PRODUCTION OF AMYLASES BY *ASPERGILLUS TAMARII*

**Fabiana Guillen Moreira; Francieli Arrias de Lima; Sophia Renata Fazzano Pedrinho;
Veridiana Lenartovicz; Cristina Giatti Marques de Souza; Rosane Marina Peralta[*]**

Departamento de Bioquímica, Universidade Estadual de Maringá, Maringá, PR, Brazil.

ABSTRACT

A strain of *Aspergillus tamarii*, a filamentous fungus isolated from soil, was able to produce both α-amylase and glucoamylase activities in mineral media supplemented with 1% (w/v) starch or maltose as the carbon source. Static cultivation led to significantly higher yields than those obtained using shaking culture. The production of amylases was tolerant to a wide range of initial culture pH values (from 4 to 10) and temperature (from 25 to 42ºC). Two amylases, one α-amylase and one glucoamylase, were separated by ion exchange chromatography. Both partially purified enzymes had optimal activities at pH values between 4.5 and 6.0 and were stable under acid conditions (pH 4.0-7.0). The enzymes exhibited optimal activities at temperatures between 50º and 60º C and were stable for more than ten hours at 55ºC.

Key words: α-amylase, *Aspergillus tamarii,* glucoamylase.

INTRODUCTION

A variety of industries (e.g. food, chemical, detergent, textile) employ microbial amylolytic enzymes to convert starch into different sugar solutions (2,13,15,18). Several types of enzymes are involved in the degradation of starch, mainly α-amylase (1,4 α-glucan glucanohydrolase, EC 3.2.1.1), β-amylase (1,4 α-glucan maltohydrolase, EC 3.2.1.2), and glucoamylase (1,4 α-glucan glucohydrolase, EC 3.2.1.3) (2,8). These enzymes are common in fungi, and *Aspergillus* sp and *Rhizopus* sp are often used as sources of industrial amylases (3). Although genetic manipulation by classical mutation techniques and recombinant DNA technology are frequently used to increase the expression levels of a large number of microbial enzymes, including amylases, in well-known microorganisms, traditional screening procedures make possible to find new attractive wild microorganisms able to produce useful enzymes. Some environments such as fertile soil, have great variety of microbial populations. Hundred of different species of fungi inhabit the soil, especially near the soil surface where aerobic conditions prevail (2). Such fungi are active in degrading a wide variety of biological materials present in the soil. They thrive on such material by secreting extracellular enzymes able to degrading large polymeric plant molecules such as cellulose, hemicellulose, starch and pectin, with subsequent assimilation of the liberated nutrients. Considering that a strain of *Aspergillus tamarii* isolated by soil during a screening programme for xylanase producing microorganisms

(7) showed great growth rate in starch as the only carbon source, the purpose of this work was to investigate the ability of *Aspergillus tamarii* to produce amylases as well as the effect of pH and temperature in the enzyme activity.

MATERIALS AND METHODS

Microorganism and culture conditions: *Aspergillus tamarii* was isolated from soil during a screening programme for xylanase-producing microorganisms (7). It was deposited in the Fundação Tropical de Pesquisas e Tecnologia André Tosello Collection, Campinas, Brazil. In the lab, it was routinely maintained on potato dextrose agar at 4°C. The cultures were developed on 250 ml Erlenmeyer flasks containing 50 ml of Vogel salts (10), and various carbon sources (1%, w/v) at 30°C on a rotary shaker at 120 rpm or under static condition. Flasks were harvested at periodic intervals, the contents filtered through tared filter paper and the mycelia dried to constant weigh at 60°C. The filtrates were assayed for α-amylase and glucoamylase activities, and protein. Results were expressed as the mean of at least three independent measurements. Residual maltose and starch concentrations in the culture filtrates were estimated using anthrone reagent (6).

