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Research Article

Production and Functional Properties of Free and Immobilized Glucoamylases of *Penicillium citrinum*

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Abstract

Amylases are enzymes with important biotechnological applications that are produced by several microorganisms in the form of filamentous fungi. *Penicillium citrinum* was recently isolated from the decomposed materials of Atlantic Forest, Brazil. It was identified by internal transcribed spacer (ITS) and beta-tubulin, and presented with excellent amylolytic levels. Thin layer chromatography showed that the amylase system was constituted by glucoamylase. This enzyme showed high activity at 60°C, pH 5.5, and an increment of 160% of the relative activity in the presence of 2 mM $MgCl_2$. The thermostability was increased after the immobilization on ionic supports. The enzyme immobilized on DEAE-sepharose retained 100% of its residual activity and when immobilized on MANAE-sepharose derivatives retained 72% at temperatures of 50°C and 60°C after 120 minutes of incubation. CNBr was used as a control. In contrast, the free enzyme only retained 50% of its residual activity after 90 minutes, at 50°C. Therefore, this work shows the production of glucoamylase by a novel isolated of *P. citrinum* with potential application in biotechnological areas as starch, food, baking, detergent and soap powder, paper and textile industries and fuel production.

Keywords: Glucoamylase; *Penicillium citrinum*; PCR; Immobilization; Enzymatic Properties

Introduction

Brazil is situated in a tropical region with hot-wet large forests, the Atlantic Forest, which is a biome with 98.000 km^2 of extension [1]. These conditions are appropriate for the development of novel and interesting species of microorganisms with attractive production of primary and secondary metabolites. Bioprospecting may be defined as the exploitation and research resources from the flora and fauna to identify the active principles for obtaining new products and processes aimed by the market [2]. In this context, the screening and identification of filamentous fungi with potential biotechnological applications have become important. According to BBC reports, the enzyme market handles about 4 billion dollars nowadays, and is expected to reach about 6 billion dollars by 2016 [3].

Starch is present in most vegetables with the initial function of storing energy collected by the photosynthesis, and is fundamental to plants, animals and microorganisms. Chemically, starch is a mixture of two polysaccharides: amylose and amylopectin. Amylose consists of linear chains of glucose

residues helically joined by α-1, 4 glycosidic bonds in which each loop of the spiral is formed by about six glucose units. Amylopectin has a highly branched structure with a chain of glucose residues linked by α-1, 4 glycosidic bonds, and the branches taking place with α-1,6 bonds every 25 glucose residues [4]. Amylase hydrolyzes the α-1, 4 glycosidic linkages from the starch molecule. First, endoamylases (EC 3.2.1.1) hydrolyze the glycosidic linkages rando mLy within the starch molecule releasing oligosaccharides. After that, exoamylases (essentially β-amylase; glucoamylase; α-glucosidase) hydrolyze the glycosidic linkages from non-reducing end of the molecule, releasing glucose and maltose [5]. The dextrins, glucose syrups, maltose syrups, maltodextrins and cyclodextrins obtained from the starch processing are used in confectionery, brewery and pharmaceutical industries [6,7]. For the wide application of these enzymes, it is relevant to find new sources of amylase, especially those with high thermostability, which are interesting for industries. The major problem regarding the application of these enzymes is the limitations due to low enzyme activity, poor stability, incomplete conversion, enzyme inhibition of glucose and maltose, microorganism contamination at low temperatures and inability to degrade a wide range of starches [8]. Enzymes that have optimal activities at extreme temperatures and pH are widely used in household detergents and in the food, textile, pulp and paper, leather processing and chemical industries [9]. Enzyme immobilization when adequately carried out satisfies both catalytic and non-catalytic requirements, including the operational process and storage stability [10].

Generally, soluble enzymes have to be immobilized for reuse for long times in industrial reactors and, in addition to that, some other critical enzyme properties have to be improved like stability, activity, inhibition by reaction products, selectivity towards non-natural substrates. In this way, immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols [11]. Immobilization process can occur by simple adsorption or by covalent bonds. The most common immobilization is occurred by adsorption, because it has a low cost and few deleterious effects on the activity and selectivity of the enzyme [12].

The aim of this work was to isolate fungi from soil, decomposing material or residues of cassava flour artisanal industry placed in the Atlantic Forest. After a screening for amylase production, *P. citrinum* was chosen by its high enzymatic levels and because data about this microorganism are rare. The results presented here have shown interesting functional properties as good thermal activity, which can be improved using immobilization procedures.

