The Journal of Microbiology, December 2005, p.561-568 Vol. 43, No. 6

Production of Saccharogenic and Dextrinogenic Amylases by *Rhizomucor pusillus* A 13.36

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(Received June 2, 2005 / Accepted October 19, 2005)

A newly-isolated thermophilic strain of the zygomycete fungus *Rhizomucor pusillus* 13.36 produced highly active dextrinogenic and saccharogenic enzymes. Cassava pulp was a good alternative substrate for amylase production. Dextrinogenic and saccharogenic amylases exhibited optimum activities at a pH of 4.0-4.5 and 5.0 respectively and at a temperature of 75°C. The enzymes were highly thermostable, with no detectable loss of saccharogenic or dextrinogenic activity after 1 h and 6 h at 60°C, respectively. The saccharogenic activity was inhibited by Ca^{2+} while the dextrinogenic was indifferent to this ion. Both activities were inhibited by Fe^{2+} and Cu^{2+} Hydrolysis of soluble starch by the crude enzyme yielded 66% glucose, 19.5% maltose, 7.7% maltotriose and 6.6% oligosaccharides.

Key words: amylase, *Rhizomucor pusillus*, thermostability

Starch is hydrolyzed to glucose, maltose and maltooligosaccharides by four groups of amylases. The endoamylases (EC 3.2.1.1) cleave α-1,4 glycosidic bonds in amylose, amylopectin and related polysaccarides but not α-1,6 linkages. The exoamylases, glucoamylase (EC 3.2.1.3), α-glucosidase (EC 3.2.1.20) and β-amylase (EC 3.2.1.2) act preferentially on α-1,4 linkages from the non-reducing end successively, resulting in products with low molecular weights. Debranching enzymes include pullulanase (EC 3.2.1.41), which act specifically on α-1,6 linkages in pullulan, starch, amylopectin and related oligosaccharides. The enzymes also effect isoamylase (EC 3.2.1.68), which hydrolyzes α-1,6 linkages in amylopectin, but has very low or even no activity on pullulan. The other group of starch-converting enzymes are transferases, which cleave the α-1,4 glycosidic bond of a donor molecule and transfer part of the donor to a glycosidic acceptor, forming a new glycosidic bond. Examples are amylomaltase (EC 2.4.1.25) and cyclodextrin glycosyltransferase (EC2.4.1.19) (van der Maarel *et al*., 2002).

Microorganims that grow in temperatures above the mesophilic range are classified as thermophilic (grow optimally between 40-60°C), and include fungi, bacteria and archaea; extreme thermophilic (optimal growth 70-80°C), represented by bacteria and archaea, and hyperthermophilic, represented by archaea and a few bacteria (optimal growth 85-110°C) (Vieille *et al*., 1996; Niehaus *et al*., 1999). Only a few species of fungi among the eukaryotic thermophilic organisms have the ability to grow at temperatures between 45 and 55°C (Maheshwari *et al*., 2000).

Enzymes from thermophilic microorganisms which are intrinsically stable and active at high temperatures offer major biotechnological advantages over their mesophilic or psychrophilic counterparts. The most important advantages are: (i) once expressed in a mesophilic host, these enzymes are easier to purify by heat treatment; (ii) their thermostability is associated with a stronger resistance to chemical denaturants (such as a solvent or guanidium hydrochloride); and (iii) performing enzymatic reactions at high temperature allows higher substrate concentrations, lower viscosity, fewer risks of microbial contaminations and higher reactions rates (Vieille and Zeikus, 2001).

This article presents data on extracellular amylase production by a newly isolated strain of the thermophilic fungus *Rhizomucor pusillus*. The influence of carbon source

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on saccharogenic and dextrinogenic α-amylase and glucoamylase production and the characteristics of these enzymes are described.

