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Novel beta-glucosidase from *Issatchenkia orientalis:* Characterization and assessment for hydrolysis of muscat wine glycosides

Stefani de Ovalle [a], Beatriz Brena [a], Laura Fariña [b], Paula González-Pombo*[a].

[a]Cátedra de Bioquímica, Departamento de Biociencias, Facultad de Química, General Flores 2124, CC1157 Montevideo, Uruguay.
[b]Sección Enología, Departamento de Ciencia y Tecnología de Alimentos, Facultad de Química, General Flores 2124, CC1157 Montevideo, Uruguay

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The current work is the first report on the isolation and characterization of an extracellular β-glucosidases from *I. orientalis*, isolated from Uruguayan Tempranillo grapes. The extracellular activity reached a maximum after 96h of growth in the presence of 20 g/L of cellobiose. β-glucosidases was purified to homogeneity by anion-exchange chromatography and its biochemical properties were studied. Optimum catalytic activity conditions were 50°C and pH 5.0. The enzyme showed wide substrate specificity and proved to be highly tolerant to the presence of wine-associated inhibitory compounds such as glucose (100 g/L) and ethanol (12%). Immobilization onto silica nano-particles greatly improved enzyme stability at low pH (240-fold) with respect to the soluble biocatalyst. In addition, it is very active on wine glycosides and shows several suitable features suggesting that it is appropriate for the release of wine aroma. The increase in concentration of free norisoprenoids, terpenes and phenols was statistically significant. The release of phenols was particularly noteworthy, especially 4-vinilguayacol which is known to provide curry and clove notes. These results suggest the potential of this new β-glucosidase as an aroma-enhancing enzyme in winemaking.

Keywords: Beta-glucosidases, enzyme characterization, *Issatchenkia orientalis*, enzyme immobilization, aroma enhancement, wine.

INTRODUCTION

The intensive research carried out over the past decades has demonstrated that, in a great number of fruit and other plant tissues, important flavor compounds are accumulate as non-volatile and flavorless glycoconjugates, which make up a reserve of aroma to be exploited (Bartowsky et al., 2004; Sarry and Günata, 2004). These odorless non-volatile glycosides can be hydrolyzed by acid or enzymatic hydrolysis. However, fast acid hydrolysis may cause rearrangements in the aglycone structure led to the formation of undesirable flavors (Günata, et al., 1988). On the other hand, enzymatic hydrolysis selectively leaves the glycosidic linkage without altering the aglycone, which makes this process more favorable (Swiegers et al., 2005) β–D-Glucosidases, found in all domains of living organisms, are the most important flavor enzymes in the process of enzymatic hydrolysis of non-volatile glycosidic precursors in fruit juices, musts, and wines (Bartowsky et al., 2004; Sarry,2004).

*Corresponding author: Telephone/Fax; +598 2 9241806/ 2 9241906 E-**mail**; pgonzale@fq.edu.uy.

However, in the stringent winemaking conditions, such as high sugar content, presence of ethanol, low pH, and high concentrations of polyphenols, most grape and microbial β-glucosidases are inhibited. For this reason, the hydrolysis of glycosylated precursors of volatile compounds by β-glucosidases from grape and most *Saccharomyces* strains is often incomplete during the winemaking process (Barbagallo et al.,2004).So, attention has been focused on the role on *non-Saccharomyces* yeasts in wine fermentations; about 20 species of non-*Saccharomyces* yeasts display β-glucosidase activity and there is a general agreement among wine microbiologists that indigenous yeasts, are essential to the authenticity of the wine, imparting distinct regional and other desirable characteristics (Harborne, 1971; González-Pombo et al., 2011; Jolly et al., 2014).

This highlights the relevance of studying β-glucosidases from the biodiversity of yeasts available in the local wine ecosystems (Cordero Otero et al., 2003; Arévalo Villena et al., 2005). Recently, we described the isolation and characterization of an interesting native β-glucosidase from *Issatchenkia terricola* isolated from Tannat grapes, which was active at acidic pH (over 3.0) and at high concentration of glucose and ethanol(González-Pombo et al., 2011). In search of other new β-glucosidases for wine improvement in this work we have studied another native enzyme: β-glucosidase from *Issatchenkia orientalis,* isolated from Uruguayan Tempranillo grapes. We report its purification and characterization, and assessed for its efficiency for the hydrolysis of wine aroma precursors.

MATERIALS AND METHODS.

