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Prospecting Filamentous Fungi for Amylase Production: Standardization of *Aspergillus japonicus* Culture Conditions

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TMP, VMB, EAM and MLTMP performed the first draft of the manuscript and he was responsible for the literature searches. Author JAJ performed the design of some experiments. Authors VMB and MLTMP managed the analyses of the study. Author MLTMP was responsible by the study and collaborated in the final writing of the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: Prospecting different filamentous fungi for high production of amylases in standard conditions for future application in biotechnology industries.
Methodology: Samples were collected in different field areas in the state of Bahia, Brazil, for isolating filamentous fungi. Fungi were grown in Petri dishes in a culture medium containing 4% Quaker® oatmeal and 2% bacteriological agar. Fungi screening was carried out in liquid medium containing 1% starch at 30°C and pH6.0 under static conditions for 4 days. Proteins and enzyme activities were determined by Bradford and DNS methods, respectively. A submerged fermentation was performed with different liquid media in order to obtain the best growth composition and enzyme production for the selected fungus. Several conditions such as time course of inactivation, pH, temperature, carbon and nitrogen sources were determined in the culture medium with the aim of

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improving amylase production and fungi growth. Various fibers and food residues were used as enzymatic inducers in a way to assess potential integration of this enzyme, producing microorganisms in the food industry.
Results: *A. japonicus* proved to be the best degrader of fibers and food residues. After 4 days, *A. japonicus* enzyme production was maximal, with 44.65(±0.49) U/ml under static conditions. The maximal enzyme activity was obtained at pH 6.0, retaining its activity even at a higher pH. The optimum temperature was 25°C. The best carbon source was potato starch and the best food residues were orange bagasse and bark for the enzyme production by *A. japonicus*.
Conclusion: The results suggest that *A. japonicus* is a good amylase producer for the degradation of fibers and food residues, indicating that it might become important for the food industry, bringing value to what is known as waste these days.

Keywords: Agro-industrial; food residues; isolation; screening; starch; waste.

1. INTRODUCTION

Enzyme technology and bio-catalysis are promising tools for generatin compounds with a high aggregated value. The interest in enzymes has increased in Brazil, which imports tons of these products. Enzymes have a large variety of applications in different industries. The global market for industrial enzymes was estimated at 3.3 billion dollars in 2010 and it is expected to reach more than 4 billion dollars by 2015 [1]. Amylases are the second largest group of enzymes used all over the world. They are widely used in biotechnology industries in starch saccharification processes, such as in textile industries, food and animal feed, detergents, fermented beverages and distilleries. In addition, they also have potential application in pharmaceutical, refined chemical and bakery industries [1]. They can also be applied in recycling and paper production as well as in the juice industry, where they are used to clarify and decrease turbidity [2]. Amylase production has been reported in several different fungi species such as *Fusarium solani* [3], *Aspergillus oryzae* [4], *Aspergillus niger* [5] and *Streptomyces erumpens* [6].

The main amylolytic enzymes are as follows: α-amylase (EC3.2.1.1;1,4-α-D-glucan glucan hydrolase), which promotes the hydrolysis of α-1,4 within the chain of amylose and amylopectin, releasing several oligosaccharide chains of different sizes that have an α-configuration at C1. Mariani et al. [7] studied an α-amylase produced by *Aspergillus niger* and reported that it was thermostable in reactions involving temperatures reaching 100ºC. These kinds of enzymes are used in the sugar liquefaction process, in the fermented drink industry, and in the production of adhesives, detergents and animal feed. Glucoamylase (EC 3.2.1.3;1, 4-α-D-glucanglucohydrolase) or amyloglucosidase is also an extremely interesting biotechnological enzyme. It is an exoamylase that is capable of hydrolyzing the α-1,4 glycosidic linkages through the successive removal of glucose units from then on-reducing end of the chain, releasing molecules of D-glucose in the β-conformation. These enzymes also hydrolyze the α-1,6 and some α-1,3 bonds but this process rarely occurs. According to recent data from the Ministry of Development, Industry and Foreign Trade, Brazil imports most of the products involving enzymatic preparations [8], as it is evident that the market for industrial enzymes is small in the country compared with global demand.

The use of enzymes as catalysts in industrial processes is crucial to obtain high-quality products with clean technologies in harmony with the technological needs of the market and the environmental preservation.

Thus, the aim of this work was to prospect various filamentous fungi isolated from different sources, seeking the maximal production of amylases. Among the diverse isolated fungi, a filamentous fungus, identified as *Aspergillus japonicus* Saito has demonstrated to be an amylase producer in liquid Khanna medium [9]. Earlier, this fungus exhibited high potential to xylanase, cellulose [10] and phytase [11] production.