Materials and Methods

Isolation of fungus

A sterile liquid medium containing of 2 g of sample of compost was used. It also included 10 g soluble starch, 1.4 g $(NH_4)_2SO_4$, 2.0 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.0016 g $MnSO_4 \cdot H_2O$, 0.0014 g $ZnSO_4 \cdot 7H_2O$, 0.002 g $CoCl_2$ per liter. The mixture was incubated at 45°C for 24 h, followed by a loop of the homogenized culture being streaked on the surface of the same medium containing 3.0% agar and incubated at 45°C for 24 to 72 h. All morphologically contrasting colonies were purified by repeated streaking. Pure cultures were subcultured onto agar slants for identification and enzyme studies. The fungi identification was based on morphological and physiological characteristics (Kirk *et al.*, 2001). The stock cultures were maintained at 7°C on medium Potato Dextrose Agar (PDA).

Effect of temperature on fungal growth

In order to investigate the strain's performance at various temperatures, mycelial hyphae from a pure culture were inoculated at points on agar plates by stabbing with twisted wires and incubated at 40, 45, 48 and 50°C. Every 12 h, the diameters of the colonies were measured.

Production of amylases in submerged fermentation (SmF)

125-ml Erlenmeyer flasks with 25ml of medium containing 10 g carbon source, 1.4 g $(NH_4)_2SO_4$, 6.0 g K_2HPO_4, 2.0 g KH_2PO_4, 0.1 Mg $SO_4 \cdot 7H_2O$, 5.0 g yeast extract, 2.0 g peptone, 2.0 g beef extract per liter (pH 5.0) were inoculated with 1-2 ml aliquots of a suspension in sterile 0.01% Tween 80 of the sporangiospores from a seven day agar slant culture, to give 10^6 spores/ml in the growth medium. The fermentation was carried out in a rotary shaker at 100 rpm at 45°C for 120 h. The biomass was separated by filtering through Whatman No. 1 paper in a Buchner funnel, washed with deionized water and dried to a constant weight at 105°C. The filtrate was used to evaluate amylase activities. The carbon sources were soluble starch (Synth, Brazil), cassava pulp and cassava processing wastes (solid and liquid wastes from the starch-extraction process), corn bran and corn processing waste generated during germ extraction. The experiments were performed in triplicate.

Enzyme assays

The α-amylase dextrinogenic activity was analyzed by the starch-iodine method at 75°C (Medda and Chandra, 1980). 0.7 ml of 0.3% (w/v) soluble starch (Reagen, Brazil) in 0.25 M acetate buffer, pH 4.5, was mixed with 0.30 ml of the crude enzyme solution and incubated at 60°C for 10 min. One unit of dextrinogenic activity (U) was defined as the amount, of enzyme that hydrolyzes 1 mg of starch in 10 min under assay conditions.

Amylase saccharogenic activity was assayed with 0.9 ml of 0.5% (w/v) soluble starch (Reagen, Brazil) in 0.25 M acetate buffer, pH 5.0, and 0.1 ml of crude enzyme solution, incubated at 75°C for 10 min. The reducing sugar released was measured by the DNS method (Miller 1959), using glucose as a standard. One unit of saccharogenic activity (U) was defined as the amount of enzyme releasing 1 μmol of reducing sugar/min. Glucoamylase activity was assayed at 60°C in a reaction mixture containing 0.50 ml of diluted crude enzyme solution and 0.5 ml of 0.50% starch in 0.25 M acetate buffer, pH 5.0. The glucose released was estimated by the glucose oxidase/peroxidase assay (Bergmeyer *et al.*, 1974). One unit enzyme activity (U) was defined as the amount of enzyme that releases 1 μmol of glucose/min. Substrate-specific assays were performed by substituting 0.5% (w/v) soluble starch by cassava, potato and corn starches, dextrins (2 to 7 glucose units) and *p*-nitrophenyl-α-D-maltoside. When the substrate was *p*-nitrophenyl-α-D-maltoside, the activity was measured in a mixture containing 0.20 ml of 0.1 M sodium acetate pH 5.0, 0.05 ml of a 2 mM substrate solution, and 0.1 ml of crude enzyme. After 10 min, of incubation at 70°C, the reaction was halted with 1 ml of 2 M Na_2CO_3, and the nitrophenol released was quantified spectrophotometrically at 410 nm.

