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Short communication

A novel extracellular β-glucosidase from *Issatchenkia terricola*: Isolation, immobilization and application for aroma enhancement of white Muscat wine

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ABSTRACT

Since most *Saccharomyces* strains show no β-glucosidase activity, the importance of non-*Saccharomyces* β-glucosidases in the development of wine aroma has been highlighted. However, only a few enzymes from yeasts isolated from grape-must and enological ecosystems are active in the stringent conditions of wine and the wine-making process (low pH, high concentrations of ethanol or glucose). A purified extract of extracellular β-glucosidase from *Issatchenkia terricola*, proved to be very active in the presence of glucose (100 g/L), ethanol (18%) and metabisulfite (60 mg/L). It is also active and relatively stable at acidic pH over 3.0. Immobilization onto Eupergit C greatly improved its stability, allowing the aromatization of white Muscat wine over a 16-day experiment. The released aroma compounds of control and treated wine were analyzed by GC–MS. The enzymatic treatment significantly increased the amount of monoterpenes and norisoprenoids, showing the potential of the immobilized enzyme for aroma development in wines.

1. Introduction

The use of β-glucosidases in the wine industry is potentially very interesting because they can promote the liberation of aromatic compounds from monoterpene glycoside precursors present in young wines [1,2].

Several advantages of immobilized enzymes such as their use in continuous processes with important cost savings as well as the preparation of enzyme free products are particularly relevant for enological processes. In addition, immobilization of β-glucosidases allows a precise management of the conversion degree, achieving a rapid and controlled liberation of terpenes, which favors a quick sale, while preserving a fraction of bound aromas as a reserve, to be released with time. There are several reports on the immobilization of fungal commercial glycosidases [3–5]. However, these preparations contain several other secondary activities that have often produced negative effects in wines and many of them perform poorly in enological conditions [6–8]. More recently, attention has been focused on the role of non-*Saccharomyces* yeasts in wine fermentation and there is general agreement among wine microbiologists that indigenous yeasts are essential to the authenticity of the wine, imparting distinct regional and other desirable characteristics [9]. On the other hand, many β-glucosidases from non-*Saccharomyces* are not active at low pH [3], as shown for *M. pulcherrima* [10], *H. uvarum* [11], *Z. baili* [12], and *P. pastoris* [13]. The activity of these enzymes in must and wine has not been extensively characterized and therefore their effects on wine flavor are still unclear [3,14].

This paper presents the isolation and characterization of an extracellular β-glucosidase enzyme from *Issatchenkia terricola* that was selected for its activity at acid pH. A simple method for its immobilization on epoxy-activated Eupergit C was developed and the immobilized biocatalyst was used for flavor enrichment of a white Muscat wine.

2. Materials and methods

2.1. Materials

The synthetic enzyme substrates: *p*-nitrophenyl-β-D-glucopyranoside, 4-methyl-β-umbelliferyl-β-D-glucoside, and D-(+)cellobiose were supplied by Sigma (St. Louis, MO). *I. terricola* isolated from grape must was supplied by the Laboratorio de Enología (Facultad de Química, Montevideo, Uruguay). The glucose determination kit was purchased from REACUR (Montevideo, Uruguay). EUPERGIT C 250L was kindly donated by Röhm Pharma (Darmstadt, Germany). Bradford's reagent was purchased from Bio-Rad laboratories (Richmond, CA). The C18 reverse phase column (Sep-Pak® cartridge) was supplied by Waters TM and the ISOLUTE® ENV+ was purchased from IST Ltd., Mid Glamorgan, U.K. All other chemicals used were of reagent grade.

