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Production and characterization of a β-glucosidase from *Issatchenkia terricola* and its use for hydrolysis of aromatic precursors in Cabernet Sauvignon wine.

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INTRODUCTION

In grapes, a major part of the aroma compounds are present as non-volatile glycosidic precursors constituting a reserve of potential active aroma molecules that can be released during the winemaking process, increasing wine complexity (Hernandez-Orte et al., 2009). The application of enzymes in oenology has increased over the past decade. Nowadays, in industrial production, the hydrolysis of aromatic precursors is often enhanced using fungal commercial enzymes preparations not adequately purified, and thus containing different glycosidase activities (Maicas & Mateo, 2005). However, such preparations are known to promote collateral reactions that damage wine quality and lead to the loss of wine typicity (Arévalo-Villena, Úbeda-Iranzo,

Cordero-Otero & Briones-Pérez, 2005; Fia, Olivier, Cavaglioni, Canuti, & Zanoni, 2016).

It is well known that in oenological ecosystems, β-glucosidases from non-*Saccharomyces* yeasts could impact in the development of varietal aroma and contribute to wine typicity (Palmeri & Spagna, 2007; Romo-sánchez, Arévalo-villena, Romero, & Ramirez, 2013). Thus, in search of alternatives to the use of commercial preparations, studies have been focused in the isolation and characterization of specific enzymes from non-*Saccharomyces* yeasts, isolated from the biodiversity of native wine ecosystems. Strains of *Issatchenkia terricola* yeast are found in soils, sea water, and spoiled fruit. They can also be part of grape native flora and often act as a spoilage yeast in fruit juices (Chavan et al., 2009). Due to its low fermentative characteristics and its capability to increase ethyl acetate concentrations, the use of *Issatchenkia terricola* in mixed fermentations has been discarded (Clemente-Jimenez, Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-Vico, 2004). A previous report of an extracellular β-glucosidase from *Issatchenkia terricola* isolated from Tannat grapes of Uruguayan vineyards showed activity on white wine glucosides, and was tolerant to acidic pH (over 3.0) and high concentrations of glucose and ethanol (González-Pombo, Fariña, Carrau, Batista-Viera, & Brena, 2011). All these properties suggest that it could be exploited to release wine aroma. However, the constitutive production of these extracellular enzymes is usually poor, which limits their applicability in biotechnological processes. Therefore, in order to carry out a successful process, the production needs to be enhanced. In the present work, the effect of environmental and nutritional conditions for the production of the β-glucosidase

from *Issatchenkia terricola* have been studied in batch and fed batch processes, and kinetic and stoichiometric parameters were determined.

The purified enzyme was biochemically characterized and its specificity towards aroma precursors as well as anthocyanin glucosides from Cabernet Sauvignon wine were also studied. Cabernet Sauvignon is originated in the Bordeaux region, France, but now it is planted in vineyards all over the world. The aroma of Cabernet Sauvignon wines is usually described as fruity or floral with roasted, wood-smoke, and cooked meat nuances (Peynaud, 1980) and often as herbaceous (Ugliano & Henschke, 2009).

2. MATERIALS AND METHODS

2.1 Chemical and reagents

The enzyme substrates: *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-L-rhamnopyranoside, *p*-nitrophenyl-α-L-arabinopyranoside, D-(+) cellobiose, sucrose, maltose and carboxymethylcellulose were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards of molecular weight were purchased from General Electric (Fairfield, CT, USA). EUPERGIT C 250L was kindly donated by RohmPharma (Darmstadt, Germany). *Issatchenkia terricola* yeast was supplied by the Laboratorio de Enología (Facultad de Química, Montevideo, Uruguay). The glucose determination kit was purchased from Spinreact (Girona, Spain). Bradford´s reagent was purchased from Bio-Rad laboratories (Richmond, CA, USA). Pure standards were purchased from Sigma-Aldrich Corp. (Milwaukee, WI, USA) and Fluka (Buchs, Switzerland). Solvents were of spectrophotometric

grade from Merck (USA). ISOLUTE ENV+ was purchased from Biotage AB (Uppsala, Sweden). All other chemicals were of analytical grade.

2.2 Culture media

A Strain of *Issatchenkia terricola,* isolated from Tannat grapes of Uruguayan vineyards, was screened at pH 4.0 for β-glucosidase activity, in Esculin Glycerol Agar medium as previously reported (Pérez et al., 2011). *I. terricola* was grown using eight cultures media with different carbon sources. Control medium (YPG): composed by (per liter) 25 g Yeast extract, 1 g peptone, 8 mL glycerol; wheat medium, composed by (per liter): 3 g wheat bran, 3 g yeast extract; 3 g KH_2PO_4, 6 g K_2HPO_4, 0.5 g $CaCl_2.2H_2O$, 0.2 g $MgSO_4.7H_2O$. For the remaining six culture media, the carbon source (per liter) was: 10 g glucose as limiting substrate (synthetic medium), 10 mL commercial vegetable juice V8 (Campbell's Oblimar, MI, USA), 8 mL glycerol, 10 g sugarcane molasses, 1.25 g hesperidin, 1.25 g naringin, respectively. Additionally, the remaining media contained (per liter): 4 g urea, 1 g K_2HPO_4, 0.45 g sodium citrate, 0.1 g, $CaCl_2$; 0.6 g $MgSO_4$ and 1 mL of vitamin solution, 1 mL trace element solution C, and 1 mL of trace element solution A. All medium were adjusted to pH 5.0. The vitamin solution contained (per liter): 6 mg folic acid, 6 mg myo-inositol, 6 mg d-biotin, 0.8 g calcium pantothenate, 0.8 g *p*-aminobenzoic acid, 0.8 g riboflavin, and 1.6 g pyridoxine. Trace element solution C contained (per liter): 0.6 g citric acid, 0.15 g $CoCl_2$, 3 g $MnSO_4 \cdot H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 15 g $FeSO_4 \cdot 7H_2O$, and 0.75 g $CuSO_4 \cdot 5H_2O$, pH 1.5. Trace element solution A contained (per liter): 0.65 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g KI, and 0.1 g H_3BO_3, pH 1.5.

