

## Localization and Partial Characterization of Thermostable Glucoamylase Produced by Newly Isolated *Thermomyces lanuginosus* TO3 in Submerged Fermentation

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### ABSTRACT

*Thermophilic Thermomyces lanuginosus* strain TO3 was isolated from compost pile samples and was used for its ability to produce considerable glucoamylase activity when growing in liquid medium at 45°C with starch as the sole carbon source. Enzyme productivity was high in submerged fermentation (SmF) with maximum activity of 13 U/mL after 168 h of fermentation. Higher quantities of glucose were released when the substrate for enzyme was soluble starch than maltose or maltooligosaccharides were used. The distribution of glucoamylase between the extracellular and cell-associated fractions varied according to fermentation time. Glucoamylase produced from *T. lanuginosus* TO3 had optimum activity at 65 °C and good thermostability in the absence of substrate, with a half-life of 6 h at 60 °C. The enzyme was stable over a wide pH range (4.0-10.0).

**Key Words:** Glucoamylase, submerged fermentation, *Thermomyces lanuginosus*

### INTRODUCTION

Filamentous fungi have an extraordinary capacity to secrete large amounts of protein and other metabolic products into the growth medium. This has been exploited by the biotechnological industry for the production of industrial enzymes (Iwashita, 2002). Glucoamylase (EC 3.2.1.3) of fungal origin cleaves  $\alpha$ -1,4 bonds, releasing D-glucose molecules in the  $\beta$ -configuration. This enzyme also attacks  $\alpha$ -1,6 bonds at the branching points in the amylopectin molecule, but much

more slowly than the  $\alpha$ -1,4 linkages (Chiba, 1997).

In industrial starch syrup production, the starch is min, followed by a second stage for 60-180 min at 95-98 °C. Thermostable  $\alpha$ -amylases are used to hydrolyze the starch during this liquefaction (Satyarayana et al., 2004). Upon completion of the liquefaction, the slurry has to be cooled to 55-60 °C before the saccharification step, since the glucoamylases from the *Aspergillus* and *Rhizopus* genera used in this step are unstable at temperatures above 60 °C (Brumm, 1998; Silva et al., 2005). When this enzyme is used to produce

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glucose, they are allowed to react at 55-60 °C for 2-4 days; consequently, industries need enzymes with high thermal stability for an extended period of time (Abraham et al., 2004). Thus, for industrial purposes, new highly thermostable and environmentally compatible glucoamylases might make an important contribution to the starch-processing industry (Gomes et al., 2005; Neves et al., 2006).

The aim of this work was to study glucoamylase production in submerged fermentation from the newly-isolated thermophilic fungal strains, the distribution of the enzyme between cell-associated and extracellular fraction and its physico-chemical properties.

## MATERIAL AND METHODS

### Isolation and identification of microorganisms

Samples of agricultural soil (2.0 g), potato tubers, compost and animal manure were homogenized in the sterile medium of pH 5.0 containing (g/L) 10 soluble starch; 1.4 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 2.0 K<sub>2</sub>HPO<sub>4</sub>; 0.2 MgSO<sub>4</sub>.7H<sub>2</sub>O; 0.0016 MnSO<sub>4</sub>.H<sub>2</sub>O; 0.0014 ZnSO<sub>4</sub>.7H<sub>2</sub>O; 0.02 CoCl<sub>2</sub>. The mixture was incubated at 45 °C for 24 h, after which a loop of the homogenized culture was streaked on the surface of the same medium containing 3.0% agar and incubated at 45 °C for 24 to 72 h. All the morphologically contrasting colonies were purified by repeated streaking. Pure cultures were subcultured on the slants of the same medium for identification and enzyme studies. Fungi were identified by their morphological and biochemical characteristics (Kirk et al., 2001). The stock cultures were maintained at 7 °C on potato dextrose agar medium (PDA-Oxoid).

