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A Specific Short Dextrin-Hydrolyzing Extracellular Glucosidase from the Thermophilic Fungus *Thermoascus aurantiacus* 179-5

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The thermophilic fungus *Thermoascus aurantiacus* 179-5 produced large quantities of a glucosidase which preferentially hydrolyzed maltose over starch. Enzyme production was high in submerged fermentation, with a maximal activity of 30 U/ml after 336 h of fermentation. In solid-state fermentation, the activity of the enzyme was 22 U/ml at 144 h in medium containing wheat bran and 5.8 U/ml at 48 h when cassava pulp was used as the culture medium. The enzyme was specific for maltose, very slowly hydrolyzed starch, dextrins (2-7G) and the synthetic substrate (α-PNPG), and did not hydrolyze sucrose. These properties suggest that the enzyme is a type II α-glucosidase. The optimum temperature of the enzyme was 70°C. In addition, the enzyme was highly thermostable (100% stability for 10 h at 60°C and a half-life of 15 min at 80°C), and stable within a wide pH range.

Keywords: glucosidase, *Thermoascus*, submerged fermentation, solid-state fermentation, thermophilic, thermostable

Glucosidases (glucohydrolases) are ubiquitous among living beings and catalyze the cleavage of glycosidic bonds in glycosides, oligosaccharides and polysaccharides. Glucosidases are involved in carbohydrate degradation and the processing of glycoproteins and glycolipids (Wang *et al*, 2004).

The enzymes that hydrolyze starch can be divided into endoamylases, exoamylases and debranching amylases. Exoamylases are glucosidases that preferentially act on α-1,4 bonds from the nonreducing end, successively resulting in low molecular weight products (James and Lee, 1997), and include α-glucosidase (EC 3.2.1.20, α-D-glucohydrolase) and glucoamylase (EC 3.2.1.3). These two enzymes cannot readily be distinguished from each other since both release D-glucose, but can be differentiated based on the anomer type produced: α-glucosidase produces α-glucose and glucoamylase produces β-glucose (Chiba, 1997).

Suggestions for applications of α-glucosidase and glucoamylase include their use in industrial starch processing (Zdzieblo and Synowiecki, 2002) and also as a guide to the structure-based design of anti-HIV inhibitors (Da Silva *et al.*, 2005). Several studies have proposed the inhibition of α-glucosidase as a potential therapy for diseases such as cancer and diabetes (Hakamata *et al.*, 2005). Therefore, it is important to establish the molecular nature, mechanisms of action and functions of this enzyme obtained from several eukaryotic organisms for industrial application and to provide a feasible pattern basis for the structural study of these enzymes and their mechanism of action. The importance of this enzyme justifies the interest in microorganisms capable of secreting it in large quantities as well the search for in-depth knowledge of its biochemical characteristics.

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Material and Methods

Microorganisms

The *Thermoascus aurantiacus* strain 179-5 used in the present study was isolated from decaying hemicellulosic material collected in the State of Amazonas, Brazil, and was maintained as a stock culture at 7°C on potato dextrose agar.

Enzyme production in submerged fermentation (SmF)

Erlenmeyer flasks (250 ml) containing 50 ml medium consisting of (w/v) 1.0% carbon source, 0.20% $(NH_4)_2SO_4$, 0.10% K_2HPO_4, 0.70% KH_2PO_4, 0.01% $MgSO_4 \cdot 7H_2O$, 0.50% yeast extract, 0.20% peptone, and 0.05% micronutrient solution (0.10% $FeSO_4 \cdot 7H_2O$, 0.005% $MnSO_4 \cdot H_2O$, 0.02% $ZnSO_4 \cdot 7H_2O$,

0.005% $CaCl_2$, 0.025% $CuSO_4.5H_2O$, pH 5.5) were inoculated with aliquots of a mycelial suspension (approximately 30 mg dry mycelial mass per ml) obtained from 4-day agar slant cultures suspended in sterile 0.01% Tween 80. Fermentation was carried out in a rotary shaker at 100 rpm for 336 h at 45°C. The biomass was separated by filtering through Whatman No. 1 paper in a Büchner funnel. The filtrate was used to evaluate amylase activities. The experiments were performed in triplicate with three replicates and the results are reported as means.