2.3 Culture conditions

Erlenmeyer flasks filled to 10% of their nominal volume were inoculated with a 72-h-old preculture grown on YPG medium and incubated in the media previously described at 28ºC with shaking at 150 rpm. Liquid samples were withdrawn at regular intervals, and used for growth monitoring by measuring optical density at 600 nm and analytical determination (pH, substrate and enzyme activity).

Batch and fed-batch cultures were carried out in a 5-litre LH-210 Bioreactor (Inceltech, Toulouse, France) with synthetic medium, with aeration of 1 vvm (volume of air per volume of medium per minute) and stirred at 650 rpm. The culture pH was measured with a glass electrode MettlerToledo (Columbus, OH, USA). The outlet gas was analyzed with a paramagnetic O_2 detector (Series 1100, Servomex, Crowborough, UK) and an infrared CO_2 detector (Pir 2000, Horiba, Japan). The O_2 uptake and CO_2 production rates were calculated according to Cooney, Wang, & Wang, 2006.

The fed-batch protocol was designed according to the equations derived from the mass balances for the substrate and biomass in carbon-limited cultures by means of the kinetic and stoichiometric parameters calculated in the batch cultures.

(Eq. 1)

where S_R is the concentration of limiting substrate in the feeding medium; X_0, X_f are the biomass concentration at the beginning and the end of the feeding phase (g/L), respectively; V_0 and V_f, the initial and final volume (L); F, the feeding rate (L/h), μ, the specific growth rate (h^{-1}); and $Y_{X/S}$, cellular yield coefficient based on carbon source consumption (g cell/g carbon source).

2.4 Enzyme assay

β-Glucosidase activity was determined using a chromogenic substrate: *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG). A sample volume of 0.1 mL of enzyme solution was added to 1.25 mL of 25 mmol/L *p*NPG in 0.1 mol/L sodium acetate buffer, pH 4.5 (activity buffer). The reaction mixture was incubated at 23° ± 1°C (room temperature). Initial velocity was determined by taking 0.5 mL aliquots of the reaction mixture at regular intervals and added to 0.5 mL of carbonate buffer (0.2 mol/L; pH 10) to stop the reaction. The liberated *p*-nitrophenol (*p*NP) was measured spectrophotometrically at 405 nm in Shimadzu UV-Visible spectrophotometer, UV-1603 (Nakagyo-ku, Kyoto, Japan). The molar extinction coefficient used was 18,300 mol/L^{-1} cm^{-1}. (Blondin, Ratomahenina, Arnaud, & Galzy, 1983; Gueguen, Chemardin, Labrot, Arnaud, & Galzy, 1997). Enzyme activity is expressed in katals.

2.5 Enzyme characterization

The following characterization studies were performed with the purified enzyme extract prepared as reported in González-Pombo et al., 2011.

Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS)-PAGE and isoelectric focusing electrophoresis (IEF) were carried out with Phast System apparatus (Pharmacia LKB, Stockholm, Sweden) SDS-PAGE was performed with Homo 12.5 Phast Gels. The isoelectric point (Ip) was determined using the broad Ip calibration kit, run on PhastGel IEF 3-9 and staining with the specific fluorogenic substrate; 4-methyl-umbelliferyl-β-D-glucopyranoside (5 mmol/L) for 10 min at 30°C. The proteins in the polyacrylamide gels were stained with Coomassie Brilliant Blue.

2.5.1 Determination of molecular weight

Enzyme molecular weight was determined by size-exclusion chromatography in AKTA system (AKTA Purifier 10, General Electric, Fairfield, CT, USA), using a Superdex 200 10/300 GL column (GE Healthcare, Fairfield, CT, USA) in sodium phosphate buffer 50 mmol/L, pH 7.0, 0.15 mol/L 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).

2.5.2 Kinetic properties

The kinetic parameters K_m (mmol/L), K_{cat} (s^{-1}) and K_{cat}/K_m were determined with using the substrate *p*NPG (in the range 1-10 mmol/L) at room temperature. The rates were measured in duplicate. K_m and K_{cat} values were determined using linear regression (Lineweaver Burk plot).

2.5.3 Effect of Metal ions and EDTA on enzyme activity

The effect of different metal ions (Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}) on enzyme activity was studied. Each cation (or EDTA) at 10 mmol/L was added to 25 mmol/L of *p*NPG, prior to enzyme activity determination. Hundred per cent of activity was defined as the activity obtained in absence of metal ions and EDTA.

