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PRODUCTION OF THERMOSTABLE GLUCOAMYLASE BY NEWLY ISOLATED *ASPERGILLUS FLAVUS* A 1.1 AND *THERMOMYCES LANUGINOSUS* A 13.37

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ABSTRACT

Thirteen thermophilic fungal strains were isolated from agricultural soil, tubers and compost samples in tropical Brazil. Two strains were selected based on of their ability to produce considerable glucoamylase activity while growing in liquid medium at 45ºC with starch as the only carbon source. They were identified as *Aspergillus flavus* A1.1 and *Thermomyces lanuginosus* A 13.37 Tsiklinsky. The experiment to evaluate the effect of carbon source, temperature and initial pH of the medium on enzyme production was developed in a full factorial design (2x2x3). Enzyme productivity was influenced by the type of starch used as carbon source. Cassava starch showed to be a better substrate than corn starch for glucoamylase production by *A. flavus* but for *T. lanuginosus* the difference was not significant. Enzyme activities were determined using as substrates 0.3% soluble starch, 0.3% maltose or 0.3% of starch plus 0.1% maltose. The enzymes from *A. flavus* A1.1 hydrolyzed soluble starch preferentially but also exhibited a significant maltase activity. Moreover higher quantities of glucose were released when the substrate used was a mixture of starch and maltose, suggesting that this fungus produced two types of enzyme. In the case *T. lanuginosus* A 13.37, the substrate specificity test indicated that the enzyme released also hydrolyzed starch more efficiently than maltose, but there was no increase in the liberation of glucose when a mixture of starch and maltose was used as substrate, suggesting that only one type of enzyme was secreted. Glucoamylases produced from *A. flavus* A1.1 and *T. lanuginous* A.13-37 have high optimum temperature (65ºC and 70ºC) and good thermostability in the absence of substrate (maintaining 50% of activity for 5 and 8 hours, respectively, at 60ºC) and are stable over in a wide pH range. These new strains offer an attractive alternative source of enzymes for industrial starch processing.

Key words: Glucoamylase, α-glucosidase, *Aspergillus, Thermomyces*

INTRODUCTION

The enzymes that hydrolyze starch can be divided into endoamylases, exoamylases and debranching amylases.

The endoamylases (EC 3.2.1.1) cleave α-1,4 glycosidic bonds in amylose, amylopectin and related polysaccharides but not α-1,6 linkages. The products of hydrolysis are oligosaccharides of varied chain lengths and have a α-configuration on the C_1 of the reducing glucose unit produced (8).

The exoamylases act preferentially on α-1,4 linkages from the nonreducing end, successively, resulting in low molecular weight products. Currently two types of exoamylases are important for starch hydrolysis. Glucoamylase (EC 3.2.1.3) of fungal origin attacks α-1,4 bonds, releasing D-glucose molecules in the ß-configuration; this enzyme also attacks α-1,6 bonds at branching points in the amylopectin molecule, but much more slowly than α-1,4 linkages. α-Glucosidase (EC 3.2.1.20) catalyzes the splitting of α-D-glucosyl residues from the nonreducing end of substrates to release α-glucose (9); ß-amylase (EC 3.2.1.2) of plant or bacterial origin acts in an exo-fashion proceeding, from the nonreducing ends of the outer starch chains or related polysaccharides, gradual removal the romoving maltose units in the ß-anomeric form (6).

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Debranching enzymes include pullulanase (EC 3.2.1.41) which acts specifically on α-1,6 linkages in pullulan, starch, amylopectin and related oligosaccharides and isoamylase (EC 3.2.1.68), that hydrolyzes α-1,6 linkages in amylopectin, but has a very low activity or no activity toward pullulan (8).

In industrial starch syrup production, the starch is liquefied in a first stage at 105-107ºC for 5-15 min, followed by a second stage for 60-180 min at 95-98ºC. Thermostable α-amylases are used for starch hydrolysis in this liquefaction step. Upon completion of liquefaction, the slurry has to be cooled at 55-60ºC before the saccharification step, since the glucoamylase from *Aspergillus niger* used in this stage is unstable at temperatures above 60ºC (2; 17).

High value is placed on the extreme thermostability and thermoactivity of amylases used in the starch bioprocessing. Thus, for industrial purposes, new highly thermostable and environmentally compatible glucoamylases may make an important contribution to the starch processing industry.

On the other hand, the discovery, isolation, and application of different amylolytic enzymes have resulted in the development of new hydrolyzed starch products and in a wide variety of different syrup properties, which can be exploited in many applications.

The objectives of this study were to evaluate glucoamylase production in various culture conditions from newly-isolated thermophilic fungi and the physico-chemical characterization of these enzymes.