2. MATERIALS AND METHODS

2.1 Isolation of the Microorganisms

Twelve samples were collected from different sources, which include cocoa fruit affected by pests (sample n° 1), cocoa leaf affected by pests (sample nº 2), cattle compost (sample nº 3), lemon trunk (sample nº 4), soursop fruit affected by pests (sample nº 5), decomposing foliage (sample nº 6), mushroom (sample nº 7), decaying coffee bean (sample nº 8), bark of eucalyptus (sample nº 9), rubber tree leaf affected by pests (sample nº 10), coconut straw (sample nº 11) and bark of jack fruit tree affected by fungi (sample nº 12). All the samples were obtained in the state of Bahia, Brazil. Samples of these materials were taken to the laboratory so that the microorganisms present in them could be cultivated in solid culture medium. The isolation of filamentous fungi was performed by the pour plating method, in which the strains were repeatedly inoculated in Petri dishes containing solid culture medium that was composed by 4% Quaker® oatmeal and 2% bacteriological agar [12] to which commercial veterinary pentabiotic Fort Dodge® was added. This procedure was repeated until there was just one fungus growing on the dish with no contamination. After that, the strains were placed at different temperatures (30, 35, 40 and 45ºC), to check the temperature dependence of fungal growth.

2.2 Screening of Microorganisms for Amylolytic Activity and Strain Maintenance

Tests were carried out to select the best fungi for amylase production. The amylase screening was performed with all the fungi isolated and grown in liquid medium described by Khanna medium [9] to which 1% starch was added at 30ºC, pH 6.0, under static conditions for 4 days in order to obtain the best amylase producer. The protein and enzyme activity was determined by Bradford [13] and DNS [14] methods, respectively. The screening revealed two fungi as the best amylase producers. After the screening, only the higher amylase producers were identified using morphological analysis and microscopic observations. The identification was carried out at Departamento de Micologia of Universidade Federal de Pernambuco (UFPE), Brazil. The strains were maintained in our laboratory in slants of solid culture medium that was composed by 4% Quaker® oatmeal and 2% bacteriological agar [12]. In intervals of 15 days, spores of *A. japonicus* were transferred into new plates containing the same culture medium. Samples of the fungus were kept at - 80ºC in a solution of 20% glycerol and/or at 4ºC in silica.

2.3 Inoculum and Composition of Different Liquid Culture Media

Cultures of *A. japonicas* were scraped and spores were re-suspended in sterile distilled water. The spores were counted in a Neubauer chamber and a solution containing 1.77 x 10^6spores/mL was obtained. A volume of one milliliter of this solution was inoculated in 25mL of liquid medium in Erlenmeyer flasks of 125mL. Five liquid culture media were tested: CP medium [15]; SR medium [16]; Adams medium [17]; Khanna medium [9] and Vogel medium [18]. The pH was adjusted to 6.0. After being prepared, all the liquid media were

autoclaved for 30 minutes at 1.5 atm and 120ºC. The best medium was chosen for the subsequent determinations mentioned.

2.4 Obtaining Mycelial Mass and the Extracellular Enzymes

The mycelial mass was obtained after its separation from the culture medium by vacuum filtration using a Büchner funnel and whatman filter paper no 1. The filtrate was used as an extracellular enzyme source. The mycelial mass was washed with two volume of distilled water, pressed between paper sheets, maintained at -18°C and further macerated and resuspended in 10 mL of 100 mM sodium acetate buffer, pH 5.0. This suspension was subjected to centrifugation at 8000xg, for 15 minutes, at 4ºC.The supernatant containing the intracellular enzymes was then separated with a view to study the intracellular amylolytic complex of the fungus.

2.5 Measurements of Protein and Enzyme Activity

The proteins present in the intracellular and extracellular samples were determined by the Bradford method [13] using bovine serum albumin as a standard. The readings were performed in a spectrophotometer at 595 nm and the results were expressed in mg/mL. The amylase activity was determined by the quantification of the reducing sugars formed during the enzyme incubation with the substrate soluble starch 1% in sodium acetate buffer 100mM pH 5.5 at 55ºC using 3,5-dinitrosalicylic, DNS [14]. The reaction consisted of 250 µl of substrate and 250 µL of the enzyme. Aliquots of 100 µL were removed after 10 minutes of the reaction and added to test tubes containing 100 µL of DNS [14]. After that, the tubes were immersed in a boiling water bath for 5 min, after which they were allowed to cool and 1 mL of distilled water was added. The absorbance for all the test tubes was measured at 540 nm in a spectrophotometer [19]. The blank consisted of 100 µL of the reaction mixture, which was immediately added to 100 µL of DNS. The method was previously standardized using glucose (0.1 to 1.0 mg/mL). The activity unit (U) was defined as the amount of enzyme that hydrolyzes one µmol of soluble starch per minute under the assay conditions.

Total activity (total U) = µmol/mL x filtrate volume. The specific activity was expressed in total U/total mg protein of the extracellular extract.