2.2. Methods

2.2.1. Yeast strain and culture conditions

The yeast strain was isolated from Tannat grapes from Uruguayan vineyards, screened at pH 4.0 for β-glucosidase activity using Esculine Glycerol Agar medium as previously reported [15]. Strain T06/21G was identificated as *I. terricola* using DNA

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isolated from fresh colonies grown on YPD (0.1 g yeast extract/L, 2 g peptone/L, 2 g glucose/L) medium at 30 °C. The quick method described by Gonzalez Techera et al. [16], was followed. DNA quality and concentration were determined in an agarose gel to adjust the appropriate dilution for the PCR reaction of the 26S rDNA gene. The yeast was grown in medium containing: 1 g peptone/L; 25 g yeast extract/L; 8 mL glycerol/L, buffered at pH 5.0 with citrate–phosphate buffer. Erlenmeyer flasks filled to 10% of their volume were inoculated with a 48-h-old culture grown on the above medium and cells were incubated at 28 °C with shaking at 100 oscillations/min for 6 days. Growth was monitored by measuring optical density at 600 nm.

2.2.2. Isolation of β-glucosidase

The cell culture from *I. terricola* was centrifuged at 5000 × *g* for 30 min and the cell free culture medium was precipitated with ammonium sulfate (70% saturation) overnight. Then it was centrifuged, dissolved in 0.1 M sodium acetate buffer, pH 4.5 (activity buffer) and dialyzed against distilled water (enzyme extract).

2.2.3. Protein and enzyme assay

β-Glucosidase activity was determined spectrophotometrically using 5 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), at 23 °C as reported [17]. We defined one unit of enzyme activity (EU) as that which releases 1 μmol *p*-nitrophenol/min under the specified enzyme assay conditions. Specific activity was expressed as enzyme activity units per mg of protein. Proteins were assayed according to the procedure of Bradford [18].

2.2.4. Electrophoresis

Native PAGE, was carried out with PhastSystem apparatus (Pharmacia LKB) with Homo 12.5 Phast Gels. The proteins in the polyacrylamide gels were silver stained.

2.2.5. Immobilization procedure

The β-glucosidase of *I. terricola* was immobilized onto Eupergit C 250L. The beads was washed with distilled water and then with 1 M sodium phosphate buffer at pH 7.0. The cell-free culture medium precipitated with ammonium sulfate was dissolved in 1 M sodium phosphate buffer pH 7.0, incubated with the gel (1 g filter dry gel: 5 mL solution) and the mixture was incubated for 24 h at 4 °C with roller shaking. The biocatalyst was then filtered, and washed with 1 M sodium phosphate buffer pH 7.0. Excess epoxide groups on the matrix were blocked by incubation with 3 M glycine in distilled water overnight at 6 °C. The immobilized enzyme was stored at 4 °C in activity buffer.

2.2.6. Enzymatic treatment of wine

The immobilized biocatalyst (1 EU) was incubated with a white Muscat wine of the variety Early Muscat from the 2009 vintage (750 mL adjusted to pH 4.0 with 2 M NaOH) at room temperature with agitation (Treated wine). A control experiment without enzyme was performed by incubating the matrix (Eupergit C with the epoxy-groups previously blocked with 3 M glycine), in the same conditions (Control wine). Aliquots were taken over time to perform glycosyl–glucose assays.

2.2.7. Glycosyl–glucose (G–G) assay

The total concentration of glycosides in Muscat wine samples was determined by the G–G assay, using a C18 reverse phase column, according to the method of Williams et al. [19]. G–G analysis was performed in duplicate.

2.2.8. Isolation of volatiles

Volatiles were adsorbed on Isolute ENV+ cartridge packed with 1 g of highly cross-linked styrene-divinyl benzene (SDVB) polymer (40–140 μm, cod. no. 915-0100-C) as previously reported [20]. The cartridges were equilibrated 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 (230 ppm 1-heptanol) was applied at 4–5 mL/min and the residue was washed with 15 mL of distilled water. The aroma components 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, stored at −10 °C, and, immediately prior to GC–MS analysis, further concentrated to 100 μL under nitrogen.

