RESEARCH ARTICLE

An alkaline β-glucosidase isolated from an olive brine strain of *Wickerhamomyces anomalus*

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Abstract

An efficient β-glucosidase (βG)-producing strain, *Wickerhamomyces anomalus* BS81, was isolated from naturally fermented olive brine and identified based on PCR/restriction fragment length polymorphism of the rDNA internal transcribed spacer and sequence analysis of the D1/D2 region of the 26S rRNA gene. The hydrolytic activity of the βG had an optimum pH of 8.5 and an optimum temperature of 35 °C. The enzyme had high substrate specificity and high catalytic efficiency (K_m 0.99 mM, V_{max} 14 U g^{-1} of cells) for *p*-nitrophenyl-β-D-glucopyranoside. The enzyme was activated by increasing concentrations of NaCl, with maximum activity at 150 g L^{-1} NaCl. Although βGs have been purified and characterized from several other sources, the *W. anomalus* βG is unique among βGs because its relative maximum activity occurs at alkaline pH and 35 °C. Moreover, the yeast strain has esterase activity that acts synergistically with βG to degrade oleuropein to debitter table olives and olive oil.

Introduction

β-Glucosidase (βG; EC 3.2.1.21) catalyses the hydrolysis of alkyl and aryl β-glycosides as well as disaccharide glucosides and gluco-oligosaccharides. The enzyme is classified into glycohydrolase families 1 and 3 (Henrissat & Bairoch, 1993, 1996). The hydrolytic activity of this enzyme has been exploited in various industrial applications (Bhatia *et al.*, 2002a).

During the processing of foods and beverages, especially in winemaking, βGs are important for the enzymatic release of aromatic compounds from glycosidic precursors present in raw materials to increase flavour potential (Gueguen *et al.*, 1998; Palmeri & Spagna, 2007). βGs are also used in the production of naturally fermented table olives and olive oil. Olives contain a bitter compound, oleuropein, that must be eliminated during the olive elaboration process. Currently, typical industrial debittering methods consist of treating the fruits with a sodium hydroxide solution that results in the hydrolysis of oleuropein. The lye treatment and subsequent washing process are a very complex mechanism for removing water-soluble compounds such as reducing sugars and organic acids as well as oleuropein from the olive fruit (Garrido-Fernandez *et al.*, 1997). In addition, the lye can dissolve the epicuticular waxy coating and enhance diffusion from the fruit flesh, resulting in softening (Sanchez-Romero *et al.*, 1998).

Furthermore, these technologies have huge drawbacks, such as the highly contaminated discharges that are generated and the large quantity of water used for rinsing. Wastewater originating from the processing of table olives poses an important environmental threat, as it contains a very high organic load and a high concentration of phenolic compounds that are toxic to living organisms. These organic compounds also represent a loss of biological value from the olive. The debittering and washing steps remove the majority of the phenols from the olive fruit, particularly hydroxytyrosol, which is present in large amounts in the waste of the washing step (Parinos *et al.*, 2007).

An alternative strategy is biological debittering, which occurs during the natural fermentation of table olives (Ciafardini & Zullo, 2000) and in the production of olive oil (Ciafardini & Zullo, 2002b) when microbial βGs and

esterases hydrolyse oleuropein (Esti *et al.*, 1998). This procedure, typical of Greek-style table olives, eliminates the wastewater problem and avoids eliminating phenolic antioxidants from the olive. In fact, many of the molecules isolated from *Olea europaea* fruits and leaves likely originated from oleuropein via elenolic acid ring opening of the aglycone, the many forms of elenolic acid and simple phenolic compounds such as hydroxytyrosol (Gariboldi *et al.*, 1986; Montedoro *et al.*, 1993). These molecules are known for their free radical scavenging activity (Visioli *et al.*, 1998; Manna *et al.*, 1999) and may have clinical applications, such as the prevention of certain tumours (prostate and colon cancers) (Lipworth *et al.*, 1997) and coronary heart disease (Keys, 1995), because they exhibit a pronounced hypolipidaemic effect, reduce the lipid peroxidation process and enhance the antioxidant defence system in an experimental atherogenic model (Jemai *et al.*, 2008). Consumption of 50 g of table olives from the Greek market provides approximately 56 mg of polyphenols, confirming the advantages of Greek-style olive processing (Boskou *et al.*, 2006).

