

## THERMOSTABLE SACCHAROGENIC AMYLASE PRODUCED UNDER SUBMERGED FERMENTATION BY FILAMENTOUS FUNGUS *Penicillium purpurogenum*

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### ABSTRACT

The effect of several nutritional and environmental parameters on *Penicillium purpurogenum* growth and saccharogenic amylase production was analyzed. High enzyme levels ( $68.2 \text{ U mg}^{-1}$ ) were obtained with Khanna medium at initial pH 6.0, incubated at  $30^\circ\text{C}$  for 144 hours. The optimum pH and temperature activities were 5.0 and  $65^\circ\text{C}$ , respectively. The enzyme presented a half-life ( $t_{50}$ ) of 60 min, at  $65^\circ\text{C}$ . Only glucose was detected after 24 hours of reaction using soluble starch as substrate.

**Key words:** amylase, *Penicillium purpurogenum*, submerged fermentation

Starch-based foods like bread, pasta, potatoes and rice contribute approximately with one third of the total weight of our dietary intake. Starch also is a major raw material for the manufacture of several products including glucose syrup and sweeteners (13). Starch is hydrolyzed to glucose, maltose and maltooligosaccharides by four groups of amylases according to Van der Maarel *et al.* (2002) (16). The first one is formed by the endoamylases (EC 3.2.1.1) which cleave  $\alpha$ -1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides, but not the  $\alpha$ -1,6 linkages. Enzymes belonging to the second group are known as exoamylases and successively act at the non-reducing end. These enzymes preferentially act either on  $\alpha$ -1,4 bonds ( $\beta$ -amylase; EC 3.2.1.2) or on both  $\alpha$ -1,4 and  $\alpha$ -1,6 glycosidic bonds (glucoamylase; EC 3.2.1.3 and  $\alpha$ -glucosidase; EC 3.2.1.20). Another group is constituted by the debranching enzymes known as pullulanase (EC 3.2.1.41) and isoamylase

(EC 3.2.1.68), which acts specifically on  $\alpha$ -1,6 bonds. The last ones are transferases that cleave the  $\alpha$ -1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor leading to the formation of a new glycosidic bond (14,16).

Amylases have been produced by bacterial, fungal and other organisms (7, 17, 18). The industrial demand for these enzymes is limited to specific applications in the food industry, where fungal amylases are preferred over other microbial sources, mainly because of their high accepted GRAS status (13). In this study we investigated the production of saccharogenic amylases by the new filamentous fungus *P. purpurogenum* strain under submerged fermentation and reported some cultivation conditions. The products of starch hydrolysis after a treatment with crude enzyme were determined.

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For this purpose the *P. purpurogenum* strain isolated from soil and identified by the laboratory of molecular genetic of microorganism (Department of Genetic, FMRP-USP, Ribeirão Preto, Brasil) was maintained at 30°C, on slants of solid 4% (w/V) oatmeal baby food (Quaker) media, at 4°C. The microorganism cultures were obtained by the addition of 1 mL solution of spore suspension ( $10^7$  conidia/mL) in Khanna medium (9) added with 1% starch (w/V) or other carbon source (rice straw, oat meal, crushed corn cob, crushed corn, “Maizena”, maltose, penetrose, amilopectin, raffinose, lactose and glucose) at different temperatures (20°C-40°C) for 144 hours under stationary condition.

After cultivation, the cultures were harvested and the filtrate was used for the determination of amylase activity using 1% (w/V) starch (Sigma® and Reagen®) as substrate (0.1 M sodium acetate buffer, pH 5.0) at 60°C. The reducing sugars were quantified according to Miller (1959) (11). The enzymatic activity was also determined using others substrates as glycogen, amylopectin, maltose, maltotriose,  $\alpha$ -PNPG and sucrose in the same concentration and conditions described for starch. One unit of activity was defined as the amount of released reducing sugar at initial rate of  $1 \mu\text{mol min}^{-1}$  in the assay condition. Protein was determined according to Lowry *et al.* (1951) (10) and expressed as mg of protein.

The enzyme stability to pH was determined at a range from 3.0 to 9.0 using 100 mM McIlvaine buffer and the stability to temperature from 55°C to 70°C for 4 hours, both in the same conditions described above. The hydrolysis products of soluble starch (1%; w/V) were analyzed by thin-layer chromatography (TLC) using buthanol/ethanol/water (5:3:2; V/V/V) as mobile phase. It was used 1% (w/V) of glucose, maltose and maltotriose solutions as standard.

All experiments were conducted in triplicate and the results were present as media values  $\pm$  standard deviation (SD).