### Production of glucoamylase in submerged fermentation (SmF)

Erlenmeyer flasks (250 mL), with 50 mL of the sterile medium containing (g/L) 10.0 of soluble cassava starch, 2.0 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.0 K<sub>2</sub>HPO<sub>4</sub>, 7.0 KH<sub>2</sub>PO<sub>4</sub>, 0.1 MgSO<sub>4</sub>.7H<sub>2</sub>O, 5.0 yeast extract, 2.0 peptone (pH 5.0) were inoculated with three disks (1.5 cm diameter x 2mm height) of the mycelium grown on the PDA. The fermentation was carried out in a rotary shaker at 100 rpm for 240 h at 45 °C. The biomass was separated by filtering through Whatman N° 1 paper in a Büchner funnel. The filtrate was centrifuged (10 min., 10,000 x g) and used to evaluate the extracellular

glucoamylase activity. The experiments were performed in duplicate with three repetitions and results presented as means.

### Enzyme activity measurements

Enzyme activity was assayed at 60 °C in a reaction mixture containing 0.1 mL of diluted crude enzyme solution and 0.4 mL of substrate solution in 0.25 M sodium acetate buffer pH 5.0. The substrates used were 0.5% soluble starch or 0.3 % maltose. The amount of glucose released was estimated by the peroxidase/glucose oxidase assay (Cereia et al., 2000). One unit of enzyme activity (U) was defined as the amount of enzyme that releases one µmol of glucose per minute per mL of reaction. Substrate-specific assays were performed by substituting the substrate above by the raw and soluble cassava, potato and corn starches or maltooligosaccharides. When the substrate was *p*-nitrophenyl-β-D-glucopyranoside, the activity was measured in a mixture containing 0.20 mL of 0.1 M sodium acetate pH 5.0, 0.05 mL of 2 mM substrate solution, and 0.1 mL of crude enzyme. After 10 min. of incubation at 70 °C, the reaction was stopped with 1 mL of 2 M Na<sub>2</sub>CO<sub>3</sub>, and the *p*-nitrophenol released was quantified spectrophotometrically at 410 nm.

### Enzyme characterization

*Optimum pH and temperature for enzyme activity:* The optimum pH was determined by measuring the activity at 60 °C, with various buffers: sodium acetate (pH 3.0-5.5), citrate-phosphate (pH 5.5-7.0) and Tris-HCl (pH 7.0-8.5). The optimum temperature was assayed by incubating each reaction mixture at optimum pH and 40-85 °C. The reaction mixture was the same described above.

*Thermostability:* A thin layer of mineral oil prevented the evaporation of the 1 mL of crude enzyme solution which was incubated at various temperatures (10 -90 °C) for 1 h at pH 5.0. Another experiment was carried out to assay the enzyme stability at 60 °C. The enzyme solution was maintained at this temperature for a period of 10 h and in the control used to evaluate possible starch and maltose chemical hydrolysis, the crude enzyme was replaced by water. In both the assays an aliquot was withdrawn and placed on ice before measuring the residual enzyme activity at the optimum pH and temperature.

*pH stability:* The crude enzyme was dispersed (1:1) in 0.1 M buffer solutions pH 3.0-8.0

(McIlvaine buffer) 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

Fungal growth was recorded in terms of dry and moist weight and total cell protein. The mycelia from 50 mL fermented medium was filtered in a Büchner funnel, washed with chilled deionized water, filtered under vacuum and its moist weight was determined. The dry weight was determined after drying the same mycelium at 65 °C till constant weight. The intracellular total protein and glucoamylase were extracted from the moist mycelium by rinsing with chilled water, freezing and crushing with glass beads on a vortex. Proteins were extracted from the disrupted cells with 100 mM 1-1 acetate buffer, pH 5.4, at 4 °C, and the crude extract was centrifuged (12,000 x g for 15 min, 4 °C). Protein concentration was determined by the Hartree (1972) method. Reducing sugar was quantified by the DNS method (Miller, 1959).

## RESULTS AND DISCUSSION

### Selection of strains with amylolytic activity

Out of 20 thermophilic fungi isolated from the material obtained in 20 collecting trips (200 samples), five *Thermomyces* strains were selected for their ability to grow at 45 °C on a liquid medium containing starch as the sole carbon source and to produce considerable glucoamylase activity under these conditions. The strain was identified as *Thermomyces lanuginosus* TO3, which secreted high glucoamylase activity, was selected in this work.