Enzyme assays: Two different methods were used to quantify the glucoamylase and α-amylase activities in the culture filtrates. Firstly, total amylase activity was estimated by analysis of reducing sugars released during hydrolysis of 0.5% (w/v) starch in 0.05 M phosphate buffer pH 6.0 at 40°C by the dinitrosalicylic acid method (9). Secondly, glucoamylase activity (EC 3.2.1.3) was estimated by determining the D-glucose content released during hydrolysis of starch by the peroxidase-glucose oxidase assay (1). Finally, α-amylase activity (EC 3.2.1.1) was estimated by deducting the reducing sugars from the values of D-glucose determined enzymatically. One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as D-glucose per min under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). Extracellular proteins were estimated as described by Bradford (4) using bovine serum albumin as the standard.

Separation of the α-amylase and glucoamylase activities of the culture filtrates: The culture filtrates were concentrated by precipitation with 3 volumes of acetone at 4°C, the pellet dissolved in 0.01M phosphate buffer, pH 6.8, and charged on to a DEAE-cellulose column (1.5 x 20 cm) equilibrated with the same buffer. The column was washed with 10 bed volumes of buffer and the protein adsorbed in the column was eluted with a linear gradient of NaCl (0-0.50 M) in the same buffer at 15 ml/h. Each fraction (2.5 ml) obtained during washing and elution was assayed for amylase activity and absorbance at 280 nm.

Chromatography of hydrolysis products: The pattern of soluble starch hydrolysis by amylolytic enzymes from *A. tamarii* was examined using paper chromatography. Amylases I and II from *A. tamarii* were incubated with 1.0 ml of solution containing 1% of starch in 0.05 M phosphate buffer, pH 6.0. After 30 and 60 min, the reactions were stopped by boiling and stored at 4°C. Samples (15 µl) were spotted onto Whatman N° 1 chromatographic paper and descending chromatography carried out at room temperature with a benzene:n-butanol:piridine:water (1:5:3:3) solvent system. The chromatograms were stained with silver nitrate (17).

Chemicals: Starch, amylopectin, amylose, glycogen, maltose, and glucose were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The ability of *A. tamarii* to grow and to produce amylases on different carbon sources was studied. Significant growth of *A. tamarii* was observed on monosaccharides, disaccharides and polysaccharides 6 days-cultures (Table 1). The existence of low level constitutive production of glucoamylase and α-amylase was detected in sucrose, cellobiose, glucose and raffinose cultures, but active synthesis of both enzymes took place only during growth on maltose, starch, amylose, amylopectin and glycogen. Although higher biomass levels were obtained with shaking cultures, a four-fold increase in both glucoamylase and α-amylase activities was observed under static conditions. For this reason, static conditions were used in all following experiments.

A typical time course of extracellular α-amylase and glucoamylase production is shown in Fig. 1A-1B. Maximal activities were attained on day 4 and 6,

Table 1. Effect of the carbon source on growth and amylase production by *Aspergillus tamarii*.

Carbon source (1%, w/v)	Static conditions		Shaking conditions	
	biomass (mg)	total amylase (U/mg)	biomass (mg)	total amylase (U/mg)
none	28± 5	15± 2	45± 5	8± 1
sucrose	260±30	24± 3	317±40	10± 2
cellobiose	250±21	25± 3	320±28	12± 2
glucose	355±30	10± 2	440±50	7± 1
raffinose	215±23	68± 8	320±40	20± 2
maltose	284±30	155±16	410±37	38± 5
glycogen	310±27	180±21	480±51	47± 6
starch	290±32	176±15	440±48	42± 4
amylopectin	289±35	170±20	490±53	40± 5
amylose	380±42	144±16	400±37	32± 2