Enzyme production in solid-state fermentation (SSF)

The carbon sources used for SSF were commercially available wheat bran and cassava pulp, the solid waste of the starch extraction process, provided by Usina Plaza, Santa Maria da Serra, SP, Brazil. The material was dried and used untreated. Fermentation was carried out in 250 ml Erlenmeyer flasks containing 5 g of the substrate and 12 ml of a mycelial suspension in nutrient solution composed by (w/v) 0.20% $(NH_4)_2SO_4$, 0.10% K_2HPO_4, 0.70% KH_2PO_4, 0.01% MgSO4·7H2O, 0.50% yeast extract, 0.20% peptone, and 0.05% micronutrient solution described above. The inoculum proportion was approximately 5 mg dry mycelial mass per gram dry substrate. The final moisture content of the medium was approximately 70%. The cultures were incubated at 45°C for 336 h. Fermented material was mixed with 40 ml distilled water at 48 h intervals, vacuum filtered, and centrifuged. The experiments were performed in triplicate with three replicates and the results are reported as means.

Effect of different carbon sources, initial medium pH and incubation temperatures on enzyme production

Corn or cassava starch (1%) was used as a carbon source. The pH of the medium was adjusted to 4.0, 5.0 or 6.0, and the incubation temperature was 40 or 45°C. A complete factorial design (2×3×2) with three replicates was used. Statistical analysis (ANOVA and Tukey test) was performed with Estat software (free).

Measurement of enzyme activity

Enzyme activity was assayed at 70°C in a reaction mixture containing 0.1 ml diluted crude enzyme and 0.4 ml substrate solution in 0.25 M sodium acetate buffer, pH 5.0. The substrates used were 0.5% soluble starch or 0.2 % maltose. The amount of glucose released was estimated by the peroxidase/glucose oxidase assay (Cereia *et al.*, 2000). One unit (U) of enzyme activity was defined as the amount of enzyme that releases one μmol of glucose per minute per ml reaction mixture.

Substrate-specific assays were performed by replacing the above substrate with raw and soluble cassava, potato and corn starch, sucrose or dextrins (two to seven glucose units). When *p*-nitrophenyl-α-D-glucopyranoside (α-PNPG) was used as substrate, activity was measured in a mixture containing 0.20 ml 0.1 M sodium acetate, pH 5.0, 0.05 ml of a 2 mM substrate solution, and 0.1 ml crude enzyme. After 10 min of incubation at 70°C, the reaction was stopped with 1 ml 2 M Na_2CO_3, and the *p*-nitrophenol released was quantified spectrophotometrically at 410 nm.

Enzyme characterization

pH and temperature for optimum enzyme activity: The optimum pH was evaluated by measuring enzyme activity at 60°C using various buffers: sodium acetate (pH 3.0-5.0), citrate-phosphate (pH 5.0-7.0), Tris-HCl (pH 7.0-8.5), and glycine-NaOH (pH 8.5-11.0) and a reaction mixture containing 0.4 ml 0.2% (w/v) maltose in 0.25 M buffer and 0.10 ml crude enzyme. The optimal temperature was determined by incubating the reaction mixture at 50-85°C and assaying the activity at the optimum pH in the same reaction mixture.

Thermostability: The crude enzyme solution was incubated at various temperatures (40-95°C) for 1 h at pH 7.0 in sealed tubes to prevent evaporation. Another experiment was carried out to determine the stability of the enzyme at 60°C. The enzyme solution was maintained at this temperature for a period of 10 h. To evaluate possible maltose hydrolysis, water was used instead of crude enzyme as a control. In both assays, an aliquot was removed and placed on ice before assaying for residual enzyme activity at the optimum pH and temperature.

pH stability: Crude enzyme was dissolved (1:1) in 0.1 M buffer solution at pH 3.0-8.0 (McIlvaine) and pH 8.0-10.0 (glycine-NaOH) and maintained at 25°C for 24 h. An aliquot was used to determine the remaining activity at the optimum pH and temperature.

Analytical methods

The growth of the culture was evaluated in terms of dry weight. Fermented material was filtered through Whatman N° 1 filter paper and the biomass was washed with chilled deionized water, vacuum filtered and dried at 65°C to a constant weight.

Protein concentration was determined by the method of Hartree-Lowry (1972). Reducing sugar was quantified by the dinitrosalicylic acid method (Miller, 1959).

