Int Microbiol (2003) 6: 269–273
DOI 10.1007/s10123-003-0140-1

RESEARCH ARTICLE

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Rhizopus microsporus var. *rhizopodiformis*: a thermotolerant fungus with potential for production of thermostable amylases

Received: 25 April 2003 / Accepted: 13 June 2003 / Published online: 15 August 2003
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Abstract The effect of several nutritional and environmental parameters on growth and amylase production from *Rhizopus microsporus* var. *rhizopodiformis* was analysed. This fungus was isolated from soil of the Brazilian "cerrado" and produced high levels of amylolytic activity at 45°C in liquid medium supplemented with starch, sugar cane bagasse, oat meal or cassava flour. Glucose in the culture medium drastically repressed the amylolytic activity. The products of hydrolysis were analysed by thin layer chromatography, and glucose was detected as the main component. The amylolytic activity hydrolysed several substrates, such as amylopectin, amylase, glycogen, pullulan, starch, and maltose. Glucose was always the main end product detected by high-pressure liquid chromatography analysis. These results indicated that the amylolytic activity studied is a glucoamylase, but there were also low levels of α-amylase. As compared to other fungi, *R. microsporus* var. *rhizopodiformis* can be considered an efficient producer of thermostable amylases, using raw residues of low cost as substrates. This information is of technological value, considering the importance of amylases for industrial hydrolysis.

Keywords *Rhizopus microsporus* · Amylase · Glucoamylase · α-Amylase · Thermotolerant fungi

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Introduction

Amylases are hydrolysing enzymes used by many organisms, such as plants for assimilation of the starch accumulated in some types of roots; animals for the digestion of starch contained in food, and many prokaryotic and eukaryotic microbes that use starch as a source of carbon. These enzymes are used in starch-processing industries, to produce ethanol, glucose and fructose syrups, and also in the textile, brewing and paper industries. Several enzymes with different specificity constitute the amylolytic complex, but α-amylases (1,4-α-D-glucan glucanhydrolase EC 3.2.1.1) and glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) are the major commercially important amylases. Glucoamylase, an exo-acting enzyme, releases β-D-glucose from the nonreducing chain ends of amylose, amylopectin, and glycogen, by consecutively hydrolysing α-1,4; α-1,6 and rare α-1,3 linkages. The endo-enzyme, α-amylase, hydrolyses the α-1,4-D-glucosidic linkage of large polymers or granular starch yielding dextrins. This action results in a rapid decrease in viscosity, and hence these enzymes are called liquefying α-amylases. Otherwise, some of them may produce short oligosaccharides from the nonreducing end of the dextrins and are thus called saccharifying α-amylases (exo-acting α-amylase) [7].

Biotechnological interest in microbial amylases has increased recently [6, 8, 9, 11, 20, 21]. Amylases produced by thermophilic and thermotolerant microorganisms are especially interesting because they are usually thermostable, and can be used in saccharification processes that occur at high temperatures [2, 3, 4, 5, 15, 20]. The potential of thermostable amylases for biotechnological applications has stimulated the search for microbial strains expressing activities with the desired features. In our laboratory, we have isolated a *Rhizopus* strain that exhibits optimal growth at 50°C. Fungi from the genus *Rhizopus* are considered good producers of amylolytic activities [12, 19]. Here, we report some

culture conditions that affect the amylolytic activity of *Rhizopus microsporus* var. *rhizopodiformis* and some characteristics of these enzymes.

Materials and methods

Organism and growth conditions

R. microsporus var. *rhizopodiformis* was isolated from soil of the Brazilian "cerrado" (Pirassununga, São Paulo) and identified by the "Instituto de Pesquisas Tropicais André Tosello" (Brazil), as described by Schipper and Stalpers [18]. The organism was maintained at 45°C on slants of solid 4% oat-meal baby food (Quaker), or tomato juice-agar medium [10]. Approximately 3×10^5 spores/ml from 15-day-old cultures were inoculated into 125 ml Erlenmeyer flasks containing 25 ml liquid Carvalho Peixoto (CP) medium: 0.8% yeast extract (w/v), 0.3% KH_2PO_4 (w/v) and 0.05% $Mg_2SO_4{\cdot}7H_2O$ (w/v), 1.5% starch (w/v) or other carbon source, pH 6.0. The cultures were incubated at 45°C, in a rotary shaker at 100 rpm for 72 h.