2.5.4 Substrate specificity

β-Glucosidase activity was assayed against aryl-glycosides: *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-α-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *o*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-α-L-rhamnopyranoside and *p*-nitrophenyl-α-L-arabinopyranoside. The activity towards aryl-glucosides was measured by the method previously described for *p*NPG. For disaccharides (D-(+) cellobiose, sucrose, maltose) and the

polysaccharide carboxymethylcellulose, the activity was determined by assaying the amount of glucose released by the glucose oxidase method (Trinder & Infirmary, 1969) using the glucose oxidase/peroxidase enzymatic assay kit.

2.6 Treatment of Cabernet Sauvignon young red wine

2.6.1 Enzymatic treatment of wine

The β-glucosidase of *I. terricola* was previously immobilized onto Eupergit C 250L according to González-Pombo et al., 2011. Immobilized enzyme (20 nkat) was incubated with a Cabernet Sauvignon red wine (500 mL adjusted to pH 4.0 with 2 mol/L NaOH) at room temperature with stirring (Treated wine). A control experiment without enzyme was performed by incubating the matrix (Eupergit C 250 L with the epoxy-groups previously blocked with 3 mol/L glycine), in the same conditions (control wine).

2.6.2 Glycosyl–Glucose (G–G) assay

The G-G assay was used to determine the total concentration of glycosides in wine samples in order to follow the time course of the enzymatic treatment. The total concentration of glycosides in Cabernet Sauvignon wine samples was determined using a C18 reverse phase column (Iland, Cynkar, Francis, Williams, & Coombe, 1995). In the first step, C18 reverse phase was activated with 10 mL of methanol followed by 10 mL distilled water. A volume of 10 mL of wine was loaded on the column and washed with 50 mL distilled water. Glycosides were eluted with 1.5 mL ethanol followed with distilled water to a final volume of 5 mL. In the second step, glycosides were hydrolyzed in acidic conditions according to the method of Iland et al., 1995. Then, samples were

neutralized using 1 mol/L of buffer Tris-HCl, pH 7.6 and 2 mol/L of NaOH solution. In the final step, the concentration of the released D-glucose was determined by spectrophotometric method using a glucose oxidase/peroxidase enzymatic assay kit. For each independent experiment, G–G analysis was performed in triplicate.

2.6.3 Isolation of volatiles

Volatiles were adsorbed on Isolute ENV+ cartridge packed with 1 g of highly cross-linked styrene-divinylbenzene (SDVB) polymer (40–140 µm, cod. no. 915-0100-C) as previously reported Boido et al., 2003. The cartridges were equilibrated sequentially with methanol (15 mL) and distilled water (20 mL). A sample of wine (50 mL diluted with 50 mL of distilled water) containing internal standard (0.1 mL of a 230 mg/L of 1-heptanol hydroalcoholic solution) was applied at 4–5 mL/min and the residue was washed with 15 mL of distilled water. The volatile fractions were eluted with 30 mL of dichloromethane; the solution was dried with Na_2SO_4 and concentrated to 1.5 mL on a Vigreux column. Samples were stored at −10º C, and further concentrated to 100 µL under nitrogen just immediately prior to GC–MS analysis.

2.6.4 Identification and quantification of aroma compounds

Extracts were analyzed by GC–MS using a Shimadzu QP 5050 mass spectrometer with reference libraries (Adams, 2001; McLafferty & Stauffer, 1991; Marais, Versini, van Wyk, & Rapp, 1992; Strauss, Gooley, Wilson, & Williams, 1987; Strauss, Wilson, & Williams, 1987) using a BP 20 (SGE, Ringwood, Australia) bonded fused silica capillary column (25m×0.25mm i.d.), coated with polyethylene glycol (0.25µm phase thickness) (Fariña, Boido,

Carrau, Versini, & Dellacassa, 2005). The identification of compounds was confirmed by injection of pure standards and comparing their retention index and relevant MS-spectra. Volatile compounds were quantified by GC, using 1-heptanol as the internal standard. In cases where pure reference compounds were not used, the identification was indicated as tentative and the quantification was performed using the characteristic fragments (Loscos, Hernandez-Orte, Cacho, & Ferreira, 2007).

2.6.5 Wine sensory analysis

The panel that carried out the sensory evaluation was composed of 14 subjects (5 women and 9 men) belonging to the staff of Enology lab of the Food Department of Faculty of Chemistry (UdelaR) and expert sommeliers. All of them participate regularly in sensory tests. Samples (30 mL, 18°C) were presented in a random order in coded tulip-shaped wine glasses covered with a Petri dish in individual testing booths. In the extended triangle test, one cup of enzymatic treated wine was confronted to two cups of the control wine (untreated wine). The panelists were instructed to smell the samples from left to right and to identify the different sample. When a significant difference was detected, the judges were asked to freely note the descriptors.

2.6.6 Analysis of wine color

Concentration of free anthocyanin was estimated by the method of sulfur dioxide (Ribéreau-Gayon, & Stonestreet, 1965). Aliquots of 1 mL of young wine and 1 mL of ethanol (containing 1 mL/L v/v of HCl) were mixed and added to 20 mL of 20 mL/L v/v, HCl. An aliquot of 10 mL of that mixture was taken and 4 mL

of 15 g/L of potassium bisulfite was added. For blank, the latter was replaced by distilled water. Absorbances at 520 nm was determined after incubated samples for 5 minutes, at 23ºC.