Results and Discussion

Enzyme production in SmF and SSF

Crude enzyme obtained in SmF showed high activity on maltose, with the peak at 336 h (30 U/ml). When

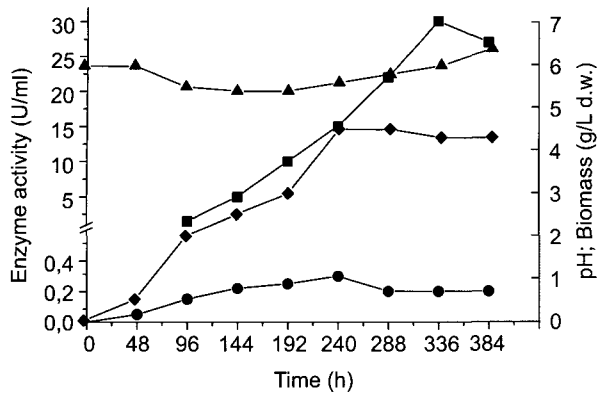

Fig. 1. α-Glucosidase production by *Thermoascus aurantiacus* 179-5 in submerged fermentation. The enzyme activity was evaluated using soluble starch (●) or maltose as substrate (■). ▲ = pH of the medium; ◆ = biomass production (g dry weight/L).

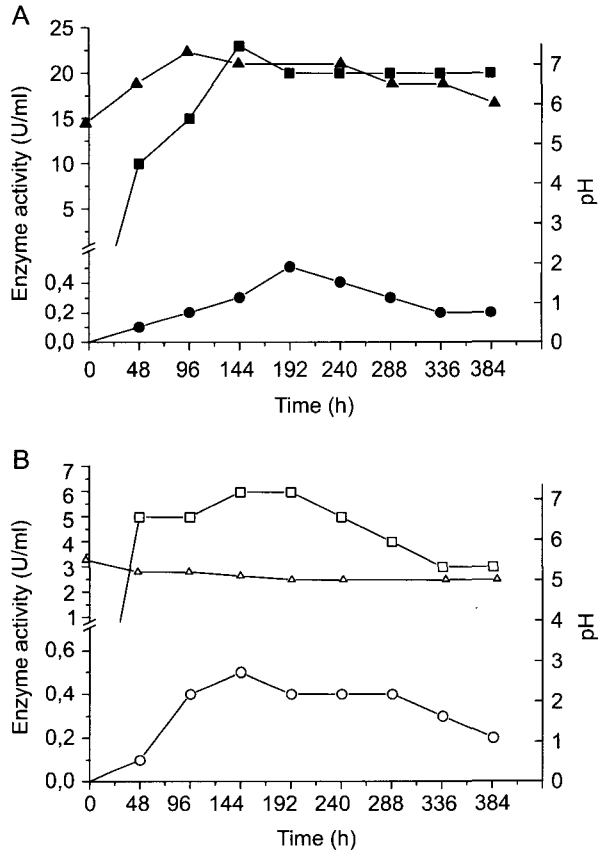

Fig. 2. Glucosidase production by *Thermoascus aurantiacus* 179-5 in solid-state fermentation using wheat bran (a) and cassava pulp (b) as substrate. △▲ = pH of the medium; ■□ = glucosidase activity on maltose; ●○ = glucosidase activity on soluble cassava starch.

soluble starch was used as substrate for the measurement of enzyme activity, the peak occurred between 192 and 240 h and maximal activity was 0.3 U/ml (Fig. 1).

The mycelial mass reached a maximum at 240 h. Comparison of enzyme activity on maltose and biomass production by *T. aurantiacus* 179-5 (Fig. 1) indicated that the largest amount of enzyme is released into the medium after the log phase. These results disagree with those reported in the literature for *Aspergillus niger* and *Thermomyces lanuginosus* in which glucosidase secretion occurred during the growth phase (Jensen and Olsen, 1996; Alabaek *et al.*, 2002; Papagianni and Moo-Yung, 2002).

During the first 96 h of growth, the pH of the medium decreased from an initial 6.0 to 5.5, followed by an increase until reaching 6.5 after 384 h. Several studies have reported that the secretion of some fungal proteins requires pH-dependent glycosylation as part of the secretory machinery (Conesa *et al.*, 2001; Wallis *et al.*, 2001). Therefore, a possible influence of medium pH on the activation/secretion processes cannot be ruled out.