Preparation of crude enzyme

Culture filtrates were obtained by filtration through Whatman No. 1 paper in a Büchner funnel. The filtrate samples were dialysed for 16 h at 4°C against distilled water, and used as a source of crude extracellular amylolytic activity. The mycelia were rinsed with chilled water, blotted onto filter paper, and ground in a mortar with the aid of glass beads. Proteins were extracted from the disrupted cells with 100 mmol l^{-1} acetate buffer, pH 6.0, at 4°C, and the crude extracts were centrifuged (12,100 *g* for 15 min, 4°C).

Enzymatic assay

Amylase activities were routinely assayed with 1% (w/v) starch in 100 mmol l^{-1} sodium acetate buffer, pH 5.0 [16]. An enzyme unit was defined as the amount that releases reducing sugar at an initial rate of 1 μmol min^{-1} at 65°C (optimal temperature—see results). Protein was determined by the method of Lowry [14] using bovine serum albumin as standard.

Identification of hydrolysis products

The hydrolysis products of amylase activity on soluble starch as substrate were analysed by thin-layer chromatography (TLC) on silica gel (DC-Alufolien Kieselgel 60, Merck). The mobile phase was: butanol/ethanol/water (5:3:2, by volume). Sugars were detected with 0.2% orcinol in a methanol-sulfuric acid (9:1) mixture [2]. Other reactions were carried out using 1% (w/v) amylopectin, 1% (w/v) amylose, 1% (w/v) glycogen, 1% (w/v) maltose, 1% (w/v) pullulan or 1% (w/v) starch in 100 mmol l^{-1} sodium acetate buffer, pH 5.0, as substrates, for 1 h at 65°C. Hydrolysis products were analysed by high pressure liquid chromatography (HPLC) using an aminex HPX-42-A column (Bio-Rad, Richmond, Calif.). The mobile phase was water at a flow rate of 0.4 ml min^{-1} at 85°C.

Reproducibility of the results

All data are the mean of at least three independent experiments showing consistent results.

Results and discussion

Optimisation of culture medium composition for amylase production

The influence of components of the culture medium on production of amylolytic activity from *R. microsporus* var. *rhizopodiformis* was investigated. The culture medium initially used was based on the composition described by Adams [1]. Other media, such as those reported by Khanna [13] and Vogel [22] did not favour enzymatic production (data not shown).

To optimise amylase production, the concentration of some components of the Adams medium, such as starch (0–3.0%, w/v), yeast extract (0–1.0%, w/v) and KH_2PO_4 (0–2.0%, w/v) was varied. Table 1 shows the results of altering the concentration of each of the components of the original medium separately, and of the combination of the three variables. The highest amylolytic activity levels were obtained with CP medium, which increased amylase activity about 5-fold. Thus, this medium was used for all subsequent experiments. Addition of different concentrations of sodium chloride, Triton, Tween-20 or Tween-80 to the culture medium did not enhance, but rather diminished the production of amylolytic activity, suggesting that the enzyme is not a membrane-bound protein (not shown).

Effect of carbon and nitrogen sources

Several carbon sources were tested as inducers of amylolytic activity (Table 2). Fungal growth was very efficient on maltose, but glucose, sucrose, starch, and agro-industrial residues also supported good growth. The highest level of amylolytic activity was found in starch-supplemented cultures, although oatmeal, cassava flour, pectin or sugar cane bagasse might also serve as alternative sources to produce amylases. This may be significant, because the use of industrial residues lowers the price of enzyme production, thus making the

Table 1 Influence of the composition of the culture medium on growth and amylase activity from *Rhizopus microsporus* var. *rhizopodiformis*. *CP* Carvalho Peixoto medium