3. RESULTS AND DISCUSSION

3.1 Optimization of culture conditions

In order to optimize the production of the extracellular β-glucosidase, different culture media, and the influence of the initial pH and incubation temperature were studied. In all the media tested, optical density increased concomitantly with β-glucosidase production reaching its maximum in the exponential phase (data not shown). So, β-glucosidase production is associated with yeast growth. As shown in Fig. 1, the culture medium had a profound effect on the amount of enzyme activity produced. For most media (wheat, cane molasse, vegetable juice and glycerol) the yeast growth was low and as consequence the enzyme production was very poor. It is well known that the presence of substrates in culture media could contribute to enzyme production (Lee, Prometto, Demirci, & Hinz, 1998), however, the natural flavonoids tested (naringin and hesperidin) did not increase the enzyme production (Fig. 1). A similar result was obtained in presence of 5 g/L of the D-(+)-cellobiose (González-Pombo et al., 2011). For the synthetic medium the production of β-glucosidase was increased two fold with respect to the YPG and 3 to 8-fold with respect to the other media assayed. The synthetic medium differs from the other media tested, mainly in the carbon source composition. The fact that it is the only medium containing glucose, suggests that it acts not only as a carbon source but also as a stimulator of β-glucosidase production. This result is remarkable since most β-glucosidases are inhibited by the presence of glucose

and means that this glucose-tolerant enzyme could be used in some glucose-rich products such as fruit juices (Sarry, & Günata; 2004). Noteworthy, in synthetic medium, maximum production was attained one day before than in the control (YPG medium) and the others culture media assayed.

FIG. 1

The productivity of the enzyme was increased by rising the culture temperature from room temperature to 28°C in synthetic medium. With respect to the influence of pH (pH 4 to 6), the use of an initial pH of 5 or 6 almost doubled the enzyme production with respect to pH 4, increasing it from 130 pkat to 250 pkat (Supplementary Material 1). Thus, the optimized culture conditions for synthetic medium were: initial pH of 5.0, 28ºC and 96 h.

3.2 Scaling up of β-glucosidase production

3.2.1 Batch cultures at bioreactor scale

For synthetic medium, the time-course of cell growth and substrate consumption (Fig. 2) as well as the rates of oxygen consumption and carbon dioxide production (Fig. 3) were studied in batch culture. The respiratory quotient was always near 1, typical of a full respiratory metabolism. The stoichiometric and kinetic parameters of the culture are reported in Table 1. The carbon and energy balances were calculated according to Erickson, Minkevich, & Eroshin, 2000. A respiratory quotient close to unity indicates that only biomass and CO_2 are produced during cultivation under these conditions. Although the β-glucosidase is an extracellular enzyme, the amount of the produced protein is low enough compared to biomass, to impact on the carbon balance.

FIG. 2

FIG. 3

TABLE 1

3.3.2 Fed-batch cultures in bioreactor

Fed-batch is known to be the optimal cultivation process to produce cell-growth associated products due to its high volumetric productivity, as well as high final product concentration, stability and reproducibility of the process (Dodge, 2009). Considering that most protein production processes are based on fed-batch protocols, and in an attempt to increase β-glucosidase productivity, a fed-batch fermentation experiment using synthetic medium was performed. Indeed, this process allows controlling the rate of glucose feeding so as to avoid accumulation of the carbon and energy source, and a consequent non-restrict growth profile. The fundamental fermentation parameters were previously estimated from the batch-culture data (Table 1). For a desired final biomass concentration of 30 g/L, according to Eq 1 and based on the physiological values in Table 1 ($Yx/s = 0.488\ g_X/g_S$ and a $\mu_{max} = 0.144\ h^{-1}$), the corresponding parameters were: X_0=6 g/L, V_0=3.0 L and V_f=4.0 L, S_F=200 g/L, F=50 mL/h, where X_0 and V_0 are biomass concentration and volume at initial condition respectively, V_f is final volume, S_F is substrate feeding concentration, F is feeding flux. To our knowledge, this is the first report of kinetic and stoichiometric studies of this yeast.

Although fed-batch system is usually the most suitable to enzyme production in submerged culture, in the case of β-glucosidase, the final enzyme activity was practically the same as in batch culture. This behavior could be due to the fact

that some enzymes are synthesized in greater quantity when the microorganism grows at high rate. This is called growth-associated enzyme production. For the production of these enzymes, batch culture is the best selection because it is easier and faster than fed-batch. Thus, in batch culture, the volumetric productivity (in katals mL^{-1} h^{-1}) resulted higher than the fed batch (Dodge, 2009).

3.4 Enzyme characterization

3.4.1 Biochemical properties

The precipitation with ammonium sulfate allowed a one-step preparation of a purified extract of the enzyme as reported in González-Pombo et al., 2011. The SDS-PAGE (Fig. 4 lane 1), shows the presence of a single band at 49 kDa confirming the purity of the enzyme preparation used. Size-exclusion chromatography revealed that the molecular weight of native β-glucosidase was of about 48 kDa, suggesting that the enzyme is monomeric. Specific staining of isoelectric focusing gels with the fluorogenic substrate 4-methyl-β-umbelliferyl-β-D-glucoside (MUG) reveals that the isoelectric point of the enzyme is 3.5. Both results are similar to those of the majority of the β-glucosidases described previously, as those enzymes are acidic and commonly have monomers no bigger than 65 kDa (Esen, 1993).

The Michaelis-Menten constant (K_m) using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) was 4.35 mmol/L. This K_m value is higher than those of other β-glucosidases from non-*Saccharomyces* yeasts. The K_{cat} value was 460 s^{-1}. K_{cat}/ K_m is 1.1 x10^5 s^{-1} $(mol/L)^{-1}$.