Enzyme production in SSF was analyzed over a period of 384 h and peaks of enzyme activity were detected at 144-192 h (Fig. 2). Glucosidase production was higher in medium containing wheat bran than in medium containing cassava pulp. The maximal activity on maltose was 22 U/ml in wheat bran medium versus 5.8 U/ml in cassava pulp medium. Similar to enzyme production in SmF, the enzyme activity was much higher on maltose compared to that on starch.

Effect of carbon source, initial medium pH and incubation temperature on enzyme production in submerged fermentation

Only activity on maltose was evaluated in this assay. Statistical analysis indicated that different carbon sources and pH significantly influenced enzyme production. The type of starch used as carbon source influenced the production of glucosidase by *T. aurantiacus* 179-5, which was higher (statistically significant at the 1% level) in media containing corn starch than in those containing cassava starch (Fig. 3 a-b). Since these types of starch differ in their amylose and amylopectin composition and lipid content (Chaves *et al.*, 2004) (corn starch has approximately 28% amylose, 72% amylopectin and 6.0% lipids while cassava starch has approximately 17% amylose, 83% amylopectin and 0.1% lipids), these results suggest that the composition and differences in the molecular structure of starch affect enzyme induction. Cruz *et al.* (1997) observed that the ability of *Rhizopus* sp. to produce amylolytic enzymes that release reducing units was higher when the carbon source was amylose rather than amylopectin, and that

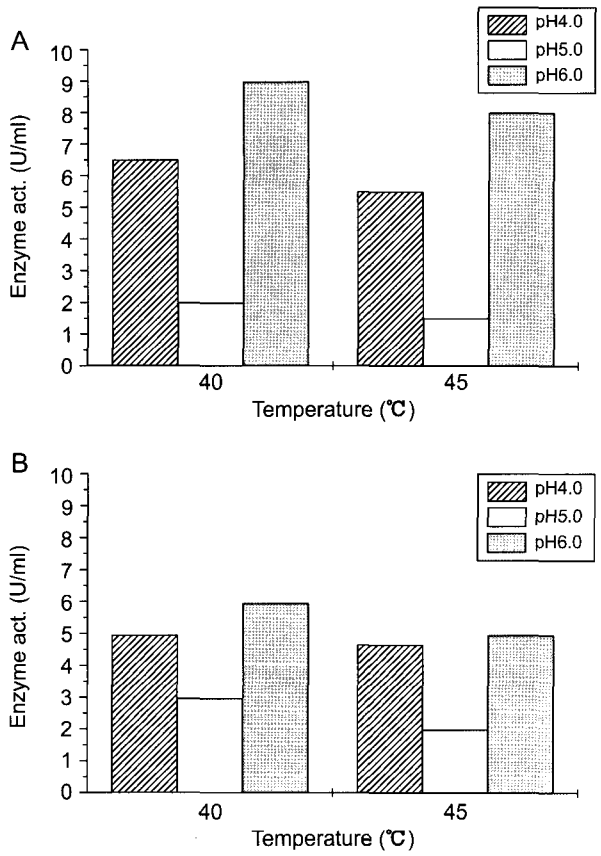

Fig. 3. Effect of pH, temperature and carbon source on glucosidase production by *Thermoascus aurantiacus* 179-5. Culture media were supplemented with 1% corn starch (a) or 1% cassava starch (b).

corn amylose was a better inducer of the enzyme than its potato homolog.

Fig. 3 shows that enzyme synthesis by the fungus grown on corn starch and cassava starch was significantly higher when the initial pH of the culture medium was 6.0 (5% level, Tukey test). However, enzyme activity was higher when the initial pH of the culture medium was 4.0 compared to pH 5.0, suggesting the synthesis of two forms of the enzyme.

Since the total growth of the microorganism was not significantly affected by medium pH (data not shown), the effect of pH observed must be related to mechanisms regulating enzyme synthesis and secretion. The effect of pH on production and secretion of glucoamylase by *A. niger* has been reported (Wallis *et al.*, 2001), with glucoamylase production being higher at pH 4.0 than at pH 5.5. Since enzyme activity was not correlated to biomass production, the authors speculated that pH and the high level of gluconic acid produced controlled some aspects of enzyme production. Characterization of the pH signal transduction pathway in *A. nidulans* has been completed at the molecular level, although the biochemical functions of the involved proteins have yet to be completely elucidated (Kaia *et al.*, 1991; Denison, 2000).