Materials and Methods

Microorganisms and maintenance medium

Microorganisms were previously collected and isolated from soil, decomposed materials or residues of cassava flour artisanal industry from the Atlantic Forest, in the Region of Paraty, Rio de Janeiro, Brazil. Fungal strains were morphologically identified and maintained at Universidade Federal de Pernambuco (UFPE), PE, Brazil and further, their identification was confirmed by molecular identification as described below. Microorganisms were maintained in PDA medium at 4°C.

Molecular characterization of the isolated fungi

Analysis based on the sequences of rDNA coding for internal transcribed spacers (ITS 1 and 4) and the intervening 5.8S gene were used for the molecular identification of the isolated fungal species. Mycelia, developed in SR (Segato-Rizzatti) medium cultures [13], were incubated at 30°C, for 4 days and used for genomic DNA extraction. 40mg of fine-powdered mycelium, obtained using the acetone-drying protocol, was suspended in 1 mL of the extraction buffer (1% SDS; 50 mM EDTA, 200 μg mL^{-1} RNase) and subjected to a conventional phenol extraction method [14]. The internal transcribed spacer sequence (ITSI-5.8S-ITSII) was amplified using universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') according to White et al. [15]. The PCR reaction mixture (50 μl) containing 2 mM $MgCl_2$, 2 μM of each primer, 100 μM dNTP mix, 1U of Taq DNA polymerase and 100ng DNA template was used. The sequence was amplified by PCR with an initial denaturation (10min at 96°C), followed by 30 cycles of denaturation (95°C for 1min), annealing (60°C for 1min), and primer extension (72°C for 1min), and a final extension step for 10min at 72°C. The amplicons were verified by electrophoresis on 1.8% (w/v) agarose gel. Beta-tubulin was amplified using primers, beta-tubulin forward (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and beta-tubulin reverse (5'-ACCCTCAGTGTAGTGACCCTTGGC-3') [16]. PCR reactions were performed in 50 μL of the reaction mixtures containing 1 μl genomic DNA (10 $ng.\mu l^{-1}$), 5 μl PCR buffer, 30 μl ultra-pure sterile water, 10 μl dNTP (1 mM), 1 μl of each primer (50 $pmol.\mu l^{-1}$) and 1 μl Taq polymerase (2.5 $U.\mu l^{-1}$ DNA). The samples were also amplified by PCR. After the initial DNA denaturation at 94°C for 1 minute, the reaction proceeded with 35 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes.

DNA sequencing was performed using an ABI PRISM 377 sequencer (Applied Biosystem, USA) following the manufacturer's protocol from the DYEnamic ET terminator cycle sequencing kits (GE Healthcare, UK). The cycle sequencing reaction mixture had a total reaction volume of 10 μl, and contained 1 μl of template DNA (10-15 $ng.\mu l^{-1}$), 4 μl Dye terminator RR mix, 4 μl ultra-pure sterile water and 1 μl primer (4 $pmol.\mu l^{-1}$).

ITS PCR products were directly sequenced using the ITS1 and ITS4 PCR primers as described above and two additional internal primers ITS2 (5' GCTGCGTTCTTCATCGATGC 3') and ITS3 (5' GCATCGATGAAGAACGCAGC 3') [17], and also beta-tubulin forward and reverse as described above. The quality of the sequences was examined by the Phred/Phrap/Consed package (University of Washington, Seattle, Wash.). Species identification was made by searching databases using the BLAST and Clustal Omega algorithms [18,19], with default settings, for sequence comparison with reference sequences from NCBI GenBank. Species identification was determined from the best-scoring reference sequence of the similarity output that had ≥ 97% identity with the query sequence. Other sequences of Penicillium ITS1 and ITS4 were obtained from GenBank for comparison and phylogenetic analyzes. A phylogram was constructed using Clustal Omega.

Culture medium and growth conditions for enzyme production

The fungus was grown in liquid standing cultures at 30°C for 144 hours. Approximately 107 spores/ mL from a 3-day-old PDA culture were inoculated into 125 mL Erlenmeyer flasks containing 25 mL of CP (Carvalho-Peixoto) medium, pH 6.0, containing 1.0% (w/v) soluble starch as carbon source, previously autoclaved for 121°C and 1 atm for 20 minutes [20]. After 144h, the mycelium was filtered and the crude extract was dialyzed overnight, at 4°C, against 100 mM sodium acetate buffer, pH 5.5 to determine the enzymatic activity levels. Dry weight was determined by drying the mycelium at 80°C up to 72 hours.

Identification of hydrolysis products

The hydrolysis products of amylase activity on 1.0 % soluble starch as substrate were analyzed using thin-layer chromatography (TLC). The mobile phase was composed by butanol/ethanol/water (5:3:2 v/v/v). Sugars were revealed in a 0.2% orcinol diluted with methanol-sulfuric acid (9:1 v/v) mixture [21], using 1.0% glucose, maltose, maltotriose, maltotetraose and maltopentaose as standards.