Characterizing microbial degrading enzymes could help identify new enzymes for commercial applications and improve the effectiveness of microbial cultures. The goal of the present study was to characterize the βG from a strain of *Wickerhamomyces anomalus* (formerly *Pichia anomala*) (Kurtzman *et al.*, 2008) isolated from naturally fermented olives. The source of the isolation, the brine, is usually characterized by different parameters, and therefore, the isolation, purification and characterization of the enzyme could provide useful information for different culture conditions and different substrates.

Materials and methods

Yeast isolation

Brine samples of naturally fermented, biologically green olives were collected throughout fermentation at 7, 15, 30, 60, 90 and 120 days and were suitably diluted and inoculated onto plates containing Sabouraud dextrose agar supplemented with 100 mg L^{-1} chloramphenicol (Oxoid, Basingstoke, UK). The plates were then incubated at 25 °C for 48–72 h. The same medium was used to isolate and purify 120 yeast colonies, which were then transferred to YPDA (g L^{-1} distilled water: yeast extract, 10; peptone, 10; dextrose, 20; agar, 20) and refrigerated (4 °C) before testing.

βG and esterase assays

Qualitative assay

Glucoside hydrolase activity was detected qualitatively in 120 yeast isolates on agar plates containing arbutin as a substrate. Solid medium, adjusted to pH 5, consisted of the following: 6.7 g L^{-1} yeast nitrogen base (YNB), 5 g L^{-1} arbutin, 0.2 g L^{-1} ferric ammonium citrate and 20 g L^{-1} bacteriological agar, as described by Rosi *et al.* (1994). Yeast strains with βG activity hydrolyse arbutin to produce a brown colour of variable intensity.

Quantitative assay

One litre of each yeast culture was grown at 25 °C for 48 h and then centrifuged at 4100 ***g*** for 20 min at 4 °C. The supernatant was ultrafiltered using a membrane Pellicon XL (Millipore, Billerica, MA) with a 10-kDa cut-off. The enzyme was quantitatively recovered from the membrane with 0.1 M citrate–citrate (C–C) buffer (pH 5.0) in a total volume of 25 mL. To correlate the OD with the dry weight of the yeast cells, the pellet was serially diluted to cover the range of absorbance measurements from 0 to a maximum value of 1 against distilled water at 600 nm.

The yeast cells in the pellets were disrupted by freezing and thawing cycles (five times in dry ice and 37 °C water bath), and the presence of βG was separately determined in the yeast pellet suspension and the supernatant. Next, 100 μL of 0.1 M citrate–phosphate buffer (C–P) pH 5.0 was added to 100 μL of enzyme solution, followed by 100 μL of the substrate (5.5 mM) dissolved in the same buffer, and the mixture was stirred at 30 °C for a reaction time that varied according to each sample. The synthetic substrate *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) was obtained from Sigma Aldrich (St. Louis, MO).

The reaction was stopped by adding 600 μL of 1 M sodium carbonate (Merck, Darmstadt, Germany) after which the amount of liberated *p*-nitrophenol is converted to the yellow coloured *p*-nitrophenolate anionic form (Fleming & Duerksen, 1967). To eliminate turbidity caused by precipitation of the proteins present in the pellets, 2.0 mL of ethanol was added, and the sample was centrifuged at 4100 ***g*** for 15 min at 4 °C.

The absorbance of the samples was read spectrophotometrically against the blank at 400 nm using an extinction coefficient of 18 300 M^{-1} cm^{-1} (Ruttersmith & Daniel, 1993). To confirm the complete inactivation of the enzyme, the absorbance was measured for further 30 min, at regular intervals of 5 min (data not shown). For the blank, the stop solution was added to the enzyme solution before the substrate to prevent reaction between the enzyme and the substrate.

One enzyme unit was defined as the quantity of enzyme required for hydrolysis of 1 μmol min^{-1} substrate under the previous experimental conditions. Activity data were expressed as specific productivity, the ratio between the enzyme units and the dry weight of the yeast cells in grams, using the following equation: $A_{600\,nm} = 2.2256 \times$ (g L^{-1} of yeast cells). All analyses were performed in triplicate.

The mean, SD, confidence interval were calculated using MICROSOFT EXCEL 2010 (Microsoft Corporation, Redmond, WA). A 95% confidence interval was used to assess the reproducibility of the experimental results.

The esterase activity was determined by the method of Blanco *et al.* (2004) modified as follows. The hydrolysis of *p*-nitrophenyl acetate (*p*-NPA) (0.4 mM in 50 mM sodium phosphate buffer pH 7.0) was assayed with 50 μL of yeast suspension diluted in phosphate buffer (50 mM, pH 7.0) at 25 °C for 30 min. The absorbance of the solution was read spectrophotometrically against a blank at 348 nm. One esterase unit corresponds to the consumption of 1 μmol *p*-NPA min^{-1} (εp-NPA = 5150 M^{-1} cm^{-1}). The activity data are expressed as specific productivity, the ratio between the enzyme units and the dry weight of the yeast cells in grams.