Regarding incubation temperature, no statistically significant differences in enzyme synthesis were detected when fermentation was carried out at 40 or 45°C, although higher activities were observed in some assays at 40°C (Fig. 3).

Substrate specificity

Figs. 1 and 2 show that the enzyme preferentially hydrolyzed maltose and exhibited a low amylolytic activity. The data regarding substrate specificity shown in Fig. 4 confirm that the enzyme preferentially acted on maltose and short dextrins as opposed to starch. Raw starch was preferred over soluble starch, although the enzyme activity was low on these substrates. Hydrolysis of α-PNPG was slow and the enzyme did not degrade sucrose.

The differences observed in the catalytic properties of enzymes as a function of the substrate suggest the presence of exo-amylases that release glucose preferentially from maltose. According to Chiba (1997), two exo-amylases, glucoamylase and α-glucosidase, are able to release glucose from maltose. However, the former preferentially hydrolyzes starch while the latter hydrolyzes soluble starch very slowly and preferentially acts on maltose and malto-oligosaccharides. α-Glucosidases are divided into three types: type I hydrolyzes heterogeneous substrates such as aryl glucosides and sucrose more efficiently than maltose; type II prefers maltose and isomaltose and shows a low activity toward aryl glucosides and heterogeneous substrates; type III has the same specificity as type II and, in addition, hydrolyzes polysaccharides such as amylose and starch (Frandsen and Svensson, 1998). Since maltose was a better substrate than starch or dextrins, the glucosidase isolated here cannot be a glucoamylase or exodextranase. Thus, the enzyme isolated from *T. aurantiacus* seems to be a type II α-glucosidase.

α-Glucosidases have been detected in the culture media of filamentous fungi such as *A. awamori*, *A. niger*, *A. oryzae*, *A. flavus*, *Thermomyces lanuginosus* and *Mucor javanicus* (Yamasaki *et al.*, 1973; Jensen and Olsen, 1996; James and Lee, 1997; Anindyawati *et al.*, 1998; Negret *et al.*, 1999; Gomes *et al.*, 2005).

Enzyme characterization

The activity on maltose of enzymes obtained by cultivation of *T. aurantiacus* 179-5 in SmF and SSF exhibited almost the same enzymatic characteristics, except for thermal stability. Although the optimum temperature (70°C) and thermal stability at 60°C were