Determination of amylase activity

The amylolytic activity was carried out using 1% soluble commercial starch in 100 mM sodium acetate buffer, pH 5.5, to determine the released reducing sugar as described by Miller [22]. A glucose (0–1 mg. mL^{-1}) standard curve was used as a control. A blank was prepared by adding DNS before the enzyme was added. One unit of enzyme was defined as the amount of enzyme that releases reducing sugars at an initial rate of 1 $\mu mol.min^{-1}$ per mL of the reaction mixture under the assay conditions.

Enzymatic characterization

The optimum pH was determined at 60°C using 100 mM McIlvaine buffer (pH range 3.0-7.5). The pH stability was determined at 30°C, up to 24 hours, after pre-incubation of the diluted enzyme in McIlvaine (100 mM) buffer at different pH values (pH range 3.0-7.5). The optimum temperature of the enzyme was determined at pH 5.5 by measuring the activity at different temperatures in the range of 30– 80°C.

The thermostability of the free amylase was determined by measuring the residual activity after incubating the diluted enzyme in the absence of substrate at 50°C and 60°C in 100 mM sodium acetate buffer pH 5.5, for 120 minutes.

Effect of some metal ions and chemicals on the enzyme activity

The effect of metal ions on the enzyme activity was separately investigated by adding $BaCl_2$, $CoCl_2$, $MgCl_2$, $CuSO_4$, $MnCl_2$, $Zn(NO_3)_2$, $AlCl_3$, NH_4Cl, NaBr, KCl, $CaCl_2$ and ethylenediamine tetra-acetic acid (EDTA) directly to the standard reaction mixture in a final concentration of 1 mM and 10 mM. The ions that improve the enzyme activity were tested in different concentrations (10^{-5}, 10^{-3}, 10^{-1}, 1, 2, 4, 6, 8, 10, 12 and 15 mM). Control was carried out with the enzyme denatured by heat, metallic ions or EDTA and soluble starch as described in the enzyme characterization.

Immobilization

Support preparation

DEAE-Sepharose was purchased from GE Healthcare Bio-Sciences. Monoaminoethyl-N-ethyl-agarose (MANAE-agarose) was prepared according to Fernandez-Lafuente et al. [23]. A volume of 27.07 mL of 2 M ethylenediamine was dissolved in 100 mL distilled water, and the pH of the solution was adjusted to 10, using HCl, and the volume adjusted to 200 mL with distilled water. The next step was to pour the solution of ethylenediamine over 35g glyoxyl agarose and constantly stirred for 2h. Then 2g of sodium borohydride (BH_4Na) was added to the vessel, and this was stirred for 2h. Finally, the gel was washed with 1L of 100 mM sodium acetate buffer, pH 4.0, and 1L of a solution of 100 mM sodium borate, pH 9.0.

Enzyme immobilization

The enzyme solution (3.13 U. mL^{-1}, 0.006 mg/ mL) at pH 7.0 was mixed with the specific amount of different supports at different times, and the samples of the supernatant and support enzyme suspension were taken, and then the activity was assayed. The immobilization was carried out with 1g of the corresponding anionic exchanger support (MANAE-agarose

and DEAE-Sepharose) with 20 mL of the enzyme solutions (0.12mg of protein) in 25 mM of sodium phosphate buffer pH 7.0. The different suspensions were gently stirred at 25°C, for 2h. Samples of the supernatant and the suspensions were periodically taken and the enzymatic activities or protein concentrations were measured. After that, the preparations were washed five-fold with 2 volumes of the same adsorption buffer and, finally, twice with 2 volumes of 25 mM sodium phosphate buffer pH 7.0. An amylase solution, used as control, was submitted to the same conditions to detect possible damages on the enzyme activity.

Desorption of enzyme from the DEAE-Sepharose and MANAE-agarose supports

The DEAE Sepharose and MANAE-agarose derivatives were suspended in a volume equivalent in 25 mM sodium phosphate buffer at pH 7 in the adsorption process carried out. Increasing concentrations of NaCl were added to the derivative, starting with 50 up to 300 mM, in sodium phosphate buffer, pH 7. Aliquots were withdrawn from both the supernatant and the suspension, containing the derivative. The measurement was performed at intervals of 30 min, and the amylase activity was determined. Desorption occurred at room temperature. The DEAE-sepharose and MANAE-agarose derivatives in 25 mM sodium phosphate buffer at pH 7 were used. The free enzyme was also used as control. Both controls were subjected to the same treatment in order to detect any possible effect of NaCl and temperature on the amylase activity.

