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Purification and Characterization of an Extracellular β-Glucosidase from *Monascus purpureus*

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An extracellular β-glucosidase produced by *Monascus purpureus* NRRL1992 in submerged cultivation was purified by acetone precipitation, gel filtration, and hydrophobic interaction chromatography, resulting in a purification factor of 92-fold. A 2^2 central-composite design (CCD) was performed to find the best temperature and pH conditions for enzyme activity. Maximum activity was observed in a wide range of temperature and pH values, with optimal conditions set at 50°C and pH 5.5. The β-glucosidase showed moderate thermostability, was inhibited by $HgCl_2$, K_2CrO_4, and $K_2Cr_2O_7$, whereas other reagents including β-mercaptoethanol, SDS, and EDTA showed no effect. Activity was slightly stimulated by low concentrations of ethanol and methanol. Hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), cellobiose, salicin, *n*-octyl-β-D-glucopyranoside, and maltose indicates that the β-glucosidase has broad substrate specificity. Apparently, glucosyl residues were removed from the nonreducing end of *p*-nitrophenyl-β-D-cellobiose. β-Glucosidase affinity and hydrolytic efficiency were higher for *p*NPG, followed by maltose and cellobiose. Glucose and cellobiose competitively inhibited *p*NPG hydrolysis.

Keywords: β-Glucosidase, *Monascus purpureus*, characterization, thermostability, substrate specificity, kinetic constants

β-D-Glucosidases (βG; E.C. 3.2.1.21) catalyze the hydrolysis of β-glycosidic linkages in a variety of glycosides. According to their properties, these enzymes could be potentially employed in a diversity of biotechnological processes. The application of microbial βGs has been studied, for instance, in the enzymatic saccharification of cellulose, in the liberation of flavor compounds in fruit juices and wines, and in the release of phenolic compounds with antioxidant activity from fruit and vegetable residues, among other applications [3, 32, 39].

The increased need for energy conserving and recycling has strongly stimulated the search for alternatives for the conversion of lignocellulosic biomass into reducing sugars for fuel production. The possibility of using microorganisms for the effective transformation of cellulose, hemicelluloses, and lignins to yield chemicals through energy-saving processes has been investigated [10, 36]. Several fungi are used for the biological treatment of agricultural wastes like wood chips, wheat straws, corn cobs, and other. These microorganisms produce cellulolytic systems consisting of three major activities (endo-β-glucanase, cellobiohydrolase and β-glucosidase), acting synergistically to hydrolyze cellulose [35]. The potential application of glycosidases for release of aromatic compounds in musts and wines has gaining relevance, and βGs of fungal origin have remarkable stability in wine [25].

Although βGs are widespread produced among filamentous fungi, including species of *Trichoderma*, *Penicillium*, *Fusarium*, and *Aspergillus* [35], this enzymatic activity was just recently reported for *Monascus purpureus* [8]. This fungus is well known as a source of pigments for the coloring of traditional foods, although other metabolic products from *Monascus* species, like organic acids, antimicrobial agents, and proteolytic enzymes, have been described [9, 19]. *M. purpureus* produces an extracellular βG in submerged cultivations with agro-industrial residues as substrates, and the production of this enzyme was shown to be inducible and controlled by carbon catabolite repression [8]. The current article presents the partial purification of this enzyme and its characterization.

Materials and Methods

Microorganism

Monascus purpureus NRRL1992 was maintained on Sabouraud dextrose agar plates at 4°C and subcultured periodically. Cultures

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reactivated by transferring onto fresh Sabouraud agar plates and cultured at 30°C for 12–14 days were used for inoculum preparation.

βG Production

Culture medium was prepared with grape waste (20 g/l) and peptone (5 g/l) in mineral medium [8]. The mineral medium contained K_2HPO_4 (5.0 g/l), KH_2PO_4 (5.0 g/l), $MgSO_4·7H_2O$ (0.1 g/l), $CaCl_2$ (0.025 g/l), $ZnSO_4·7H_2O$ (0.01 g/l), and $MnSO_4$ (0.01 g/l). The initial pH of the medium was adjusted to 6.0.

Erlenmeyer flasks (250 ml) containing 50 ml of culture medium were inoculated with 500 µl (1%, v/v) of a conidial and mycelial suspension with an OD_{620} of 0.4. For inoculum preparation, 12–14-day-old cultures were scraped from the agar surface, added to a 0.85% NaCl sterile solution, and mixed until a homogeneous suspension was obtained. The inoculated flasks were incubated at 30°C on a rotatory shaker at 100 rpm for 9 days.

βG Activity Assay

β-Glucosidase activity was assayed by a modified method [13]. The reaction mixture (200 µl) contained 90 µl of sodium citrate buffer (250 mM, pH 4.5), 10 µl of *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; 4 mg/ml), and 100 µl of enzyme-containing sample. After incubation at 37°C for 10 min, the reaction was stopped by adding 1 ml of cold sodium carbonate buffer (500 mM, pH 10). The activity of βG was estimated spectrophotometrically by reading the absorbance of the liberated *p*-nitrophenol (*p*NP) at 405 nm (ε= 18,700). One unit (U) of βG activity was defined as the amount of enzyme required for the hydrolysis of 1 µmole of *p*NP per minute, under the assay conditions.

Purification Protocol

After the incubation period, the culture was filtered, centrifuged (10,000 ×*g* for 15 min), and the supernatant (crude extract) utilized as enzyme source for the purification process (Table 1). Cold acetone (80%, v/v) was added to the crude extract and maintained in an ice bath with constant agitation for one hour. This mixture was centrifuged (10,000 ×*g* for 30 min) and the pellet was resuspended in phosphate buffer (20 mM; pH 6.25). This material was applied to a Sephadex G-100 gel filtration column (20×0.8 cm) with a flow rate of 22.2 ml/h. The column was eluted with sodium phosphate buffer (20 mM; pH 6.25) and a total of 25 fractions of 1 ml were collected. Each fraction was assayed for βG activity and protein estimation at 280 nm. The fractions with enzyme activity were pooled and ammonium sulfate was added to a final concentration of 55 mM. This pool was applied to a Phenyl Sepharose hydrophobic interaction column (10.5×0.65 cm) with a flow rate of 16.8 ml/h, and a total of 20 fractions of 1 ml were collected. The first five fractions were eluted with sodium phosphate buffer (250 mM; pH 6.25) containing 55 mM ammonium sulfate, and the subsequent fractions were eluted with the same buffer without ammonium sulfate. Each fraction was assayed for βG activity and protein estimation at 280 nm. The fractions with enzymatic activity were pooled and submitted to the βG activity assay and protein determination. The resulting pool was utilized for the characterization of the extracellular β-glucosidase.

The protein content at each stage of enzyme purification was determined according to Lowry *et al.* [21] with bovine serum albumin as the standard.