2.6 Time Course and Physical Condition for Growth and for Amylolytic Production by the Selected Fungus

The fungus was incubated in liquid medium described by Khanna medium [9] that was supplemented with 1% soluble starch as carbon source, at 30ºC and pH 6.0.The fungus was incubated for a period of 1 to 9 days under static conditions in a microbiological incubator and for 1 to 6 days under stirring condition in a shaker (120 rpm), with a relative humidity of around 70%. Every day, one sample of each, static and stirring condition was removed from the incubator and subjected to vacuum filtration. The protein and enzymatic measurements were performed with the extracellular enzyme.

2.7 Effect of pH on the Enzyme Production by *A. japonicus*

The selected fungus was incubated in liquid medium described by Khanna medium [9] that was supplemented with 1% soluble starch as carbon source, at 30ºC using the following pH values of 3.5; 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; 9.0 and 9.5 for 4 days under static

condition. Samples were subjected to vacuum filtration. The protein and enzymatic measurements were performed with the extracellular enzyme.

2.8 Effect of Temperature and Nitrogen Sources on the Growth and Production of Amylolytic Enzyme by *A. japonicus*

The fungus was incubated using the following temperature values: 25, 30, 35 and 40ºC in liquid medium described by Khanna medium [9] with 0.1% yeast extract at each experimental temperature and in a second part of the experiment, the fungus was incubated with the same conditions but with 0.1% peptone as a nitrogen source at all tested temperatures for 4 days, under static condition, pH 5.5. Samples were subjected to vacuum filtration as described. The protein and enzymatic measurements were performed with the extracellular enzyme.

2.9 Effect of Different Carbon Sources on the Amylase Production

The fungus was incubated in liquid medium described by Khanna medium [9] that was supplemented with 1% of different carbon sources under static conditions for 4 days, at 25ºC and pH 5.5. The different carbon sources used were as follows: amylopectin, corn starch, glucose, maltose, pectin, potato amylose, potato starch, raffinose, rice starch and starch Merck™, starch Reagen®. The control used was composed by the liquid medium described by Khanna medium [9] that was supplemented with 0.1% peptone and no carbon source was added. Samples were subjected to vacuum filtration and protein and enzymatic measurements were performed with the extracellular enzyme.

2.10 Cultivation of the Selected Fungus in Fiber and Food Residues

The selected fungus was cultivated in liquid medium described by Khanna medium [9] consisting of one of the following carbon sources at the concentration of 1% cassava flour, rye flakes, oat flour, bark and orange bagasse, corn cob, corn mashed, wheat bran, glucose or peanut hulls for 4 days, under static conditions, at 25ºC and pH 5.5. Samples were subjected to vacuum filtration and protein and enzymatic measurements were performed with the extracellular enzyme.

2.11 Statistical Analysis

All data were independently performed in triplicate. The mean was calculated from these triplicates. The standard deviation was performed using Excel 2007.

3. RESULTS AND DISCUSSION

3.1 Isolation and Temperature-dependence Study of the Filamentous Fungi

Twelve samples were collected from a specific rural area of the state of Bahia, Brazil, and twenty fungal strains were entirely isolated using solid culture medium at 30ºC (Fig.1). All the isolated fungi were maintained at different temperatures (30, 35, 40 and 45ºC), and all of them grew at 30ºC; 3 of them grew very well at 35°C but 8 of them exhibited slow growth in this condition; in general, the fungal growth diminished at 40ºC, except by 5D, which showed morphological changes in sporulation as well as in growth. Interestingly, at 45ºC, only the fungus named 5B was able to grow but at 30, 35 and 40ºC, minimal growth was observed.

Thus, it is possible to classify this fungus as a thermophilic microorganism, as Madigan et al. [20] classified microorganisms according to their growth temperature: psychrophilic (low temperatures), mesophilic (median temperatures), thermophilic (high temperature) and hyper thermophilic (high temperatures). Furthermore, only thermophilic fungi have the exceptional ability to grow at high temperatures but not at lower temperatures. Fungus 4A Fig.1 exhibited maximum mycelial growth after 48 hours at 30, 35 and 40ºC, occupying the entire dish and so, it may be considered a thermotolerant fungus. Besides, fungi 3B and 6B exhibited immense development at 30ºC and 35ºC and minimal growth at 40ºC, which is characteristic of mesophilic fungi [20]. The least growth at 30°C was observed for fungus 10A. Fungi 12B, 12F, 8A, 7A, 3A and 8B exhibited immense growth at 30°C and 35ºC, but did not grow at 40ºC and 45ºC. Fungi 12A, 11A, 5B, 5A, 9A, 9D,12E and 5H exhibited good growth only at 30ºC and it was not possible to verify their growth in other temperatures. These data showed that most of these fungi are mesophilic [20].