Reproducibility

All results are the means of at least three independent experiments.

Results

Identification and Growth

According to Figure 1, of 19 isolated fungi, three were separated as the best producers of amylase (Figure 1). *Penicillium citrinum* was the best producer with an amylase activity of 1.10 U. mL^{-1}, and *Aspergillus japonicus* produced 0.86 U. mL^{-1}, which was considered good enzymatic levels as well. The amylase production by *A. japonicus* was well described by [24], and it will be considered for further studies. These fungi were morphologically identified by Universidade Federal de Pernambuco, PE, Brazil, by Dr. Cristina Maria de Souza Mota, and the molecular identification was performed in our laboratory using ITS1, ITS4 and beta-tubulin as markers. Two other fungi named 3B1 and 2B1 also showed high amylase activity levels, but they will be investigated in further studies. The fungi were identified based on the sequences previously deposited in GenBank as shown in Table 1. Based on the genetic analysis of the fungi shown in Table 1, the topology of a phylogram was assembled using Cluster Omega (Figure 2). It is observed that all species of *Penicillium* were grouped separately from the representatives of other genera (*Paecilomyces* and *Aspergillus*), as expected. However, it is noteworthy that among the Penicillium species, there are higher similarities forming monophyletic groups between *P. radicum* and *P. pinophilum*, and between *P. crustosum* and P. commune. *P. citrinum* was not grouped with any other *Penicillium* analyzed.

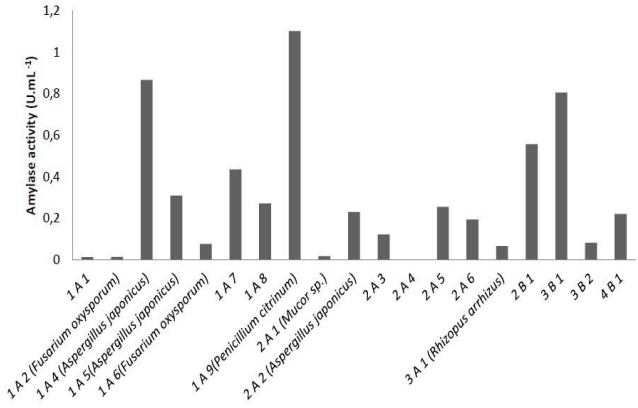

Figure 1. Enzymatic activity of the collected fungi. Fermentation occurred in a liquid medium (CP) with three days of cultivation at 30°C.

Table 1. Fungi strains used in this study.

Fungus	GenBank accession number	Reference
Penicillium citrinum	JQ666004.1	25
Penicillium crustosum	JN226946.1	26
Penicillium radicum	AB457007.1	27
Penicilium pinophilum	JN222359.1	28
Penicillium janthinellum	JX111908.1	-
Penicillium commune	HM366606.1	29
Paecilomyces variotti	FJ895878.1	30
Aspergillus niger	FJ810501.1	30
Aspergillus kawachii	AB573884.1	31

*Fungi from the collection of the microbiology laboratory in FFCLRP-USP.

Table 2 shows the time-course of glucoamylase production and mycelial dry weight. *P. citrinum* showed an exponential growth (the log phase) up to 72h. From this period, the fungus entered into the stationary growth phase up to 144h, where a decrease of dry weight was observed suggesting the beginning of the death phase. In contrast, the highest glucoamylase levels were observed with 168h, and although at this time, the enzyme showed the highest activity (4.7 U. mL^{-1}), these were not significantly different from those enzymatic level found with 144h (4.1 U. mL^{-1}), so this was chosen for the fermentation time to the characterization step.

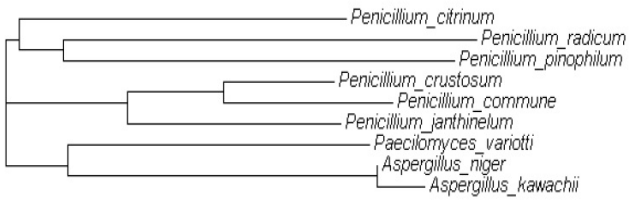

Figure 2. Phylogram after CLUSTAL OMEGA multiple alignments of the ITS-1-5.8S-ITS4 sequence regions of the nine fungal strains studied in this work in comparison to their respective sequences obtained from NCBI GenBank by BLASTn searching. Every sequence access numbers are indicated according to table 1.