Table 2. βG activity in different temperature and pH combinations of a 2^2 central-composite design.

Treatment	Independent variables[a]		Enzymatic activity (U/ml)[c]
	Temperature (T)[b]	pH	
1	71.3 (+1)	7.28 (+1)	0.101
2	71.3 (+1)	3.72 (−1)	0.984
3	28.7 (−1)	7.28 (+1)	0.270
4	28.7 (−1)	3.72 (−1)	0.505
5	80 (+1.41)	5.50 (0)	1.341
6	20 (−1.41)	5.50 (0)	1.045
7	50 (0)	8.00 (+1.41)	0.209
8	50 (0)	3.00 (−1.41)	1.058
9	50 (0)	5.50 (0)	5.912
10	50 (0)	5.50 (0)	5.878
11	50 (0)	5.50 (0)	5.919
12	50 (0)	5.50 (0)	5.986

[a]Codified values are in parenthesis.
[b]Temperature actual values are expressed in °C.
[c]Dependent variable.

Zymogram-PAGE

The enzyme was electrophoresed on a 10% native polyacrylamide gel according to standard procedures [18]. The detection of βG activity was based on the method of Badhan *et al.* [1]. The gel was incubated with sodium citrate-phosphate buffer (250 mM; pH 5.5) containing 10 mM of 4-methylumbelliferyl-β-D-glucoside (4-MUG) for 10 min at 37°C. Then the gel was observed under UV light where fluorescent bands indicate the release of methylumbelliferone due to enzyme activity.

Determination of Temperature and pH Optima

The temperature and pH optima were determined with the aid of a 2^2 central-composite design (CCD) with temperature (T) and pH as independent variables [4]. Five levels were set for each variable, with a total of 12 treatment combinations. Table 2 shows the real and codified levels of each variable, as well as the response (β-glucolytic activity) for each treatment. Different pH values were obtained with sodium citrate-phosphate buffer (3.00, 3.72, and 5.50), sodium phosphate buffer (7.28), and Tris-HCl buffer (8.00).

Table 1. Beta-glucosidase purification summary.

Purification step	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification factor
Crude extract	2.40	2.60	0.92	1.0
Acetone precipitation	36.34	4.16	8.73	9.5
Gel filtration	4.23	0.36	11.75	13.0
Hydrophobic interaction	2.53	0.03	84.34	92.0

The results of the CCD were analyzed using the software Statistica 5.0 (Statsoft, Tulsa, OK, U.S.A.). The coefficients were generated by regression analysis, and the fit of the models was evaluated by determination coefficient (r^2) and analysis of variance (ANOVA).

Thermostability

For thermal stability evaluation, the enzyme was incubated at 50, 60, 65, and 70°C for 0 to 120 min. Storage stability at 5°C and -15°C was evaluated in a period of 60 days. After the respective incubation/storage period, the enzymatic assay was performed at temperature and pH optima. Activity at time zero was taken as the control (100%).

Effect of Reagents on βG Activity

The effect of different reagents was tested by the pre-incubation (37°C for 10 min) of the properly diluted enzyme with the respective reagent. The working concentrations of metal ions and carbohydrates were set at 5 and 10 mM, respectively. After pre-incubation, the enzymatic assay was performed under optimal conditions, and the enzyme activity was expressed as percentage of the activity observed without additions.

Enzyme Specificity

Enzyme specificity was tested with various chromogenic and nonchromogenic substrates. Hydrolysis rates of chromogenic substrates at 1 mM final concentration were evaluated by liberation of *p*NP or *o*-nitrophenol (*o*NP) as described in the βG activity assay, in optimal conditions of temperature and pH.

For the analysis of hydrolysis rate of nonchromogenic substrates, the reaction mixture (200 µl) consisted of 100 µl of sodium citrate-phosphate buffer (250 mM; pH 5.5) containing the respective substrates in the final concentrations presented in Table 5, and 100 µl of the conveniently diluted enzyme. After incubation of 30 min at temperature and pH optima, the reaction was stopped by incubation at 100°C for 5 min in a dry-bath. When oligo- or disaccharides were the substrates, the liberated glucose was evaluated by the glucose-oxidase method with a commercial kit (Wiener Lab S.A.I.C., Rosario, Argentina). When the substrate was a polysaccharide, reducing sugars liberation was determined by the 3,5-dinitrosalicylic acid (DNS) method. One βG unit (U) was considered as the amount of enzyme required for the liberation of 1 µmole of glucose/reducing sugar per minute, under the above conditions.

Kinetic Constants

The influence of substrate concentration on the reaction velocities of the partially purified βG was studied with *p*NPG (0.039–25 mM), cellobiose (2.5–75 mM), and maltose (2.5–75 mM). In all cases, the enzymatic activity was assayed under temperature and pH optima. The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined from Lineweaver-Burk plots. Alternatively, data were fitted to the Michaelis-Menten equation to obtain the kinetic constants by nonlinear regression, and curve fitting was carried out using the software Statistica 5.0 (Statsoft, Tulsa, OK, U.S.A.), which utilized the Levenberg-Marquardt algorithm.

Inhibition of *p*NPG hydrolysis was studied in the presence of glucose (0, 10, and 20 mM) and cellobiose (0, 10, and 20 mM). Kinetic constants (K_m and V_{max}) were determined from Lineweaver-Burk plots, and inhibition constants (K_i) were obtained by plotting K_m/V_{max} versus inhibitor concentration.

RESULTS AND DISCUSSION

Enzyme Purification

The βG produced by *M. purpureus* NRRL1992 in submerged cultivations with grape residue and peptone as substrates [8] was submitted to a purification protocol. The purification involved acetone precipitation and liquid chromatography steps. A summary of this process is presented in Table 1. Acetone precipitation resulted in a 9.5-fold purification factor, with a recovery of 60% of the enzyme present in the crude extract.