2.2.9. Identification and quantification

GC–MS analyses were conducted using a Shimadzu QP 5050 mass spectrometer with reference libraries [21,22] using a BP 20 (SGE, Ringwood, Australia) bonded fused silica capillary column (25 m × 0.25 mm i.d.), coated with polyethylene glycol (0.25 μm phase thickness). The extract was analyzed by GC–MS as previously described [23]. The identification of compounds was confirmed by injection of pure standards and comparison of their retention index and relevant MS-spectra. Volatile compounds were quantified by GC, by the internal standard method. The terpenes analyzed were: linalool, α-terpineol, nerol, geraniol, ho-diol I (trans-3,7-dimethyl-1,5-octadien-3,7-diol) and II (trans-3,7-dimethyl-1,7-octadien-3,6-diol) whereas norisoprenoids were: 3-oxo-α-ionol and vomifoliol.

2.2.10. Statistical analysis

One-way analysis of variance (ANOVA) was carried out by using a statistical program (STATISTICA 5.1).

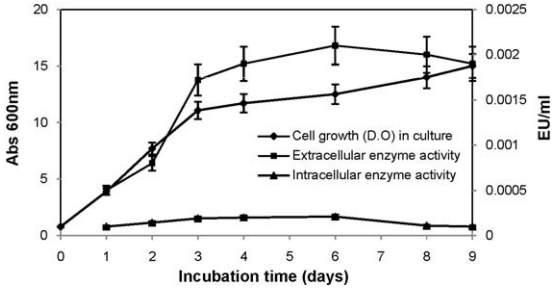

Fig. 1. Kinetics of β-glucosidase production by *Issatchenkia terricola* during cell growth on culture medium, without cellobiose. (▲) Intracellular enzyme activity towards *p*-nitrophenyl-β-D-glucopyranoside (pNPG) (enzyme units per mL) determined in the cell pellet, after cell disruption; (■) extracellular enzyme activity towards pNPG (enzyme units per mL), measured in the cell free culture medium after centrifugation; (◆) cell growth ($A_{600\,nm}$) in culture. The results are mean values from triplicate experiments.

3. Results

3.1. Isolation and characterization of β-glucosidase

The production of β-glucosidase by *I. terricola* in relation to the physiological stage of the cell culture is shown in Fig. 1. The extracellular activity, which was markedly higher than intracellular activity, reached a maximum at the end of the growth phase (0.002 EU/mL) and then decreased slowly. As the inclusion of cellobiose (0.5%) did not change the production of intra- and extracellular enzymes we can conclude that the enzyme was not induced by cellobiose.

The extracellular enzyme was isolated from the cell free culture medium by precipitation with ammonium sulfate at 70% saturation (enzyme extract). Non-denaturing PAGE and specific staining using a fluorogenic substrate, revealed only one band which corresponded to the β-glucosidase activity (Fig. 2 lane A). Thus, precipitation with ammonium sulfate allowed a one-step preparation of a purified extract of the enzyme of interest.

3.2. Immobilization

The β-glucosidase was immobilized onto epoxy activated Eupergit C, one of the most useful carriers for covalent immobiliza-

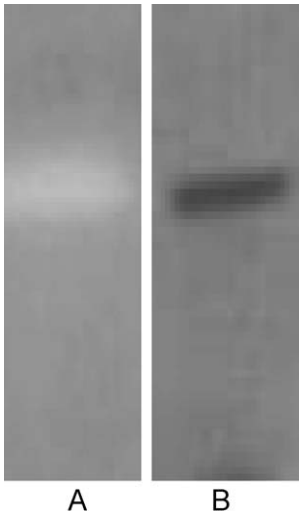

Fig. 2. Native homogeneous 12.5% PAGE of the *I. terricola* enzyme extract. β-Glucosidase activity band was developed by incubating the gel with a 5 mM solution of 4-methyl-β-umbelliferyl-β-D-glucoside for 10 min at 30 °C (lane A), and afterwards proteins were silver stained (lane B).

Table 1
Influence of enzyme load on the immobilization of β-glucosidase from *I. terricola*.