Table 2. Time-course of glucoamylase production and dry weight of *P. citrinum.*

Cultivation time (hours)	Dry weight (mg)	Glucoamylase activity (U/mL)	Specific activity (U/mg dry weight)
48	0.01	0.00	0.00
72	12.35	2.67	0.22
96	13.10	3.60	0.27
120	12.96	3.08	0.24
144	13.53	4.10	0.30
168	11.96	4.70	0.39

* *P. citrinum* was cultivated in CP medium supplemented with soluble starch up to 168h, 30ºC, in static conditions.

Thin layer chromatography on silica (TLC) of the products of starch hydrolysis

The released products of starch hydrolysis were analyzed and identified by TLC using glucose, maltose, maltotriose, maltotetraose and maltopentose as standards (Figure 3). In the first minutes of incubation, only glucose formation was observed, which indicates the presence of a glucoamylase.

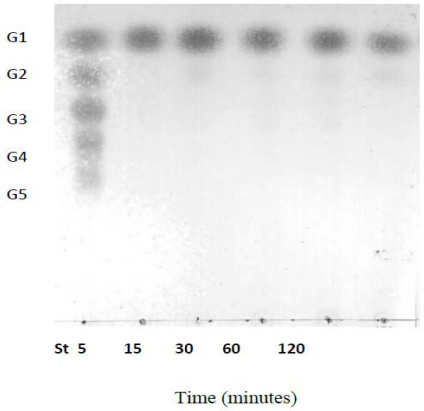

Figure 3. Thin-layer chromatography of the reaction products of the soluble starch hydrolyzed by *Penicillium citrinum* glucoamylase at different times. Standard (St) was a mixture of 1 mg/mL of glucose (G1), maltose (G2), maltotriose (G3), maltotetraose (G4), and maltopentaose (G5).

Enzymatic characterization

Figure 4 shows the effects of temperature on the activity of the glucoamylase of *P. citrinum*. In order to determine the optimum temperature of the enzyme, the tests were carried out at temperatures ranging from 30 to 80°C. From 30°C (4.5 U. mL^{-1}) to 60°C (6.0 U. mL^{-1}), the activity gradually rose up to 60°C and after it was observed a slight fall at 65°C (4.0 U. mL^{-1}). From this moment, the activity sharply decreased until it no longer was detected at temperatures higher than 70°C, probably a denaturant condition to the glucoamylase produced by *P. citrinum*.

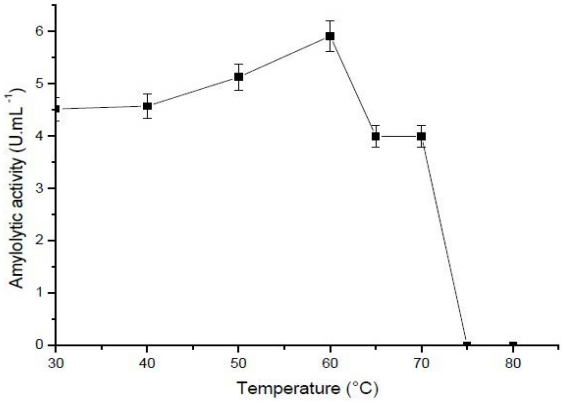

Figure 4. Effect of temperature on glucoamylase activity. The enzyme was assayed at 100 mMMcIlvaine buffer, pH 5.5. The glucoamylase activity was estimated by the DNS method using a 1% starch solution as substrate.

Aiming for improving the glucoamylase thermostability, the enzyme was immobilized on DEAE-Sepharose and MANAE-agarose, both resins of adsorption column with weak ionic interaction charges [10]. The enzyme was immobilized on DEAE-Sepharose and MANAE-agarose retained 100% and 72%, respectively. Both had 100% of recovery activity when used 200 mM and 300 mM NaCl, respectively (Table 3).

Table 3. Adsorption and desorption of the enzyme and the expressed activity on different supports.

Support	Immobilization (%)	Recovery activity [a] (%)	Concentration of NaCl[b](mM)
DEAE-sepharose	100	100	200
MANAE-agarose	72	100	300

[a] The percentage of the recovery activity on the support with the enzyme is relative to the initial activity.

[b] The desorption was carry out for the two better immobilized supports. Increasing concentrations of NaCl were used.

The glucoamylase thermostability was investigated by incubating the enzyme at 50°C and 60°C for 2 hours. The enzyme was incubated on ice bath, and then the activity was tested under standard conditions. The free amylases showed to be sensitive to the temperature at the first minutes of incubation, presenting approximately 78% of residual activity at 50°C and 60°C after 50 minutes, respectively. Besides, the immobilized

enzymes were completely stable at both temperatures. DEAE-agarose and MANAE-agarose derivatives retained 100% of residual activity with them. Whereas the temperature of 50°C the control (CNBr) of immobilization reached the half-life to 70 minutes. Already at 60°C, CNBr reached the half-life of approximately 25 minutes (Figures 5A and B).