MATERIALS AND METHODS

Isolation and identification of microorganisms

Two g samples of agricultural soil, potato tubers, compost and animal manure were homogenized in sterile medium of pH 5.0 containing 1.0% soluble starch; 0.14% $(NH_4)_2SO_4$; 0.20% K_2HPO_4; 0.02% $MgSO_4.7H_2O$; 1.60 mg/L $MnSO_4.H_2O$; 1.40 mg/L $ZnSO_4.7H_2O$; 2.0 mg/L $CoCl_2$. After incubation at 45ºC for 24 h, a loop of the homogenized culture was streaked on the surface of the same medium containing 3.0% agar and incubated at 45ºC for 24 to 72 h. All morphologically contrasting colonies were purified by repeated streaking. Pure cultures were sub-cultured on slants of the same medium for identification and enzyme studies. The identification of the fungi was based on morphological and biochemical characteristics (12). The stock cultures were maintained at 7ºC on Potato Dextrose Agar medium (PDA).

Production of amylases in submerged fermentation (SmF)

Erlenmeyer flasks with 25 mL of medium containing 1.0% carbon source, 0.14% $(NH_4)_2SO_4$, 0.60% K_2HPO_4, 0.20% KH_2PO_4, 0.01% $MgSO_4\ 7H_2O$, 0.50% yeast extract, 0.20% peptone, 0.20% beef extract (pH 5.0) were inoculated with aliquots of mycelial suspensions (approx. 5.0 mg/mL) of *Thermomyces* or spore suspensions (10^6 spores/mL) of *Aspergillus*, obtained from 7-day agar slant cultures suspended in sterile 0.01% Tween 80 solution. The fermentation was carried out in a rotary shaker at 100 rpm for 96 h at the temperatures described below. The biomass was separated by centrifugation at 10000 g for 20 min and the supernatant was used to evaluate amylase activities.

Effect of carbon source, initial pH of the media and temperature of incubation on enzyme production

Corn or cassava starches (1.0%) were used as carbon sources. The media pH was adjusted to 4.0, 5.0 or 6.0 and incubation temperatures were 40 or 45ºC. The experiment was developed in a full factorial design (2x3x2) with three repetitions. For statistical analysis (ANOVA and Tukey test) the face software Estat was used.

Enzyme activity measurement

Enzyme activity was assayed at 60ºC in a reaction mixture containing 0.5 mL of diluted crude enzyme solution and 0.5 mL of substrate solution in 0.25 M sodium acetate buffer pH 5.0. The substrates used were 0.3% soluble starch, 0.3% maltose or a mixture of 0.3% soluble starch plus 0.1% maltose (16). The amount of glucose released was estimated by the peroxidase/glucose oxidase assay (4). One unit of enzymatic activity (U) was defined as the amount of enzyme that releases one μmol of glucose per minute per mL of reaction.

Enzyme characterization

Optimum pH and temperature for enzyme activity: The optimum pH was determined by measuring activity at 60ºC 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). The optimum temperature was assayed by incubating each reaction mixture at 40ºC up to 90ºC.

Thermostability: a thin layer of mineral oil prevented evaporation of the crude enzyme solution which was incubated at various temperatures (10-90ºC) for 1 h at pH 5.0. Another experiment was carried out to assay the enzyme stability at 60ºC. The enzyme solution was maintained at this temperature for a period of 9 h. A control to evaluate a possible maltose hydrolysis was carried out using water instead crude enzyme. In both assays an aliquot was withdrawn and placed on ice before assayed for residual enzyme activity at the optimum pH and temperature.

pH stability: Crude enzyme was dispersed (1:1) in 0.1 M buffer solutions pH 3.0-5.0 (sodium acetate), pH 5.0-7.0 (citrate-phosphate), pH 7.0-8.5 (tris-HCl) and pH 8.5-11.0 (glycine-NaOH) and maintained at 25ºC for 24 h. An aliquot was used to determine the remaining activity at the optimum pH and temperature.

RESULTS AND DISCUSSION

Selection of strains with amylolytic activity

Among the 13 thermophilic fungi isolated from the tested materials, two strains were selected for their ability to grow at 45ºC on a liquid medium containing starch as the only carbon source and to produce considerable glucoamylase activity under these conditions. These strains were identified as *Aspergillus flavus* A1.1 and *Thermomyces lanuginosus* A13.37.

Effect of carbon source, media initial pH and temperature of incubation on glucoamylase production in submerged fermentation

The statistical analysis indicated that variables carbon source, pH and temperature influenced significantly the enzyme production. The type of starch source used as carbon source influenced the glucoamylase production by *A. flavus* A1.1, which was higher (with statistical significance at 1% level) in media containing cassava starch than in those with corn starch (Fig. 1a-f). No statistical difference was observed in the amounts of enzyme synthesized by *T. lanuginosus* A13-37 on the two carbon sources, but it can be observed that enzyme activity was higher in the medium containing cassava starch (Fig 2a-f). Since there are differences in the amylose and amylopectin composition and lipid contents of these starches (3,5) (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 the starch could affect the enzyme induction. Cruz *et al*. (7) observed that the ability of *Rhizopus* sp to produce amylolytic enzymes that liberate reducing units was higher when the carbon source was amylose rather than to amylopectin, and that corn amylose was a better inducer of enzyme than its homologue from potatoes.