### Glucoamylase (GA) production in SmF

Glucoamylase activity in the fermentation medium maximum (extracellular) was between 168 and 192 h, with a maximal activity of 13 U mL<sup>-1</sup> when soluble starch was used as substrate for enzyme activity assay, and 6 U mL<sup>-1</sup>, when the substrate was maltose. The data in Fig. 1a indicated that GA activity preferentially hydrolyzed starch and exhibited lower maltase activity (Fig. 1a).

When evaluated in terms of the cell protein, the mycelial production reached a maximum at 48 h (Fig. 2b). The hyphae accumulated the protein

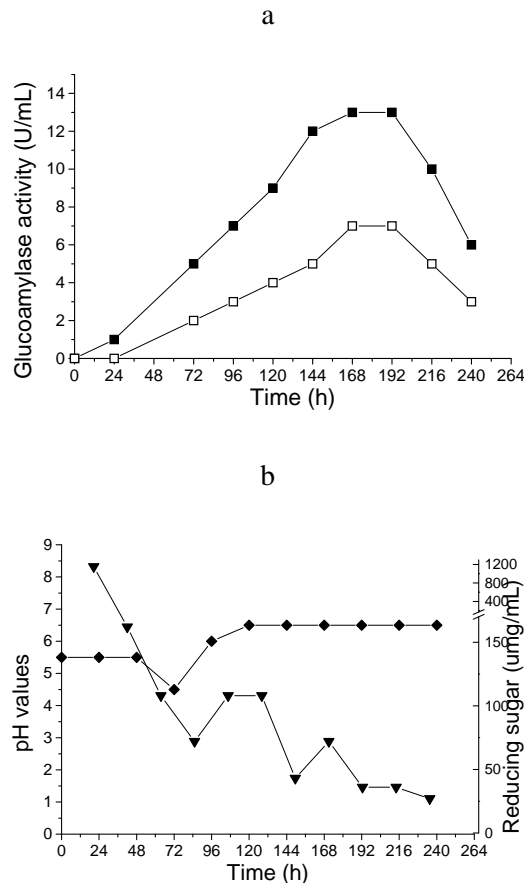
during the log phase, which was then released or degraded after 72 h of fermentation. When moist biomass was the parameter for measuring the growth-rates, the peak occurred between 48 and 72 h. However, when the growth was assessed as dry weight, the peak was observed at 72 h. The decrease in the moist biomass and protein was significant at 96 h (more than 50%), dry weight decreased only 25%; nevertheless, the fall in the cell protein content was not correlated with a rise in the extracellular protein (Fig 2c). These results could not be attributed to hyphal rupture or proteolytic action since the hyphae remained intact up to 168h (according to microscopic analysis), and no significant protease activity was detected in the medium (data not shown). These data demonstrated that the evaluation of the fungal growth by measuring the cell protein may not be accurate in all cases.

Comparing the extracellular GA activity (Fig. 2c) with the biomass production by *T. lanuginosus* TO3 (Fig 2b) showed that most of the enzyme was released into the medium after the log phase. This result disagree with those reported for *A. niger* in which secretion of GA and production of total protein, in similar experiment conditions, occurred mainly in the growth phase (Aalbaek et al., 2002; Papagianni and Moo-Yung, 2002).

For the purpose of comparing the extracellular and cell-associated GA activities, the amount of enzyme has been expressed in units per mycelial dry weight and units per total protein (mg) in the medium and in the cell, respectively. The fermentation procedure was the same described in material and methods. Both the cell-associated and extracellular GA production exhibited two peaks (Fig. 2a and 2c). The first peak of cell-associated GA occurred at 72 h growth, decreasing to a minimum at 96 h, exactly when the first peak of the extracellular GA occurred. The same was observed for the second peak: cell-associated GA was maximum at 168 h and extracellular GA at 192 h of fermentation, when cellular activity was declining. These data suggested a coordinated control of the activation and secretion of cell associated GA throughout the fermentative process. Since the method used for the extraction of the cell protein did not permit the separation of enzyme present in the cytoplasm or associated with the cell wall, it was not possible to infer a possible mechanism of activation of the enzyme in

the intracellular or periplasmatic compartments. It was not inconceivable that the protein synthesis occurred in the stationary phase, therefore the assumption of the post-translational modification

effecting enzyme activation of the cell-associated enzyme and its secretion might then explain the observed pattern (Wallis et al, 1999).