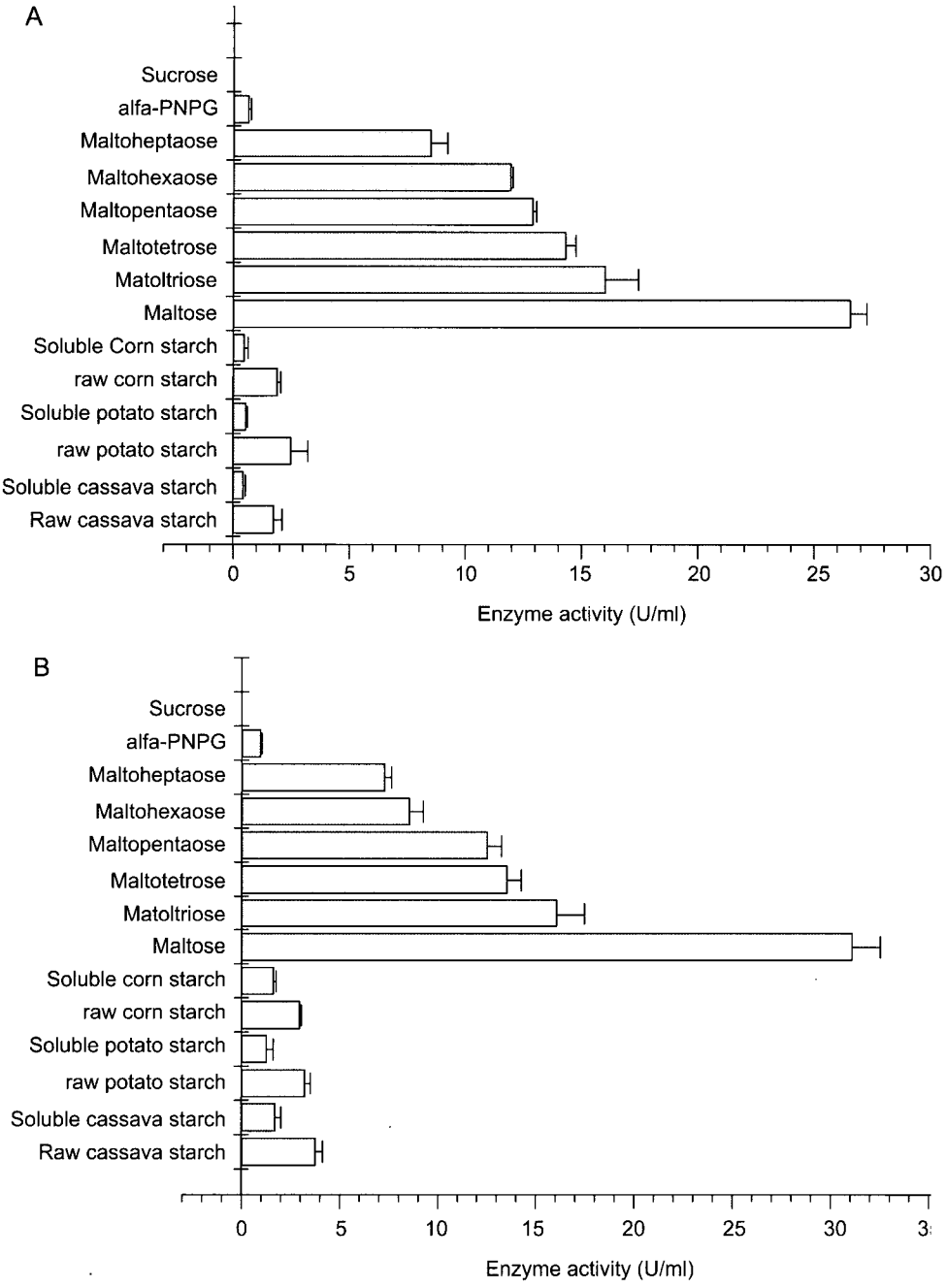

Fig. 4. Glucosidase activity on different substrates. a = enzyme produced in solid-state fermentation; b = enzyme produced in submerged fermentation.

similar for both enzymes (Fig. 5a-c), the enzyme produced in SSF showed an 80% loss of activity during incubation for 1 h at 70°C in the absence of substrate, while the enzyme produced in SmF presented a decrease of only 34% under the same conditions (Fig. 5d). The half-life at 80°C was 7.5 min for the SSF enzyme and 15 min for the SmF enzyme (Fig. 5d). Extracellular fungal enzymes have been shown to exist in multiple forms whose enzymatic properties such as thermal stability and Km values vary depending on whether they are produced in SSF or SmF (Hata *et al.*, 1997). Ishida *et al.* (1998) demonstrated the presence of two glucoamylase-encoding genes in *A. niger* which are differently expressed in solid-state and submerged culture. The higher thermal stability observed for the enzyme produced by *T. aurantiacus* in submerged culture than that obtained in SSF might be due to the