Applied activity (EU/g support)[a]	Immobilization yield (%)[b]	Gel bound activity (EU/g)	Immobilization efficiency (%)[c]
0.1 ± 0.006	100 ± 6.0	0.10 ± 0.008	100 ± 6.0
0.2 ± 0.012	90 ± 5.4	0.14 ± 0.01	80 ± 4.8
0.4 ± 0.024	60 ± 3.6	0.12 ± 0.009	50 ± 3.0

Specific activity of the enzyme extract: 0.1 EU/mg of protein.
The results are mean values from triplicate experiments.
[a] Enzyme units (EU) per gram of dried gel.
[b] Calculated from the difference between applied enzyme activity (EU_A) and recovered activity in the supernatant and washings (EU_R).
[c] Calculated using the expression $[EU_I/(EU_A - EU_R)] \times 100$; where, EU_I, is the gel-bound activity.

Table 2
Physicochemical and kinetic characteristics of β-glucosidase from *I. terricola*.

Physicochemical parameters		Inhibition		
Optimum pH[a]	Optimum temperature (°C)[b]	Glucose (100 g/L)	Ethanol (18%, w/v)	Sodium metabisulfite (60 mg/L)
4.5	50	20%	No	No

[a] Studied in the range of pH 3–10.
[b] Studied in the range 23–80 °C.

tion of enzymes due to its ability to stabilize protein conformation by multi-point attachment [24,25], its good chemical and mechanical properties and also because it is acceptable in the food industry [26–29]. The coupling conditions require long incubation times under alkaline conditions and high ionic strength to favor hydrophobic interactions, previous to the covalent reaction between the protein and the epoxide reactive groups [25,26]. Thus, we could perform the immobilization directly, with the cell-free culture medium precipitated with ammonium sulfate and dissolved in 1 M sodium phosphate buffer pH 7.0.

As shown in Table 1, the maximum enzyme activity immobilized (0.14 EU/g support) was achieved when 0.2 enzyme units (2 mg of protein) were applied per gram of support. This biocatalyst was used in the experiments reported below.

3.3. Enzyme characterization

The physicochemical and kinetic characteristics of β-glucosidase from *I. terricola* are shown in Table 2. Noteworthy, at pH values over 3.5 and at room temperature, the enzyme showed more than 30% of the maximum activity (not shown). Thus, the enzyme has the potential to be compatible with enological conditions.

The immobilization process has notoriously improved the enzyme pH stability (Fig. 3). The half life of the immobilized enzyme in model wine media at pH 3.0 was increased 25-fold while at pH 4.0, a steady enzyme state with 80% the initial activity was established after the second day, and continued up to the end of the experiment. In Muscat wine at pH 4.0 the half life of the immobilized biocatalyst at room temperature was 40 days (960 h). In terms of storage stability, the immobilized biocatalyst maintained 75% of the initial activity after 10 months at 6 °C. These results demonstrate that immobilization of *I. terricola* β-glucosidase had a strong stabilizing effect which allowed the use of the immobilized biocatalyst under wine conditions.

3.4. Application of the immobilized β-glucosidase in white Muscat wine

The incubation of Muscat wine with immobilized biocatalyst achieved a 50% decrease in the amount of wine glycosides from 130 to 66 μM (G–G values) after 16 days. The corresponding G–G value of the "control wine" (treated with blocked matrix) remained unchanged. So, this enzyme activity is expected to release monoterpenes and norisoprenoids and to increase the varietal aroma of the wine. Consistently, the GC–MS analysis evidenced an important release of geraniol (data not shown). ANOVA analysis showed that the enzyme treatment had a significant effect on the hydrolysis of glycosides showing an increase in monoterpenes (from 1420 to 1914 μg/L, $p < 0.05$) and norisoprenoids (from 27 to 99 μg/L, $p < 0.01$) with respect to control wine. After this incubation, the immobilized biocatalyst was re-used preserving the conversion rate.

