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

4

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13

14 Keywords: non-*Saccharomyces*, enzyme production, bioreactor, sensorial
15 analysis, aroma.

16
17 ## INTRODUCTION

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

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

30 It is well known that in oenological ecosystems, β-glucosidases from non-
31 *Saccharomyces* yeasts could impact in the development of varietal aroma and
32 contribute to wine typicity (Palmeri & Spagna, 2007; Romo-sánchez, Arévalo-
33 villena, Romero, & Ramirez, 2013). Thus, in search of alternatives to the use of
34 commercial preparations, studies have been focused in the isolation and
35 characterization of specific enzymes from non-*Saccharomyces* yeasts, isolated
36 from the biodiversity of native wine ecosystems. Strains of *Issatchenkia*
37 *terricola* yeast are found in soils, sea water, and spoiled fruit. They can also be
38 part of grape native flora and often act as a spoilage yeast in fruit juices
39 (Chavan et al., 2009). Due to its low fermentative characteristics and its
40 capability to increase ethyl acetate concentrations, the use of *Issatchenkia*
41 *terricola* in mixed fermentations has been discarded (Clemente-Jimenez,
42 Mingorance-Cazorla, Martínez-Rodríguez, Las Heras-Vázquez, & Rodríguez-
43 Vico, 2004). A previous report of an extracellular β-glucosidase from
44 *Issatchenkia terricola* isolated from Tannat grapes of Uruguayan vineyards
45 showed activity on white wine glucosides, and was tolerant to acidic pH (over
46 3.0) and high concentrations of glucose and ethanol (González-Pombo, Fariña,
47 Carrau, Batista-Viera, & Brena, 2011). All these properties suggest that it could
48 be exploited to release wine aroma. However, the constitutive production of
49 these extracellular enzymes is usually poor, which limits their applicability in
50 biotechnological processes. Therefore, in order to carry out a successful
51 process, the production needs to be enhanced. In the present work, the effect of
52 environmental and nutritional conditions for the production of the β-glucosidase

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53 from *Issatchenkia terricola* have been studied in batch and fed batch processes,
54 and kinetic and stoichiometric parameters were determined.
55 The purified enzyme was biochemically characterized and its specificity towards
56 aroma precursors as well as anthocyanin glucosides from Cabernet Sauvignon
57 wine were also studied. Cabernet Sauvignon is originated in the Bordeaux
58 region, France, but now it is planted in vineyards all over the world. The aroma
59 of Cabernet Sauvignon wines is usually described as fruity or floral with roasted,
60 wood-smoke, and cooked meat nuances (Peynaud, 1980) and often as
61 herbaceous (Ugliano & Henschke, 2009).
62

63 **2. MATERIALS AND METHODS**

64 **2.1 Chemical and reagents**

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

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

80 **2.2 Culture media**

81 A Strain of *Issatchenkia terricola,* isolated from Tannat grapes of Uruguayan
82 vineyards, was screened at pH 4.0 for β-glucosidase activity, in Esculin Glycerol
83 Agar medium as previously reported (Pérez et al., 2011). *I. terricola* was grown
84 using eight cultures media with different carbon sources. Control medium
85 (YPG): composed by (per liter) 25 g Yeast extract, 1 g peptone, 8 mL glycerol;
86 wheat medium, composed by (per liter): 3 g wheat bran, 3 g yeast extract; 3 g
87 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
88 six culture media, the carbon source (per liter) was: 10 g glucose as limiting
89 substrate (synthetic medium), 10 mL commercial vegetable juice V8
90 (Campbell's Oblimar, MI, USA), 8 mL glycerol, 10 g sugarcane molasses, 1.25
91 g hesperidin, 1.25 g naringin, respectively. Additionally, the remaining media
92 contained (per liter): 4 g urea, 1 g K_2HPO_4, 0.45 g sodium citrate, 0.1 g, $CaCl_2$;
93 0.6 g $MgSO_4$ and 1 mL of vitamin solution, 1 mL trace element solution C, and 1
94 mL of trace element solution A. All medium were adjusted to pH 5.0. The
95 vitamin solution contained (per liter): 6 mg folic acid, 6 mg myo-inositol, 6 mg d-
96 biotin, 0.8 g calcium pantothenate, 0.8 g *p*-aminobenzoic acid, 0.8 g riboflavin,
97 and 1.6 g pyridoxine. Trace element solution C contained (per liter): 0.6 g citric
98 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
99 0.75 g $CuSO_4 \cdot 5H_2O$, pH 1.5. Trace element solution A contained (per liter): 0.65
100 g $Na_2MoO_4 \cdot 2H_2O$, 0.1 g KI, and 0.1 g H_3BO_3, pH 1.5.