Yeast identification

Strain BS81, which exhibited the highest enzymatic activity, was identified by PCR/restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) regions (Esteve-Zarzoso *et al.*, 1999). The yeast strain was grown overnight in liquid YPD at 28 °C with stirring. DNA was extracted from 3 mL of this culture according to the method of Hoffman & Winston (1987) with a few modifications (Pulvirenti *et al.*, 2001). The rDNA ITS regions (Molina *et al.*, 1992) were amplified using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and purified DNA. The DNA was suspended in a solution containing recombinant Taq polymerase (Invitrogen, Carlsbad, CA).

The amplification conditions were the following: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 s. The digestion was performed directly on 5–10 μL of amplified DNA in a final volume of 20 μL with HaeIII, HinfI, HhaI and MspI (New England BioLabs, Beverly, MA). Restriction fragments were separated for 1.5 h in a 2% Nusieve 3 : 1 agarose gel containing ethidium bromide in 0.5 × TBE buffer. Strain CBS 5759 of *W. anomalus* was used as a reference.

To confirm the species attribution of the investigated strain, the D1/D2 region of the 26S rRNA gene was amplified by PCR with the primers NL1 5′-GCATATCAA TAAGCGGAGGAAAAG-3′ and NL4 5′-GGTCCGTGTTT CAAGACGG-3′ (Kurtzman & Robnett, 1998). The 26S rRNA gene PCR product obtained from the isolate was commercially sequenced by Biodiversity S.P.A. (Brescia, Italy), and the sequence data were compared with the sequences present in public data libraries (GenBank) using the BLAST search program to determine the closest known relatives (Altschul *et al.*, 1997; http://www.ncbi.nlm.nih.gov/BLAST/).

Properties of the βG from the identified strain of *W. anomalus*

The βG of the BS81 yeast strain was characterized with respect to the following parameters: optimum pH (between 2.0 and 11.5 in 0.1 M C–P and P buffer) at 30 °C, optimum temperature (between 10 and 60 °C) and inhibition by NaCl (between 50 and 300 g L^{-1}). The kinetic parameters K_m and V_{max} (at pH 5.0 and 30 °C) were determined from Lineweaver–Burk plots using standard linear regression techniques (Lineweaver & Burk, 1934).

Results

βG screening

Of the 120 yeast isolates, 40 strains were able to hydrolyse arbutin on the agar plates, producing a brown colour of variable intensity.

Both the liquid fraction and the pellets of the culture medium of the βG-positive strains were analysed by a quantitative assay. The βG activity of all strains was found exclusively in the pellets, confirming that the activity was not exogenous. Strain BS81 exhibited the highest enzymatic activity (13 U g^{-1} of cells).

Strain identification

The BS81 strain was identified at the species level by analysing the restriction pattern of the rDNA ITS regions, and the results were compared with those of the *W. anomalus* type strain and those reported in the literature. The amplified fragment was 620 bp, and the restriction profile was identical to the type strain CBS 5759. HaeIII did not digest amplified DNA from the isolated strain or the *W. anomalus* comparison strain. The restriction enzymes HinfI and HhaI cut once in the defined region. HinfI produced two fragments of equal size (310 bp); HhaI produced two fragments of different sizes (580 and 60 bp). The HinfI fragments were the same as those reported by Esteve-Zarzoso *et al.* (1999), whereas slight differences were found in the size of the amplicon (620 vs. 650 bp reported); in the previous report, HaeIII produced two fragments, one of which was only 50 bp long and was barely detectable by conventional gel electrophoresis. Similarly, the 60-bp fragment produced by HhaI was not detectable by electrophoresis.

The results of PCR-RFLP were confirmed by sequencing the D1/D2 region of the 26S rRNA gene. The sequence of the PCR product obtained from the isolate was compared with the sequences present in GenBank using the BLAST search program, and the closest match was *W. anomalus* (accession number GU225759.1) at 99%.

Properties of βG and assay for esterase activity

The activity of βG was monitored at increasing substrate concentrations, resulting in typical Michaelis–Menten-type kinetics with a K_m of 0.99 mM and a V_{max} of 14 U g^{-1} of cells (Table 1). The βG of the BS81 strain was activated by NaCl, with a maximum activity at 15% NaCl but a 50% reduction in enzymatic activity at 30% NaCl (Fig. 1).