Analysis of Fig. 1 indicated that enzyme synthesis by *A. flavus* A1.1 grown on cassava starch was significantly higher when the initial pH of the culture medium was 6.0 (5% level by Tukey test). However the enzyme activity was higher when the initial pH of the culture medium was 4.0 than when it was 5.0 (Fig. 1c to 1f), indicating that two forms of enzyme may have been produced. Enzyme synthesis by *T. lanuginosus* A.13.37 was higher at pH 5.0 and 6.0 than at pH 4.0 for both substrates.

Considering that the total growth of the microorganisms was significantly not affected by the pH of the media (data not shown), the effect of pH must be related to mechanisms regulating enzymatic synthesis and secretion. A similar effect of pH on production and secretion of glucoamylase (GA) by *A. niger* was described by Wallis *et al*. (22). They demonstrated that GA production was higher at pH 4.0 than at pH 5.5. Since enzyme activity was not correlated with biomass production, the authors speculated that pH and the high level of produced gluconic acid controlled some aspects of enzyme production. Characterization of the pH signal transduction pathway in *A. nidulans* has been completed at the molecular genetics level, although the biochemical functions of the proteins involved still have yet to be elucidated (10).

Regarding the incubation temperature no statistically significant differences were detected in the enzyme synthesis when fermentation was carried out at 40 or 45ºC, although higher activities were observed in some assays at 40ºC (Figs. 1 and 2).

To test the substrate-specificity, enzyme activities were determined with 0.3% soluble starch, 0.3% maltose or 0.3% starch plus 0.1% maltose as substrate. The results shown in Fig. 1 indicated that the enzymes produced by *A. flavus* A1.1 preferentially hydrolyzed starch (significantly 1 % level) and exhibited a low maltase activity. On the other hand, crude enzyme released the highest quantities of glucose when the substrate used was a mixture of starch and maltose (Fig. 1e, 1f).

In the case of enzymes from *T. lanuginosus* A 13.37, the data of Fig. 2 indicate that the enzyme was able to hydrolyze starch more efficiently than maltose, but differently from *A. flavus* enzyme its activity on maltose was considerable. Moreover, there was no increase in the glucose liberated when a mixture of starch and maltose was used as substrate.

Although α-glucosidase and glucoamylase are essentially differentiated by the produced anomer (α-glucosidase produces α-glucose and glucoamylase, β-glucose (6)), according to Saha and Zeikus (16) the α-glucosidase activity can be determined in the presence of glucoamylase by comparing the results of incubating the enzyme with starch or with starch plus maltose. The higher yield of glucose from the second substrate gives evidence of the presence of α-glucosidase. In this way, the results indicated that both glucoamylase and α-glucosidase may be secreted into the cultivation medium by *A. flavus*. The presence of both enzymes has been reported in culture media of *A. awamori, A. niger* and *A. oryzae* (18,19,20) and it was determined that the α-glucosidase from these fungi hydrolyses not only maltose and malto-oligosaccharides, but also hydrolyses soluble starch very slowly, while glucoamylase is a starch-hydrolase capable of also degrading starch (6).

On the other hand, Tosi *et al.* (21) reported that a purified glucoamylase from *H. grisea* showed considerable maltase activity, however the optima pH, profiles response temperature and also the activation energies for hydrolysis of starch and maltose were different. Similar characteristics were described for glucoamylase from *Scytalidium thermophilum* (4). 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 in the enzyme was also demonstrated for α-glucosidase from *Mucor javanicus* and *A. niger. M. javanicus* α-glucosidase has high activity on maltooligosaccharide and on soluble starch. This enzyme