Materials

The enzyme substrates and tetramethylorthosilicate were supplied by Sigma-Aldrich (St. Louis, MO, USA). The enzymatic glucose determination kit was purchased from Spinreact S.A (Spain). Bradford´s reagent was purchased from Bio-Rad laboratories (Richmond, CA, USA). The C18 reverse phase column (Sep-Pak® cartridge) was supplied by Waters TM, USA, and the ISOLUTE® ENV+ was purchased from IST Ltd., Mid Glamorgan, U.K. *Issatchenkia orientalis* isolated from grape must was supplied by the Laboratorio de Enología (Facultad de Química, Montevideo, Uruguay). All other chemicals used were of reagent grade.

Methods

Enzyme production

A collection of 160 Saccharomyces and non-Saccharomyces indigenous yeasts strains, isolated from grapes and musts from Uruguayan vineyards, was screened for β-glucosidases activity using esculine as substrate at pH 4.0 (Pérez et al., 2011). The selected yeast, *I. orientalis,* was identified by analysis of the variable domain D1 / D2 from 26S ribosomal RNA subunit (Kurtzman and Robnett, 1998). A 48-h-old pre-culture in YEPD medium was added to Erlenmeyer flasks with an initial optical density (O.D) of about 0.2, incubated aerobically at 200 rpm for 6 days at 28ºC. Growth was monitored by absorbance determination at 600 nm. The extracellular activity was determined in the supernatant medium after centrifugation at 5000g for 30 min at 4ºC. The cell free culture medium was precipitated with ammonium sulfate (70 % saturation) overnight (4ºC) and centrifuged as previously described. The precipitate was dissolved in 0.1 M sodium acetate buffer, pH 4.5 (activity buffer) and then dialyzed (crude extract).

Protein and enzyme assay

Protein was quantified by Bradford and activity was measured by quantifying the hydrolysis of p-nitrophenyl-β–D-glucopyranoside (pNPG) in activity buffer as reported in González-Pombo et al. (2011). We defined one unit of enzyme activity (EU) as that which releases 1 µmol p-nitrophenol/ min under the specified enzyme assay conditions.

Enzyme purification

The desalted enzyme extract was purified using anion-exchange chromatography with a Mono Q_{TM} 5/50 GL column (Amersham Biosciences) equilibrated with 20 mM pH 6.0 sodium acetate buffer (buffer A). A sample volume of 0.5 mL of the enzyme extract was loaded onto the column at a flow rate of 0.5 mL/min. Afterwards, the column was washed with ten column volumes of buffer A. Proteins were eluted with ten column volumes of buffer A, supplemented with NaCl 1M, at linear velocity of 0.5 mL/min. Fractions of 0.5 mL were collected and analyzed for protein and β-glucosidase activity. All chromatography experiments were carried out at room temperature (23ºC) using an ÄKTA explorer chromatography system (Amersham Biosciences) controlled by Unicorn software 5.31. The enzyme characterization and was performed with the purified fractions eluted from the Mono Q_{TM} 5/50 GL column.

Polyacrylamide gel electrophoresis (PAGE)

Native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulfate (SDS)-PAGE and isoelectric focusing were carried out with Phast System apparatus. Proteins were silver stained as reported in González-Pombo et al. (2011).

Determination of molecular weight

Size-exclusion chromatography (SEC) was performed in an AKTA purifier system (AKTA Purifier10, GeneralElectric),

using a Superdex 200 10/300 GL column (GE Healthcare) in sodium phosphate 50 mM, pH 7.0, 0.15 M NaCl at 0.25 mL per minute. The following molecular weight standards were used: Blue Dextran (MW > 2000 kDa), Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa), Aldolase (158 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43kDa), and Ribonuclease (13.7 kDa).

Kinetic properties

The kinetic parameters Vmax (µmolpNP/mL. min) and K_m (mM) were determined using Michaelis-Menten plots. Activity on pNPG (in the range 1-10 mM) was measured for both soluble and immobilized enzyme. The rates were measured in duplicate. Vmax and K_m values were determined using linear regression (Lineweaver Burk) using Microcal Origin 6.0.

Immobilization procedure

The beta-glucosidase from *I. orientalis* was immobilized onto silica nanoparticles synthesized as reported inLuckarift et al. (2004). The dialyzed crude extract was incubated with the support in activity buffer during 30 minutes at room temperature. After centrifugation (1 min at 1300 g and room temperature), the pellet was collected and washed with activity buffer. Then, the immobilized enzyme was agitated with 0.5 % v/v of glutaraldehyde solution (prepared in 25 mM buffer sodium-phosphate pH 7.0) during an hour at room temperature, further washed and stored at 4º C during 16 hours (López-Gallego et al., 2013).