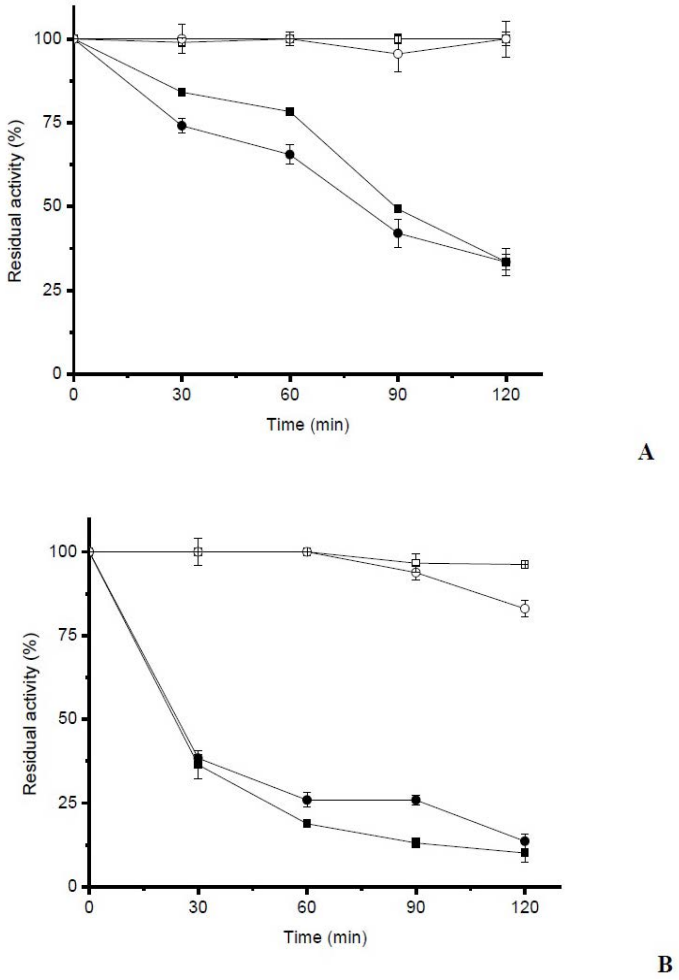

Figure 5. Effect of thermal stability on glucoamylase activity.(A) Thermal stability at 50°C; (B) thermal stability at 60°C.Symbols: (-■-) free enzyme; (-•-) CNBr derivative; (-□-) DEAE-sepharose derivative; (-○-) MANAE-sepharose derivative.

The results of thermostability of the derivatives were with the free enzyme. The DEAE-sepharose and MANAE-agarose derivatives showed excellent thermostability as compared to the free enzyme when the CNBr derivative was used as a control. At 60°C (Figure 5B), DEAE-sepharose and MANAE-agarose derivatives retained 100% of their residual activities even after 120 minutes of incubation, while the free enzyme and CNBr derivative retained only about 50% of the residual activity when incubated at 50°C for 90 minutes (Figure 5A).

pH effect

Aiming to verify the optimum pH of the *P. citrinum* glucoamylase, the enzymatic reaction was carried out in the pH range of 3–7.5 using McIlvaine buffer (citrate-phosphate). Tests have shown that the best values were obtained at pH 6.0 (Figure 6), which is slightly higher than glucoamylase from *Aspergillus niveus* (pH 5.5) [32].