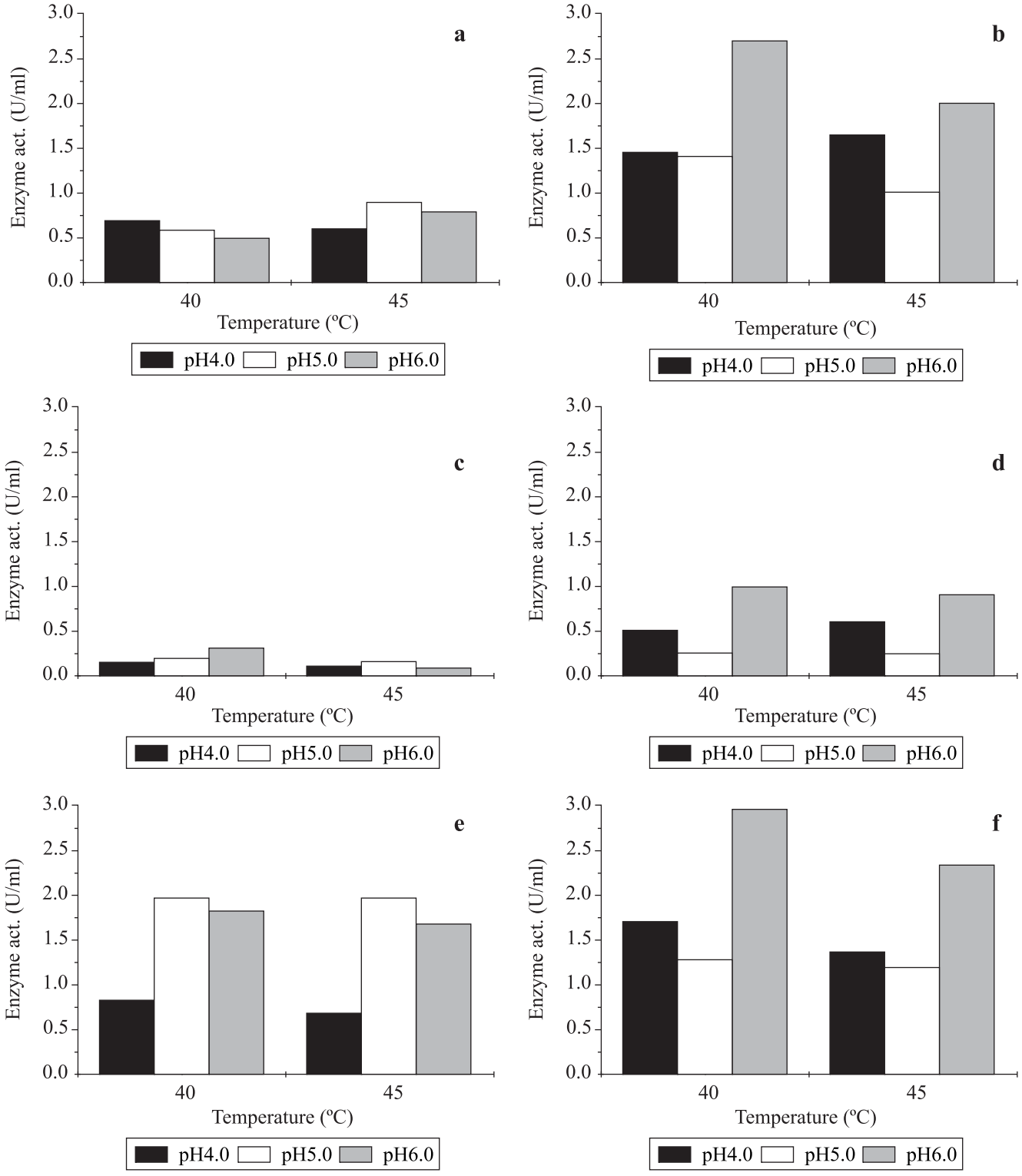

Figure 1. Effect of pH, temperature and carbon source as a function of temperature on the glucoamylase production by *Aspergillus flavus* A1.1 determined with 0.3% starch (a, b), 0.3% maltose (c, d) and 0.3% starch plus 0.1% maltose (e, f). Culture media were supplemented with 1% corn starch (a, c, e) or 1% cassava starch (b, d, f).