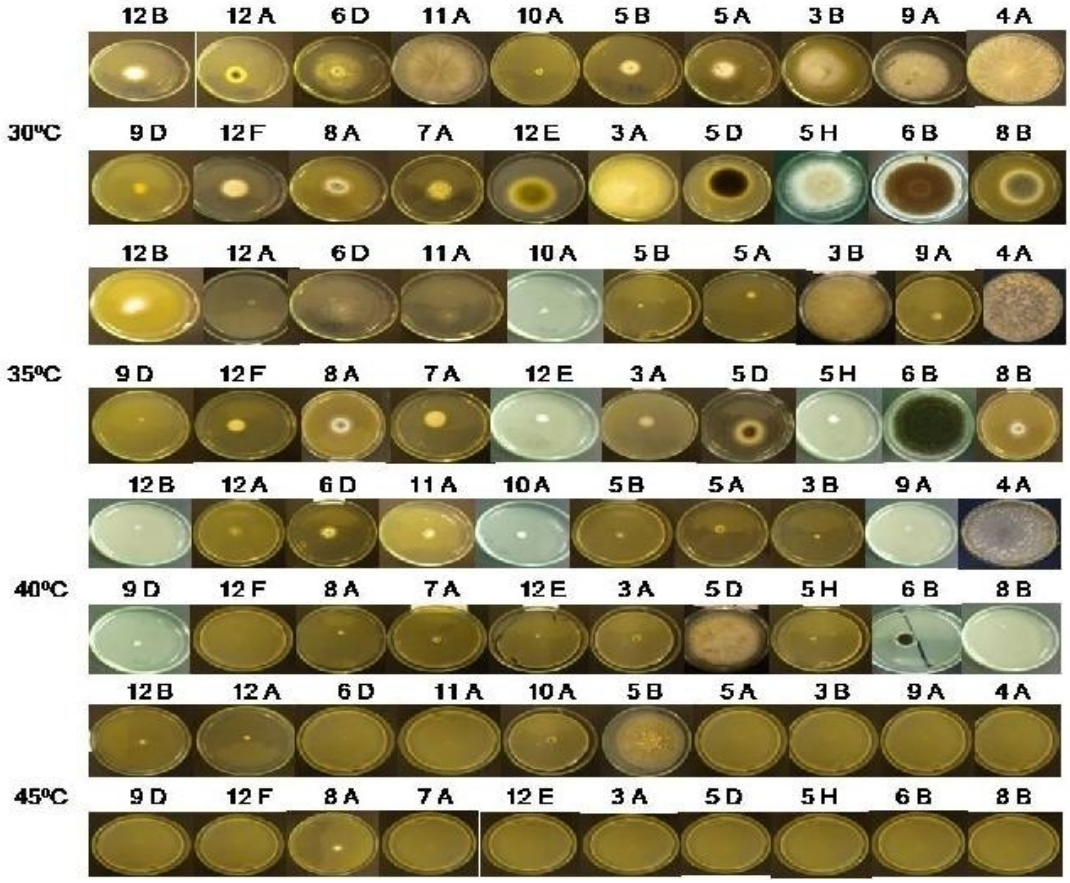

Fig. 1. Growth of the isolated fungi at different temperatures on solid culture medium

As shown in Table 1, some fungi exhibited immense growth: 11A, 4A, 7A and 6B for which the halo size was greater than 2.5cm. Fungus 11A did not exhibit good growth at 35ºC; whereas fungi 7A and 6B exhibited minimal growth at this temperature, though with a slight decrease, with a halo around 1.0 cm. Fungus 4A maintained the growth, showing a halo with 3.0 cm, at 40ºC. Fungus 6B grew with its halo reaching about 1.0 cm at 40ºC but it presented no growth at 45ºC. Based on these results it can be inferred that the majority of

the isolated fungi are mesophilic and some of them can be considered thermotolerant, because they grow at higher temperatures than those reported in the literature [20]. These data are important while highlighting some aspects with regard to the biology and physiology of filamentous fungi and they also demonstrate the need to determine the optimum temperature for fungal growth, which is the aim of biotechnological applications.

Table1. Analysis of the halo length (cm) of the fungi cultivated in 48 hours

Fungi*	Mycelial halo length (cm)			
	30ºC	35ºC	40ºC	45ºC
12B	1.00	1.00	0.30	0.00
12A	1.00	0.00	0.00	0.00
6D	2.30	1.10	0.80	0.00
11A	2.80	1.00	0.30	0.00
10A	0.50	0.00	0.00	0.00
5B	1.00	0.30	0.30	2.00
5A	1.00	0.30	0.50	0.00
3B	2.50	2.00	0.30	0.00
9A	1.50	0.30	0.30	0.00
4A	3.00	3.00	3.00	0.00
9D	1.00	0.30	0.30	0.00
12F	1.30	1.00	0.00	0.00
8A	1.40	1.00	0.30	0.00
7A	1.60	1.40	0.30	0.00
12E	2.00	0.50	0.00	0.00
3A	2.50	0.50	0.30	0.00
5D	1.90	1.00	2.00	0.00
5H	2.50	0.30	0.00	0.00
6B	2.90	2.80	0.80	0.00
8B	1.80	1.00	0.00	0.00