Fig. 4

3.4.2 Effect of metal ions and EDTA on activity

As shown in Table 2**,** 10 mmol/L of K^+ practically did not affect enzyme activity, however, K_2SO_4 showed a stimulating effect (30% increased) suggesting that K^+ could have a stimulatory effect, depending on its concentration. The stimulatory effect of K^+ in the activity of β-glucosidases has been previously reported (Souza et al., 2010). The presence of 10 mmol/L of Ca^{2+}, Mg^{2+} and Na^+ cations did not influence the enzyme activity. These results are similar to other β-glucosidases (Chen, Hayn, & Esterbauer, 1992) but different to those observed for β-glucosidase from *Issatchenkia orientalis,* in which Ca^{2+} and Mg^{2+} ions increased enzyme activity (de Ovalle, Brena, Fariña, & González-Pombo, 2016). Similarly to other β-glucosidases, the presence of Co^{2+} decreased the activity by approximately 30 % (Baffi et al., 2013). An analogous behavior to the Co^{2+}, was observed in the presence of Mn^{2+}. Like other non-Saccharomyces β-glucosidases, the chelating agent EDTA practically did not affect enzyme activity, indicating that divalent cations are not required for enzyme activity (Chen, Li, & Zong, 2012; González-Pombo et al., 2008; de Ovalle et al., 2016).

Table 2

3.4.3 Substrate specificity

Concerning specificity for synthetic substrates, the enzyme was much more active on *p*-nitrophenyl-β-D-glucopyranoside than on other nitrophenyl-glucosides of α and β configurations (Table 3). So, both the sugar moiety and the type of glycosidic linkage are essential to substrate recognition. The enzyme strongly preferred glucose over other monosaccharides, and there was also striking specificity difference between *p*-nitrophenyl-β-D-glucopyranoside over the corresponding an isomer (*p*-nitrophenyl-α-D-glucopyranoside). This

suggests that this enzyme is much more specific for β (1→4) bonds, as compared to α (1→4) linkages. Accordingly, the enzyme was quite active on the disaccharide cellobiose containing β (1→4) linkages and only slightly active on maltose with α (1→4) glucosidic linkages. However, it was active on sucrose containing α (1→2) linkages and it did not hydrolyze the polysaccharide carboxymethylcellulose, with β (1→4) glucosidic bonds. Clearly, the β-glucosidase from *I. terricola* showed to be more selective than the one from *I. orientalis,* which showed a broad range of activity against different substrates (de Ovalle et al., 2016).

Table 3

3.5 Hydrolysis of aromatic precursors in Cabernet Sauvignon young wine.

The activity of β-glucosidase on the aromatic precursors was tested by the incubation of immobilized biocatalyst with a Cabernet Sauvignon wine. The activity of the β-glucosidase on red wine aroma precursor was tested during 19 days using the enzyme immobilized on Eupergit C 250L. The enzyme-treated wine showed a significant effect with respect to the control, decreasing 40% the amount of wine glycosides, from a G-G value of 500 µmol/L to 290 µmol/L.

After the enzyme treatment of the wine, the concentration of acids, esters and alcohols remained unchanged (Table 4). The enzyme treatment had a significant effect on the release of different aglycones and resulted in increased phenols and norisoprenoids with respect to control wine (Table 4). The volatile levels of both phenols and norisoprenoids increased significantly. Phenols increased (83%), from 607 µg/L to 1113 µg/L and norisoprenoids increased 65%, from 17 µg/L to 28 µg/L with respect to control wine. Concerning phenols, guaiacol is an established indicator of the smoke taint and at low levels it could

add complexity to wine flavor, however at higher concentration it may cause undesirable aromas (Kennison, Wilkinson, Pollnitz, Williams, & Gibberd, 2009; Parker et al., 2012; Ristic et al., 2011). As a result of the enzymatic treatment although an increase of its concentration around its threshold was observed (Table 4), its presence was not detected by the judges in the sensorial extended triangle test.

As for norisoprenoids and other carotenoid-derived aroma compounds they are recognized as aroma contributors in both, red and white wines and in grape juices, including the Chardonnay, Chenin blanc, Semillon, Sauvignon blanc, Riesling, Cabernet Sauvignon, and Shiraz varieties (Winterhalter & Rouseff, 2002). After enzymatic treatment of wine, the norisoprenoids, such as vomifoliol and 3-oxo-alpha ionol presented significant differences with respect to the control. Even though the threshold of these compounds has not been reported, they are known to be very low, and norisoprenoids have been characterized as enhancers of fruity, dried raisin or red plum notes (Escudero, Campo, Fariña, Cacho & Ferreira, 2007; Wang, Kang, Xu, & Li, 2011). Consistently, the judges of the triangle test, detected raisin and dried fruits notes in the treated wine.

The concentration of C6 compounds particularly (Z)-3-hexen-1-ol, was increased significantly by 30% (from 68 to 89 ug/L) with respect to control. Some authors describe C6 compounds as contributors of vegetative and green flavor attributes (Escudero et al., 2007). However, the contribution of C6-derived compounds with green attributes in wines is poorly understood (Bindon et al., 2014). Anyway, as odor threshold of (Z)-3-hexen-1-ol (400 ug/L) was not reached after the enzyme treatment, its contribution to wine aroma is expected

to be insignificant (Fariña et al., 2015). Accordingly, herbaceous aromas were not detected in the sensorial test in any of the wines.