The dependence of the enzyme activity on pH was profiled between pH 2.0 and 11.5 (Fig. 2), with an optimum value at pH 8.5 in C–P buffer with *p*NPG as the substrate. The activity of the enzyme increased as the pH increased from 5 to 8.5, with a drastic loss of activity at pH values higher than 9.0.

The activity of the enzyme increased as the temperature increased from 30 to 35 °C, at which temperature the highest activity was observed (Fig. 3). The activity decreased considerably as the temperature increased to 45 °C, then remained constant between 45 and 60 °C.

The strain BS81 was assayed for esterase activity in the presence of *p*-NPA as described in the 'Materials and methods', and the specific esterase activity was 0.33 U g^{-1} of cells.

Discussion

Olive flesh contains phenolic compounds including oleuropein (a heterosidic ester of elenolic acid and hydroxytyrosol), hydroxytyrosol 4-β-D-glucoside (4-β-D-glucosyl-3-hydroxyphenylethanol) and hydroxytyrosol (3,4-dihydroxyphenylethanol) as well as other minor compounds such as tyrosol, tyrosol glucoside, rutin and verbascoside (Romero *et al.*, 2002), which can be degraded through the concerted action of different enzymes such as βGs and esterases (Marsilio *et al.*, 1996b). Esterases hydrolyse the ester bonds of oleuropein, producing hydroxytyrosol and glucosyl derivatives (Capozzi *et al.*, 2000); βGs break the bond between glucose and the aglycone, which, in turn, may react with esterases to release hydroxytyrosol and elenolic acid (Briante *et al.*, 2004; Mazzei *et al.*, 2006).

Especially at the beginning of the fermentation process of olives not treated with alkali, the hydrolysis of oleuropein can be attributed to the βG produced by oleuropeinolytic microorganisms. Because oleuropein induces leakage of glutamate and inorganic phosphate from the bacterial cell as well as degradation of the cell wall itself (Ruiz-Barba *et al.*, 1990), biodegradation of polyphenols by yeast would promote the growth of lactic acid bacteria.

Recently, Kachouri & Hamdi (2004) attempted to improve olive oil quality by increasing the bioconversion of healthy phenolic compounds and their solubility in olive oil and preventing the oxidation of triglycerides. Incubation of olive oil samples with fermented olive mill wastewater (OMW) from *Lactobacillus plantarum* caused polyphenols to decrease in OMW and increase in oil with multiple biological effects. However, only yeast can survive in the microdroplets of vegetation water present in newly produced olive oil because the water contains various simple and complex phenolic compounds with high antimicrobial activity (Ciafardini & Zullo, 2002a). A yeast strain such as

Table 1. Chemical and physical characteristics of the βG from the BS81 yeast strain

V_{max}	K_m	Optimum pH	Optimum temperature (°C)	Relative activity at 150 g L^{-1} NaCl
14	0.99	8.5	35	100

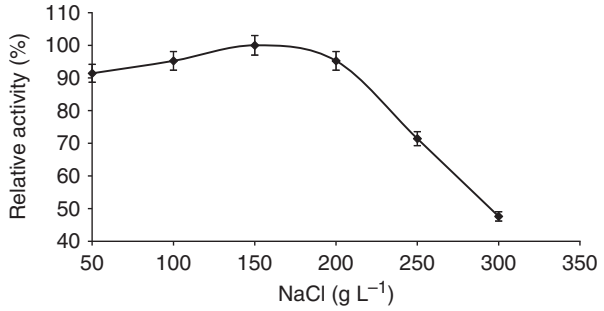

Fig. 1. Effect of NaCl concentration on relative βG activity.

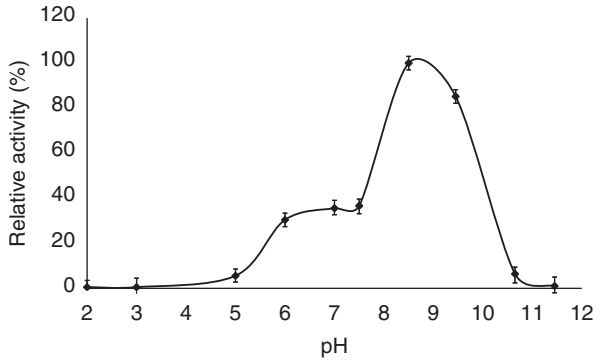

Fig. 2. Effect of pH on relative βG activity.