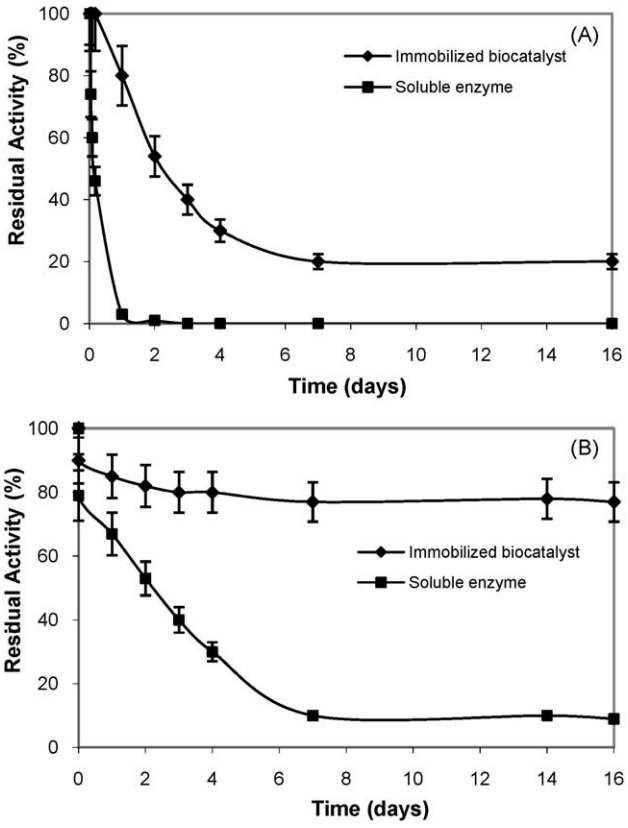

Fig. 3. Stability of β-glucosidase in model wine at pH 3.0 (A) and pH 4.0 (B), at room temperature. Model wine consisted of ethanol–water 12:88 (v/v) containing 3.5 g/L of tartaric acid, 2.5 g/L of malic acid and 60 mg/L of sodium metabisulfite.

4. Discussion

We aimed at contributing to the research on the role and possible exploitation of non-*Saccharomyces* yeasts in the development of wine aroma by focusing on the characterization of a new, purified β-glucosidase from a native strain of *I. terricola* isolated from grapes. To our knowledge, this is the first report on the isolation and immobilization of β-glucosidase from *I. terricola* as well as its application for aroma development in wine.

This enzyme has several features that make it an interesting candidate for contributing to the development of wine aroma with possible technological impact. In the first place, in contrast to the majority of the β-glucosidases from non-*Saccharomyces* yeasts [6], this enzyme is secreted into the medium and it shows a better tolerance to ethanol than most reported yeast β-glucosidases [3,10,30,31], similar to that of the *Saccharomyces cerevisiae* enzyme [29,33]. Many β-glucosidases are inhibited by glucose during the fermentation process, which restricts their activity and the liberation of aroma volatile compounds. Unlike most grape and fungal β-glucosidases, the enzyme from *I. terricola* was highly active at high glucose concentrations [29,32,33] and it performed better than many yeast β-glucosidases, preserving 80% of the original activity at 100 g glucose/L [34]. Moreover, while most of the reported yeast β-glucosidase enzymes show poor performance at pH values usually found in wines (between 3.0 and 4.0), *I. terricola* β-glucosidase is active and relatively stable at acidic pH over 3.0.

Our immobilized biocatalyst shows notoriously better storage stability than previously reported immobilized β-glucosidases from yeast [35] and from fungal commercial preparations [36]. Furthermore, the half life of *I. terricola* immobilized biocatalyst in wine conditions (960 h), is highly competitive with that of immobilized β-glucosidase from Cytolase PCL 5 (1200 h), one of the most frequently commercial preparations used in enology [2]. Finally the immobilized *I. terricola* β-glucosidase was active on the aromatic glycosidic precursors. Further studies involving engineering the enzyme gene into well known yeasts such as *S. cerevisiae*, will allow a better understanding of this enzyme and its potential for increasing the aroma of grape juice during fermentation processes.

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