Enzyme characterization

Optimal pH and temperature for activity

The enzyme activity at 75°C was assayed at various pH values, using the 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) and reaction mixture containing 0.7 ml of 0.3% (w/v) cassava starch in 0.25 M buffer and 0.30 ml of crude enzyme solution. The optimal temperature was determined by incubation of the reaction mixture at 40-90°C and assaying the activity at the optimum pH in the same reaction mixture.

Thermostability

The enzyme solution was incubated at various temperatures (10°C - 90°C) for 1 h at a pH of 5.0, covered by a thin layer of mineral oil to prevent evaporation. In a separate experiment, the enzyme stability at 60°C was determined in the presence and absence of 5 mM Ca^{2+}. The enzyme solution was maintained at this temperature for 9 h. In both experiments aliquots were withdrawn and placed on ice for subsequent assaying for residual enzyme activity at 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 (gly-

cine-NaOH) and maintained at 25°C for 24 h. Aliquots were taken to determine the remaining activity at the optimum pH and temperature.

Effect of Ca^{2+} and other metal ions

The dextrinogenic and saccharogenic activities were measured at a pH of 5.0 and 75°C in the presence of Ca^{2+}, in concentrations varying from 2.0 to 60 mM, and other metal ions at 10 mM. A sample of the crude enzyme was dialyzed against 10 mM sodium-acetate buffer (pH 5.0) containing 1 mM of EDTA before the experiments began.

Identification of hydrolytic products

The products obtained by enzymatic hydrolysis of soluble starch, were analyzed by paper chromatography on Whatman No. 1 paper, with ethyl acetate / isopropanol / water (6:3:1, by vol) as the mobile phase. This also included running high performance liquid chromatography (HPLC) tests, using a PU-980 pump (Jasco, Italy), an Aminex HPX-42A column (Bio Rad, USA) and detector Shodex RI-72, the mobile phase was water fat 0.7 ml/min, at 80°C.

Results

Selection of strains with amylolytic activity and assessment of thermophilic feature

Of the 13 thermophilic fungal strains isolated from 20 collecting trips (200 samples), one was selected for its ability to grow on a liquid medium containing starch as the sole carbon source at 45°C, and to produce a significant level of amylase activity in the medium. The culture was identified as *Rhizomucor pusillus* A13.36 (Fungi; Zygomycetes; Mucorales; Mucoraceae). The effect of incubation temperature on its growth on the solid medium is presented in Fig. 1. The maximum growth-rate at 45°C confirms the thermophilic character of the fungus (Maheshwari *et al*., 2000). According to Mouchacca's taxonomic review (1997), fungi with optimal growth temperatures above the mesophilic range consist of a few Rhizomucorales, Eurotiales, Sphaeriales and Hyphomycetes, and an agonomycete species. Among Rhizomucorales, there are thermophilic species in the genus Rhizomucor and the monospecific genus *Thermomucor* Thermophilic and mesophilic Rhizomucorales have been described as amylase-producing (Adams and Deploy, 1978; Somkuti and Steinberg, 1980; Adams, 1994; Mohapatra *et al*., 1998).

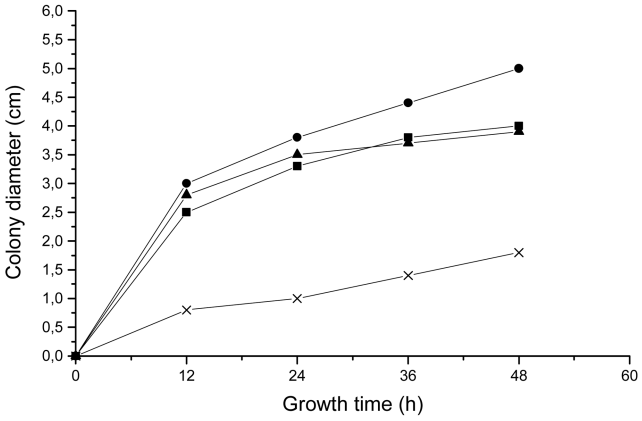

Fig. 1. Effect of temperature on *Rhizomucor pusillus* 13.36 growth on solid malt agar medium. Square=40°C; circle=45°C; triangle=48°C; cross=50°C

Amylase production in liquid (SmF)