The acetone precipitation sample was applied to a gel-filtration column (Sephadex G-100) and 25 fractions of 1 ml were collected. A typical elution pattern is showed in Fig. 1A. Two protein peaks were observed (fractions 4 and

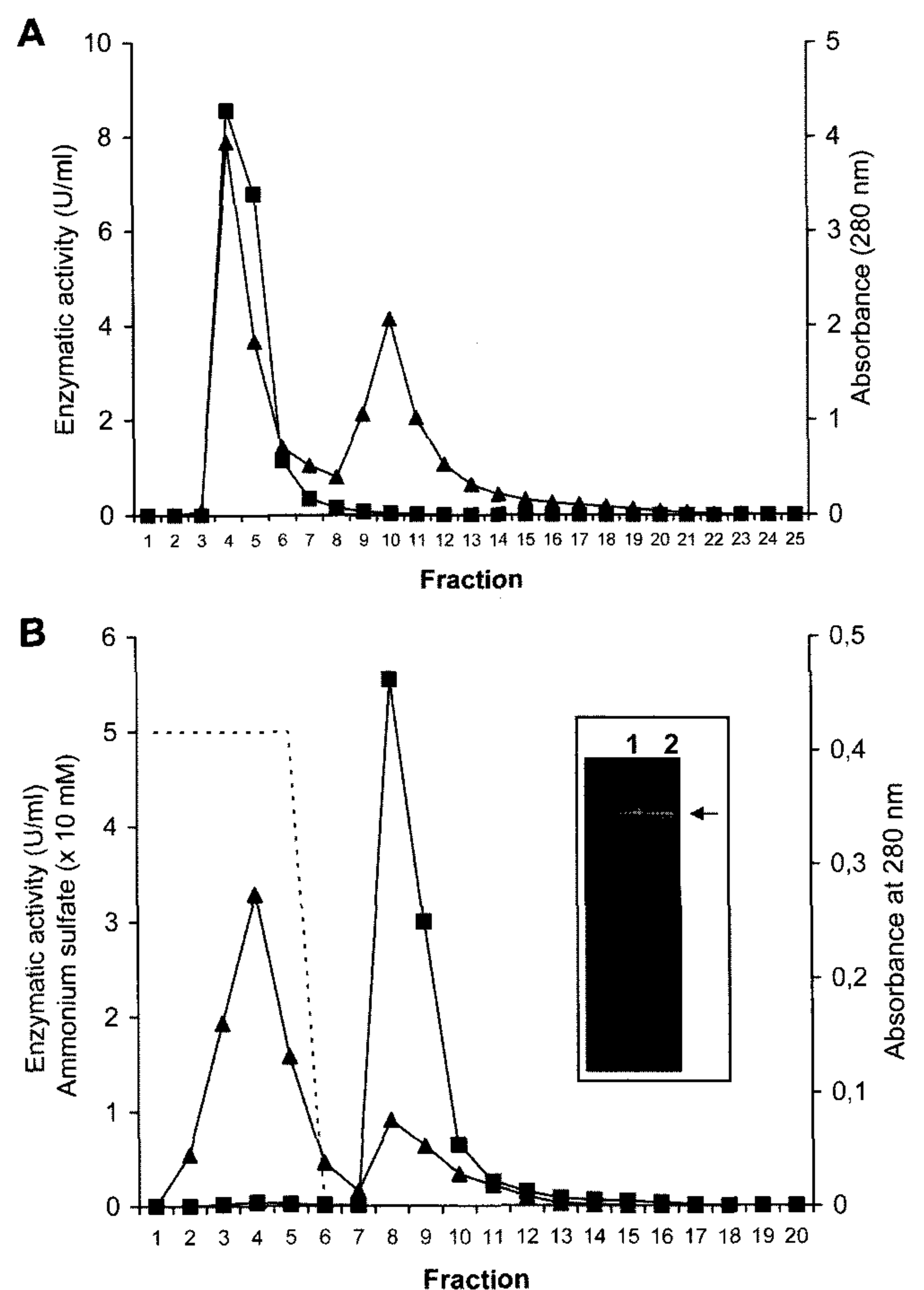

Fig. 1. Partial purification of *M. purpureus* βG.
A. Gel-filtration chromatography of the acetone-concentrated culture supernatant using a Sephadex G-100 column. **B.** Hydrophobic interaction chromatography of the active fractions collected from the gel-filtration column (fractions 4–7) using a Phenyl Sepharose column. (▲) Protein elution profile, (■) enzymatic activity, and (---) ammonium sulfate concentration (×10 mM). *Inset*: zymogram with the substrate 4-MUG; lane 1: sample from gel-filtration cromatography; lane 2: sample from hydrophobic interaction chromatography; the arrow indicates βG activity observed under UV light.

10) and only one activity peak was detected, coinciding with the first protein peak. Gel-filtration separates proteins by size and molecular mass, with larger molecules migrating at a higher speed than smaller ones [33]. The elution of the enzymatic activity in the fractions 4–7 could indicate that the enzyme has a high molecular mass (probably higher than 100 kDa), or that the enzyme was present in aggregates or associated with other molecules. In this sense, *Monascus* pigments can react with amino groups of proteins and amino acids present in the medium to form hydrosoluble pigments [9].

Fractions with activity in the gel-filtration step (4–7) were pooled, added with ammonium sulfate (55 mM), and applied to a hydrophobic interaction column (Phenyl Sepharose). This chromatography promotes the protein separation through interactions between immobilized hydrophobic ligands and nonpolar regions in the surface of proteins [28]. In the first five fractions, eluted with phosphate buffer containing ammonium sulfate, a peak of proteins that did not associate with the column was observed (fraction 4). The suppression of ammonium sulfate from the elution buffer resulted in the disassociation of proteins attached to the column, resulting in a second protein peak (fraction 8) that coincided with the activity peak (Fig. 1B).

In summary, the purification process resulted in a 92-fold purification factor and a final recovery (yield) of 23% of the enzyme present in the crude extract. The partially purified βG was submitted to a zymogram-PAGE protocol with the substrate 4-MUG, and a single activity band was visible under UV light (Fig. 1B, inset).

Optimal Activity

Optimal enzymatic activity was studied with different temperature and pH combinations in a CCD (2^2) leading to a set of 12 experiments. Results of this experiment are shown in Table 2. βG activity, measured by *p*NPG hydrolysis, varied markedly within the conditions evaluated, and the higher enzyme activities were observed at the central point of this experiment (50°C and pH 5.5). All variables were significant at 95% confidence level (Table 3). The temperature (T) increase resulted in higher enzyme activity; however, when the upper limit (80°C) was approached, the enzyme activity diminished, indicating thermal denaturation. On the other hand, the pH increase had a negative effect on *p*NPG hydrolysis, indicating that acidic-to-neutral pH values are better suited for enzyme activity. Significant interaction was observed between temperature and pH, and in this context, CCD is advantageous in comparison with conventional statistical approaches that changes one parameter per trial [16].

The regression analysis of the obtained data was performed and the following model was generated [Eq. (1)]:

$$A=5,925+0,0912.T-2,485.T^2-0,290.pH -2,767.pH^2-0,162.T.pH \quad (1)$$

where A is the enzymatic activity (U), T is the temperature, and pH is the pH value as codified values. The statistical significance of the generated model was analyzed by ANOVA and Fischer's F-test. The obtained F-value (249.3) was much higher than the F tabulated ($F_{0.95,5;6}$=4.39), indicating that the regression model is significant at 95% confidence level. The R^2=0.995 indicates that 99.5% of the variation is explained by the model, and a good correlation between the experimental and predicted values was obtained.