For the extended triangle test, in a total of 28 trials, 21 found differences in the treated wine with respect to the control (level of significance < 0.001). The panel of judges considered that the control wine was sweet and fruity whereas the treated one had notes of dried fruits and raisins. As previously mentioned, the latter notes are in agreement with the increase in the concentration of norisoprenoids. This increase occurs slowly during the aging of wine (Loscos, Hernández-Orte, Cacho, & Ferreira, 2010), in a process that takes until 6 months in barrel (Oberholster et al., 2015). Noteworthy, these notes were achieved in a very short time (19 days), using β-glucosidase from *I. terricola*

Table 4.

Besides aroma profiles, color in red wines is one of the main attributes and anthocyanins are the major pigment compounds (Corduas, Cinquanta, & Ievoli, 2013). Since the main anthocyanins are mono-glucosides, attention has been focused in the role of β-glucosidases in the decrease of red wine color (Barbagallo, Palmeri, Fabiano, Rapisarda, & Spagna, 2007). *I. terricola* β-glucosidase had no activity onto anthocyanin glucosides since the concentration of anthocyanin after the enzymatic treat-wine remained unchanged (210 ± 22 mg/L). The high selectivity shown by this enzyme represents an advantage for its application since it could be used to develop aroma without compromised wine color.

4. CONCLUSIONS

The current work contributes to the investigation of the role and the assessment of the potential applications of native β-glucosidases to release aromatic compounds in wines. The analysis of the released aglycones after the enzymatic hydrolysis, revealed significant increases in the concentration of several volatile compounds. β-Glucosidase showed high ability to liberate norisoprenoids and phenols from their precursors, resulting in a wine with dried fruits and raisins notes without compromised red wine color. These results reinforce those previously obtained in Muscat wine (González-Pombo et al., 2011) and suggest that *Issatchenkia terricola* β-glucosidase, may be an approach to develop aroma in both white and red wines in very short times. For industrial application, even though there was an increase in enzyme production using optimal cultivation conditions, there is still the need to improve enzyme yield by means of for example recombinant DNA-technology using heterologous expression in *Saccharomyces cerevisiae*. The strain of *Issatchenkia terricola* yeast was not patented and its genome has not been sequenced yet.

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TABLES

Table 1. Stoichiometric and kinetic parameters of the *I. terricola* culture using synthetic medium. $Y_{x/s}$ and $Y_{CO2/s}$ represent, cellular and CO_2 yield coefficient based on carbon source consumption, respectively; b is the moles of O_2 consumed related with substrate consumption; Carbon balance and Reduction degree balance represent the way that carbon and energy of the substrate are distributed in the products of the growth reaction; μ is the specific growth rate (in h^{-1}).

$Y_{x/s}$	$Y_{CO2/s}$	b	Carbon balance	Reduction degree balance	μ (h^{-1})
0.488	0.567	0.511	1.055	1.023	0.144

Table 2. Effect of metal ions on the activity of *I. terricola* β-glucosidase. Residual activity (%) in presence of 10 mmol/L of different cations and EDTA. Assays were performed in triplicates.

Compound (10 mmol/L)	**% Residual activity* ± S.D**
$CaCl_2$	113 ± 13
$MgCl_2$	114 ± 18
KCl	106 ± 9
NaCl	113 ± 10
K_2SO_4	131 ± 8**
$MnCl_2$	70 ± 4**
$CoCl_2$	72 ± 5**
EDTA	91 ± 8

*residual activity value of 100% ($2x10^{-9}$ katals) was determined in absence of these compounds using 10 mmol/L *p*NPG in 0.1 mol/L of sodium acetate buffer, pH 4.5. **Values with significant differences with respect to the activity in the absence of metals ($p<0.05$).

Table 3. Substrate specificity of β-glucosidase from *I. terricola* against different substrates. Each substrate was tested at a concentration of 10 mmol/L, except for Carboxymethylcellulose (5 g/L). Assays were performed in triplicates.

Substrate	Glycosidic linkage	% Relative activity* ± S.D.
p-nitrophenyl-β-D-glucopyranoside	(1→4) – β	100 ± 5.0
p-nitrophenyl-α-L-arabinopyranoside	(1→6) – α	5.0 ± 1.0
p-nitrophenyl-β-D-galactopyranoside	(1→4) – β	3.0 ± 0.5
o-nitrophenyl-β-D-galactopyranoside	(1→4) – β	1.0 ± 0.1
p-nitrophenyl-α-D-glucopyranoside	(1→4) – α	<1
p-nitrophenyl-α-L-ramnopyranoside	(1→6) – α	<1
D-(+) Cellobiose	(1→4) – β	10 ± 1.0
Sucrose	(1→2) – α	12 ± 1.0
Maltose	(1→4) – α	3.0 ± 0.2
Carboxymethylcellulose	(1→4) – β	<1

*Relative activity value of 100% was determined using 10 mmol/L pNPG in 0.1 mol/L sodium acetate buffer, pH 4.5.

Table 4. Concentration of free volatile compounds (in µg/L) for both, control and treated-wine. Odor threshold (in µg/L) and descriptors of some compounds are shown. [a; b] Letters indicate the level of significant difference ($p<0.05$) according to a LSD test of ANOVA. N/A represent not available data. LRI refers to lineal retention index. Assays were performed in duplicates.