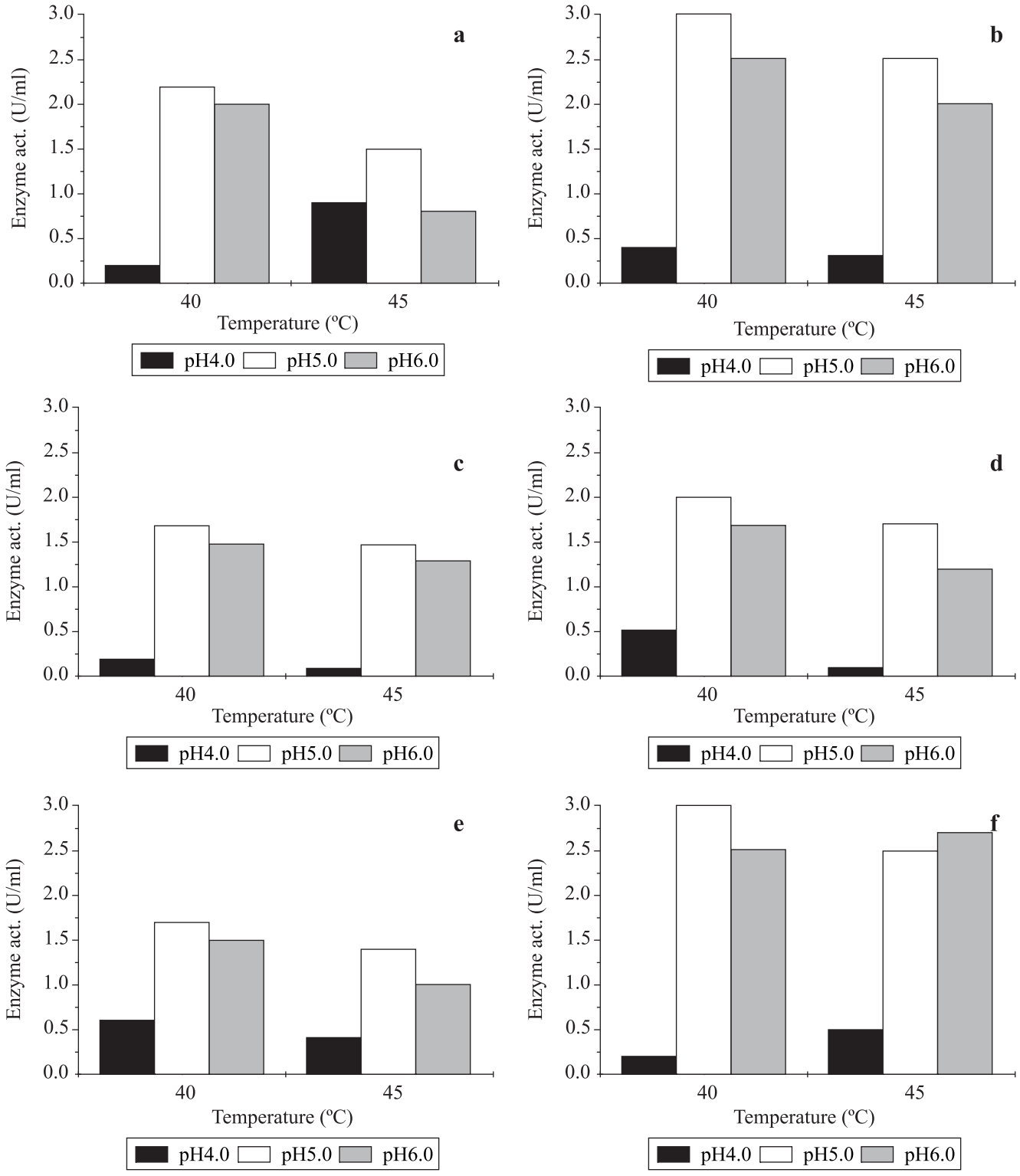

Figure 2. Effect of pH, temperature and carbon source as a function of temperature on the glucoamylase production by *Thermomyces lanuginosus 14.37* determined with 0.3% starch (a, b), 0.3% maltose (c, d) and 0.3% starch plus 0.1% maltose (e, f). Culture media were supplemented with 1% corn starch (a, c, e) or 1% cassava starch (b, d, f).

hydrolyses maltose and soluble starch at a single active site and has six subsites, which are more extended than other described subsites. This extended subsite structure binds the maltoligosaccharide firmly on the bond-cleavage site; however, the smallest substrate, maltose, would be bound in a non-productive configuration and would be slowly hydrolyses when compared with larger maltooligosaccharides (18). In α-glucosidase from *A. niger*, three subsites have been determined; this enzyme preferentially hydrolyses maltooligosaccharide and maltose and hydrolyses soluble starch slightly (10). This difference in substrate specificity is due to subsite structure (19).

Our results support that *A. flavus* produces two different enzymes while *T. lanuginosus* seems to produce one enzyme with a preference for soluble starch, but it is not possible to infer whether it is a glucoamylase or α-glucosidase. Further investigations are required.

Enzyme characterization

The glucoamylase activities in the crude filtrates obtained from *Aspergillus flavus* and *Thermomyces lanuginosus* cultivation were characterized in terms of pH and temperature optima and stability. The enzyme from *A. flavus* exhibited maximum activity at pH 4.0. Approximately 86% of this activity was observed at pH 3.5 and 57% at pH 7.0, indicating the enzyme showed activity over a wide pH range (Fig. 3a). The enzyme showed optimum activity at 65ºC and 40% of this activity at 70ºC.

Enzyme from *T. lanuginosu* showed maximum activity at pH 4.5 and 67% of this activity at pH 4.0 and at 5.5 (Fig. 3a). Optimum temperature for its activity was determined to be 70ºC and the enzyme showed 67% of maximum activity at 75ºC (Fig. 3b).