The contour plot is shown in Fig. 2. Maximal enzymatic activity was observed in a wide range of temperatures (35–65°C) and pH values (4.2–6.7) including the central points (50°C and pH 5.5). In this sense, the conditions of temperature and pH of the central points were selected as enzyme optimal. The wide range of optimal conditions establishes the potential versatility of this enzyme for use in various processes. Most microbial βGs exhibit optimum temperature at 40–60°C and optimum pH values from 4.0 to 6.0 [3, 32, 39]. In particular, βG from *Chalara paradoxa* presented maximum activity at 45°C and pH 4.0–5.0 [22], whereas *Aspergillus foetidus* βG exhibited optimal activity at 65°C and pH 4.6 [13], comparable to that reported for *A. japonicus* (65°C and pH 5.0) [31], *A. niger* (60°C and pH

Table 3. Effects and interactions of independent variables on βG activity.

Variables	Effect (U/ml)	Std. Err.	*p*-value
Mean	5.925	0.02	<0.000000*
Temperature (T)	0.182	0.03	0.010718*
(T)×(T)	-4.970	0.03	0.000001*
pH	-0.580	0.32	0.000366*
(pH)×·(pH)	-5.533	0.35	0.000001*
(T)×(pH)	-0.324	0.45	0.005592*

*Significant factors at 95% confidence level (p<0.05).

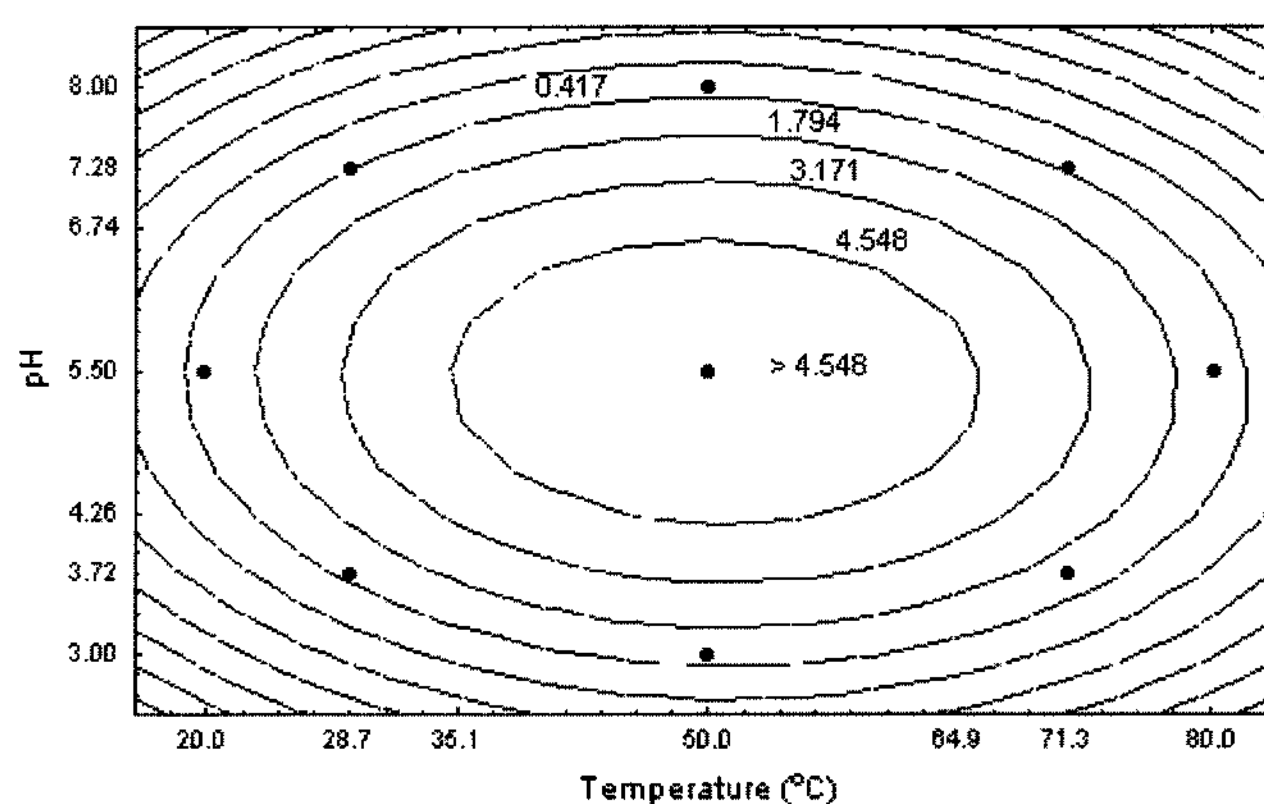

Fig. 2. Contour plot for the effects of temperature and pH on βG activity.