101 **2.3 Culture conditions**

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102 Erlenmeyer flasks filled to 10% of their nominal volume were inoculated with a
103 72-h-old preculture grown on YPG medium and incubated in the media
104 previously described at 28ºC with shaking at 150 rpm. Liquid samples were
105 withdrawn at regular intervals, and used for growth monitoring by measuring
106 optical density at 600 nm and analytical determination (pH, substrate and
107 enzyme activity).

108 Batch and fed-batch cultures were carried out in a 5-litre LH-210 Bioreactor
109 (Inceltech, Toulouse, France) with synthetic medium, with aeration of 1 vvm
110 (volume of air per volume of medium per minute) and stirred at 650 rpm. The
111 culture pH was measured with a glass electrode MettlerToledo (Columbus, OH,
112 USA). The outlet gas was analyzed with a paramagnetic O_2 detector (Series
113 1100, Servomex, Crowborough, UK) and an infrared CO_2 detector (Pir 2000,
114 Horiba, Japan). The O_2 uptake and CO_2 production rates were calculated
115 according to Cooney, Wang, & Wang, 2006.

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

120 (Eq. 1)

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

126 **2.4 Enzyme assay**

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127 β-Glucosidase activity was determined using a chromogenic substrate: *p*-
128 nitrophenyl-β-D-glucopyranoside (*p*NPG). A sample volume of 0.1 mL of
129 enzyme solution was added to 1.25 mL of 25 mmol/L *p*NPG in 0.1 mol/L
130 sodium acetate buffer, pH 4.5 (activity buffer). The reaction mixture was
131 incubated at 23° ± 1°C (room temperature). Initial velocity was determined by
132 taking 0.5 mL aliquots of the reaction mixture at regular intervals and added to
133 0.5 mL of carbonate buffer (0.2 mol/L; pH 10) to stop the reaction. The liberated
134 *p*-nitrophenol (*p*NP) was measured spectrophotometrically at 405 nm in
135 Shimadzu UV-Visible spectrophotometer, UV-1603 (Nakagyo-ku, Kyoto,
136 Japan). The molar extinction coefficient used was 18,300 mol/L^{-1} cm^{-1}. (Blondin,
137 Ratomahenina, Arnaud, & Galzy, 1983; Gueguen, Chemardin, Labrot, Arnaud,
138 & Galzy, 1997). Enzyme activity is expressed in katals.

139 **2.5 Enzyme characterization**

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

142 **Polyacrylamide gel electrophoresis (PAGE)**

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

150 **2.5.1 Determination of molecular weight**

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151 Enzyme molecular weight was determined by size-exclusion chromatography in
152 AKTA system (AKTA Purifier 10, General Electric, Fairfield, CT, USA), using a
153 Superdex 200 10/300 GL column (GE Healthcare, Fairfield, CT, USA) in sodium
154 phosphate buffer 50 mmol/L, pH 7.0, 0.15 mol/L NaCl at 0.25 mL per minute.
155 The following molecular weight standards were used: Blue Dextran (MW > 2000
156 kDa), Thyroglobulin (669 kDa), Ferritin (440 kDa), Catalase (232 kDa), Aldolase
157 (158 kDa), Bovine Serum Albumin (67 kDa), Ovalbumin (43kDa), and
158 Ribonuclease (13.7 kDa).

159 **2.5.2 Kinetic properties**

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

164 **2.5.3 Effect of Metal ions and EDTA on enzyme activity**

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

169 **2.5.4 Substrate specificity**

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

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

180 **2.6 Treatment of Cabernet Sauvignon young red wine**

181 **2.6.1 Enzymatic treatment of wine**

182 The β-glucosidase of *I. terricola* was previously immobilized onto Eupergit C
183 250L according to González-Pombo et al., 2011. Immobilized enzyme (20 nkat)
184 was incubated with a Cabernet Sauvignon red wine (500 mL adjusted to pH 4.0
185 with 2 mol/L NaOH) at room temperature with stirring (Treated wine). A control
186 experiment without enzyme was performed by incubating the matrix (Eupergit C
187 250 L with the epoxy-groups previously blocked with 3 mol/L glycine), in the
188 same conditions (control wine).