Stability of Soluble and Immobilized Enzyme.

Aliquots of soluble enzyme or immobilized enzyme suspension (0.1 UE and 0.5 mg) were incubated in model wine at pH 4.0 (ethanol-water 12:88 v/v containing 3.5 g/L of tartaric acid, 2.5 g/L of malic acid and 60 mg/L of sodium metabisulfite) at 23ºC under gentle stirring. Aliquots were taken at regular intervals, and the residual activity was determined.

The stability of the immobilized enzyme was also studied in model wine at pH 4.0 with the addition of previously isolated glycosides from white Muscat wine. Experiments were performed in triplicate.

Isolation of volatiles and bound glycosides of Muscat wine

Volatiles and bound glycosides were isolated from 50.00 mL of Muscat wine as previously reported (Boido et al., 2003; González-Pombo et al., 2011).

Enzymatic hydrolysis of bound glycosides

The immobilized biocatalyst (0.2 EU/g) was incubated with bound glycosides extract re-suspended in 50 mL in model wine at pH 4.0 and room temperature with agitation. A control experiment without enzyme was performed by incubating the matrix (silica nanoparticles) in the same conditions (Control wine). Aliquots were taken over time to follow the progress of the hydrolysis by Glycosyl–Glucose assay. Experiments were performed in duplicate.

Glycosyl-Glucose (G-G) assay

The concentrations of glycosides were determined according to the method ofIland et al. (1995).

Identification and Quantification of volatile compounds.

Released volatile compounds were quantified by GC-MS using a Shimadzu QP 5050 according to Fariña et al. (2005).

RESULTS

Enzyme production, purification and characterization

Issatchenkia orientalis, isolated from Tempranillo grapes was selected due to its large diameter of iron-esculetin diffusion zone that reveals high β–glucosidase activity at pH 4.0. The production of β–glucosidase was followed during the different physiological stages of the cell culture. As shown in Figure 1 in the absence and in the presence of cellobiose (20 g/L), the extracellular activity was higher than the intracellular one. The activity of β–glucosidase increased six-fold in the presence of cellobiose, reaching a maximum at the end of growing phase (6 x 10 -3 EU/mL) and then decreasing sharply to 1x10-3 EU/mL (Figure 1A).

The β–glucosidase was purified upon elution from the Mono Q column with 1.0 M NaCl (Figure 2). The enzyme was purified 17-fold, with a yield of 71 %. The specific activity increased from 6.4 E-3 EU/mg in the culture to 0.11 EU/mg.