**References of these numbers are in Methods item 2.1.*

3.2 Screening of the Filamentous Fungi

The selection of microorganisms that produce good levels of amylolytic enzymes constituted an important step. For this purpose, submerged fermentations were performed using the liquid medium described by Khanna medium [9] under stationary conditions at 30ºC for 4 days, with an initial pH of 6.0. Analyzing Table 2, it was found that fungi 12E and 6B exhibited higher amylase production (47.93 and 42.73 U/mL, respectively). After the screening, only the higher amylase producers were identified by morphological analysis as cited in the “Methods” section. Furthermore, it is possible to say that according to Table 2, the amylases produced by fungi 6B caused the acidification of the medium, implying that they have maximum amylolytic activity at low pH, as the enzymes produced by fungi 12E caused a slight decrease in the pH of the extract, indicating that the amylase has maximum enzymatic activity between pH 5.0 and 6.0. These results can be explained by the oxalic acid production that has chelating properties and may increase the availability of metal ions such as iron and calcium [21]. Alternatively, oxalic acid formation may be involved in biological competition [22], facilitating the enzyme activity for the glucose formation from the hydrolysis of the substrate, as the optimal pH of the enzyme is between 5 and 6. Oxalic acid production by fungi is most efficient at a pH from 5 to 8 and is completely absent below pH 3.0 [22]. Another important acid produced by fungi is citric acid, which has the same function

of oxalic acid; its production begins at pH 3.0 and is optimal just below pH 2.0 [23] Therefore, media acidification is caused by secondary metabolites (acids) production, which serves as an important phenomenon for fungus to survive.

Table 2. Determination of final pH quantification and determination of extra- and intracellular amylases produced by the isolated fungi

Fungus	Enzyme Activity (U/mL)			Protein (mg/mL)	
	pH	Intracellular	Extracellular	Intracellular	Extracellular
12B	2.45	1.77 (±0.12)	1.65 (±0.32)	0.51 (±0.06)	0.14 (±0.04)
12A	5.25	1.46 (±0.10)	0.82 (±0.05)	0.19 (±0.03)	0.09 (±0.02)
6D	3.20	0.69 (±0.07)	1.14 (±0.22)	1.15 (±0.20)	0.06 (±0.01)
11A	2.22	7.11 (±0.50)	12.38 (±0.87)	0.94 (±0.07)	0.13 (±0.02)
10A	3.65	0.76 (± 0.05)	0.12 (±0.01)	0.78 (±0.10)	0.24 (±0.04)
5B	4.98	0.82 (± 0.03)	0.63 (±0.04)	0.50 (±0.02)	0.18 (±0.02)
5A	3.85	3.04 (±0.32)	0.57(±0.02)	0.15 (±0.03)	0.12 (±0.01)
3B	2.62	1.01 (±0.21)	1.58 (±0.07)	0.20 (±0.08)	0.21 (±0.03)
9A	3.40	0.88 (±0.07)	6.15 (±1.29)	0.34 (±0.04)	0.14 (±0.02)
4A	2.90	3.11 (±0.14)	14.47 (±3.85)	0.21 (±0.01)	0.15 (±0.04)
9D	3.0	2.03 (±0.11)	3.11 (±1.03)	0.18 (±0.05)	0.23 (±0.02)
12F	2.52	1.90 (±0.04)	7.04 (±2.87)	0.33 (±0.12)	0.18 (±0.07)
8A	5.55	3.42 (±0.32)	7.04 (±1.46)	0.09 (±0.02)	0.31 (±0.10)
7A	3.23	4.06 (±0.17)	2.47 (±0.68)	0.23 (±0.03)	0.23 (±0.06)
12E	5.45	38.09 (±1.23)	47.93 (±1.94)	0.59 (±0.04)	0.29 (±0.02)
3A	5.55	2.34 (±0.63)	3.49 (±0.13)	0.24 (±0.06)	0.10 (±0.05)
5D	2.43	3.15 (±0.05)	6.15 (±0.82)	0.34 (±0.11)	0.14 (±0.05)
5H	3.62	1.39 (±0.19)	11.04 (±1,16)	0.21 (±0.07)	0.05 (±0.01)
6B	3.05	9.33 (±1.76)	42.73 (±2.06)	0.52 (±0.02)	0.18 (±0.04)
8B	3.26	5.65 (±0.40)	7.93 (±0.80)	0.09 (±0.03)	0.10 (±0.07)

These results represent mean and the standard deviations.

According to the results of Table 2, fungi 12E and 6B, which are considered the best amylase producers were identified by morphological analysis as *Aspergillus parasiticus* and *Aspergillus japonicus* Saito, respectively. Although *A. parasiticus* presented 37% more extracellular protein than did *A. japonicus*, it also exhibited toxicity according to Okoth et al. [23] and thus, it was discarded, as one of the target applications of this work is the food industry. Thus, *A. Japonicus* was the chosen fungus for further studies, as no information related to its toxin production has been reported in the literature according to Spadaro et al. [24], as has been shown for *A. parasiticus.* In addition, there are not many studies about the applications of *A. Japonicus* enzymes in industries, which is the purpose of this study.