189 **2.6.2 Glycosyl–Glucose (G–G) assay**

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

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

205

206 **2.6.3 Isolation of volatiles**

207 Volatiles were adsorbed on Isolute ENV+ cartridge packed with 1 g of highly
208 cross-linked styrene-divinylbenzene (SDVB) polymer (40–140 μm, cod. no. 915-
209 0100-C) as previously reported Boido et al., 2003. The cartridges were
210 equilibrated sequentially with methanol (15 mL) and distilled water (20 mL). A
211 sample of wine (50 mL diluted with 50 mL of distilled water) containing internal
212 standard (0.1 mL of a 230 mg/L of 1-heptanol hydroalcoholic solution) was
213 applied at 4–5 mL/min and the residue was washed with 15 mL of distilled
214 water. The volatile fractions were eluted with 30 mL of dichloromethane; the
215 solution was dried with Na_2SO_4 and concentrated to 1.5 mL on a Vigreux
216 column. Samples were stored at −10º C, and further concentrated to 100 μL
217 under nitrogen just immediately prior to GC–MS analysis.

218 **2.6.4 Identification and quantification of aroma compounds**

219 Extracts were analyzed by GC–MS using a Shimadzu QP 5050 mass
220 spectrometer with reference libraries (Adams, 2001; McLafferty & Stauffer,
221 1991; Marais, Versini, van Wyk, & Rapp, 1992; Strauss, Gooley, Wilson, &
222 Williams, 1987; Strauss, Wilson, & Williams, 1987) using a BP 20 (SGE,
223 Ringwood, Australia) bonded fused silica capillary column (25m×0.25mm i.d.),
224 coated with polyethylene glycol (0.25μm phase thickness) (Fariña, Boido,

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

232

233 **2.6.5 Wine sensory analysis**

234 The panel that carried out the sensory evaluation was composed of 14 subjects
235 (5 women and 9 men) belonging to the staff of Enology lab of the Food
236 Department of Faculty of Chemistry (UdelaR) and expert sommeliers. All of
237 them participate regularly in sensory tests. Samples (30 mL, 18°C) were
238 presented in a random order in coded tulip-shaped wine glasses covered with a
239 Petri dish in individual testing booths. In the extended triangle test, one cup of
240 enzymatic treated wine was confronted to two cups of the control wine
241 (untreated wine). The panelists were instructed to smell the samples from left to
242 right and to identify the different sample. When a significant difference was
243 detected, the judges were asked to freely note the descriptors.

244

245 **2.6.6 Analysis of wine color**

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

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

253 3. RESULTS AND DISCUSSION

254 3.1 Optimization of culture conditions

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

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

279 **FIG. 1**

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

286

287 **3.2 Scaling up of β-glucosidase production**

288 **3.2.1 Batch cultures at bioreactor scale**

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

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300 **FIG. 2**

301 **FIG. 3**

302 **TABLE 1**

303

304 **3.3.2 Fed-batch cultures in bioreactor**

305 Fed-batch is known to be the optimal cultivation process to produce cell-growth
306 associated products due to its high volumetric productivity, as well as high final
307 product concentration, stability and reproducibility of the process (Dodge, 2009).
308 Considering that most protein production processes are based on fed-batch
309 protocols, and in an attempt to increase β-glucosidase productivity, a fed-batch
310 fermentation experiment using synthetic medium was performed. Indeed, this
311 process allows controlling the rate of glucose feeding so as to avoid
312 accumulation of the carbon and energy source, and a consequent non-restrict
313 growth profile. The fundamental fermentation parameters were previously
314 estimated from the batch-culture data (Table 1). For a desired final biomass
315 concentration of 30 g/L, according to Eq 1 and based on the physiological
316 values in Table 1 (Yx/s = 0.488 g_X/g_S and a μ_{max} = 0.144 h^{-1}), the corresponding
317 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,
318 where X_0 and V_0 are biomass concentration and volume at initial condition
319 respectively, V_f is final volume, S_F is substrate feeding concentration, F is
320 feeding flux. To our knowledge, this is the first report of kinetic and
321 stoichiometric studies of this yeast.

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

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325 that some enzymes are synthesized in greater quantity when the microorganism
326 grows at high rate. This is called growth-associated enzyme production. For the
327 production of these enzymes, batch culture is the best selection because it is
328 easier and faster than fed-batch. Thus, in batch culture, the volumetric
329 productivity (in katals mL^{-1} h^{-1}) resulted higher than the fed batch (Dodge,
330 2009).