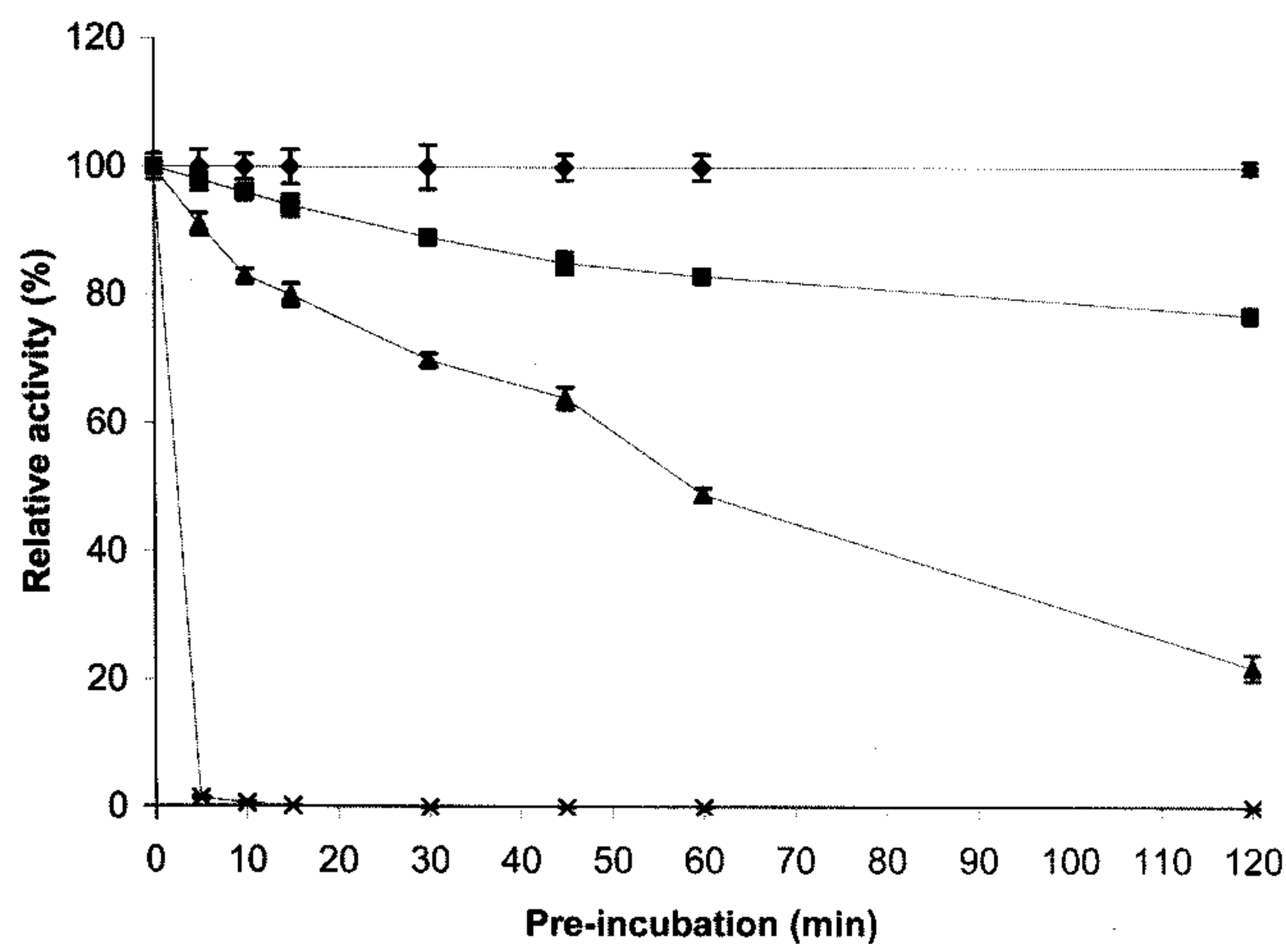

Fig. 3. βG thermal stability. Pre-incubation at (◆) 50°C, (■) 60°C, (▲) 65°C, and (×) 70°C. Each point is the mean of triplicate experiments.

Table 4. Effects of several reagents on βG activity.

Reagent[a]	Concentration	Relative activity (%)[b]
Control	none	100.0
$HgCl_2$	5 mM	14.5
$K_2Cr_2O_7$	5 mM	49.0
K_2CrO_4	5 mM	80.0
Glucose	10 mM	42.0
Glucose 6-phophate	10 mM	88.0
Cellobiose	10 mM	68.5
Maltose	10 mM	85.0

[a]No considerable effect on βG activity was observed with the addition of KCl, LiCl, NaCl, $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $MgSO_4$, $MnSO_4$, $NiCl_2$, $SrCl_2$, $ZnSO_4$, $AlCl_3$, KBr, KI, NaF, β-mercaptoethanol, EDTA, SDS, sodium azide, trichloroacetic acid, Triton X-100, Tween 20, Tween 80, arabinose, fucose, mannose, xylose, fructose, galactose, glucuronic acid, glyceraldehyde, *N*-acetyl glucosamine, *N*-acetyl galactosamine, lactose, saccharose, and methyl α-D-glucopyranoside.
[b]Each value is the mean of triplicate experiments.

4.5) [40], and *Fusarium oxysporum* (60°C and pH 5.0–6.0) [7].

Thermal Stability

The thermal stability of the partially purified βG was studied at 50, 60, 65, and 70°C (Fig. 3). The enzyme showed to be completely stable after 2 h at 50°C. At 60°C, the enzyme maintained 83% of the initial activity after 1 h and 78% after 2 h, presenting a half-life of 315 min. At 65°C, the enzyme presented a half-life of 57 min, showing 50% and 22% of the initial activity after 1 h and 2 h of pre-incubation, respectively. However, the half-life of this enzyme at 70°C was only 1.8 min. Similarly, extracellular βGs of *Aspergillus phoenicis*, *A. niger*, and *A. carbonarius* showed to be stable at 50°C for 2 h, maintaining 87%, 64%, and 53% of enzyme activity after 2 h at 60°C, respectively, and being inactivated after 2 h at 70°C [14]. In *Trichoderma reesei*, βG I retained 40% of initial activity after 1 h at 65°C while βG II was completely inactivated [6]; the βG from *T. harzianum* type C-4 maintained only 36% of its initial activity after 15 min at 60°C [41].

The storage of the enzyme at 5°C or −15°C for 60 days had not significantly affected the enzyme activity (results not shown). Similar results were obtained with the extracellular βG of *Debaryomyces hansenii* [29]; furthermore, *A. oryzae* βG retained full activity after storage of 6 months at 4°C [30].

Effect of Chemicals on βG Activity

βG activity was assayed in the presence of various reagents (Table 4). Among the salts tested, considerable loss of activity was observed only with $HgCl_2$, K_2CrO_4, and $K_2Cr_2O_7$. The inhibition caused by Hg^{2+}, a known oxidant of sulphydryl groups, could indicate the presence of important -SH groups at the enzyme catalytic site. However, this result together with the lack of effect of β-mercaptoethanol, a reducing agent of thiol groups, suggests that -SH groups are not essential for catalytic activity, but necessary for the maintenance of the enzyme three-dimensional structure [22, 30]. Alternatively, Hg^{2+} could also react with important tryptophan residues and carboxyl groups in amino acids in the enzyme [23]. The negative effect of chromium on the βG activity could be due to the oxidation of enzyme sulphydryl and carboxyl groups [5].

EDTA does not affect βG activity, indicating that the enzyme does not require metallic ions for activity [30, 40]. The addition of EDTA reversed the negative effect of $HgCl_2$ (result not shown), as reported for *A. japonicus* extracellular βG [31]. In the same way, SDS, Triton X100, Tween 20, and Tween 80, in the concentrations employed, showed no significant influence on βG activity. In microbial βGs, the negative effect of SDS is commonly reported and is related to protein denaturation [2, 37].

Glucose and cellobiose reduced *p*NPG hydrolysis by 58% and 31.5%, respectively (Table 4). Glucose and cellobiose competitively inhibited *p*NPG hydrolysis, with K_i values of 4.24 and 11.51, respectively. Competitive inhibition of *p*NPG hydrolysis by glucose is commonly observed in microbial βGs, with typical K_i values ranging from 0.5 to 14 mM [40, 41]. However, βG from *A. oryzae* showed K_i for glucose of 1.36 M [30]. Zanoelo *et al.* [42] reported competitive inhibition of *Scytalidium thermophilum* βG by cellobiose, with a K_i value of 1.32 mM. Glucose-6-phosphate and maltose slightly reduced β-glucolytic activity (12% and 15%, respectively) whereas the other carbohydrates tested presented only slight effects.