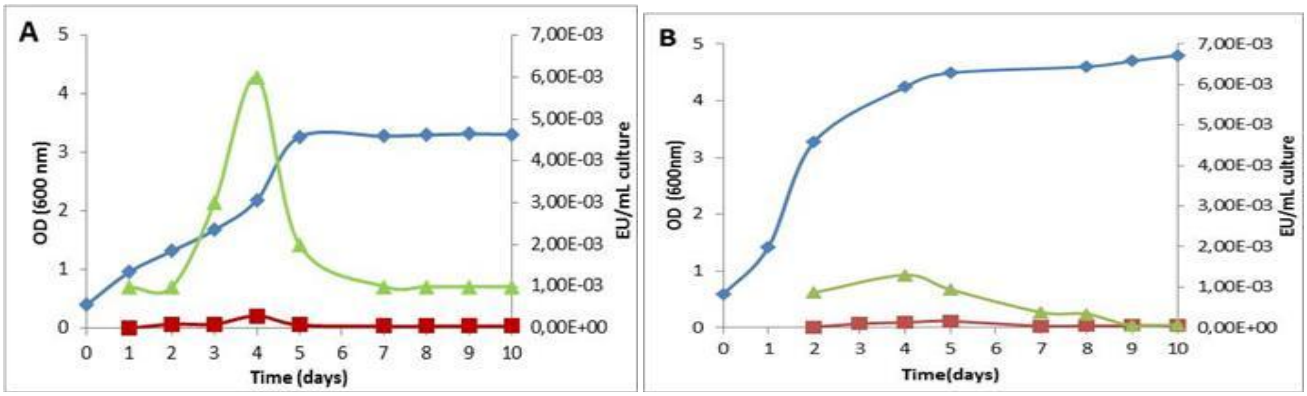
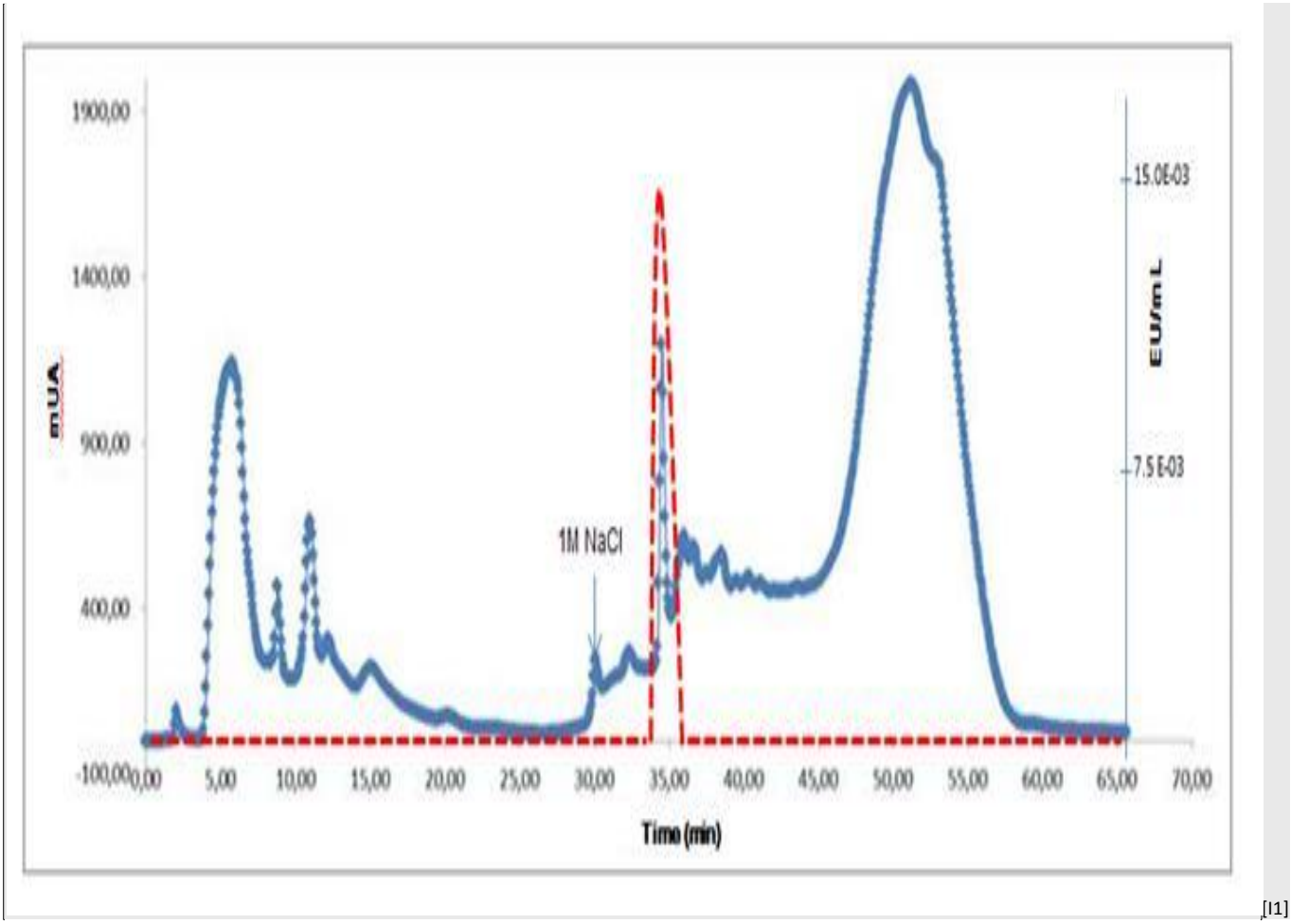


Figure 1: Kinetics of β-glucosidases production by *Issatchenkia orientalis* during cell growth on culture medium with cellobiose 20g/L (A) and without (B) cellobiose, Extracellular enzyme activity towards p-nitrophenyl-β-D-glucopyranoside (pNPG) (enzyme units); ▪, intracellular enzyme activity towards pNPG (enzyme units); ♦ Cell growth(Abs_{600nm})
Extracellular β-glucosidases activity was measured in the culture supernatant fluid after centrifugation and intracellular activity was determined in the cell pellet, after cell disruption.
The results are mean values from duplicate experiments.