This species produces black colonies with a white mycelium. Its mycelium is dense, basal, with the absence of exudates and soluble pigment. This fungus is completely covered by black or dark brown spores, with a round shape, occasionally elliptical and uniseriate.

3.3 Testing Different Culture Media

A submerged fermentation was performed in five different liquid media. The incubation temperature was 30ºC and pH 6.0, under static conditions, for 4 days of growth. The CP, Khanna and SR liquid media exhibited better amylolytic production compared with other media. These results were similar to those of Rizzatti et al. [16], who used Khanna medium to produce xylanase from *A. phoenicis* and to those of Facchini [25], who used the SR medium for the production of CMCase and xylanase from *A. japonicus*. The CP medium

presented high protein values, which diminished the specific amylase activity. Khanna medium was selected to follow amylase production by *A. japonicus* because of the lower quantity of proteins in the filtrate, offering a high specific activity, which facilitated further enzyme purification studies by chromatography procedures. The percentage of extracellular amylase activities compared with the intracellular activities was 95.5% for the Khanna medium, 88% for the CP medium and 87.5% for the SR medium (Table 3). The protein secreted by the fungi on the culture medium is a positive parameter for industrial application, as the extracellular enzyme can be easily extracted and the cellular maceration is not necessary. The liquid medium described by Khanna medium [9] was selected as the best composition for amylase production with *A. Japonicus* presenting a lower quantity of proteins, thus making it an important result to perform chromatographic processes while aiming at future enzyme purification.

Table 3. Enzyme production using different culture media

Media	Extracellular Enzymes		Intracellular Enzymes	
	Enz. Activ (U/mL)	Prot. (mg/mL)	Enz. Activ (U/mL)	Prot. (mg/mL)
SR	45.96(±0.76)	0.48(±0.06)	6.51(±0.43)	0.20(±0.02)
CP	46.82(±0.43)	0.69(±0.08)	6.27(±0.10)	0.30(±0.05)
Vogel	37.67(±1.20)	0.21(±0.03)	4.88(±0.54)	0.34(±0.04)
Adams	29.45(±1.09)	0.21(±0.03)	2.71(±0.32)	0.23(±0.03)
Khanna	46.35(±0.87)	0.25(±0.01)	2.24(±0.43)	0.13 (±0.02)

These results represent mean and the standard deviations. The mean standard deviations were ± 0.61 for the enzyme activity and ± 0.03 for the protein.

3.4 Time Course and Physical Condition for the Growth of *A. japonicus* and for Amylolytic Production

After standardizing the medium, it was important to verify whether the incubation time and the physical conditions of the cultivation that had been used (4 days) and physical condition (static) were appropriate. For this purpose, *A. japonicus* was incubated in Khanna medium that was supplemented with 1% soluble starch which served as carbon source, at 30ºC and pH 6.0 for a period of 1 to 9 days in a microbiological incubator (static condition) and for a period of 1 to 6 days in a shaker (120 rpm, stirring condition), with a relative humidity of around 70%. The enzymatic activity in the stirring condition decreased after 5 days and was not necessary for the experiment, because these results were obtained for approximately 6 days of growth. While comparing the two physical conditions, it was possible to observe that the amylase production was higher in the static condition, but the fungus maintained approximately the same extracellular activity from 4 to 9 days of growth, with the maximum levels being on the fourth day of growth 44.65 (±0.49) U/mL; while in the stirring condition, the maximum amylase production was obtained on the fifth day (35.85 U/mL ± 0.32). The percentage of extracellular enzymatic activity in the static condition, with 4 days of growth, was 75%, whereas the intracellular activity was 25% (Fig. 2). These results can be explained by the stress caused when the fungus is grown in the stirring condition; this condition makes the mycelium formation more difficult, because it cannot be easily grouped, thus resulting in pellets and requiring longer periods of time to grow and secrete the enzyme to the external medium. Furthermore, the stirring during fermentation can inhibit lactic acid formation, thus extending the fermentation time and possibly altering the quality of the enzyme. Stirring may also cause the incorporation of air into the system, interfering with the fermentation dynamics, which is an anaerobic event [26].These data were observed in this experiment. These results are in disagreement with those obtained by Michelin [27], in which the fungus *Paecilomyces variotii* reached its maximum growth after 3 days of growth in SR

medium [16] (pH 6.0), at 40ºC, with maximum extracellular amylolytic activity on the 8th day of growth. According to these results and the stirring problem observed, the static condition and 4 days of growth were selected as being the ideal ones for the amylase production by *A. japonicus.*