**Figure 1** - Glucoamylase production by *Thermomyces lanuginosus* TO3 in submerged fermentation. a) Filled square = glucoamylase activity on soluble starch; open square = glucoamylase activity on maltose; b) full triangle= reducing sugar ( $\mu\text{g/mL}$ ) in SMF; diamond=pH variation in SmF medium.

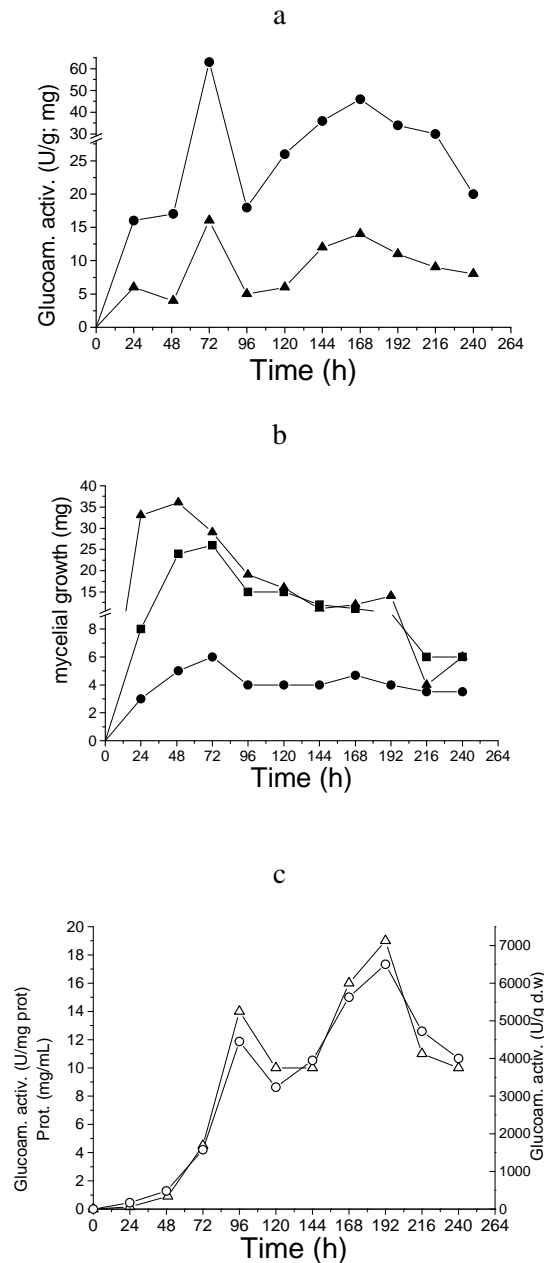
Two peaks of extracellular and cell-associated GA suggested the presence of GA isoforms. The majority of the published data about the GA isoforms refer to extracellular enzymes. Data on GA from *Aspergillus* species seem to be concerned mainly with the processing of the enzyme in the culture medium by proteases or enzymatic deglycosylation (Paszczyński et al., 1985; Neustroev et al., 1993; Nascimento et al., 1998; Dubey et al., 2000; Suthirak et al., 2005), although synthesis from different mRNA derived from one primary transcript has been considered (Boel et al, 1984). Also, specific production of different forms

of GA in the culture medium of *A. awamori* depending on the growth phase has been proposed (Hayashida et al., 1988).