The βG activity was slightly stimulated by low concentrations of ethanol and methanol. Considering the activity without alcohols as 100%, ethanol at a final concentration of 10 mM increased the enzyme activity

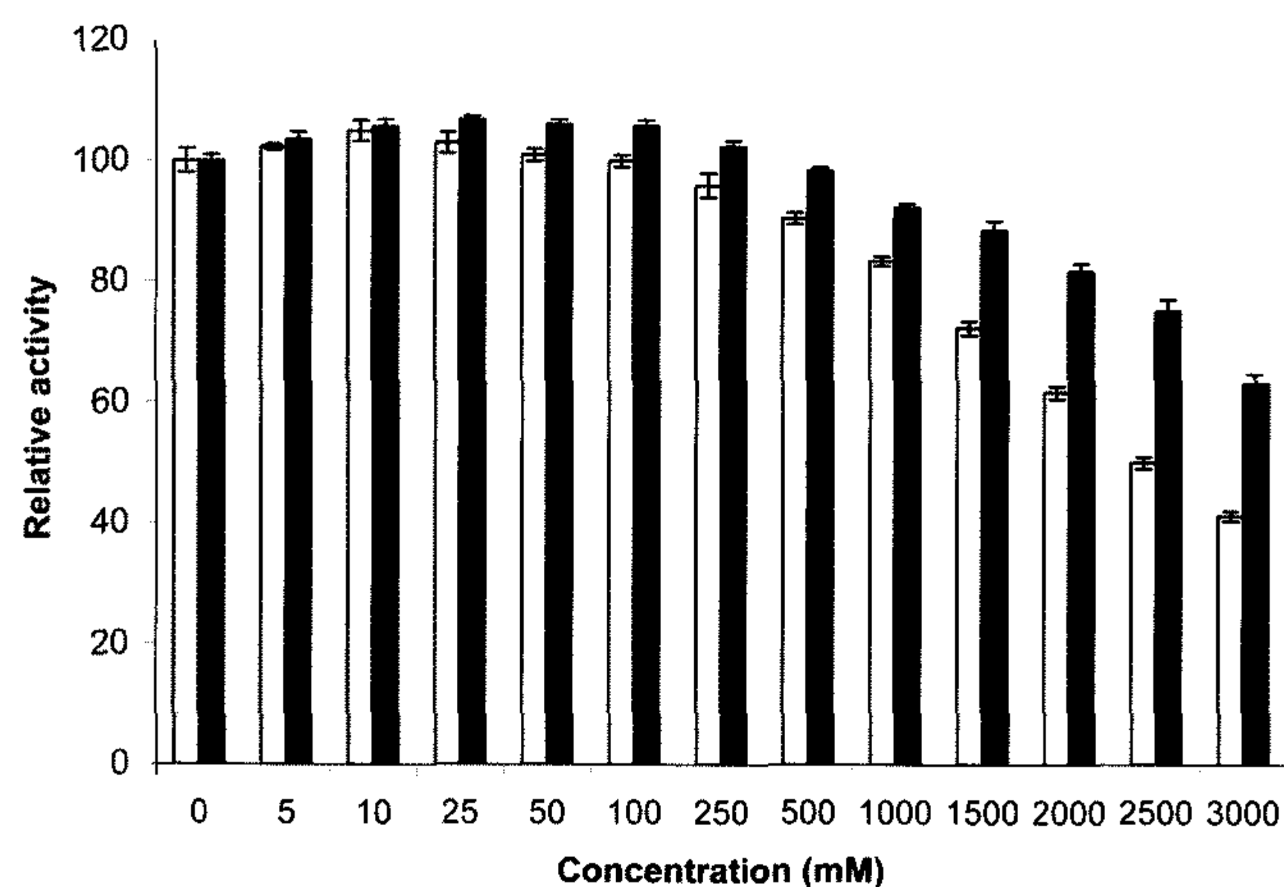

Fig. 4. Effect of ethanol (white columns) and methanol (black columns) on βG activity.
Each point is the mean of triplicate experiments.

by 5%, whereas methanol at 25 mM final concentration increased βG activity by 6.8%. The βG maintained between 83–41% and 92–63% of the original activity at final concentrations of 1–3 M of ethanol and methanol, respectively (Fig. 4). The higher βG activity observed in the presence of alcohols could be attributed to its function as a β-glucosyltransferase. Alcohols may increase reaction rates by acting as preferential acceptors of key glycosyl intermediates during catalysis, replacing water in the reaction. On the other hand, the adverse effect of a high alcohol concentration on βG activity is probably due to effects in the enzyme structure brought on by changes in the polarity of the medium [27].

The stimulation or inhibition of βGs by carbohydrates and ethanol is particularly important in vinification processes since these enzymes could be potentially employed in wine aroma optimization [32]. In some filamentous fungi, such as *Fusarium oxysporum* [7], the βG activity is hardly inhibited by ethanol but highly reduced by glucose, making the enzyme treatment impracticable in the must but viable at the end of the fermentation [11]. On the contrary, some yeasts produce βGs weakly inhibited by glucose, but the strong inhibitory effect of ethanol restricts their utilization to the first stages of must fermentation [24].

Substrate Specificity

The specificity of the partially purified βG was studied with chromogenic and nonchromogenic substrates. Considering the hydrolysis rate of *p*NPG as 100%, the hydrolysis rate of the chromogenic substrates *p*NP-β-D-cellobioside (*p*NPβCb) and *p*NP-β-D-cellotrioside (*p*NPβCt) were 22% and 5.9%, respectively (Table 5). Christakopoulos *et al.* [7] reported that the hydrolysis rate of these substrates, measured by *p*NP release, diminished with the increase in the chain length. Some studies indicate that the hydrolysis

Table 5. βG substrate specificity.

Substrates[a]	Relative activity (%)[b]
Chromogenic substrates (1 mM)	
*p*NP-β-D-glucopyranoside (control)	100.00
*p*NP-β-D-glucopyranoside	2.00
*o*NP-β-D-galactopyranoside	4.00
*p*NP-α-D-galactopyranoside	2.75
*p*NP-*N*-acetyl-β-D-glucosaminide	4.00
*p*NP-*N*-acetyl-β-D-galactosaminide	2.25
*p*NP-β-D-cellobioside	22.00
*p*NP-β-D-cellotrioside	5.90
Nonchromogenic substrates (5 mM)	
Cellobiose (control)	100.00
Maltose	125.00
n-Octyl-β-D-glucopyranoside	75.00
Salicin	70.00

*No considerable βG activity (0–4%) was observed against *p*NP-β-D-xylopyranoside, *p*NP-α-D-xylopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-α-D-fucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-*N*-acetyl-α-D-galactosaminide, *p*NP-β-glucuronide, *p*NP-β-galacturonide, *p*NP-β-D-lactopyranoside. No activity was detected against esculin, lactose, methyl α-D-glucopyranoside, raffinose, saccharose, trehalose, starch (0.1% w/v), Avicel (0.1% w/v), CMC (0.1% w/v), and xylan (0.1% w/v).
[b]Each value is the mean of triplicate experiments.