As illustrated in Fig. 3a, enzyme from *A. flavus* was stable over wide pH range, with 100% stability at values of 5.0-9.0 and 80% of this activity retained at pH 3.0 and 90% at pH 10.0. Glucoamylase from *Thermomyces* was more stable at pH 7.0-8.0, retaining 55% of its activity at pH 3.0 and 75% at pH 10.0.

Enzyme produced by *Aspergillus* lost only 20% of the initial activity after 1h at 60ºC with total inactivation at 80ºC, while glucoamylase from *Thermomyces* showed an enhanced of 30% in relation to the original activity, after being maintained at 50ºC for 1h. After 1h at 60ºC, the activity of enzyme was 20% higher than the initial activity.

The thermostability of enzymes at 60ºC was high. The enzyme from *Aspergillus* retained 85% of this activity after 1 h and 45% after 7 h at this temperature. The glucoamylase produced by *Thermomyces* increased its activity (20%) when maintained for 1h at 60ºC. After 8 h at 60ºC, 55% of the initial activity was observed (Fig. 3c).

The optimum pH found for the *Aspergillus* glucoamylase is comparable to those from other *Aspergillus* species which have optimum activity in the pH range 4.0-5.5 such as the enzymes from *A. terreus* and *A. tamari* (pH 4.0) and *A. niger* (pH 5.0)

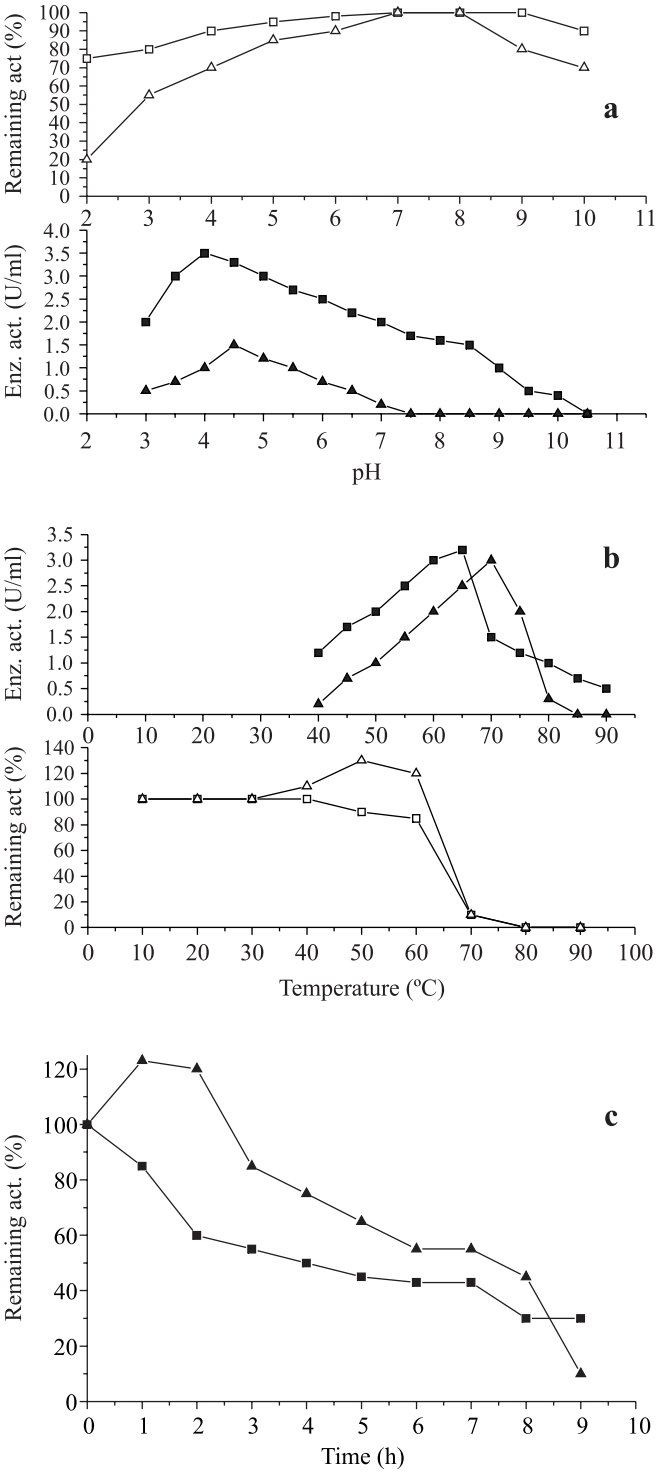

Figure 3. Physico-Chemical characterization of enzymes produced by *A. flavus* (square) and *T. lanuginosus* (up to triangle): Effect of pH (a) and temperature (b) on the enzymes stability in absence of substrate (open symbol) and activity in presence of substrate (full symbol); stability of enzyme at 60ºC (c).

(1; 13). However, for *Thermomyces* species, glucoamylases have been described with which neutral pH optimum, such as those from *H. grisea* (pH 6.0) and *H. lanuginosus* (pH 6.6) (4).