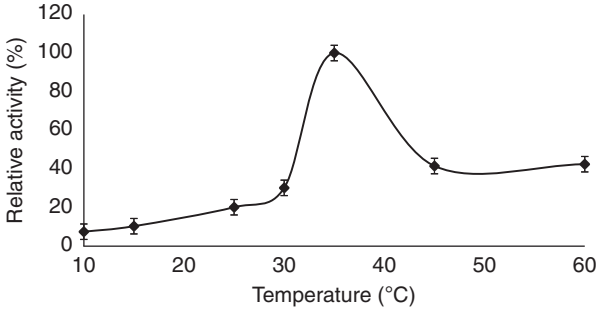

Fig. 3. Effect of temperature on relative βG activity.

BS81 that possesses both βG and esterase activities could inoculate olive oil to promote its functional properties.

An additional important aspect of the production of naturally fermented brined olives or olive oil is wastewater management, which is a serious environmental problem in almost all Mediterranean countries due to its high organic and phenolic content. The toxicity of polyphenolic compounds to microorganisms (i.e. methanogenic bacteria) could be a limiting factor in conventional methods of microbial degradation that are usually applied to OMWs. Some strains of the yeasts *Candida tropicalis* and *Yarrowia lipolytica* can reduce the chemical oxygen demand, monophenols and polyphenols in OMW (Lanciotti *et al*., 2005; Martinez-Garcia *et al*., 2009). *Candida rugosa*, *Candida cylindracea* and *Y. lipolytica* can grow on OMW-based medium and produce high-value compounds while degrading this waste (Brozzoli *et al*., 2009; Gonçalves *et al*., 2009). Thus, there is a potential demand for yeast strains that can reduce the amount of polyphenols in olive wastewater, which cannot be released into the environment unless it is suitably treated first (Amaral *et al*., 2008). Rapid polyphenol degradation may positively impact the environmental problems of OMW management because it can also act as the first step of effluent treatment.

In the present study, the biochemical parameters of the βG from a *W. anomalus* strain were characterized. The kinetic parameters were similar to those of the intracellular βG from the methylotrophic yeast *Komagataella pastoris* (Turan & Zheng, 2005).

The βG isolated from BS81 was activated at low NaCl concentrations and inhibited at high NaCl concentrations, similar to the βG isolated from *Aspergillus niger* (Rashid & Siddiqui, 1997).

βGs from yeast strains have not been previously shown to have optimal activity at alkaline pH; the optimum pH range for yeast βG is generally between 3.5 and 7.0. In particular, for *W. anomalus*, the βG pH optimum was 5.5 (Jijakli & Lepoivre, 1998; Spagna *et al*., 2002a). A maximum activity at approximately pH 8.0 has been reported for βGs isolated from *Agrobacterium tumefaciens* (Singh *et al*., 1995) and *Mucor miehei*. Moreover, Ciafardini & Zullo (2001) isolated a strain of *Leuconostoc mesenteroides*, associated with the fermentation of Coratina cultivar olives, endowed with βG activity with a maximum catalytic activity at pH 8, likely reflecting the influence of the isolation source on the enzyme pH optimum.

The high optimum temperature (35 °C) and the activity throughout the temperature range between 40 and 60 °C were similar to those of βGs isolated from *K.* (*Pichia*) *pastoris* (Turan & Zheng, 2005) and other microorganisms such as the cellobiose-fermenting yeast *Candida wickerhamii* and the yeast *Metschnikowia pulcherrima*, which has potential applications in winemaking (Singh & Hayashi, 1995; González-Pombo *et al*., 2008). Experimental data and theoretical considerations suggest that the two main contributors to thermal stability are an increased number of hydrogen bonds and salt bridges (Macedo-Ribeiro *et al*., 1996; Vogt *et al*., 1997).

In addition, this is the first report of the contemporary presence of esterase and βG activities in a *W. anomalus* strain isolated from olive brine. The esterase and βG from BS81 strain of *W. anomalus* are not either exogenous or cytoplasmatic, suggesting a localization at periplasmic or cell wall level, and their concerted action could contribute positively to the biological debittering of olives by promoting the transformation of oleuropein into hydroxytyrosol (Capozzi *et al*., 2000; Segovia-Bravo *et al*., 2009). This could be of biotechnological interest because both activities are involved in the degradation of oleuropein for the debittering of table olives and olive oil (Marsilio & Lanza, 1998; Capozzi *et al*., 2000) and in improving the flavour of plant-derived foods and beverages (Lomolino *et al*., 2005, 2006).

The unique characteristics of the βG from *W. anomalus*, such as its relative activity maximums at pH 8.5 and 35 °C with activities of 165 and 64 U mg^{-1}, respectively, could lead to important practical applications in food processing.

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