proceeds more efficiently from the nonreducing end of these molecules [20, 26]. In the present study, the hydrolysis of *p*NPβCb was carried out and both the liberation of *p*NP and glucose were assayed (Fig. 5). Results showed the immediate release of glucose and a lag period in the liberation of *p*NP, demonstrating that the hydrolysis of glucose from the nonreducing end of the molecule was more efficient than the *p*NP release and explaining the behavior of *p*NPβCb and *p*NPβCt hydrolysis measured by *p*NP liberation. The hydrolysis of other glycosides was

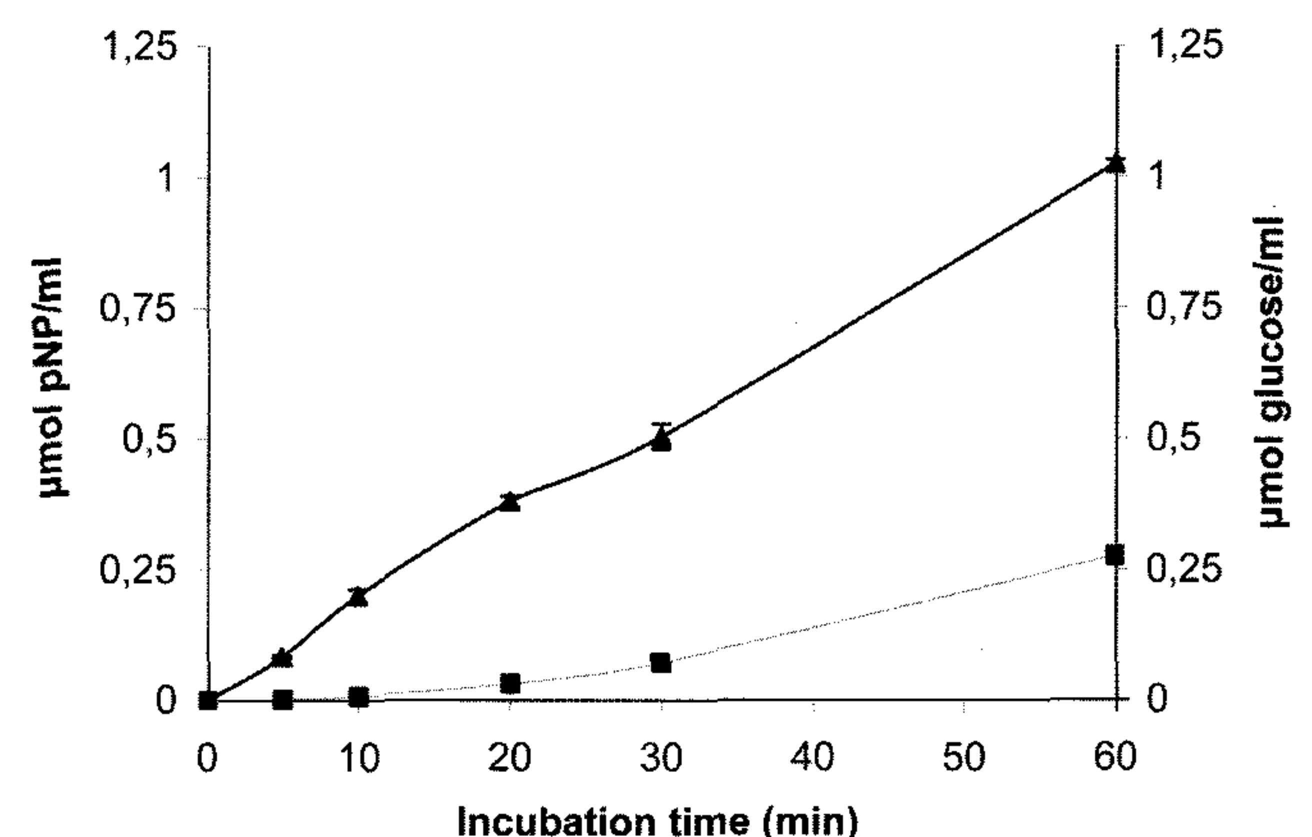

Fig. 5. Hydrolysis of *p*NPβCb (2 mM) by the partially purified βG.
(■) *p*NP release; (▲) glucose release. Each point is the mean of triplicate experiments.

also observed, although at slower rates (Table 5). βGs may frequently hydrolyze various β-glycosidic and also α-glycosidic linkages [6, 27, 29, 42].

In comparison with the hydrolysis rate of cellobiose (considered as 100%), the βG also hydrolyzed the nonchromogenic substrates salicin (70%) and *n*-octyl-β-D-glucopyranoside (75%) (Table 5). The hydrolysis of *p*NPβG, salicin, and *n*-octyl-β-D-glucopyranoside demonstrates the specificity of the enzyme for β-glucosidic linkages and the tolerance for a variety of aglycons [12, 22].

The hydrolysis rate of cellobiose was 35% from that of *p*NPG. Other nonchromogenic substrates including saccharose, lactose, esculin, methyl-α-D-glucopyranoside, Avicel, CMC, and starch were not hydrolyzed. However, surprisingly, the hydrolysis rate of maltose was higher (125%) than that of cellobiose (100%), a typical substrate of β-glucosidases. The hydrolysis of β-glucosidic linkages (*p*NPG and cellobiose) and α-glucosidic linkages (mainly maltose) indicates the relaxed specificity of the enzyme for the anomeric configuration of the glucosidic linkage to be hydrolyzed [12, 27].

βGs could be separated in three groups according to their substrate specificity: (i) aryl-β-glucosidases, which hydrolyze exclusively aryl-β-glucosides; (ii) cellobiases, which hydrolyze only oligosaccharides; and (iii) broad-specificity βG, which hydrolyzes both substrate types [3]. In this way, the partially purified extracellular βG of *M. purpureus* NRRL1992 may be classified in the last group.

Kinetic Constants

The hydrolysis of cellobiose and maltose, in the concentrations employed (2.5–75 mM), obeyed Michaelis-Menten kinetics (Fig. 6B). However, *p*NPG hydrolysis evidenced a deviation from pure Michaelis-Menten kinetics, since some inhibition of *p*NP release was observed at high substrate concentrations (Fig. 6A), a fact also reported by other authors [37, 40]. This hydrolysis inhibition may also occur using cellobiose and maltose as substrates; however, the final concentrations employed in this study were possibly not high enough for inhibition to be observed. The decline in reaction velocities with substrate concentration increases may be attributed to substrate inhibition mechanisms [15]. Nevertheless, this may be a simplistic explanation for this putative activity inhibition. Besides the hydrolytic cleavage of glycosidic linkages, βGs can simultaneously synthesize these bonds through reverse hydrolysis or transglycosylation under conditions such as high substrate concentration [3, 7]. In transglycosylation, particularly, the glycosyl portion of the substrate is transferred to a hydroxyl-containing compound other than water, and this reaction inhibits hydrolysis proportionally with an increase in substrate concentration. Thus, transglycosylation can mimic substrate inhibition in the reaction of retaining glycoside hydrolases [17].