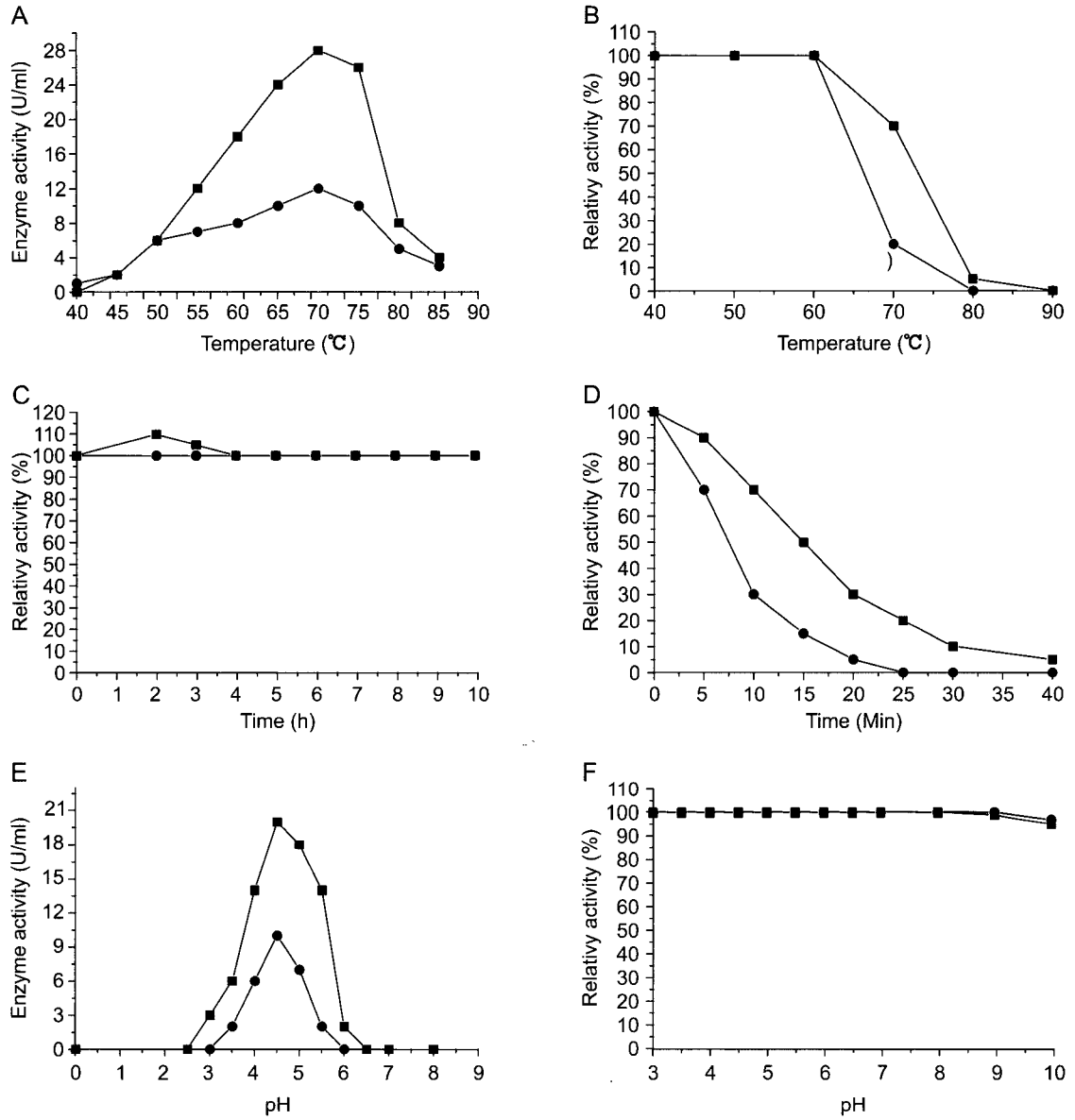

Fig. 5. Glucosidase properties. a = effect of temperature on enzyme activity in the presence of substrate; b = effect of different temperatures on enzyme stability in the absence of substrate at pH 5.0; c and d = enzyme stability at 60°C and 80°C, a respectively, at pH 5.0; e = effect of pH on enzyme activity in the presence of substrate; f = effect of different pHs on enzyme stability in the absence of substrate. Square = enzyme from submerged fermentation; circle = enzyme from solid-state fermentation.

expression of different genes or variations in post-translational processing (Hata *et al.*, 1997; Dubey *et al.*, 2000). However, purification of the enzyme and some molecular studies are necessary to confirm this.

The optimal temperature of the present enzyme (70°C) was similar to that reported for other glucosidases from thermophilic fungi such as *Talaromyces duponte*, *H. grisea* and *T. lanuginosus* with temperature optima of 75, 60 and 70°C, respectively (James and Lee, 1997), but the thermal stability in the absence of substrate was much higher than that observed for other enzymes of eukaryotic cell origin.

The optimum activity of both enzymes was observed at pH 4.5 (Fig. 5e). This value agrees with that reported for α-glucosidase from *Aspergillus* species (Kita *et al.*, 1991, Gomes *et al.*, 2005) but disagrees with those found for α-glucosidase II from *Candida albicans* (Torre-Bouscoulet *et al.*, 2004) and α-glucosidase I from *Saccharomyces cerevisiae* (Faridmoayer and Scaman, 2004) and *Mortierella*

alliacea (Tanaka *et al.*, 2002), whose optimum activities were determined in the neutral pH range (6.0-7.5). The present enzymes were stable over a wide pH range, with 100% stability at a pH of 3.0-10.0 (Fig. 5f).

Since the industrial use of enzymes in bioprocesses requires crude or partially purified enzymes, determination of the optimal temperature and thermostability of enzymes under these conditions is important. In addition to thermostability, stability and activity over a wide pH range are important properties of the enzymes produced by the strain studied here.

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