The media containing cassava pulp and corn bran as carbon sources afforded higher saccharogenic and dextrinogenic amylase activities, while glucoamylase production was higher in the media with soluble starch and cassava pulp (Table 1). Cassava pulp is a solid waste produced during the extraction of starch from cassava, containing a significant amount of starch granules and fiber (68% and 27% dry basis, respectively) (Sriroth *et al*, 2000). Its disposal causes environmental problems/degradation and the production of enzymes from this residue might help the environment, adding value to the pulp. It has been confirmed that using starch-processing wastewater to produce fungal protein and enzymes results in a reduction of Chemical Oxygen Demand (COD) and suspended solids in the effluent (Friendrich *et al*., 1987; Jamuna and Ramakrishna, 1989; Jin *et al*., 1999).

Although enzyme production could be greatly improved by optimizing fermentation conditions, this was not within the scope of this study. Additional experiments will be required.

Enzyme characterization

The *Rhizomucor pusillus* amylase activity in the SmF

Table 1. Effect of cultivation medium on amylase production by Rhizomucor pusillus 13.36

Carbon source (1%)	Amylase activities (U/ml)		
	Dextrinogenic	Saccharogenic	Glucoamylase
Soluble starch	4.9 ± 0.80	16.5 ± 1.3	0.65 ± 0.08
Cassava pulp	6.8 ± 0.90	20.5 ± 0.98	0.81 ± 0.04
Cassava processing waste	4.5 ± 0.95	11.8 ± 1.2	0.32 ± 0.02
Corn bran	6.3 ± 0.67	19.0 ± 1.4	0.16 ± 0.03
Corn processing Waste	1.2 ± 0.05	1.8 ± 1.0	0.05 ± 0.01

Dextrinogenic=assayed by iodine method; Saccharogenic=assayed by reducing sugar determination

medium was characterized directly in the crude filtrates obtained from the 120 h cultures. The dextrinogenic amylase activity indicated a peak at a pH of 4.5. Approximately 95% of this activity was observed at a pH of 3.5 and 81% at a pH of 5.0, indicating an enzyme with activity over a broad pH range (Fig. 2A). This enzyme exhibited optimal activity at 75 - 80°C and 45% of this activity at 90°C (Fig. 2B).

When assayed by the release of a reducing sugar, the enzyme indicated maximum (saccharogenic) activity at a pH of 5.0 and 85% of this at a pH of 6.0 (Fig. 2A). The optimal temperature for saccharogenic activity was found to be 75°C and the enzyme indicated 20% of maximum activity at 90°C (Fig. 2B).

As illustrated in Fig. 2A, the dextrinogenic enzyme was 100% stable at a pH of 3 - 4. It retained 80% of this activity up to pH 9.0, when maintained for 24 h in the absence of substrate. The saccharogenic activity was stable at a pH of 5.0-7.0, maintaining 70% of its activity at a pH of 10.0. The dextrinogenic and saccharogenic activities both remained inalterable after 1 h at 60°C. The dextrinogenic activity was totally lost at 80°C, whereas the saccharogenic activity indicated a different profile of temperature