High levels of amylase production ( $68.2 \pm 1.8 \text{ U mg}^{-1}$ ) were obtained when the fungus was grown in Khanna medium (9) added of 1% (w/V) soluble starch as the only carbon source

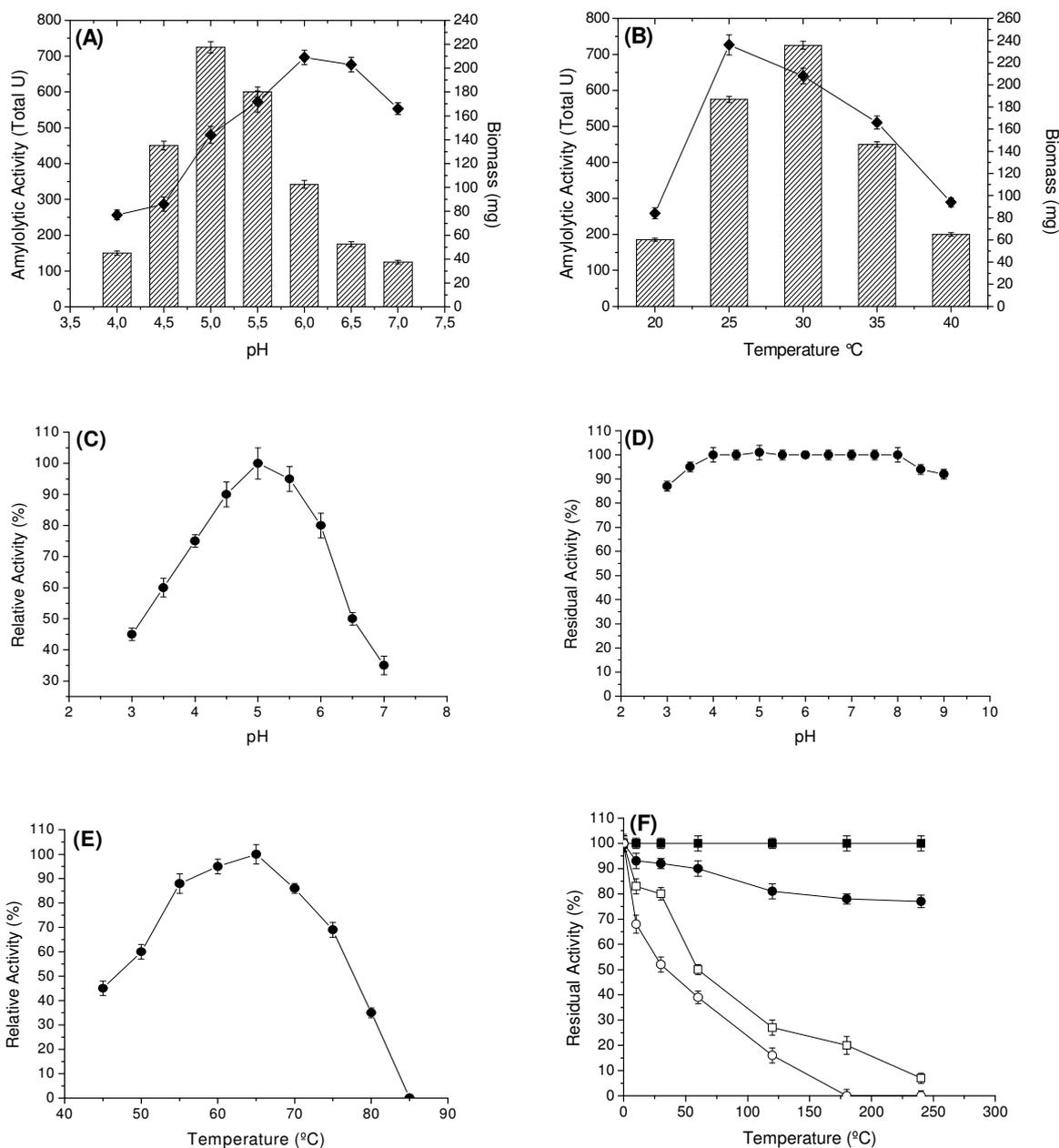
in stationary condition for 120 hours. This time is relatively short, what can carry lower cost of production in a wide scale. The production in this condition was 1.6-fold higher than the one obtained in agitation condition. On the other hand, great quantities of glucoamylase were also secreted using amylopectin ( $63.2 \pm 1.7 \text{ U mg}^{-1}$ ), maltose ( $36.0 \pm 0.9 \text{ U mg}^{-1}$ ), ground corn ( $32.5 \pm 0.8 \text{ U mg}^{-1}$ ) and oatmeal ( $20.0 \pm 1.0 \text{ U mg}^{-1}$ ) as carbon source. Despite, the production was also observed with “Maizena”, rice straw and corncob. Starch and derivates have been described as the most adequate carbon source for amylase production. In this case, the utilization of agricultural residues or starch processing, which contain enough quantities of residual starch and the low cost of process, constitute one of the most viable option. Thus, the results showed for the amylase production by *P. purpurogenum* on media supplemented with soluble starch, amylopectin, maltose and penetrose are in accordance with the literature.

The effect of initial pH of culture medium on the production of amylases, at a range of 4.0 - 7.0 was determined and the highest enzymatic production by the fungus was obtained with initial pH adjusted for 5.0 (Fig. 1A). These results are similar to those reported for *Aspergillus* sp. A3 (3) and contrasting to those obtained for amylases produced by *A. fumigatus* (6) and *A. oryzae* Ahlburg (Cohen) 1042.72 (1). In addition, the maximum enzyme production was at 30°C (Fig. 1B), although the highest fungal growth was obtained at 25°C (data not shown). This fact shows that the fungus can be considered mesophilic.