In Fig. 1b, the pH of the culture medium is seen to drop from an initial 5.5 to 4.2 within 72 h and then to rise to 6.5 at 96 h of fermentation, remaining unchanged thereafter. Several authors have reported that the fungal protein secretion required glycosylation as part of secretion machinery and that the regulation of glycosylation was pH-dependent (Wallis et al., 2001; Conesa et al., 2001). Besides this it has been demonstrated that GA isoforms from *A. niger* were modified by the

extracellular acid proteases after the pH of the culture medium was lower from 5.0 to 3.0 and that this acidification induce the secretion of acid proteases (Aalbaek et al., 2002). A possible influence of the pH of the medium in activation/secretion processes can not be

discarded. Although the data presented suggested the presence of control mechanisms for the activation of cell-associated GA and the secretion of this enzyme throughout the fermentative process, at this stage further research wont be required.



**Figure 2** - Evaluation of intra and extra-glucoamylases production by *Thermomyces lanuginosus* TO3 in SmF. a) filled triangle = intracellular glucoamylase activity (U/mg cell protein); filled circle= intracellular glucoamylase activity (U/g dw);b) filled square = moist weight (mg/mL of medium); filled circle = drying weight (mg/g dw); filled up triangle= intracellular protein (mg/g d.w);c) opened circle: extracellular glucoamylase activity (U/g dw); opened triangle = extracellular glucoamylase activity (U/mg protein of the medium).

### Enzyme characterization: substrate specificity and physico-chemical properties

The substrate-specificity results presented in Fig. 3 confirmed that the enzyme acted preferentially on starch, compared to maltose and maltooligosaccharides. Soluble starch was hydrolyzed better than the raw starch, although the

enzyme activity detected on the raw starch was still appreciable. However, the hydrolysis of  $p$ -nitrophenyl- $\alpha$ -D-glucopyranoside by this enzyme was very slow and not was observed activity on sucrose.

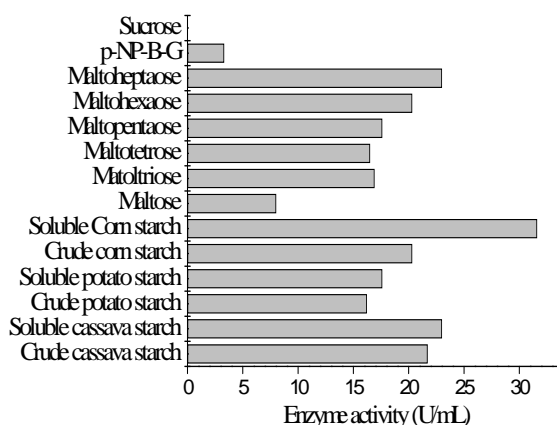


Figure 3 - Glucoamylase activity on different substrates.

The glucoamylase activities in the crude filtrates obtained from *T. lanuginosus* TO3 exhibited different pH and temperature optima, according to the substrate used. The optimum activity on maltose was observed at pH 4.5 and that on starch, at pH 5.5, while the temperature optima were 70 °C and 65 °C on starch and maltose, respectively (Table 1).

This variation in the catalytic properties of the crude enzyme as a function of substrate could reflect the presence of a mixture of enzymes. According to Chiba (1997) two exo-amylases were capable to release glucose from the starch or maltooligosaccharides: glucoamylase and  $\alpha$ -glucosidase or maltase. The first acts preferentially on starch while the second hydrolyses the soluble starch very slowly, acting preferentially on maltose and maltooligosaccharides (Saha and Zeikus, 1989). The presence of both the enzymes has been reported in the culture media of *A. awamori*, *A. niger*, *A. oryzae* and *A. flavus* (Hayashida et al., 1988, James and Lee, 1997; Negret et al., 1999; Gomes et al., 2005). However, Tosi et al. (1993) reported that a purified glucoamylase from *H. grisea* showed considerable maltase activity, and that the optima pH, temperature profile and also the activation energies for hydrolysis were different for the