In comparison with the thermal characteristics of known glucoamylases, the enzymes differed in terms of their thermal stabilities and optimum temperatures (65 and 70ºC) from those of several mesophilic fungal species whose optimum temperatures are between 40 and 50ºC, e.g. glucoamylases from *Aspergillus* and *Rhizopus* strains which are described as susceptible to denaturation at temperatures above 60ºC (9,20). The enzymes were similar to glucoamylases from thermophilic fungi such as *Talaromyces duponte, Thermomyces lanuginosus* and *H. grisea* that have optima at 75, 70 and 60ºC, respectively (15).

Since any industrial process is based on the use of crude or partially purified enzymes, it is important to determine the optimum temperature for activity and thermostability under these conditions. Although α-amylases used in the commercial production of sugar syrups from starch are sufficiently stable at the high operating temperatures, the enzymes employed for saccharification and for debranching of amylopectin are more thermolabile. Thus, glucoamylases with greater thermostability and active at higher temperatures are required.

Besides thermostability, the stability and activity over a wide pH range are important properties of the enzymes produced by the strains studied in this work. During saccharification, the pH of the liquefied slurry is adjusted down to 4.2-4.5, to move it closer to pH optimum for *Aspergillus niger* GA. Using enzymes that function over a wide pH range would eliminate this adjustment step.

In conclusion, glucoamylases produced from *A. flavus* A1.1 and *T. lanuginosus* A.13-37 have high optimal temperatures (65ºC and 70ºC) with good thermostability in the absence of substrate (they maintained 50% of activity for 5 and 8 h respectively at 60ºC) and stable activity over a wide pH range. With these characteristics they offer an attractive alternative source of enzymes for industrial starch processing.

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RESUMO

Produção e glucoamilase por *Aspergillus flavus* A1.1 e *Thermomyces lanuginosus* A13.37

Entre 13 linhagens de fungos filamentosos isolados a partir de amostras de solo agrícola, tubérculos e de material em compostagem, duas foram selecionadas em função da capacidade de crescer em meio líquido contendo amido como única fonte de carbono, a 45ºC, e produzir consideráveis quantidades de glucoamilase. Essas linhagens, identificadas como *Aspergillus flavus* A1.1 e *Thermomyces lanuginosus* A13.37, foram utilizadas para desenvolvimento de experimentos para avaliar os efeitos do tipo de amido (milho e mandioca), do pH inicial do meio de cultura (4,0; 5,0 e 6,0) e da temperatura de incubação (40 e 45ºC), em um modelo fatorial (2x3x2), sobre a produção da glucoamilase. O tipo de amido usado como fonte de carbono para o cultivo dos fungos influenciou significativamente a produção de glucoamilase por *A. flavus*, sendo obtida uma maior quantidade da enzima em meio contendo amido de mandioca do que em meio com amido de milho. A produção da enzima por *T. lanuginosus* também foi maior em meio contendo amido de mandioca, porém, a diferença não foi estatisticamente significativa. As atividades enzimáticas sobre amido (0,3%), maltose (0,3%) ou sobre mistura de 0,3% de amido com 0,1% de maltose, indicaram que as enzimas de *Aspergillus* hidrolisaram, preferencialmente, o amido, embora tenham mostrado atividade considerável sobre a maltose. A maior liberação de glicose a partir da mistura de substratos sugeriu que o fungo em questão possa secretar dois tipos diferentes de enzimas. Enzimas produzidas por *T. lanuginosus* hidrolisaram o amido e a maltose e não liberaram maiores teores de glicose quando o substrato constou de mistura de amido e maltose. As enzimas de *Aspergillus* e *Thermomyces* apresentaram elevada temperatura ótima de atividade (65 e 70ºC, respectivamente) com boa termoestabilidade na ausência de substrato (manutenção de 50% da atividade por 5 e 8h respectivamente), 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.

Palavras-chave: Glicoamilase, α-glicosidase, *Aspergillus*, *Thermomyces*

REFERENCES

1. Ali, S.; Hossain, Z. Characteristics of glucoamylase from *Aspergillus terreus*. *J. Appl. Bacteriol.*, 71: 144-146, 1991.
2. Brumm, P.J. Enzymatic production of dextrose. *Cereal Food World*, 40: 804-804-807, 1998.
3. Cereda, M.P. Propriedades gerais do amido, Fundação Cargill, Campinas, 2001, v.1, 224 p.
4. Cereia, M.; Terenzi, H.F.; Jorge, J.A.; Greene, L.J.; Rosa, J.C.; Polizeli, M.L.T.M. Glucoamylase activity from the thermophilic fungus *Scytalidium thermophilum*. Biochemical and regulatory properties. *J. B. Microbiol.*, 40: 83-92, 2000.
5. Chavez, R.A.P.; Carvalho, J.C.M.; Perego, P.; Tavares, L.C. Production of alpha-amylase and glucoamylase from different starches by a new *Trichoderma* sp isolate. *Ann. Microbiol.*, 54: 169-180, 2004.
6. Chiba, S. Molecular mechanism in α-glucosidase and glucoamylase. *Biosci. Biotech. Biochem.*, 61: 1233-1239, 1997.
7. Cruz, R.; Souza, E.L.; Hoffmann, E.H.E.; Bellini, M.Z.; Cruz, V.A.; Vieira, C.R. Relationship between carbon source, production and pattern action of α-amylase from *Rhizopus sp. Rev. Microbiol.*, 28: 101-105, 1997.