Conditions	Total cell protein (mg)	Specific activity (U/mg protein)
Original medium (OM)[a]	1.30	53.62
Modified 1: OM +1.5% starch	1.70	63.00
Modified 2: OM +0.8% yeast extract	1.80	59.50
Modified 3: OM +0.3% KH_2PO_4	2.10	53.87
Modified 4: CP	3.80	88.25

[a] 1.0% yeast extract (w/v), 0.1% KH_2PO_4 (w/v), 0.05% $Mg_2SO_4{\cdot}7H_2O$ (w/v), 1.0% starch (w/v)

Table 2 Effect of carbon source on growth and amylase activity from *R. microsporus* var. *rhizopodiformis*

Carbon source (2% w/v)	Total cell protein (mg)	Specific activity (U/mg protein)
Glucose	13.00	2.45
Maltose	29.92	0.47
Trehalose	4.64	8.44
Arabinose	2.96	9.29
Sucrose	12.16	6.65
Raffinose	3.44	2.18
Avicel	7.68	3.69
Starch	11.52	26.04
+0.5% Glucose	4.50	15.56
+1.0% Glucose	7.50	4.80
+1.5% Glucose	7.60	3.03
+2.0% Glucose	7.65	2.09
Polygalacturonic acid	2.72	0.92
Pectin	3.92	17.01
Oatmeal	14.22	5.21
Wheat (raw)	12.13	2.20
Cassava flour	10.26	6.50
Sugar cane bagasse	15.01	3.40

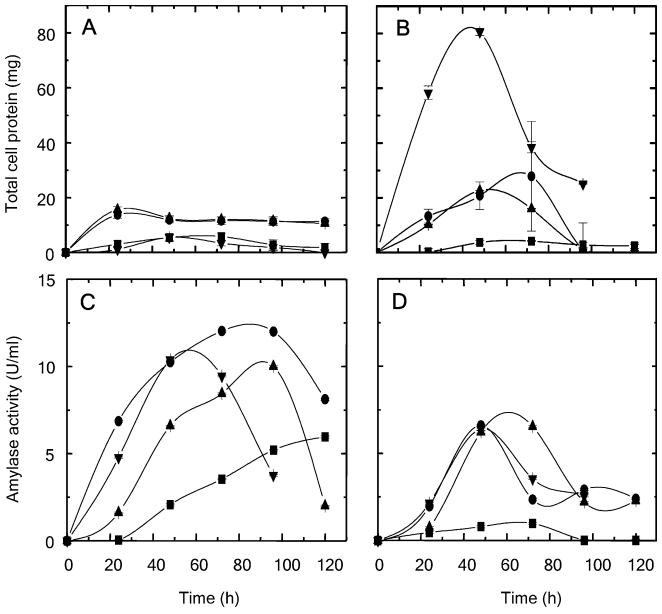

Fig. 1 Time-course of growth (**A**, **B**) and amylase production (**C**, **D**) at different temperatures, in agitated (**A**, **C**) or standing (**B**, **D**) cultures. *Squares* 30°C, *triangles pointing up* 40°C, *circles* 45°C, *triangles pointing down* 50°C. Enzymatic assay was carried out at 65°C as described in Materials and methods

commercial product more competitive. The enzymatic levels observed from *R. microsporus* were higher than those previously reported by Adams [1] from *R. pusillus*, *Papulaspora thermophilia*, *Humicola grisea* var. *thermoidea* and *Humicola lanuginosa*. The values attributed to *Myceliophthora thermophila* [17] were also lower than those observed for *R. microsporus*.

Production of amylolytic activity in starch-supplemented cultures ceased when glucose was also added to the culture medium (Table 2). Similar results are reported for the fungus *M. thermophila* [17]. Nitrogen sources including ammonium acetate, ammonium nitrate and urea were used at different concentrations (0–2.0%). Ammonium nitrate improved fungal growth, doubling the cell mass at 2.0% concentration. On the other hand, supplementation with urea or ammonium acetate drastically diminished the growth of the fungus. Regarding amylolytic activities, an increase of approximately 16% was observed (data not shown).