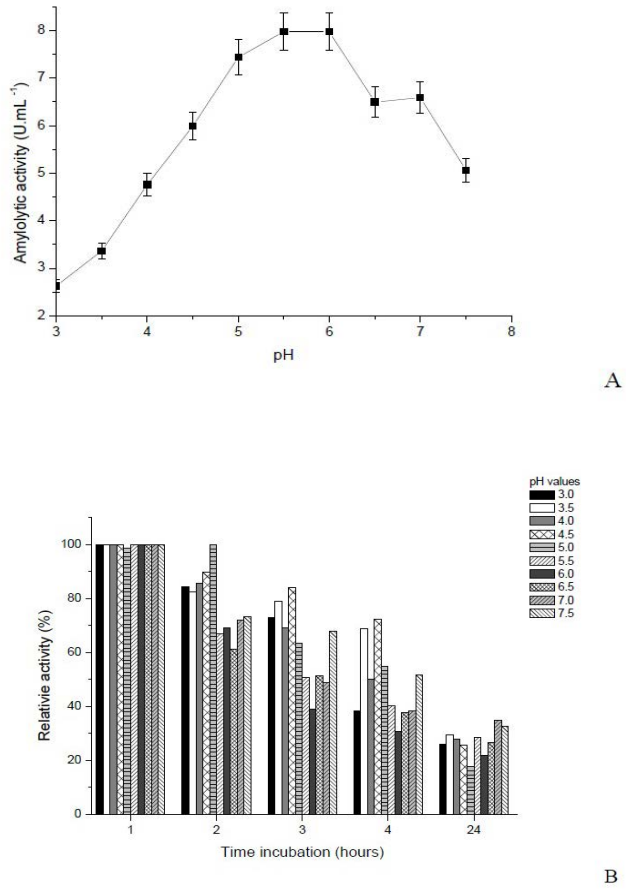

Figure 6. Effect of pH on enzyme activity (A). The reactions were performed at 60ºC at pH values (3.0 – 7.5) in McIlvine buffer. Glucoamylase stability in different pH (B). The pH stability was determined at 30°C, up to24 hours, after pre-incubation of the diluted enzyme in 100 mM McIlvaine buffer at different pH values (showed on right side of Figure B). All the glucoamylase activity was estimated by the DNS method using starch (1%) as substrate.

The pH stability was investigated by incubating the enzyme up to 24h at the same pH range. The remaining activity was measured under standard conditions (Figure 6B). The relative activity of *P. citrinum* glucoamylase was stable only for the incubation time of one hour during the entire range of pH analyzed. For the remaining times (2 hours), the stability declined with pH values above and below 5.0. Several reports showed that the best microbial glucoamylase activities are included in pHs below 7.0, but, in general, the enzyme showed to be more

stable at acidic pH as seen with *P. citrinum* glucoamylase (Table 4).

Table 4. Fungi with similar activity in relation to *P.citrinum* glucoamylase concerning the pH variations. The residual activity values are shown in % and their respective pH.

Fungi	Residual activity (%)	Optimum pH	Reference
Penicillium pinophilum MS 20	100 (pH 5.0)	5.0	28
Penicillium janthinellum	90 (pH 5.0)	5.0	33
Paecilomyces variotii	100 (pH 5.5)	4.0	34
Aspergillus niveus	100 (pH 4.0-8.0)	5.5	32
Aspergillus kawachii	90 (pH 2.0, 30 min)	5.0	35
Penicilium citrinum HBF 62	80 (pH 5.5)	5.5	36
Penicilium citrinum	100	5.5	present paper

Effects of metal ions on enzyme activity

In order to determine the influence of metal ions on the activity glucoamylase, the enzyme samples were incubated in the presence of several metallic ions and EDTA at the concentrations of 1 mM and 10 mM. The enzyme activity was inhibited by ions Cu^{2+}, Ba^{2+} and Mg^{2+}, and EDTA. A significant increase in glucoamylase activity was observed after the addition of 10 mM divalent ions, such as manganese (165%), calcium (33.6%) and cobalt (30%) (Table 5). EDTA diminished the glucoamylase activity at 40–35%. This result indicates the dependency of this enzyme to metal ions. Due to the increase in glucoamylase activity, it was interesting to test a larger range of ions as Ca^{2+}, Co^{2+} and Mn^{2+} (Figure 7).

Table 5. Effect of metallic ions and EDTA on glucoamylase activity.

Composts	Relative activity (%)	
	1.0 mM	10 mM
Control	100	100
$CuSO_4$	65.22	27.15
$BaCl_2$	65.22	83.87
$MgCl_2$	81.88	97.31
NaBr	100.72	102.96
KCl	100.00	122.58
$AlCl_3$	104.71	108.87
$Zn(NO_3)_2$	107.25	113.71
NH_4Cl	107.97	114.78
$CaCl_2$	111.23	133.60
$CoCl_2$	113.04	130.11
$MnCl_2$	125.72	265.86
EDTA	65.94	76.34

* Control: the enzymatic assay was carried out with the inactivated enzyme by boiling.

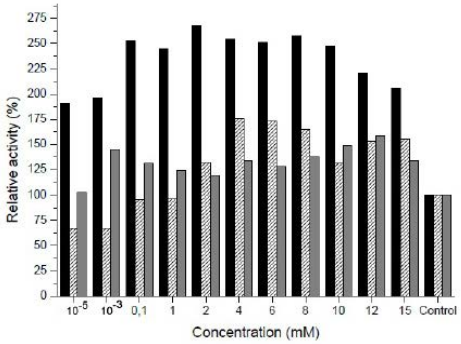

Figure 7. Effect of different concentration of ions on glucoamylase activity. Black bars correspond to $MnCl_2$, hatch bars correspond to $CoCl_2$ and gray bars correspond to $CaCl_2$.