	Volatile compounds	LRI	Identity assign-ment [c]	Control µg/L ±S.D.	Treated wine µg/L ±S.D.	Odor threshold µg/L	Odor Descriptor
Acids	butyric acid	1670	B (1)	322 ±15	387 ± 2		
	isovaleric acid	1705	B (1)	685 ± 3	725 ± 27	33	sweat, acid, rancid
	hexanoic acid	1845	A	904 ± 66	1032 ± 91	420	fatty, cheese
	octanoic acid	2072	A	990 ± 37	930 ± 236	500	fatty
	SUBTOTAL			22889 ± 1006	28926 ± 5556		
Alcohols	isobutyl alcohol	1093	A	3704 ± 222	3684 ± 233	40.000	fuel
	1-butanol	1155	A	138 ± 15	195 ± 26	150.000	like wine, medicine
	2-phenylethanol	1918	A	19988 ± 885	25852 ± 5200	N/A	N/A
	3-methyl-1-butanol	1221	A	94469 ± 1973	107957 ± 2386	30.000	whisky, malt, smoked
	tyrosol	2999	B (4)	8175 ± 300	9380 ± 417	N/A	N/A
	benzyl alcohol	1882	A	122 ± 12	145 ± 24	200.000	floral, rose, phenolic, balsamic
	SUBTOTAL			106608 ± 2522	121361 ± 3086		
Esters	ethyl lactate	1353	A	213216 ± 5123	250722 ± 6225	60.000	strawberry, rapsberry
	ethyl-3-hydroxybutyrate	1527	A	280 ± 7	324 ± 11	N/A	N/A
	diethyl succinate	1714	A	5383 ± 19	6115 ± 300	100.000	overripe melon, lavender
	diethyl malate	2058	A	368 ± 4	383 ± 35	760.000	green
	ethyl succinate	2370	B (1)	57507 ± 1937	85516 ± 20000	1.000.000	toffee, coffee
	ethyl hexanoate	1237	A	51 ± 17	32 ± 12	14	green apple
	ethyl octanoate	1436	A	42 ± 4	40 ± 7	500	sweet, banana, pineapple
	ethyl decanoate	1684	A	10 ± 2	12 ± 3	200	sweet, hazelnut oil
	SUBTOTAL			276857 ± 7113	343144 ± 26593		
C6	1-hexanol	1368	A	742 ± 16	781 ± 14	2500	grass just cut
	(Z)-3-hexen-1-ol	1382	A	68 ± 2 [a]	89 ± 3 [b]	400	green, kiwi
	SUBTOTAL			810 ± 18	870 ± 17		
Noriso-prenoids	vomifoliol	3167	B (2)	9 ± 1 [a]	15 ± 1 [b]	N/A	N/A
	3-oxo-alpha-ionol	2651	B (3)	8 ± 1 [a]	13 ± 1 [b]	N/A	honey, apricorts
	SUBTOTAL			17 ± 2 [a]	28 ± 2 [b]		
Phenols	2,6-dimethoxyphenol	2240	A	595 ± 82 [a]	1039 ± 189 [b]	570	nutty, smoky
	guaiacol	1855	A	15 ± 6 [a]	71 ± 1 [b]	75	smoky
	SUBTOTAL			610 ± 88 [a]	1110 ± 190 [b]		

[c] A: identities confirmed by comparing mass spectra and retention times with those of authentic standards supplied by Aldrich (Milwaukee, WI) and Fluka (Buchs, Switzerland), B: identities tentatively assigned by comparing mass spectra with those obtained from the literature [(1) Adams, R. P. (2001). Identification of essential oil components by gas chromatography/ quadrupole mass spectroscopy. (2nd ed.). Stockton: Allured-Carol Steam IL, (469 pp); McLafferty, F. W., & Stauffer, D. B. (1991). The Wiley/NBS Registry of Mass Spectral Data. (5th ed.). New York: Wiley and Sons, (7872 pp). (2) Strauss, C. R., Wilson, B., & Williams, P. J. (1997). 3-Oxo-α-Ionol, Vomifoliol and Roseoxide in *Vitis Vinifera* Fruit. *Phytochemistry*, *26*, 1995–1997. (3) Marais, J., Versini, G., Van Wyk, C. J., & Rapp, A. (1992). Effect of region on free and bound monoterpene and C_{13}-norisoprenoid concentrations in Weisser Riesling wines. *South African Journal for Enology and Viticulture*, *13*, 71–77.(4) Strauss, C. R., Gooley, P. R., Wilson, B., & Williams, P. J. (1987). Application of Droplet Countercurrent Chromatography to the Analysis of Conjugated Forms of Terpenoids, Phenols, and Other Constituents of Grape Juice. *Journal of Agricultural and Food Chemistry*, *35*(4), 519–524.].