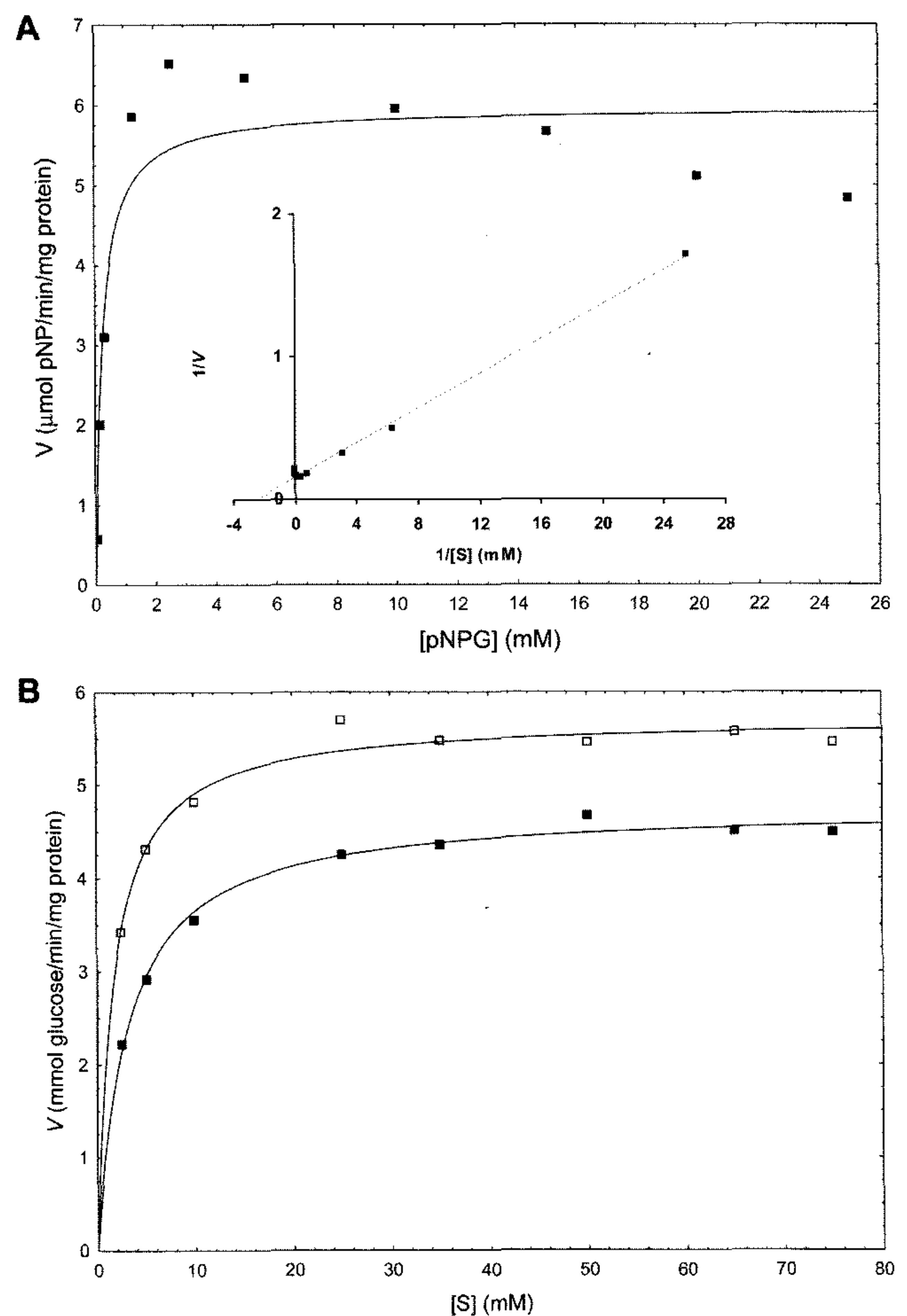

Fig. 6. Influence of substrate concentrations on βG reaction rates.

A. Nonlinear regression of *p*NPG hydrolysis. *Inset*: Lineweaver-Burk plot (1/V *vs* 1/[S]) of *p*NPG hydrolysis. **B.** Nonlinear regression of (■) cellobiose and (□) maltose hydrolysis. In nonlinear regressions, the Michaelis-Menten equation was used, and curve fitting was performed with the Levenberg-Marquardt algorithm. Each point is the mean of triplicate experiments.

At this point, the phenomenon taking place is unclear; therefore, these hypothetic assumptions (substrate inhibition, transglycosylation, or other mechanism) are worthy of further meticulous investigations. Despite the unknown βG catalytic behavior, the apparent K_m and V_{max} values were tentatively determined from both linear and nonlinear regressions using *p*NPG, cellobiose, and maltose as substrates (Fig. 6; Table 6). The lower apparent K_m indicates that the partially purified βG has a higher affinity for *p*NPG. The efficiency of substrate utilization was estimated by V_{max}/K_m ratios (Table 6), and the hydrolysis efficiency of *p*NPG was at least 10-fold and 5-fold higher than that of cellobiose and maltose, respectively.

In conclusion, the partially purified βG showed optimal activity in a wide range of temperatures and pH values and was demonstrated to be moderately thermostable. According

Table 6. Kinetic constants of the partially purified βG.

Substrate	Linear regression[a] (Lineweaver-Burk plot)			Nonlinear regression[b] (Michaelis-Menten equation)		
	K_m (mM)	V_{max} (U/mg protein)	V_{max}/K_m	K_m (mM)	V_{max} (U/mg protein)	V_{max}/K_m
*p*NPG	0.39	6.51	16.7	0.23	5.96	25.9
Cellobiose	2.86	4.71	1.65	3.05	4.76	1.56
Maltose	1.67	5.73	3.43	1.65	5.72	3.47

[a]R^2>0.98 for *p*NPG, cellobiose, and maltose.
[b]R^2=0.88 for *p*NPG, 0.98 for cellobiose, and 0.96 for maltose.

to the observed characteristics, this enzyme may be well suited for application to the release of terpenes and other aromatic compounds in wines and also to the liberation of antioxidant phenolic compounds from fruit and vegetable residues. Since *Monascus* species are generally considered as safe and widely used food microorganisms [38] and grape pomace represents a valuable source of phenolic compounds [34], the combination of *M. purpureus* NRRL1992 growth on grape pomace and βG production could be potentially explored to liberate free antioxidant phenols from this substrate.

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