Determination of physiological parameters

The influence of temperature and aeration (shaking versus standing conditions) on growth and production of amylolytic activity by the fungus was monitored for 5 days. Quite large differences were observed when comparing growth (total cell protein; Fig. 1A, B) and amylase production (Fig. 1C, D), for agitated and standing cultures.

For standing cultures, temperatures over 40°C enhanced growth. At 50°C, growth was about 4-fold higher than that of the agitated culture. These results suggested that *R. microsporus* var. *rhizopodiformis* might be a thermotolerant fungus, which grow better under lower oxygen tension at higher temperatures. Similar effects were observed when the speed of agitation of the cultures was gradually reduced (data not shown). In addition, amylase activity levels were higher for cultures incubated with agitation. With agitation and at 45°C the amylolytic activity reached its maximum between 70 h and 100 h of incubation (Fig. 1C). This period coincided with a sharp fall in cell protein content (Fig. 1A, B), suggesting that some nutrients in the culture medium had been exhausted and the hyphae were suffering autolysis.

Hydrolysis products analysis

TLC of the starch hydrolysis products formed after different reaction periods (15–240 min) revealed mainly glucose as the end product, suggesting the main enzymatic component to be glucoamylase. Only traces of maltooligosaccharides were detected, suggesting low levels of α-amylase activity (Fig. 2). These results were confirmed by HPLC analysis (Table 3). The amylolytic activities preferentially hydrolysed the substrates amylopectin, starch, glycogen, maltose and amylose, producing glucose as the main end product. Pullulan hydrolysis was practically negligible, suggesting the absence of pullulanase activity. The hydrolysis of maltose was already anticipated, since other glucoamylases with maltase activity have been reported previously [2, 5, 21].

Effects of temperature and pH on amylolytic activity

The optima of temperature and pH for extracellular amylolytic activity were 65°C and 5.0, respectively

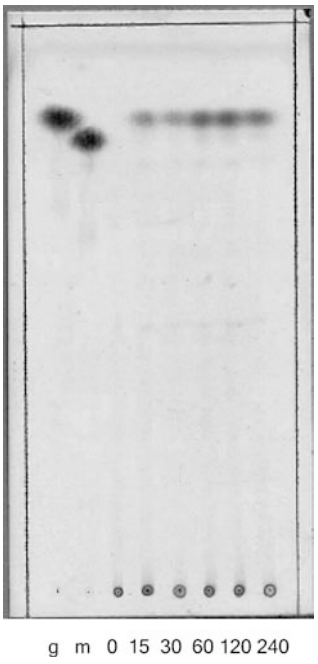

Fig. 2 Analysis of the hydrolysis products released by amylase acting on 1.0% (w/v) starch for different times (0, 15, 30, 60, 120, 240 min). The fungus was cultivated with shaking (100 rpm), at 45°C for 72 h. *g* Glucose (1% w/v), *m* maltose (1% w/v). Enzymatic assay was carried out at 65°C as described in Materials and methods

Table 3 High-pressure liquid chromatography analysis of final products of amylases produced by *R. microsporus* var. *rhizopodiformis*. *M3* Maltotriose, *M4* maltotetraose, *M5* maltopentaose, *oligo* oligosaccharide, *poly* non-degraded polysaccharide

Substrate	Products (%)						
	Glucose	Maltose	M3	M4	M5	oligo	poly
Amylopectin	62.4	2.1	–	0.6	0.4	0.5	34
Amylose	42.6	–	–	–	–	1.2	56.2
Glycogen	49.9	–	–	–	–	0.2	49.9
Maltose	45.7	53.6	0.7[a]	–	–	–	–
Pullulan	0.8	–	–	–	–	2.6	96.6
Starch	57.5	2.9	–	0.5	0.2	0.5	38.4

[a]Minor contaminants present in maltose

(Fig. 3A). Amylolytic activity was also rather stable, maintaining 50% activity after 120 min at 60°C (Fig. 3B). At a higher temperature, 65°C, the amylase showed instability, but addition of starch (1% w/v) in the reaction mixture protected the enzyme from thermal inactivation. Similar results have been reported by others [2, 21]. At 25°C, the enzyme was completely stable for at least 3 days. The amylase activity was also acid- and alkali-tolerant, maintaining its stability for 2 h over a wide range of pH (2.5–7.5; Fig. 3C), a fact that reinforces the potential value of *R. microsporus* amylase for industrial applications, since this thermostable enzyme may be employed without the need for pH adjustments.

