimum temperatures for the different activity assays were determined (Table 4). For *H. uvarum* I and *T. delbrueckii*, the enzymatic activity increased with temperature up to 30–40°C and then decreased; for other isolates, maximum activities were reached at 28°C. At 20 and 60°C the enzymatic activity was only between 15 and 20% of the maximum one, except for *P. anomala*; the activity at 20°C for this isolate is very similar to maximum. This is a very important fact because winemaking is nowadays conducted at low temperatures, so that it is important to obtain enzymes with high activity under these temperature conditions.

Effect of EDTA on activity

EDTA is employed in many biochemical tests in order to decrease the action of proteases in crude extracts because of its ability to chelate metallic ions. Nevertheless, its use can be a problem if the enzyme studied is metal ion-dependent. We tested the influence of several concentrations of EDTA on the activities. Figure 4 shows the results obtained for *H. uvarum* (I) isolate; the other isolates studied

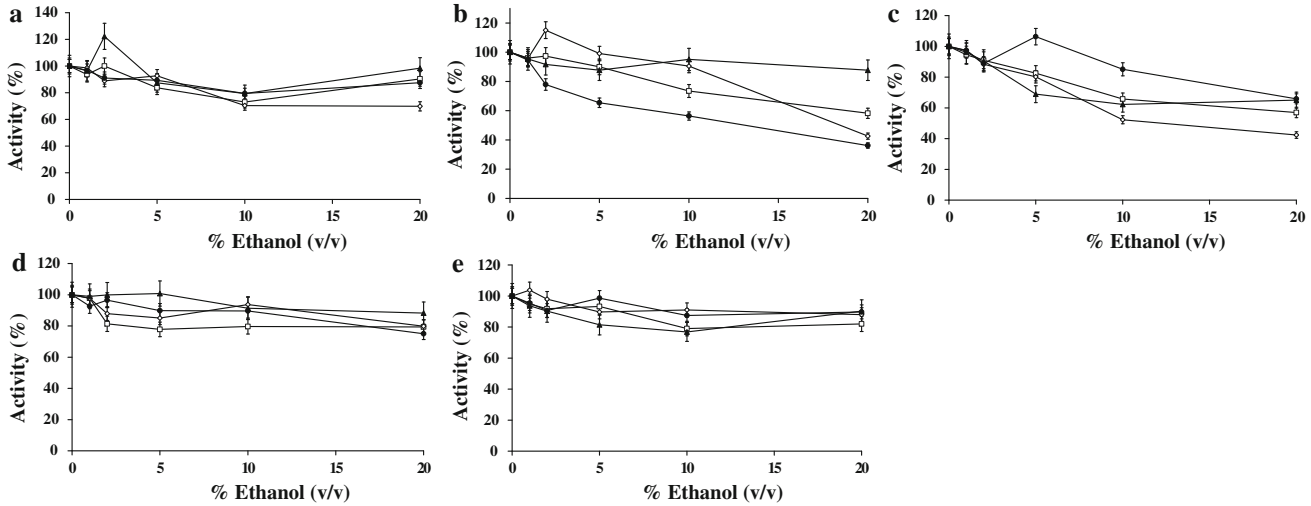

Fig. 3 Effect of ethanol concentration (% v/v) on the glycolytic activities from **a** *H. uvarum* (I), **b** *H. uvarum* (II), **c** *H. vineae*, **d** *P. anomala*, **e** *T. delbrueckii*. α-Arabinosidase (*squares*), β-glucosidase (*diamonds*), α-rhamnosidase (*triangles*), β-xylosidase (*circles*). Maximum values are those reported in Table 2. Assays were performed in triplicate

Table 4 Optimum temperature (±0.5°C) for the different activities in the five isolates studied

Activity	*H. uvarum (I)*	*H. uvarum (II)*	*H. vineae*	*P. anomala*	*T. delbrueckii*
β-Glucosidase	40	40	28	28	40
α-Arabinosidase	40	40	28	28	40
β-Xylosidase	40	28	28	28	28
α-Rhamnosidase	40	28	28	28	28

showed similar results that are not shown. With small concentrations of EDTA (0–0.02 M), the enzymatic activity increases with increasing EDTA concentration. This result can be explained by some inhibition of the proteases by the action of EDTA. The inhibition action of the chelating agent EDTA allowed us to conclude that the active site of these enzymes could be dependent on divalent cations for enzyme activation; these results agreed with those previously reported by Mateo and Di Stefano [20]. On the other hand, the influence of the various EDTA concentrations is the same on the hydrolysis of all the glycolytic activities.