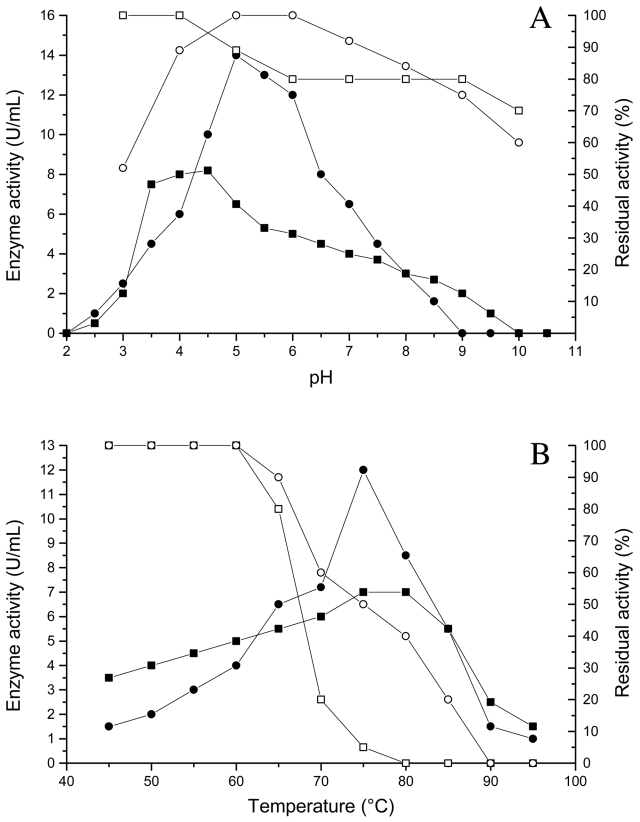

Fig. 2. Physicochemical characterization of enzymes produced by *Rhizomucor pusillus* 13.36: Effect of pH (A) and temperature (B) on the enzyme activity (open symbol) and stability in absence of substrate (filled symbol) of substrate. circle=saccharogenic activity; square= dextrinogenic activity.

sensitivity in the absence of substrate, losing 10% of its original activity at 65°C, but retaining 40% at 80°C.

The optimum pH value found for the amylases agrees with those reported in the literature for other fungal sources such as *Aspergillus niger* (Vandersall *et al.*, 1995) and *Mucor* sp. (Mohapatra *et al.*, 1998). On the other hand, the enzymes had greater thermostability than α-amylases obtained from thermophilic fungi such as *Rhizomucor pusillus*, *Scytalidium thermophilum*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus*, whose activity maxima were detected at 60-70°C (Jayachandran and Ramadran, 1970; Taylor *et al.*, 1978; Somkuti and Steinbertg, 1980; Campos and Félix, 1995; Arnesen *et al.*, 1998; Cereia *et al.*, 2000; Aquino *et al.*, 2003).

Effect of metal ions

None of the metal ions indicated stimulation of dextrinogenic activity and the EDTA treated amylase did not show any activity inhibition, demonstrating that the enzyme did not require metal ions for activity and stability (Table 2). Al^{3+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and monovalent cations inhibited from 15 to 75% of this activity. Saccharogenic activity was inhibited by all the ions except Ba^{2+} and Co^{2+} which were found to slightly activate this reaction, increasing the rate by 15% and 34%, respectively. There was marked inhibition by Ag^{+}, Hg^{2+}, Cu^{2+}, and Fe^{2+}: the enzyme activity fell to around 10% of the control with the first two ions and to 50% in the presence of Fe^{+2}. Heavy metal ions such as Hg^{2+}, Ag^{+}, and Cu^{2+} were reported, as inhibited other thermostable α-amylases (Takasaki, 1982; Shaw *et al.*, 1995; Mamo and Gessesse, 1999). Since one of the protein thermostabilization mechanisms is disulfite bridges, the presence of this group (cysteines residues) is higher in themostable molecules.

Table 2. Effect of metal ions on amylase activities

Metal ions	Relative saccharogenic activity (%)	Relative dextrinogenic activity (%)
Al^{3+}	57	67
Cr^{3+}	50	97
Mg^{2+}	60	100
Mn^{2+}	55	100
Zn^{2+}	45	100
Ba^{2+}	115	100
Ni^{2+}	70	100
Co^{2+}	134	100
Hg^{2+}	12	85
Fe^{2+}	54	25
Cu^{2+}	8	69
K^{+}	75	90
Ag^{+}	12	88
EDTA	100	100