Most of fungal amylases are produced by mesophilic organisms growing in a range of temperature varying from 25°C to 37°C (7). Same optimal temperature for amylase production was observed for mesophilic molds *A. oryzae* and *A. tamarii*, 35°C for the enzyme of *Rhizopus oligosporos* and *Aspergillus* sp. and 25°C for the amylase of *Cryptococcus flavus* (2,4,8,12,18). Optima of pH and temperature were estimated as 5.0 and 65°C, respectively (Fig. 1C and 1E). In addition, the enzyme was stable in a wide range of pH

(4.0 – 8.0) (Fig. 1D). The enzyme was completely stable for four hours when incubated at 55°C and showed a good stability

at 60°C. The enzyme maintained 50% of activity in 60 min at 65°C and 30 min at 70°C (Fig. 1F).

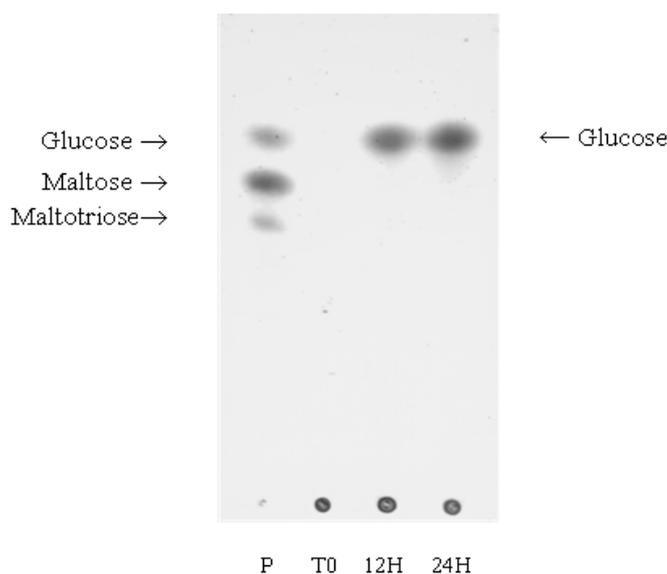


**Figure 1.** Influence of the initial pH (A) and temperature (B) on the amylase production and biomass (●), optimum of pH (C) and temperature (E) for enzymatic activity and enzyme stability to pH (D) and temperature (F). Symbols for (F): 55°C (■), 60°C (●), 65°C (□) and 70°C (○).

The determination of physical-chemical characteristics of the enzymes involved in biotechnological processes is important, having in mind the optimization of the activity, with possibilities for future applications in industrial scale. Each enzyme presents a great pH of performance, which is directly related with the native three-dimensional conformation that is influenced by pH and by ions in the solution. Fungal glucoamylases are usually active in acid pH. The optimum pH for the saccharogenic amylase of *P. purpurogenum* was 5.0, similar to the one observed for glucoamylase from *A. terreus* (5). The activity was acid-alkaline tolerant, maintaining itself stable by two hours in pH from 4.0 to 9.0. These data are interesting and they make the use of this enzyme in process which involves possible pH variations. The optimal temperature for the saccharogenic amylase from *P. purpurogenum* was 65°C. This temperature is superior to those presented for glucoamylases from *A. terreus* (60°C) (5) and *Aspergillus* sp. AS-2 (50°C) (15). The amylase was totally stable for 4 hours at 55°C, maintaining 80% of its activity after

4 hours and 50% after 60 min, at 65°C. These results reinforce the potential of this enzyme for the applications in processes that involve elevated temperatures.

The enzyme showed excellent capacity to hydrolyze different substrates with a higher action on soluble starch (Sigma® and Reagen®), glycogen, amylopectin, maltose, maltotriose and amylose (data not shown). The capacity to hydrolyze starches of long chain and glycogen allows inferring that this enzyme is a glucoamylase, but on the other hand the inability to hydrolyze  $\alpha$ -PNPG and sucrose excludes the possibility of the amylase to be characterized as  $\alpha$ -glucosidase, once these enzymes have specific action on these substrates. Other evidence that shows that the enzyme produced by *P. purpurogenum* is a glucoamylase was the presence of glucose as the only hydrolysis end-product from starch, after 24 hours of assay (Fig. 2). The capacity of breaking the starch in just glucose after long periods, and the incapacity of breaking synthetic substrate ( $\alpha$ -PNPG), assures that the enzyme is a glucoamylase (1,4- $\alpha$ -D-glucan glucohydrolase).



**Figure 2.** Analysis by TLC of the hydrolysis products of the soluble starch by the saccharogenic amylase produced by *P. purpurogenum*. S- standard; time of reaction: 0 hours (T0), 12 hours (12H) and 24 hours (24H). The mobile phase was buthanol/ethanol/water (5:3:2; v/v/v). Sugars were determined using 0.2% orcinol in a methanol-sulfuric acid (9:1; v/v) solution. Glucose, maltose and maltotriose were used as standard.

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