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with various carbon sources at 1% (w/v) under static or shaking conditions for 6 days at 30ºC. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60ºC. One unit of total amylase (glucoamylase plus α-amylase) was defined as the amount of enzymes that releases 1 µmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments.
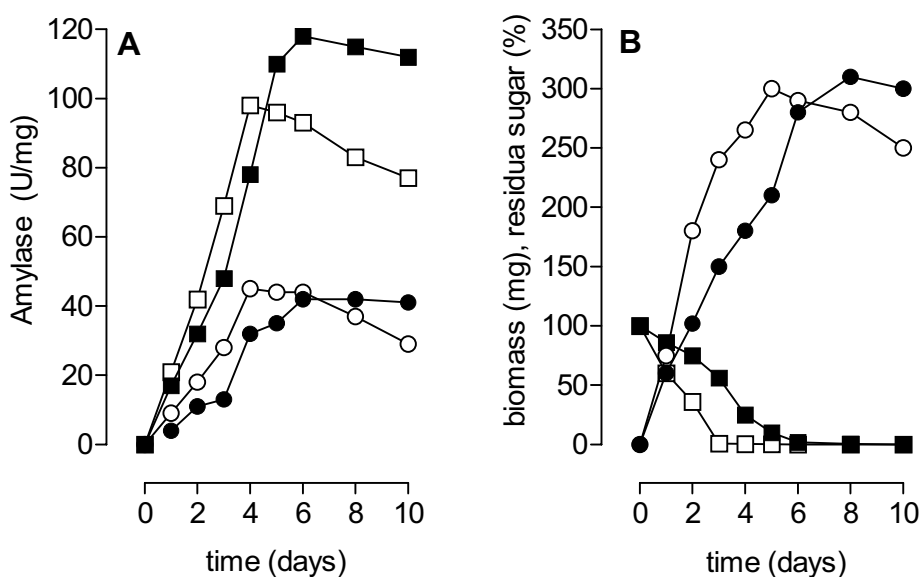
Figure. 1. Time course of amylase and biomass production by *Aspergillus tamarii*.
A: (■) α-amylase activity on starch culture; (□) α-amylase activity on maltose culture: (●) glucoamylase activity on starch culture; (○) glucoamylase activity on maltose culture; B: (●) biomass production on starch culture; (○)biomass production on maltose culture; (■) residual starch; (□) residual maltose.

respectively for the maltose and starch cultures, at the same time of the peak of maximal biomass production. A decrease in enzyme production was observed once the stationary phase of the culture had began. Similar results were obtained in several other microorganisms (12,14,16). Less than 5% of the initial concentration of maltose or starch was present in the filtrate cultures after 4 days of incubation (Fig. 1B). No significant amounts of glucose were detected in the culture filtrates, suggesting that this sugar was rapidly consumed by the fungus (data not shown).

Studies on the effect of the initial pH and temperature on the enzyme production and growth were carried out within the pH 3 to 12 and temperature range 28 to 45°C. At 30°C, growth and enzyme production were inhibited when the initial pH of the medium was above 10 or below 4 (Table 2). The optimal temperature for growth and enzyme production was 30°C (Table 3). Temperatures between 30° and 42°C had little influence on growth and amylase production. However, the fungus did not grow at temperatures above 42°C.

Table 2. Effect of initial pH on amylase production by *A. tamarii*

Initial pH	final pH	biomass (mg)	glucoamylase (U/mg)	α-amylase (U/mg)
3	4.35	ND	ND	ND
4	5.38	248±20	128±	30±3
5	6.28	279±32	129±	31±4
6	6.90	295±28	136±	35±3
7	7.10	256±29	130±	32±5
8	7.18	254±21	131±	32±3
9	7.47	213±26	125±	29±3
10	7.68	208±29	121±	28±4
11	9.20	ND	ND	ND

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with 1% (w/v) starch under static conditions for 6 days at 30°C at different initial pH. After autoclaving the medium, pH was adjusted using sterilized 10% (w/v) Na$_2$CO$_3$ solution or 5N HCl. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60°C. One unit of amylase (glucoamylase and α-amylase) was defined as the amount of enzymes that releases 1 μmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments. ND= not determined.

The filtrates of starch and maltose cultures were dialyzed against water, the proteins concentrated by precipitation with acetone, and then loaded on to a DEAE-cellulose column at pH 6.8. In each case, the amylase activity was eluted in two peaks, designated as amylase I and II (Fig. 2). Approximately 70% of the total amylase activity was due to amylase II, and 30% was due to amylase I. The fractions with activity were pooled and concentrated by lyophilization. The enzymes were incubated with starch as substrate and, samples withdrawn periodlically from the reaction mixture were analysed by descending paper chromatography (Fig. 3). A mixture of sugars, larger oligosaccharides, traces of glucose and mainly maltose, was obtained from starch when the hydrolysis was carried out using the amylase II,