331

332 **3.4 Enzyme characterization**

333 **3.4.1 Biochemical properties**

334 The precipitation with ammonium sulfate allowed a one-step preparation of a
335 purified extract of the enzyme as reported in González-Pombo et al.,
336 2011. The SDS-PAGE (Fig. 4 lane 1), shows the presence of a single band at
337 49 kDa confirming the purity of the enzyme preparation used. Size-exclusion
338 chromatography revealed that the molecular weight of native β-glucosidase was
339 of about 48 kDa, suggesting that the enzyme is monomeric. Specific staining of
340 isoelectric focusing gels with the fluorogenic substrate 4-methyl-β-umbelliferyl-β-
341 D-glucoside (MUG) reveals that the isoelectric point of the enzyme is 3.5. Both
342 results are similar to those of the majority of the β-glucosidases described
343 previously, as those enzymes are acidic and commonly have monomers no
344 bigger than 65 kDa (Esen, 1993).

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

349 Fig. 4

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350 **3.4.2 Effect of metal ions and EDTA on activity**

351 As shown in Table 2, 10 mmol/L of K^+ practically did not affect enzyme activity,
352 however, K_2SO_4 showed a stimulating effect (30% increased) suggesting that
353 K^+ could have a stimulatory effect, depending on its concentration. The
354 stimulatory effect of K^+ in the activity of β-glucosidases has been previously
355 reported (Souza et al., 2010). The presence of 10 mmol/L of Ca^{2+}, Mg^{2+} and
356 Na^+ cations did not influence the enzyme activity. These results are similar to
357 other β-glucosidases (Chen, Hayn, & Esterbauer, 1992) but different to those
358 observed for β-glucosidase from *Issatchenkia orientalis,* in which Ca^{2+} and Mg^{2+}
359 ions increased enzyme activity (de Ovalle, Brena, Fariña, & González-Pombo,
360 2016). Similarly to other β-glucosidases, the presence of Co^{2+} decreased the
361 activity by approximately 30 % (Baffi et al., 2013). An analogous behavior to the
362 Co^{2+}, was observed in the presence of Mn^{2+}. Like other non-Saccharomyces β-
363 glucosidases, the chelating agent EDTA practically did not affect enzyme
364 activity, indicating that divalent cations are not required for enzyme activity
365 (Chen, Li, & Zong, 2012; González-Pombo et al., 2008; de Ovalle et al., 2016).

366 **Table 2**

367

368 **3.4.3 Substrate specificity**

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

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

385 **Table 3**

386 **3.5 Hydrolysis of aromatic precursors in Cabernet Sauvignon young wine.**

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

393

394 After the enzyme treatment of the wine, the concentration of acids, esters and
395 alcohols remained unchanged (Table 4). The enzyme treatment had a
396 significant effect on the release of different aglycones and resulted in increased
397 phenols and norisoprenoids with respect to control wine (Table 4). The volatile
398 levels of both phenols and norisoprenoids increased significantly. Phenols
399 increased (83%), from 607 μg/L to 1113 μg/L and norisoprenoids increased
400 65%, from 17 μg/L to 28 μg/L with respect to control wine. Concerning phenols,
401 guaiacol is an established indicator of the smoke taint and at low levels it could

402 add complexity to wine flavor, however at higher concentration it may cause
403 undesirable aromas (Kennison, Wilkinson, Pollnitz, Williams, & Gibberd, 2009;
404 Parker et al., 2012; Ristic et al., 2011). As a result of the enzymatic treatment
405 although an increase of its concentration around its threshold was observed
406 (Table 4), its presence was not detected by the judges in the sensorial extended
407 triangle test.
408 As for norisoprenoids and other carotenoid-derived aroma compounds they are
409 recognized as aroma contributors in both, red and white wines and in grape
410 juices, including the Chardonnay, Chenin blanc, Semillon, Sauvignon blanc,
411 Riesling, Cabernet Sauvignon, and Shiraz varieties (Winterhalter & Rouseff,
412 2002). After enzymatic treatment of wine, the norisoprenoids, such as vomifoliol
413 and 3-oxo-alpha ionol presented significant differences with respect to the
414 control. Even though the threshold of these compounds has not been reported,
415 they are known to be very low, and norisoprenoids have been characterized as
416 enhancers of fruity, dried raisin or red plum notes (Escudero, Campo, Fariña,
417 Cacho & Ferreira, 2007; Wang, Kang, Xu, & Li, 2011). Consistently, the judges
418 of the triangle test, detected raisin and dried fruits notes in the treated wine.
419 The concentration of C6 compounds particularly (Z)-3-hexen-1-ol, was
420 increased significantly by 30% (from 68 to 89 ug/L) with respect to control.
421 Some authors describe C6 compounds as contributors of vegetative and green
422 flavor attributes (Escudero et al., 2007). However, the contribution of C6-derived
423 compounds with green attributes in wines is poorly understood (Bindon et al.,
424 2014). Anyway, as odor threshold of (Z)-3-hexen-1-ol (400 ug/L) was not
425 reached after the enzyme treatment, its contribution to wine aroma is expected