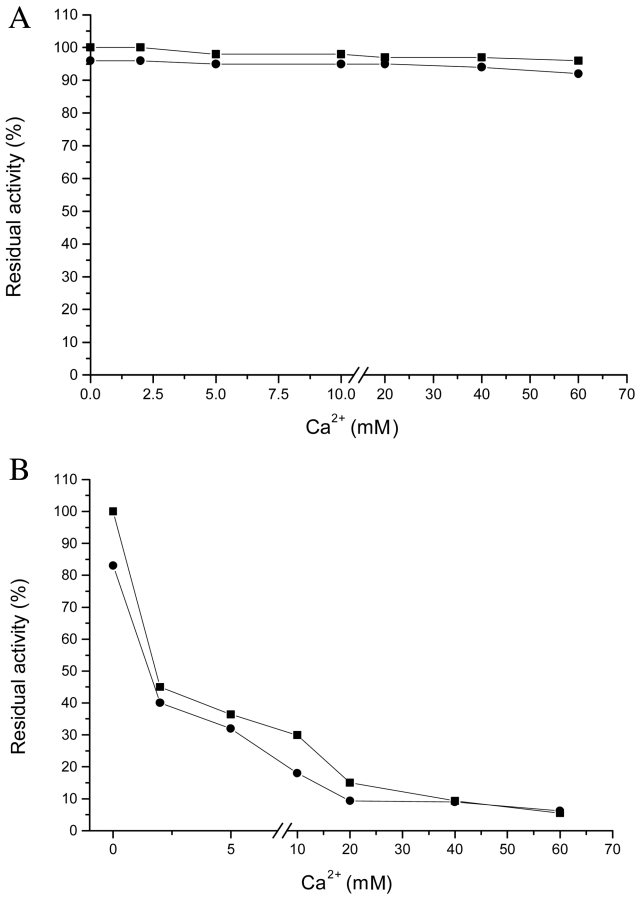

Fig. 3. Effect of Ca^{2+} concentration on the amylase activities. A, dextrinogenic activity; B, saccharogenic activity; circle=dialyzed enzyme; square=not dialyzed enzyme.

Consequently, they can be more susceptibly to inhibition forthese ions.

The effect of oxidative ions on the stability of the enzyme has been attributed to cysteine oxidation that leads to the formation or reorganization of intramolecular and intermolecular disulfide bridges, besides the formation of sulfenic acids that result in structural changes in the molecule (Vieille and Zeikus, 2001). The almost complete inhibition of saccharogenic activity by Hg^{2+} and Cu^{2+}reinforces the hypothesis that a cysteine residue can be involved in the enzyme activity. The low inhibition of dextrinogenic activity by this ion suggests that different molecules of enzymes are present in the medium.

In Fig. 3 it is shown that 2.5 mM Ca^{2+} reduced saccharogenic activity by 60% (Fig. 3B), while even 60 mM did not affect dextrinogenic activity (Fig 3A). The removal of calcium by dialysis against EDTA did not interfere in the results. Inhibition by calcium was reported for thermophilic α-amylases from fungi and hyperthermophilic Archaea and Bacteria, including *Rhizomucor pusillus*, *Pyrococcus furiosus*, *Thermatoga litoralis* (Somkuti and Steinberg, 1980; Takasaki, 1982; Koch *et al*., 1990; Laderman *et al*., 1993), unlike the α-amylases obtained from mesophilic sources such as *Aspergillus oryzae*, *A. niger*, *Bacillus licheniformis* and *B. subtilis*, which require Ca^{2+} (Joyet *et al*., 1992; Gupta *et al*. 2003).

Attempts to influence the stability of amylase at 60°C with Ca^{2+} are presented in Fig. 4. The results suggest that the enzyme thermostability was indifferent to this ion. It has been reported that calcium confers a rigid structure on the amylase molecule. This is necessary for it to function effectively at high temperatures, by forming an intramolecular metal-chelate structure (Hsiu *et al*., 1964) that acts as a salt/ion bridge between two adjacent residues, stabilizing an α-helical structure (Koch *et al*., 1990; Koch *et al*., 1991). According to Brown and Kelly (1993), Ca^{2+} is also involved in facilitating substrate binding with the active site. It has been suggested that a calcium requirement is the prerogative of amylase from mesophilic microorganisms, since there are various reports of thermophilic amylases being inhibited by or indifferent to calcium (Saboury and Karbassi, 2000). Our results suggest that Ca^{2+} may inhibit saccharogenic activity by a possible interference with the active site. According to Lévêque *et al*. (2000), a metal ion present at high concentrations might compete with another metal present at a lower concentration and replace it at a metal-binding site, even if its affinity for the binding site is lower. This would lead to a modification of the enzyme activity. However, non-inhibition of enzyme activity by EDTA treatment, seems to exclude this hypothesis.