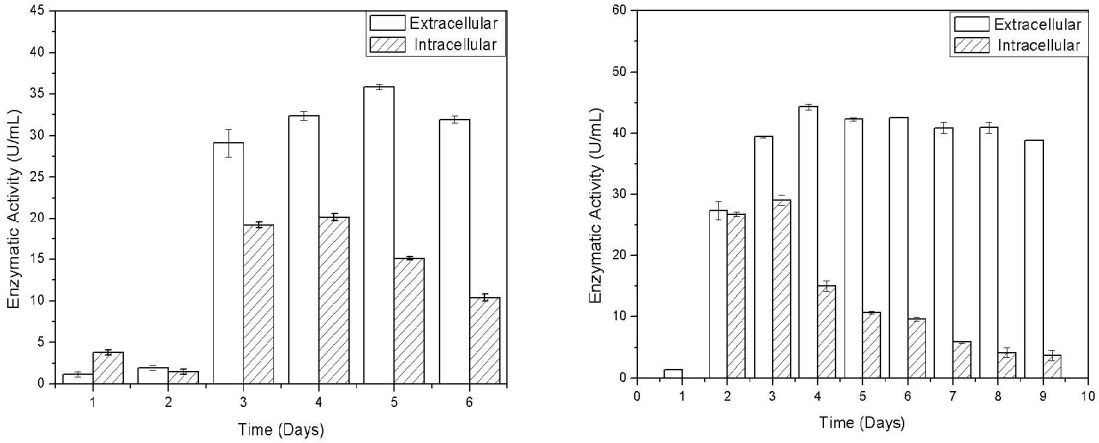

Fig. 2. Time course of amylolytic production in (A) static and (B) stirring conditions
The mean standard deviations were of ± 0.51.

3.5 Effect of pH on the Enzyme Production by *A. japonicus*

The influence of the initial pH of the medium for the production of amylases by *A. japonicus* that were cultivated for 4 days in static condition at 30ºC was verified. It was observed that pH 5.5 was the best initial pH for amylase production. Results slightly differed from the ones reported by Facchini [25], who used *Aspergillus japonicus* for the production of xylanases and CMCase and who standardized 6.0 and 7.0 as the best initial pHs for the cultivation of the fungus, thus aiming at the production of these enzymes. However, it is important to mention that in all cultures the final pH was around 2.5–3.0 but the enzyme did not lose its activity during this pH range. These results can be compared with those reported by Maktouf et al. [28], who studied an amylase produced by *Bacillus sp* and obtained maximal activity at pH 5, which efficiently hydrolysed starch and yielded glucose as the end product. From pH 3.5 to 8.5, enzyme activity was maintained high and maximum activity was obtained at pH 5.5. No significant decay was observed after this pH value, which suggested a higher tolerance in different pH (Fig. 3). Oyeleke and Oduwole [29] reported similar findings when they studied an amylase produced by *Bacillus subtilis* that exhibited activity in a pH range of 6–8. Oyeleke et al. [30] found an amylase with maximum activity in *A. niger* at pH 5; whereas at pH 4–7, the fungus also exhibited amylase production similar to the amylase obtained in this study. The vast activity of amylase at various pH suggests the wide application nature of the amylase identified [31].

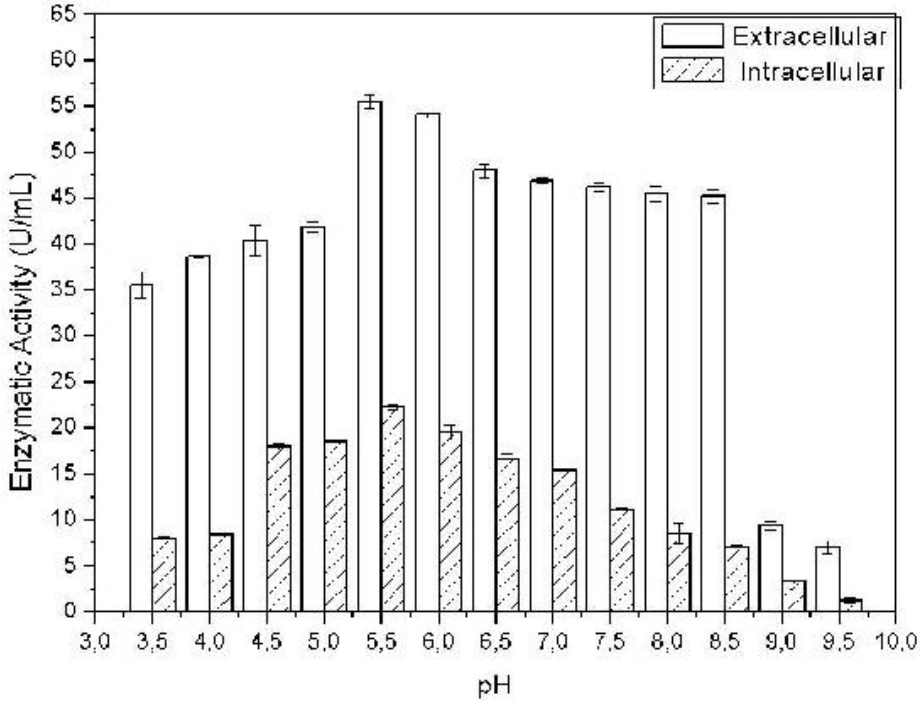

Fig. 3. Effect of pH on amylase production. The pH range considered was from 3.0 to 9.5

The mean standard deviation was ± 0.54 for the enzyme activity

3.6 Effect of Temperature and Nitrogen Sources on the Growth and Production of the Amylolytic Enzyme by *A. japonicus*

Higher amylase levels were observed in cultures grown at 25ºC. The addition of peptone instead of yeast extract resulted in a slight increase in activity, suggesting that the nitrogen sources tested did not have a significant effect on amylase production.