[I1]

Figure 2: Elution profile of β-glucosidase extract from Mono Q ionic exchanger by 1.0 M NaCl:
•••, proteins; - - -, enzyme activity on pNPG.

Native PAGE electrophoresis of the crude extract shows multiple protein bands and only one with β–glucosidase activity, as revealed with the fluorescent substrate 4-methyl-β-umbelliferyl-β-D-glucoside (MUG) (Figure 3A). As shown in lane 2, the ionic exchange purification procedure achieved a homogeneous enzyme preparation in a single step. SDS−PAGE of the purified fractions (Figure 3B) showed the presence of a single band at 60 kDa (Figure 3B lane 2). Size-exclusion chromatography revealed that the molecular weight of the native β–glucosidase was of about 58 kDa, suggesting that the enzyme is monomeric. The physicochemical and kinetics properties are shown in Table 1. In the presence of ethanol (0 to 12 % v/v) the β–glucosidase activity was not affected. Moreover, the presence of 20% ethanol v/v increased the apparent enzyme activity by 20% (Figure 4).

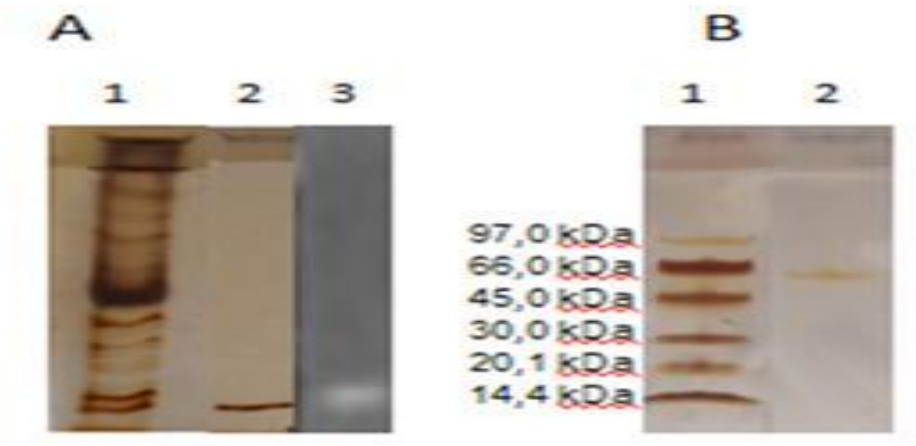


Figure 3: Native PAGE Gradient with 8-25 Phast Gels: crude *I. orientalis* extracellular extract (lane 1); purified β-glucosidase after ionic exchange chromatography (lane 2); zymogram of the crude extract using a 5mM solution of 4-MUGfor 10 min at 30°C (lane 3). B) SDS-PAGE with Homo12.5 Phast Gels: molecular weight markers (lane 1), purified β-glucosidase (lane 2).

Table 1: Physicochemical and kinetic characteristics of β-glucosidases from *I. orientalis*.

Physicochemical characterization			**Inhibition**		**Kinetics parameters**	
Optimum pH [a]	*Optimum temp (°C)* [b]	*Isoelectric point (pI)*	*Glucose (100 g/L)*	*Ethanol ≤12% (v/v)*	*Km (mM)* [c]	V Max[c] (µmoles de *p*NP/mL. min)
5	50	4.0	25%	No	0.83	50 E-3

[a]Studied in the range pH 3–10.
[b]Studied in the range 23–80°C.
[c]Using different concentrations of pNPG (range 1-10 mM) as a substrate at pH 4.5 and 23°C.