426 to be insignificant (Fariña et al., 2015). Accordingly, herbaceous aromas were
427 not detected in the sensorial test in any of the wines.
428 For the extended triangle test, in a total of 28 trials, 21 found differences in the
429 treated wine with respect to the control (level of significance < 0.001). The panel
430 of judges considered that the control wine was sweet and fruity whereas the
431 treated one had notes of dried fruits and raisins. As previously mentioned, the
432 latter notes are in agreement with the increase in the concentration of
433 norisoprenoids. This increase occurs slowly during the aging of wine (Loscos,
434 Hernández-Orte, Cacho, & Ferreira, 2010), in a process that takes until 6
435 months in barrel (Oberholster et al., 2015). Noteworthy, these
436 notes were achieved in a very short time (19 days), using β-glucosidase from *I.*
437 *terricola*
438
439 Table 4.
440
441 Besides aroma profiles, color in red wines is one of the main attributes and
442 anthocyanins are the major pigment compounds (Corduas, Cinquanta, & Ievoli,
443 2013). Since the main anthocyanins are mono-glucosides, attention has been
444 focused in the role of β-glucosidases in the decrease of red wine color
445 (Barbagallo, Palmeri, Fabiano, Rapisarda, & Spagna, 2007). *I. terricola* β-
446 glucosidase had no activity onto anthocyanin glucosides since the concentration
447 of anthocyanin after the enzymatic treat-wine remained unchanged (210 ± 22
448 mg/L). The high selectivity shown by this enzyme represents an advantage for
449 its application since it could be used to develop aroma without compromised
450 wine color.
451 **4. CONCLUSIONS**

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452
453 The current work contributes to the investigation of the role and the assessment
454 of the potential applications of native β-glucosidases to release aromatic
455 compounds in wines. The analysis of the released aglycones after the
456 enzymatic hydrolysis, revealed significant increases in the concentration of
457 several volatile compounds. β-Glucosidase showed high ability to liberate
458 norisoprenoids and phenols from their precursors, resulting in a wine with dried
459 fruits and raisins notes without compromised red wine color. These results
460 reinforce those previously obtained in Muscat wine (González-Pombo et al.,
461 2011) and suggest that *Issatchenkia terricola* β-glucosidase, may be an
462 approach to develop aroma in both white and red wines in very short times. For
463 industrial application, even though there was an increase in enzyme production
464 using optimal cultivation conditions, there is still the need to improve enzyme
465 yield by means of for example recombinant DNA-technology using heterologous
466 expression in *Saccharomyces cerevisiae*. The strain of *Issatchenkia terricola*
467 yeast was not patented and its genome has not been sequenced yet.

468
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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

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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 ($p<0.05$).

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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.

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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.].

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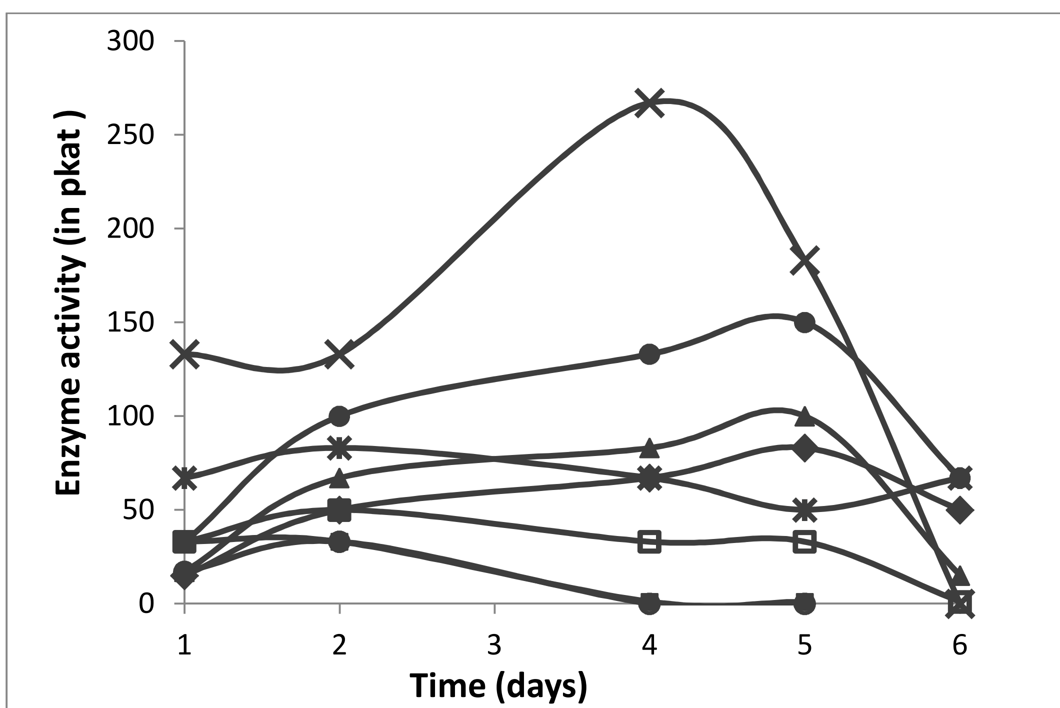