In any event, the present data indicates the excellent thermostability of these enzymes: 100% saccharogenic activity was retained after 1 h at 60°C and 80% after 2 h in the absence of substrates, while dextrinogenic activity remained at 100% of the original after 5 h at 60°C and 80 % after 7 h.

Specificity by substrate

The study of the substrate specificity indicated that dextrinogenic α-amylase liquefied cassava starch most efficiently, while saccharification was most efficient on potato starch (Table 3). The glucoamylase activity was highest on corn starch. There are often variations in the composition of amylose and amylopectin and the quantity of lipids in starch, depending on its origin (cereal starch has approximately 28% amylose, 72% amylopectin and 6.0% lipids, while tuber starch has approximately 20% amylose, 80% amylopectin and 0.1% lipids) (Aberle *et al*., 1994; Hoover, 2001). The results suggest that the composition and variation in the molecular structure of the starch could affect the enzyme action (Cruz *et al*., 1997). The lower affinity of the enzymes for low-molecular-weight substrates than for highly polymerized glucan is consistent with the properties of α-amylase and glucoamylase. In addition, the absence of any action on *p*-nitrophenyl-α-D maltoside excludes the presence of α-amylase in the crude enzyme solution.

Table 3. Substrate specificity of the amylases from *Rhizomucor pusillus* A 13.36

Substrate	Enzyme activities (U/ml)		
	Dextrinogenic	Saccharogenic	Glucoamylase
Corn starch	10.0 ± 0.80	11.5 ± 0.90	1.34 ± 0.08
Cassava starch	12.0 ± 0.50	11.0 ± 0.70	0.81 ± 0.07
Potato starch	7.5 ± 0.60	12 ± 0.89	0.35 ± 0.04
Maltoheptaose	ND	ND	ND
Maltohexaose	ND	ND	ND
Maltopentaose	ND	ND	ND
Maltotetraose	ND	ND	ND
Maltotriose	ND	ND	ND
Maltose	ND	ND	0.08
p-nitrophenyl-α-D-maltoside	ND	ND	ND

ND=Not detected

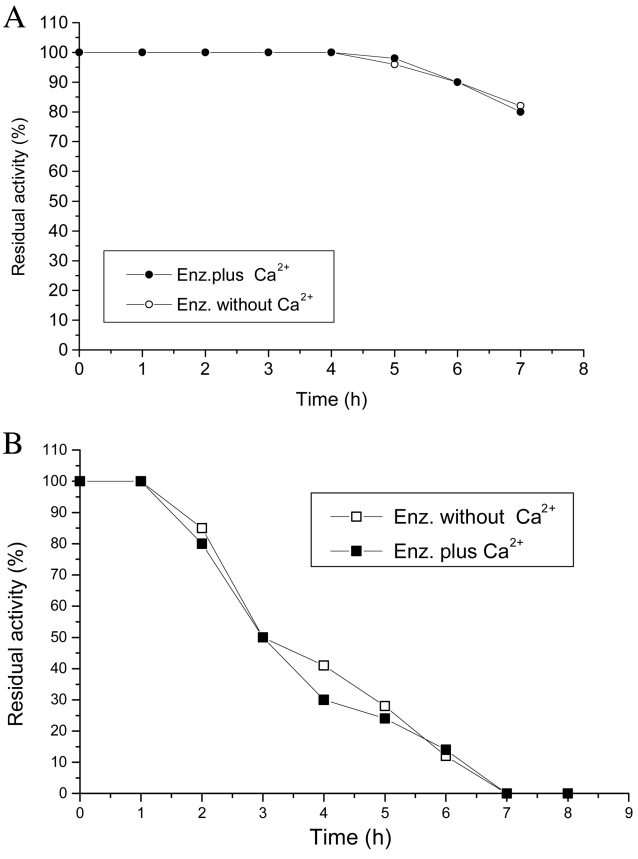

Fig. 4. Thermostability of dextrinogenic (A) and saccharogenic (B) amylase at 60°C in the presence (full symbol) and absence (open symbol) of calcium.