8. Guzman-Maldonato, H.; Paredes-Lópes O. Amylolytic enzymes and products derived from starch: a review. *Crit Rev. Food Sc. Nut.*, 35: 373-403, 1995.
9. James, J.A.; Lee, B.H. Glucoamylases: Microbial sources, industrial application and molecular biology-A review. *J. Food Biochem.*, 21: 1-52, 1997.
10. Kaia, A.; Matsui, H.; Somoto, A.; Kimura, A.; Takata, M.; Chiba, S. Substrate specificity and subsite afinities of cristaline α-glucosidase from *Aspergillus niger*. *Agric. Biol. Chem*., 55: 3237-2335, 1991.
11. Kaur, P.; Satyanarayana, T. Production and starch saccharification by a thermostable and neutral glucoamylase of a thermophilic mould *Thermomucor* indicae-seudaticae. *World J. Microbiol. Biotecnol*., 20: 419-425, 2004.
12. Kirk, P.M.; Cannon, P.F.; Daird, J.C.; Stalpers, J.A. Ainsworth Bisby´s *Dictionary of the Fungi*. CAB International, Wallingford. 9ª ed. 2001. 655 p.
13. Moreira, F.G.; Lima, F.A.; Pedrinho, S.R.F.; Lenatorviez, V.; Souza, C.G.M.; Peralta, R.M. Production of amylases by *Aspergillus tamari. Rev. Microbiol.*, 30: 157-162, 1999.
14. Negret Urtasun, S.; Reiter, W.; Diez, E.; Denison, S.H.; Tilbur, J.; Espeso, E.A.; Penalva, M.A.; Arst, H.N. Ambient pH signal transduction in *Aspergillus:* completion of gene characterization. *Mol. Microbial*., 33: 994-1003, 1999.
15. Nguyen, Q.; Rezessy-Szabó, J.M.; Claeyssens, M.; Stals, I.; Hoschke, Á. Purification and characterization of amylolytic enzymes from thermophilic fungus *Thermomyces lanuginosus* strain ATCC 34626. *Enz. Microbial Technol.*, 31: 345-352, 2002.
16. Saha, B.C.; Zeikus, J.G. Microbial glucoamylases: biochemical and biotechnological features. *Starch/Stärk*, 41: 57-64, 1989.
17. Satyarayana, T.; Noorwez, S.M.; Kumar, S.; Rao, J.L.M.; Ezhilvannan, M.; Kaur, P. Development of an ideal starch saccharification process using amylolytic enzymes from thermophiles. *Biochem. Soc. Trans*., 32: 276-278, 2004.
18. Sugimoto, M.; Ohta, T.; Kawai, F. Change in maltose and soluble activities of chimeric α-glucosidase of *Mucor javanicus* and *Aspergillus oryzae*. *Biochem. Bioph. Acta*, 1645: 1-5, 2003.
19. Sugimoto, M.; Suzuki, Y. Hydrolytic action on the mixture of maltose and soluble starch by α-glucosidase from *Mucor javanicus* IFO 4570. *Biosci. Biotech. Bichem*., 58: 1535-1536, 1994.
20. Swift, R.H.; Wiebe M.G.; Robson G.D.; Trinci A.P.J. Recombinant glucoamylase production by *Aspergillus niger* B1 in chemostat and pH auxostat cultures. *Fungal Gen. Biol.*, 25: 100-109, 1998.
21. Tosi, L.R.O.; Terenzi, H.F.; Jorge, J.A. Purification and characterization of an extracellular glucoamylase from the thermophilic fungus *Thermomyces grisea* var. *termoidea*. *Can. J. Microbiol.*, 39: 846-852, 1993.
22. Wallis, G.L.F.; Swift, R.J.; Atterbury, R.; Trappe, S.; Rinas, U.; Hemming, F.W.; Wieb, M.G.; Trinci, A.P.J.; Peberdy, J.F. The effect of pH on glucoamylase production, glicosylation and chemostat evolution of *Aspergillus niger. Bioch. Bioph. Acta*, 32: -112-122, 2001.