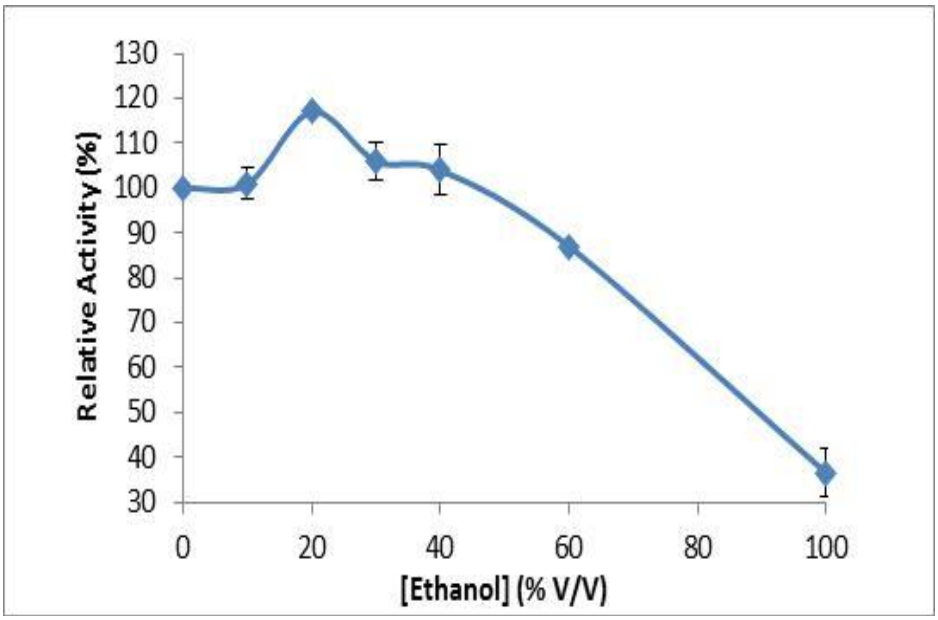


Figure 4: Relative activity (%) of soluble β-glucosidases from I. orientalis, at different ethanol concentrations in the range 0-100 % v/v . The enzyme activity at 0% ethanol (100%) was 0.1 EU/mL.

The β–Glucosidase was also highly tolerant to glucose: it was fully active in the presence of 5 g/L glucose (concentration found in wines) and there was only a minor enzyme inhibition (25%) at the very high concentrations of glucose in wine musts (100 g/L).

The effect of several cations and chemical reagents on β–Glucosidase activity is shown in Table 2. The Monovalent ion K^+ has a significant but minor positive effect on enzyme activity. The di- and trivalent ions such as Mn^{2+}, Ca^{2+} and Fe^{3+} showed important increases in the enzyme activity which suggests that they can interact with the enzyme. On the other hand, Zn^{2+} showed a detrimental effect with complete loss of enzyme activity. Since EDTA had no effect on the activity it appears that the enzyme does not depend on a metallic co-factor.

Table 2: Effect of metal ions on β-glucosidases from *I. orientalis*.

Compound (10mM)	Relativeactivity (%)* ± S.D.
$MgCl_2$	119±25
$FeCl_3$	169±20**
$CaCl_2$	146±30**
EDTA	86±18
$MnSO_4$	123±20**
KCl	115±5**
NaCl	111±15
$ZnCl_2$	0±0**

*relative activity value of 100% (0.1 EU/mL) in the absence of metals was determined in 0.1 M sodium acetate buffer, pH 4.5 (activity buffer).
**Values with significant differences with respect to the activity in the absence of metals ($p< 0.05$). The results are the average of three experiments.

The β–Glucosidase from *I. orientalis* was active against different glucosides with α-(1-4) or β-(1-4) configuration and was also able to hydrolyze β-(1-2)glycosides (Table 3).

The activity towards aryl-glycosides (10mM) was measured by the method previously described for pNPG. For disaccharides (10mM) and polysaccharides (5 g/L) the activity was determined by assaying the amount of reducing sugars released by the glucose oxidase method (Trinder, 1969). The relative activity values were determined with respect p-nitrophenyl-β-D-glucopyranoside.

Table 3: Action of β–Glucosidaseof *I. orientalis* on aryl-glycosides, disaccharides and polysaccharides.

Substrate	*Configuration of glycosidelinkage*	*Relative Activity (%) ± S.D.*
p-nitrophenyl-β-D- glucopyranoside	(1→4)-β	100±2
o-nitrophenyl-β-D-glucopyranoside	(1→4)-β	7±2
p-nitrophenyl-α-D- glucopyranoside	(1→4)-α	17±3
p-nitrophenyl-β-D-galactopyranoside	(1→4)-β	18±2
o-nitrophenyl-β-D-galactopyranoside	(1→4)-β	18±1
p-nitrophenyl-α-L-ramnopyranoside	(1→6)-α	11±1
p-nitrophenyl-α-L-arabinopyranoside	(1 →6)-α	14±1
Cellobiose	(1→4)-β	18±2
Saccarose	(1→2)-β	56±5
Maltose	(1→4)-α	25±4
Esculine	(1→6)-β	31±3
Carboxymethylcellulose	(1→4)-β	27±2

Immobilization results

Immobilization onto nanoparticles (NPs) offers many advantages such as high surface area and volume ratio which results in higher enzyme loadings with lower diffusional limitations (Jackson et al., 2015). The immobilization process of the *I. orientalis* enzyme was very efficient: 85 % of the applied protein was immobilized. Moreover, the immobilized enzyme was very active, expressing 67 % of the retained activity.