Some wine producers are interested in native, non-inoculated yeast fermentations, partly because of the perception of enhanced aroma complexity. A wide range of yeasts have been found on grapes and in wines due to variations in wine age, geography, variety, harvest, and winemaking methods. In addition to *Saccharomyces cerevisiae*, it is known that other yeast species can grow during the early stages of fermentation. Frequently isolated indigenous species include: *Hanseniaspora uvarum, Metschnikowia pulcherrima, Candida stellata, Pichia membranifaciens, Hansenula anomala* as well as *Cryptococcus* ssp., *Rhodotorula* ssp., and *Saccharomyces* sp. [29]. Non-inoculated fermentations occur as a succession of

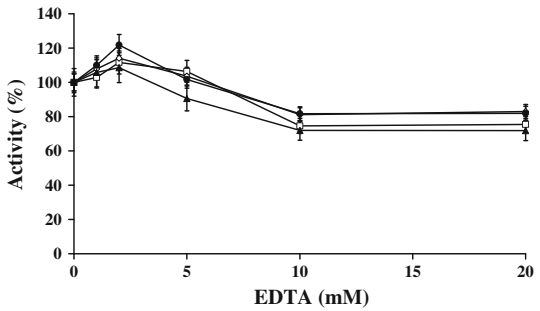

Fig. 4 Effect of EDTA concentration (M) on the glycolytic activities from *H. uvarum* (I). α-Arabinosidase (*squares*), β-glucosidase (*diamonds*), α-rhamnosidase (*triangles*), β-xylosidase (*circles*). Maximum values are those reported in Table 2. Assays were performed in triplicate

yeast populations beginning with relatively low, although numerically superior species present on the fruit. These yeasts are sensitive to increasing alcohol levels and are not so alcohol-tolerant as strains of *S. cerevisiae*, although more complex explanations have been discussed [27]. Over time the activity of indigenous species of non-*Saccharomyces* species declines and indigenous populations of *S. cerevisiae* are established and finish the fermentation. Such fermentations may be completed by as many as ten

strains of *S. cerevisiae* [24]. An explanation for the possible enhancement of aroma in wines produced by indigenous yeasts is that many different species/strains are involved [9]. Investigations of the effects of yeasts on wine aroma have confirmed the diversity of strains with respect to higher alcohol and ester production. However, there is limited information about the production of grape-derived aroma from non-*Saccharomyces* yeasts.

The optimal activity conditions for glycolytic activities studied are similar to those previously reported [33], namely pH 4–5 at 30–40°C. The higher specific activity at pH lower than the optimum might be due to an acid–base catalytic mechanism [16]. Moreover, the decrease in activity at pH lower than 3 might be correlated with the isoelectric point of the enzymes, with the increasing number of intra- and intermolecular interactions protecting its three-dimensional structure from a lower unfolding rate (thermal denaturation) [25].

From the technological point of view, some characteristics of non-*Saccharomyces* strains could justify their utilization in industrial production of more aromatic wines. The significance of glycosidases for the wine industry lies in their potential for releasing flavor compounds from glycosidically bound nonvolatile precursors in wine [18, 37]. Some papers have reported that non-*Saccharomyces* wine yeasts contain glycosidase activity not inhibited by glucose. It was reported that although the β-glucosidase from *Hansenula* sp., isolated from fermenting must, was capable of liberating aroma substances in must, it was less effective in wine [3, 23]. An intracellular β-glucosidase from *Debaryomyces hansenii* Y-44 was purified and used in the fermentation of Muscat wines [37]; a wine with considerable increase in the concentration of monoterpenols was produced. According to some reports, some strains of *S. cerevisiae* also possess β-glucosidase activity [5, 20], but this activity appears to be very limited and therefore recent studies have rather focused on non-*Saccharomyces* yeasts.

Some non-*Saccharomyces* yeasts produce undesirable concentrations of acetic acid and ethyl acetate from sugars; as a consequence they had always been regarded as unsuitable for winemaking [29]. However, some species present in mature grape berries and at the beginning of alcoholic fermentation seem to play an important role in the aromatic profile of the wines. These findings have practical enological importance, suggesting the potential of a reliable mixed culture fermentation strategy for producing a greater flavor diversity in wine [9]. The potential use of non-*Saccharomyces* yeast strains has been explored since the 1990s [22], but only recently has their importance been accepted [15]. As a result of their sensitivity to ethanol, non-*Saccharomyces* yeast could be employed at the beginning of winemaking as mixed cultures with *Saccharomyces* strains or in sequential non- *Saccharomyces*/*Saccharomyces* inoculation protocols [9]. The results obtained in this study allow us to think in an additional and more advantageous way of using these non-*Saccharomyces* strains.

It is well established that wine fermentations, whether they develop naturally or after inoculation with *S. cerevisiae*, are characterized by the significant growth of indigenous species of non-*Saccharomyces* yeasts. It has been proven that these yeasts must affect the chemical composition and final sensory quality of wine. Our study has revealed the potential of these yeasts to produce diverse glycolytic enzymes: this finding indicates the need to determine the potential impact of such activities on the sensory properties of wine. Future research focussed on the activity of these enzymes in wine fermentation and gaining better knowledge of the physiological and metabolic features of non-*Saccharomyces* yeasts is required.

On the basis of the results obtained in this work, *P. anomala* isolate seemed to be the most interesting candidate to be used to obtain enzymatic preparations for increasing the varietal flavor of wine. Optimal pH and temperature (4.2 and 28°C, respectively) and lower inhibition rate by glucose and ethanol make this yeast a good candidate for development of the varietal characteristics of wine and fruit juices. Additional work is required to determine the influence of these enzymes on wine aroma.

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