Table 3. Effect of temperature on amylase production by *A. tamarii*

temperature (°C)	biomass (mg)	glucoamylase (U/mg)	α-amylase (U/mg)
25	210±18	129±15	30±4
28	280±24	128±11	31±3
30	295±28	136±12	35±3
32	300±31	130±16	32±5
35	292±30	129±10	32±4
37	289±31	120±15	30±3
39	276±30	125±15	28±2
40	280±25	122±12	25±4
42	230±21	119±10	20±3
45	ND	ND	ND

The cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml minimal medium supplemented with 1% (w/v) starch under static conditions for 6 days at different temperatures. The mycelia (biomass) were separated by filtration and dried to constant weigh at 60°C. One unit of amylase (glucoamylase plus α-amylase) was defined as the amount of enzymes that releases 1 μmol of reducing sugar as D-glucose per min. under the assay conditions. The results are presented as specific activity (U/mg extracellular protein). The results express the media±SD of three different experiments. ND= not determined
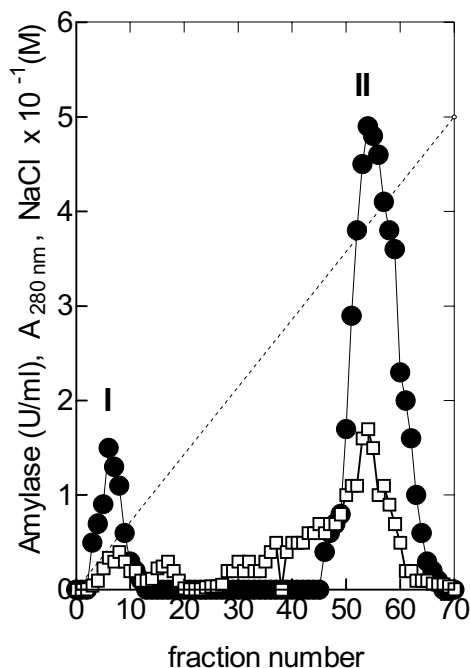

Figure 2. Distribution of protein and total amylase activities after DEAE-cellulose chromatography. (●) amylase activity; (□) absorbance at 280 nm; (---) NaCl concentration.
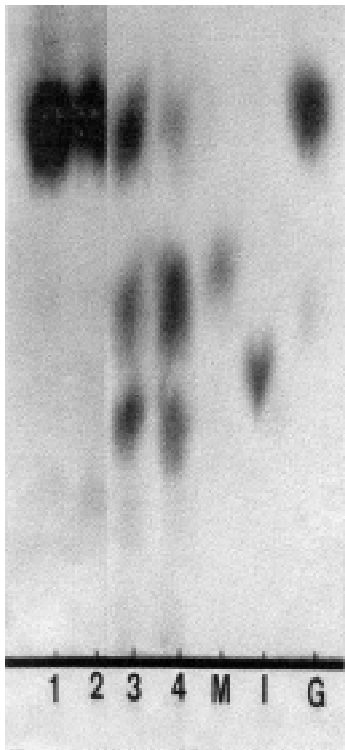
Figure 3. Paper chromatography of hydrolysis products from starch by amylase I and II. 1 and 2: starch plus amylase I (glucoamylase) after 30 and 60 min. of incubation, respectively as described in MATERIALS AND METHODS; 3 and 4: starch plus amyalse II (α-amylase) after 30 and 60 min. of incubation, respectively. M, I, and G are maltose, isomaltose and glucose standards, respectively.

suggesting that this fraction contained an α-amylase. Amylase I released only glucose from starch, indicating that this fraction contained a glucoamylase.