Stability of Soluble and Immobilized Enzyme

In order to mimic enological conditions, the stability of soluble and immobilized *I. orientalis* β-glucosidase (0.1 EU, about 0.5 mg of protein) was studied in a model wine at pH 4.0, 23º C in triplicate experiments. The half-life of the immobilized enzyme under these conditions (360 hours, 15 days) was 240-fold higher than that of the soluble enzyme (1.5 hours). Furthermore, the addition of wine glycosides to the model wine produced an extra stabilization factor of 2.3-fold, which increased the half-life of the immobilized enzyme to 34 days (Figure 5).

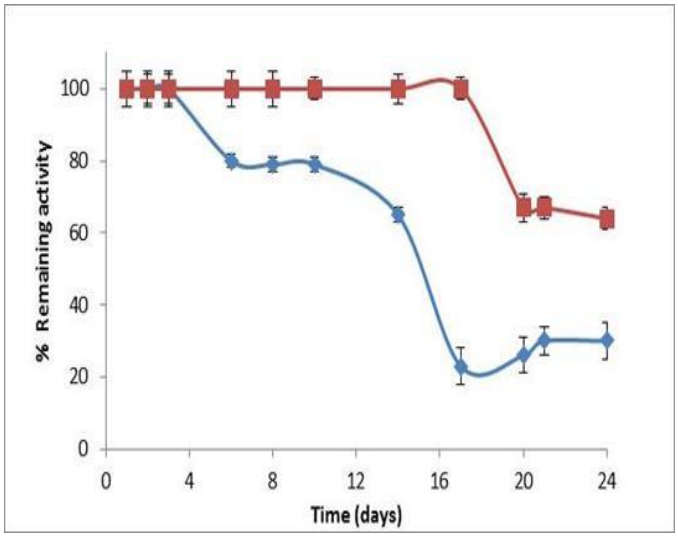


Figure 5: Effect of the addition of wine glycosides on the stability of the immobilized enzyme in model wine at pH 4.0 and 23°C:♦, without glycosides; with glycosides.

Hydrolysis of Muscat wine glycosides

The enzyme was very active on wine glycosides. While the G-G values in the control experiment remained unchanged, the immobilized biocatalyst (1.0 EU in 50 mL model wine) achieved a 40% reduction inbound glycosides. The G–G value of the enzyme treated wine decreased from 907 to 525 µM after 40 days. Consistently, upon enzymatic hydrolysis of the glycosides, the GC-MS analysis evidenced a significant and very important increase of several volatile compounds (Figure 6). The treatment with the *I. orientalis* enzyme achieved a significant increase in norisoprenoids (from 0 to 8 µg/L, $p < 0.05$) and terpenes (from 18 to46 µg/L, $p < 0.01$). An additional feature of this enzyme is the very relevant increase of phenols (from 25 to 102 µg/L, $p < 0.01$), which might have an impact in the aroma of the wine. In particular, the release of 4-vinilguayacol (from 24 to 90 µg/L, $p < 0.02$) is worthy of note, because it is known to confer species notes.

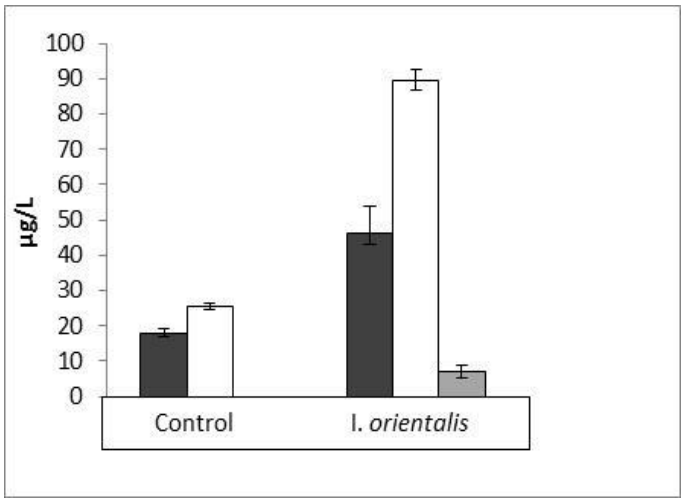


Figure 6: GC-MS analysis of volatile compounds (µg/L)after enzymatic hydrolysis of glycoside extracts in model wine. The control group was assayed in same conditions in presence of the matrix used in the immobilization (silica nanopraticles). Terpens; Phenols; Norisoprenoids.