starch and maltose substrates. Similar characteristics were described for a glucoamylase from *Scytalidium thermophilum* (Cereia et al., 2000). This pattern of activity in glucoamylase has been attributed to the existence of a common catalytic site for starch and maltose hydrolysis, but specialized subsites for each of these substrates. The presence of various subsites was also demonstrated for glucoamylase from *Mucor javanicus* and *A. niger*. These enzymes hydrolyse maltose and soluble starch at a single active site and have extended subsites that bind the maltooligosaccharide firmly on the bond-cleavage site; however, the smallest substrate, the dimmer maltose, would be bound in a non-productive configuration and would be hydrolysed slowly compared to larger maltooligosaccharides (Sugimoto et al., 1994; Sugimoto et al., 2003; Swift et al., 1998, Kaia et al., 1991). The present results supported the idea that *T. lanuginosus* TO3 produce one enzyme with a preference for soluble starch, but it was not possible to infer whether the fungus produced glucoamylase and  $\alpha$ -glucosidase. Further investigations involving enzyme purification are required.

The optimum pH found for the *T. lanuginosus* TO3 glucoamylase (5.5) agree with those reported for GA from other strains of *Thermomyces lanuginosus*, whose pH optima were determined in the acid- neutral range, such as those from *T. lanuginosus* ATCC 34626, with optimal pH of 4.5-5.6 (Nguyem et al., 2002), *T. lanuginosus* ATCC 44008 and *T. lanuginosus* ATCC 28083 with pH 4.5 (Ronaszéki et al., 2000), *T. lanuginosus* A13.37 with pH 4.0 (Gomes et al., 2005) and *T.*

*lanuginosus* and *T. lanuginosus* K13/1 (*Humicola lanuginosa*) with pH 6.6 (Cereia et al.,2000).

The thermostability of the enzyme was high, with preservation of 100% of the initial activity after 1 h at 60 °C in the absence of substrate and the half-life of the enzyme was 6h. The enzyme was stable over a wide pH range, with 100% stability between at values of 4.0-10.0 (Table 1).

**Table1** - Properties of crude glucoamylase produced by *Thermomyces lanuginosus* TO3 in SmF, on soluble starch and maltose as substrates.

Enzyme Properties	Substrate	
	Soluble starch	Maltose
Optimum pH	5.5	4.5
Optimum temperature (°C)	70	65
Half- life at 60°C (h)	6.0	6.0
pH range which preserving 100% of initial activity for 1h	4.0-10.0	4.0-10.0
Temperature range which preserving 100% of initial activity for 1h.	20-60	20-60

In comparison with the thermal characteristics of known glucoamylases, the enzyme differed in the thermal stability and optimum temperatures (65 and 70 °C) from several mesophilic fungal species, whose optimum temperatures were between 40 and 50 °C, e.g. glucoamylases from *Aspergillus* and *Rhizopus* strains described as susceptible to denaturation at temperatures above 60 °C (Ali and Hossaim, 1991). The enzymes thermostability were similar to glucoamylases from thermophilic fungi such as *Talaromyces duponte* and *H. grisea* and other *T. lanuginosus* strains that had optima activity at 75, 60 and 70 °C, respectively (James and Lee, 1997). Since any industrial process is based on the use of crude or partially purified enzymes, it is important to determine the optimum temperature for the activity and thermostability under these conditions.

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## RESUMO

O fungo termofílico *Thermomyces lanuginosus* TO3 foi isolado a partir de amostras de material de pilhas de compostagem, com base em sua capacidade de crescer em meio líquido contendo amido como única fonte de carbono, a 45 °C, e produzir considerável quantidade de glucoamilase. A produção da enzima por fermentação submersa FSm foi alta, com um máximo de 13 U/mL em 168 h de fermentação. A atividade enzimática foi

maior sobre amido do que sobre a maltose e maltooligosacarídeos. As atividades de glucoamilase extra e intracelular variaram com o tempo de fermentação. A glucoamilase produzida por *T. lanuginosus* TO3 apresentou elevada temperatura ótima de atividade (65 - 70 °C) com boa termoestabilidade em ausência de substrato, apresentando uma meia vida de 6 h a 60°C, além de estabilidade em ampla faixa de pH. Os resultados apresentados indicam uma importante fonte alternativa de glucoamilase para uso no processamento industrial de amido.

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