Some properties of amylases were studied. The effect of pH on the glucoamylase and α-amylase was studied using starch as the substrate in McIlvaine buffers (Fig. 4). The initial rate of starch hydrolysis at 40°C was maximal at pH 4.5-5.0, but substantial activities were detected at pH values ranging from 4.0 to 7.0 (Fig. 4A). At 40°C and pH 4.0 to 7.2, the enzymes were stable for 2 days (data not shown). These data are in agreement with the general behaviour reported for other fungal amylases (5,11) The recommended pH range for hydrolysis of starch with commercially available fungal amylases is between 4.0 and 4.5 (3) and the higher pH range observed may have the advantage of requering less adjustment of pH between sequential α-amylase (liquefaction) and glucoamylase (saccharification) treatments. The enzymes were highly active over a broad temperature range of 40-70°C. At pH 6.0, the optimal activity was observed at a temperature of 50-55°C for both enzymes (Fig. 4B) Thermal inactivation of the partialy purified amylases was investigated by incubating the enzymes at 55°C and 65°C in 0.05 M sodium phosphate buffer, pH 6.0, without substrate (Fig. 4C). The enzymes were stable at 55°C for more than 10 hours, and at 65°C exhibited an half-life of 30 min. (amylase I) and 45 min. (amylase II).
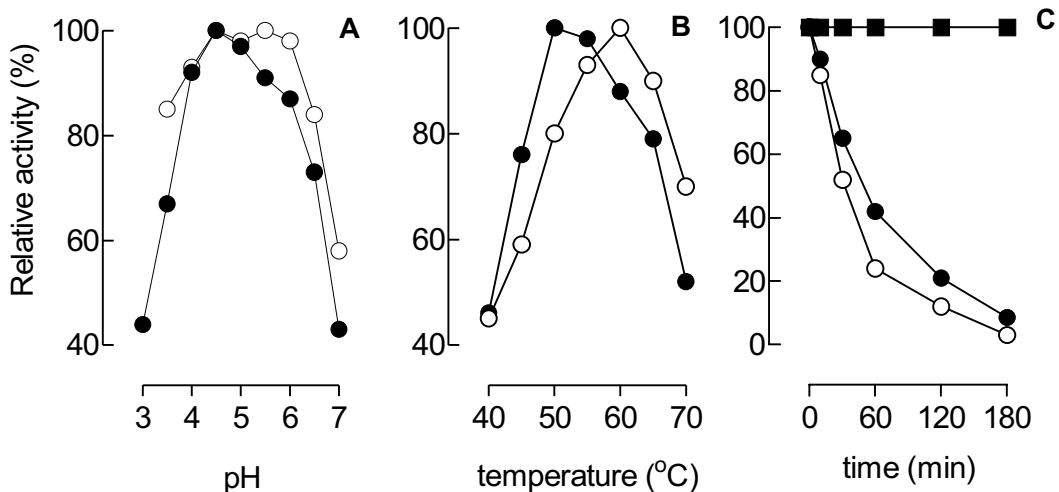

Figure 4. Effect of pH (A) and temperature (B) on the activity and effect of temperature on the stability (C) of amylases from *Aspergillus tamarii*. A and B: (●) glucoamylase activity; (○) α-amylase activity; C: (■) residual glucoamylase and α-amylase activities at 55°C; (●) residual α-amylase activitiy at 65°C; (○) residual glucoamylase activity at 65°C.

Some characteristics of *A. tamarii* described in this paper, such as easy cultivation, production of two types of amylases (glucoamylase and α-amylase) on cultures at different pH and temperature conditions, and stability of the enzymes at an acidic range of pH, make this fungus a potential source of amylase for future biotechnological applications. Purification of these amylases is in progress in our laboratory.

ACKNOWLEDGEMENTS

RESUMO

Produção de amilases por *Aspergillus tamarii*

Uma cepa de *Aspergillus tamarii*, um fungo filamentoso isolado do solo, foi hábil em produzir α-amilase e glucoamilase em meio mineral suplementado com amido ou maltose a 1% (p/v) como fonte de carbono. A produção de amilases em culturas estacionárias foi significativamente maior do que em culturas sob agitação. Uma ampla faixa de pH inicial (de 4 a 10) e temperatura (de 25 a 42°C) pode ser utilizada nos cultivos para a produção das amilases. Duas amilases, uma α-amilase e uma glucoamilase foram separadas por cromatografia de troca iônica. As enzimas parcialmente purificadas exibiram máxima atividade na faixa de pH entre 4,5-6,0 apresentando grande estabilidade sob condições ácidas (pH 4 a 7). Máxima atividade foi obtida nas temperaturas entre 50 e 60°C, e as enzimas foram estáveis por mais de dez horas à 55°C.

Palavras-chave: α-amilase, *Aspergillus tamarii*, glucoamilase.

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