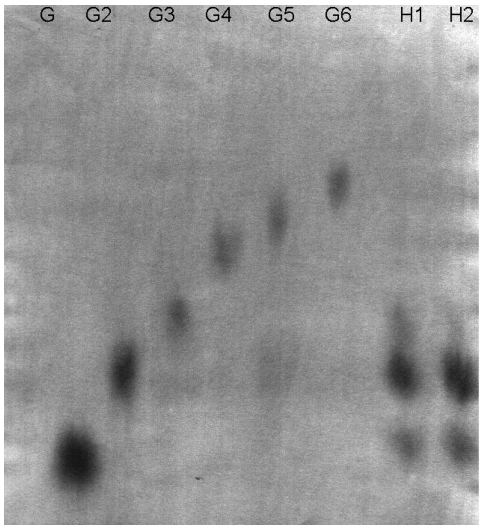

Fig. 5. Paper-chromatographic analysis of hydrolysis products of amylase from *Rhizomucor pusillus* 13.36, acting on 1.0 % (w/v) soluble starch at 75°C, for 10 and 30 min.
G=Glucose; G_2=Maltose; G_3=Maltotriose G_4=Maltotetraose; G_5=Maltopentaose; G_6=Maltohexaose; H_1=Hydrolyzed for 10 min; H_2=Hydrolyzed for 30 min.

Analysis of hydrolytic products

Paper chromatography of the soluble starch hydrolysis products formed after 10 and 30 min of reaction which mainly revealed glucose, maltose and maltotriose as end products (Fig. 5). These results were confirmed by HPLC analysis (Table 4). After 1 h of reaction, glucose represented 64% of the hydrolyzed starch and maltose and maltotriose, 19% and 7.0%, respectively. A low proportion of maltooligosaccharides was detected (6.0%). The data indicated an excellent saccharification of the starch, resulting in a syrup whose glucose concentration was higher than those described for conventional enzymatic hydrolysis of starch (Brumm, 1998).

The different responses to pH, temperature, cations and alternative substrates, observed for dextrinogenic and saccharogenic activities, indicate the presence of at least two α-amylases besides glucoamylase activity. It is widely accepted that α-amylases can be divided into two categories according to the degree of hydrolysis of the substrate. Saccharogenic α-amylase hydrolyzes 50-60% of starch producing low-molecular-weight oligosaccharides

Table 4. High-performance liquid chromatography analysis of final products from 1% soluble starch hydrolysis using crude enzyme from Rhizomucor pusillus A 13.36

Hydrolysis time (min)	Composition of hydrolyzed									
	Glucose		Maltose		Maltotriose		Maltotetraose		≥Maltopentaose	
	(%)	(mg/ml)	(%)	(mg/ml)	(%)	(mg/ml)	(%)	(mg/ml)	(%)	(mg/ml)
10	14.0	2.4 ± 0.2	4.5	0.8 ± 0.7	2.6	0.5 ± 0.05	0	0.1 ± 0.008	3.3	0.6 ± 0.06
30	19.0	3.0 ± 0.3	8.4	1.4 ± 0.8	4.0	0.7 ± 0.08	1.4	0.2 ± 0.009	0.1	0.02 ± 0.002
60	64.0	10.8 ± 0.8	19.0	3.2 ± 0.7	7.0	1.2 ± 0.07		ND	6.0	1.0 ± 0.05
240	66.0	11.2 ± 0.6	19.5	3.3 ± 0.4	7.7	1.3 ± 0.08		ND	6.6	1.1 ± 0.04

(Crude enzyme activity; dextrinogenic=11 U/ml; amylase saccharogenic=14 U/ml glucoamylase=1.0 U/ml). The result were main of three assays. ND=Not detected

(glucose and maltose) while dextrinogenic α-amylase hydrolyzes about 30 to 40% of starch, resulting in oligosaccharides from maltopentaose upwards (Vihinen *et al*., 1989; Nigam and Singh, 1995; Peixoto *et al*., 2003).

The presence of glucoamylase, associated with dextrinogenic and saccharogenic α-amylase activities, as well as the high optimal temperatures, superior thermostability in the absence of substrate and stability over a wide pH range of these enzymes are valuable characteristics that may offer great potential for industrial application.

Acknowledgements

The authors wish to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for their financial support.

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