DISCUSSION

Considering the promising results obtained previously with the β-glucosidases from *I. terricola* and its role in wine improvement (González-Pombo et al., 2011), we decided to characterize another β-glucosidases from the genus *Issatchenkia* which also presented very high enzyme activity in agar esculine medium. To our knowledge, this is the first report on the study of a β-glucosidases from *Issatchenkia orientalis,* a yeast strain previously reported to be tolerant to ethanol, pH and temperature (Hisamatsu et al., 2006; Kitagawa et al., 2010). Indeed, the ethanol stability of this enzyme is in agreement with these antecedents. Furthermore, in the presence of 20% ethanol (v/v), the apparent enzyme activity was increased by 20%, probably due to a transglycosylation process in which ethanol could act as an alternative acceptor for the glycosyl-enzyme intermediate complex during substrate hydrolysis. Unlike many β-glucosidases from non-*Saccharomyces* yeasts the *I. orientalis* enzyme is practically not inhibited by glucose: it retains 75 % of its activity at 100 g/L of glucose(Harborne, 1971). The glucose tolerance is an extremely valuable additional feature of this enzymewhich is notoriously more resistant than the enzyme from *Metschnokowia pulcherrima* (retains 25% activity)and other yeast β-glucosidases such as *Hanseniaspora sp.*(40%) and *P. anomala* (0%) (González-Pombo et al., 2008; Swangkeaw et al., 2011). In regard to the glucose tolerance, the*I. Orientalis* enzymeis similar to the one from *Issatchenkia terricola* (80% retained activity), *S. pararosesus* (69%) (Baffi et al., 2011; González-Pombo et al., 2011)and very few other non-*Saccharomyces* yeasts(Gueguen et al., 1997). These properties are very relevant for technological applications in oenology: both activity and stability in the stringent conditions found in wines are two key enzyme properties that determine the applicability. Concerning kinetics parameters, the K_m value of the β-glucosidases from*I. orientalis* (0.83 ± 0.03 mM) is lower than the enzyme from *I. terricola* (4.35 ± 0.15 mM) and is similar to other K_m values reported in non-*Sachharomyces* β-glucosidases, such as *Pichiaanomala* (0.47 ± 0.08) and *Hanseniaspora ovarum* (0.61 ± 0.14) (González-Pombo et al., 2011; Swangkeaw et al., 2011). This low K_m value shows that the enzyme acts at low substrate concentrations, which represents an advantage to its possible application.

The selectivity of the β-glucosidases depends on the species and strain. This enzyme showed broad substrate specificity on aryl-β-D-glycosides with exceptional activity towards phenol glycosides such as 4-vinilguayacol, known to confer interesting curry and clove notes. The enzyme presents also cellobiase activity, which could be potentially interesting in other food processing and fermentation industries where the hydrolysis of cellobiose is a key step, such as bioethanol production (Isono et al., 2012).

It is known that most of the reported yeast β-glucosidases show poor performance at the low pH of wines (3.0-4.0) and this is unfavorable to their possible industrial applications. So, there is the need to stabilize the biocatalysts to improve functionality (Verma et al., 2013). Immobilization of the *I. orientalis* enzyme onto nanoparticles, greatly improved its stability at low pH (240 fold stabilization factor) which would allow its successful application in oenology. Moreover, the protective effect of the substrates was very relevant: in the presence of wine glycosides at low pH, the stability of the immobilized enzyme was additionally increased by more than two-fold. The immobilization did not change Enzyme properties

such as optimum pH and temperature. However, the K_m value of the immobilized enzyme (7.44 ± 0.35 mM) was higher than that of the soluble enzyme and this is probably due to the glutaraldehyde cross linking which can lead to an enzyme structure distortion thus, reducing accommodation and accessibility of the substrate (Migneault et al., 2004).

CONCLUSIONS

The current work is the first report on the isolation and characterization of an extracellular β-glucosidases from *I. orientalis*, a yeast species known to be multi-stress tolerant. The enzyme withstands elevated concentrations of ethanol and glucose, typical of the winemaking process and it was further stabilized by immobilization to silica nanoparticles. In addition, it is very active on wine glycosides and shows several suitable features suggesting that it is appropriate for the release of wine aroma.

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