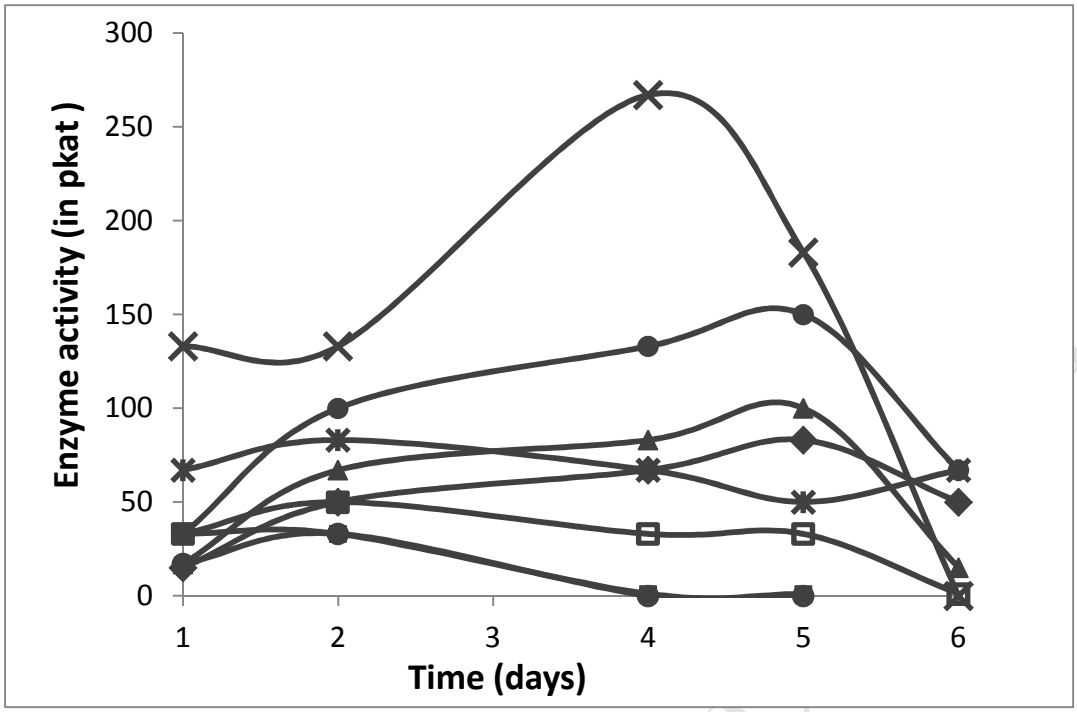

Figure 1. Extracellular Activity of β-glucosidase from *Issatchenkia terricola* in different culture media: (—×—) Synthetic; (—●—) YPG; (—▲—) Wheat; (—◆—) Orange; (—✱—) Hesperidin; (—□—) Cane molasse; (—■—) Vegetable juice and (—○—) Glycerol. All cultures were performed in Erlenmeyer flasks at 150 rpm, 28ºC and pH 5.

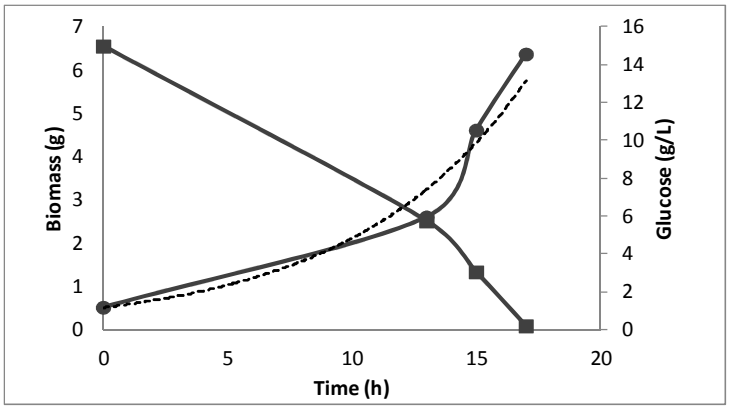

Figure 2. Time course of substrate consumption (—■—) and biomass production (—●—) in batch culture at bioreactor scale, using synthetic medium. Exponential distribution (------) of biomass conversion equation is $Y= 0.4984e^{0.1439x}$, $r^2=0.983$.

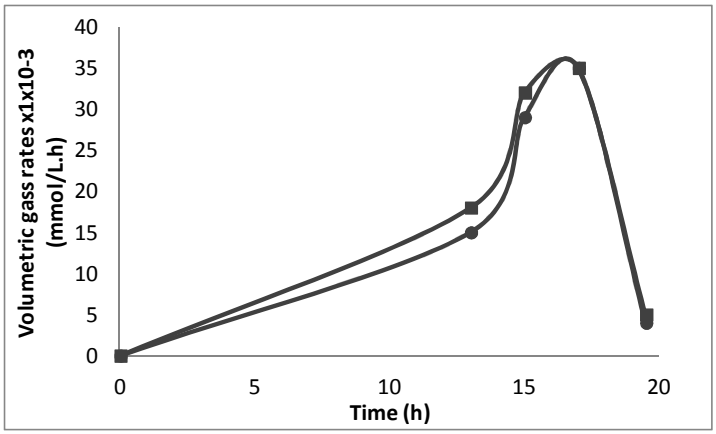

Figure 3. Time course of O_2 consumption (—●—) and CO_2 production (—■—) in batch culture at bioreactor in synthetic medium.

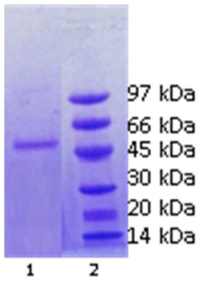

Figure 4. Sodium dodecyl sulfate electrophoresis in polyacrylamide in a Phast gel (Homo12.5%) Lane 1: purified enzyme extract; Lane 2: molecular weight marker.

1. The production of β-glucosidase from *Issatchenkia terricola* was optimized.
2. β-Glucosidase was very active on the hydrolysis of red wine glucosides.
3. GC-MS analysis of treated wine revealed the release of several volatile compounds.
4. Sensory evaluation showed significant differences between treated and control wine.
5. β-Glucosidase developed wine aroma without compromising its color.