It was observed that lower concentrations as 2.0 mM $MnCl_2$ and 4.0 mM $CoCl_2$ increased glucoamylase activity by 8.3% and 34% more than the percentage observed for the same metallic ions at 10mM. Variations in the concentration of $CaCl_2$ were slightly less significant, but 12 mM $CaCl_2$ increased the glucoamylase levels by 6%.

Discussion

Based on the genetic analysis of the fungi shown in Table 1, the topology of a phylogram was assembled using Cluster Omega (Figure 2). It is observed that all species of *Penicillium* were grouped separately from the representatives of other genera (*Paecilomyces* and *Aspergillus*), as expected. However, it is noteworthy that among the *Penicillium* species, there are higher similarities forming monophyletic groups between *P. radicum* and *P. pinophilum*, and between *P. crustosum* and *P. commune. P. citrinum* was not grouped with any other *Penicillium* analyzed, but the characterization of *P. citrinum* amylases showed to be similar to other species of fungi.

Till date, only two papers have reported amylases production by *P. citrinum*. Metin et al. [36] described a characterization of an α-amylase from *P. citrinum* with optimum temperature and pH similar to the data presented in this report. Zu-Tong and Zeng-Mei [37] related the production of α-amylase and glucoamylase from three variant forms of *P. citrinum* but nevertheless there is no information regarding physical chemical characteristics from those amylases.

The thermostability of these enzymes was similar to the studies reported by Nahas and Waldemarin [38]. Aiming to improve the glucoamylase thermostability, the enzyme was immobilized on DEAE-sepharose and MANAE-agarose, both resins of adsorption column with weak ionic interaction charges [11]. The immobilization on DEAE-sepharose showed to be more efficient (100%) when compared to the MANAE-agarose (72%). Both

had 100% of recovery activity when 200 mM and 300 mM NaCl were, respectively, used (Table 3). This probably happened because the composition of the support used, DEAE-sepharose is a anion-exchange reactive group, diethylaminoethyl (DEAE) covalently linked to Sepharose (a polysaccharide polymer). Besides MANAE-sepharose (monoamino-ethyl-N-aminoethyl) can to be prepared with different degrees of activation, becoming less in this particular case.

The thermostability of the derivatives was investigated by incubating the enzyme at 50°C and 60°C for 120 minutes, and the results were compared to the free enzyme. The DEAE-sepharose and MANAE-agarose derivatives showed excellent thermostability as compared to the free enzyme and when the CNBr derivative was used as a control. At the temperature of 60°C (Figure 5B), DEAE-sepharose and MANAE-agarose derivatives retained 100% of their residual activities even after 120 minutes of incubation, while the free enzyme and CNBr derivative retained only about 50% of the residual activity when incubated at a temperature of 50°C for 90 minutes (Figure 5A).This result was similar to the studies reported by Benassi et al. [39] in which higher level of stabilization must be due to the abundance of large areas on the surface of the enzyme with very high density of negative charges and furthermore, these same regions are important for stabilizing their three-dimensional structure. It also was observed in this work that the immobilization process protected the enzyme against heat inactivation, and suggested possible perspectives of using this immobilized catalyst in a biotechnological process [39].

Enhancement of glucoamylase activity, such as the ones observed with Mn^{2+}, Ca^{2+}, Co^{2+}, Fe^{3+} and Ba^{2+} ions could be based on its ability to interact with negatively charged amino acid residues, such as aspartic and glutamic acid [40]. Enhancing the activity of glucoamylase could be observed in other starch degrading enzymes. The affinity of Ca^{2+} to amylase is much stronger than with other ions. Similar data have been reported by Metin et al. [36], where the α-amylases produced by *P. citrinum* showed an increase in activity after the addition of Mn^{2+} (183%), Ca^{2+} (139%) and Co^{2+} (138%) ions. Other authors have also reported increases in microbial activity of amylase manganese-dependent [36,41,42].

Conclusions

Penicillium citrinum is identified as a versatile producer of enzymes that hydrolyze starch polymers, releasing glucose, which suggests the presence of a glucoamylase. The enzymes operate in acidic pH and in a wide temperature range (30°C to 60°C). These characteristics fit into the sought by industry for processing starch, detergent, pulp paper and other biotechnological applications. The addition of divalent metal ions can significantly increase the enzyme activity, making them more suitable for industrial use. The DEAE-sepharose derivative showed 100% of immobilization and maintained an excellent activity. Furthermore, the immobilized glucoamylase on DEAE-sepharose was more thermostable than the free enzyme and the immobilized on MANAE-sepharose. All these results contribute for better understanding properties free and immobilized of *P. citrinum* glucoamylase.

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