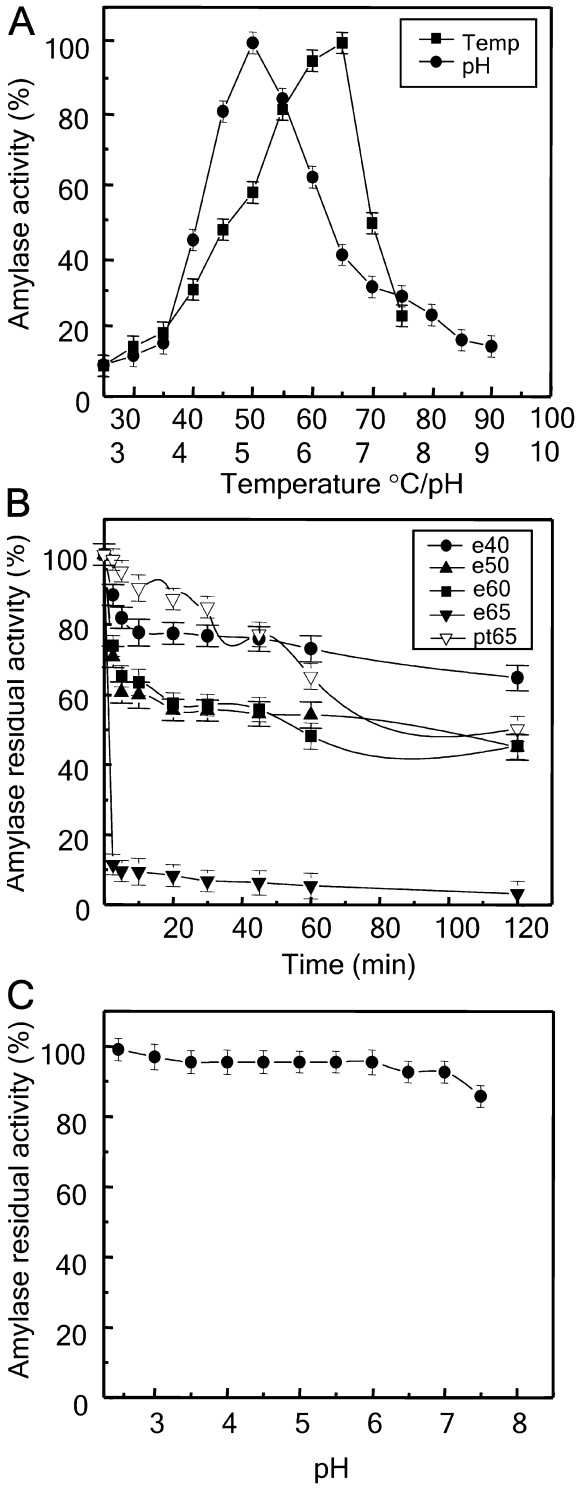

Fig. 3A Influence of temperature and pH on amylolytic activity. **B** Stability of the amylolytic activity at different temperatures: *circles* 40°C, *triangles pointing up* 50°C, *squares* 60°C, *triangles pointing down* 65°C, *open triangles* 65°C protected with starch. **C** Stability of the amylolytic activity at different pH values. The fungus was cultivated with shaking (100 rpm), at 45°C, for 72 h. Enzymatic assay was carried out at 65°C as described in Materials and methods

Acknowledgements This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho de Desenvolvimento Científico e Tecnológico (CNPq). H.F.T., J.A.J., and M.L.T.M.P. are Research Fellows of CNPq. S.C.P. is a recipient of a CAPES Fellowship. This work is part of a Master Dissertation submitted to the Departamento de Biologia da Faculdade de Filosofia Ciências e Letras de Ribeirão Preto—Universidade de São Paulo. We thank Ricardo F. Alarcon and Mauricio de Oliveira for technical assistance.

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