Figure 1. Extracellular Activity of β-glucosidase from *Issatchenkia terricola* in different culture media: ( ) Synthetic; ( ) YPG; ( ) Wheat; ( ) Narangin; ( ) Hesperidin; ( ) Cane molasse; ( ) Vegetable juice and ( ) Glycerol. All cultures were performed in Erlenmeyer flasks at 150 rpm, 28ºC and pH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

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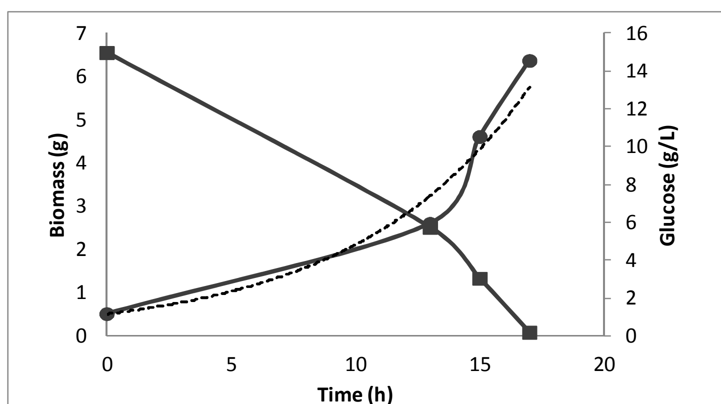

Figure 2. Time course of substrate consumption (—■—) and biomass production (—●—) in batch culture at bioreactor scale, using synthetic medium. Exponential distribution (------) of biomass conversion equation is $Y= 0.4984e^{0.1439x}$, r^2=0.983.

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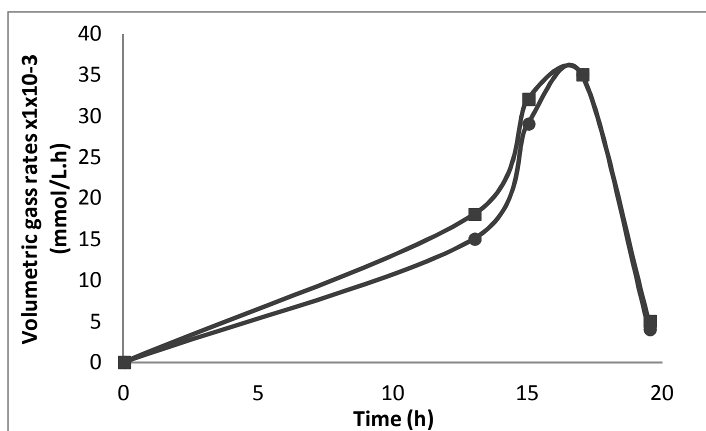

Figure 3. Time course of O_2 consumption (—●—) and CO_2 production (—■—) in batch culture at bioreactor in synthetic medium.

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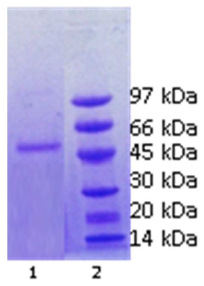

Figure 4. Sodium dodecyl sulfate electrophoresis in polyacrylamide in a Phast gel (Homo12.5%) Lane 1: purified enzyme extract; Lane 2: molecular weight marker.

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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.

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