With regard to the temperature, the cultures incubated at 25ºC exhibited higher amylase activity, with the enzymatic levels being 50% greater than the amylases synthesized at 40ºC (Fig. 4). This result is consistent with that described in the literature, in which *A. japonicus* produces higher enzymatic activity at room temperature; for example, we can mention the production of pectinases according to Teixeira et al. [32], who used 30°C as growth temperature, and the production of β-xylosidase according to Wakyiama et al. [33], who also used 30°C. Binupriya et al. [34] also reported on the strain of *A. japonicus* grown at room temperature (27ºC and 28ºC). According to Madigan et al. [20], who correlated the temperature with the collection site, it could be said that *A. japonicus* which was isolated from the soil has excellent temperature close to room temperature growth; it is considered a mesophilic microorganism and its extracellular enzymes can be seen as being thermotolerant, which will be confirmed in the future during the characterization stage.

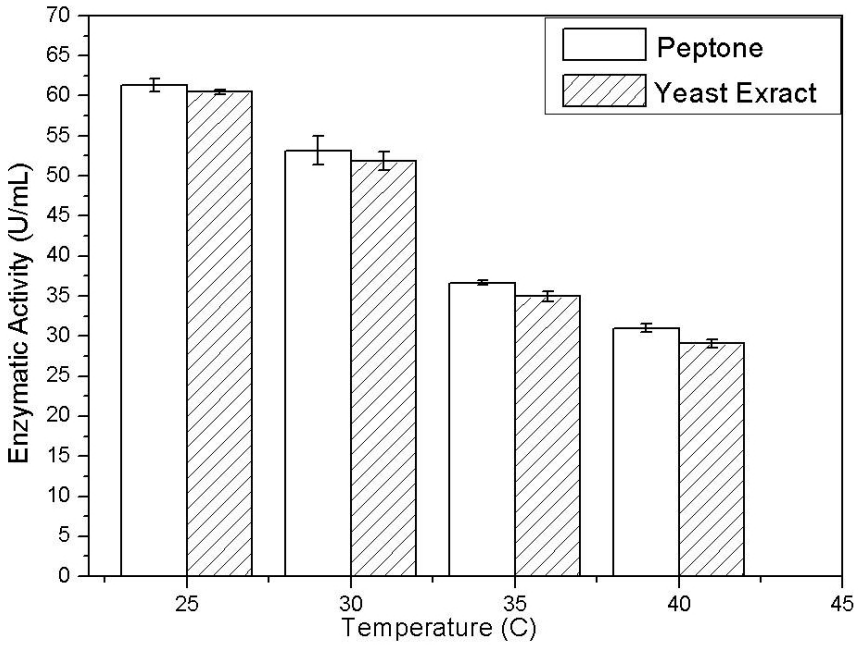

Fig. 4. Effect of temperature and nitrogen source on the cultivation of *A. japonicus*
The mean standard deviation was ± 0.76.

3.7 Effect of Different Carbon Sources on Amylase Production

Starches from many sources are frequently used in food industries. With an aim to study the repressor or inductor effect of carbon sources on amylase production, *A. japonicus* cultures were prepared in Khanna medium that was supplemented with 1% of different carbon sources and incubated under static conditions for 4 days, at 25ºC. An identical medium without the supplementation of an additional carbon source was used to compare the activity.

With regard to the enzymatic activity (U/mL), it could be inferred that the carbon source which provided the highest amylase production when compared with the medium without the supplementation of carbon source was potato starch (7.46-fold), maltose (7.26-fold) and rice starch (6.90-fold). Starch from Merck™, glucose and starch from Reagen ® exhibited on an average 6.62-fold more activity than the medium without supplementation; whereas corn starch, potato, amylopectin and amylose exhibited on an average 5.02-fold more activity than the medium without carbon source. Other carbon sources, such as raffinose and pectin, had about 4-fold more activity than the medium without carbon source. The high amylase level may be attributed to the exhaustion of this compost in the medium after four days, resulting in limited growth and in the secretion of constitutive enzyme (Table 4).

These results are particularly interesting, as starch potato and maltose can induce the synthesis of amylase in high amounts. The action of amylolytic synthesis induction verified by maltose is mediated by a transport system of this disaccharide by permeases. The genes required for the amylolytic enzyme production using maltose are found in the locus MAL (MAL 1, MAL 4 and MAL 6). Physical and genetic analyses of MAL have shown that each active locus includes three genes which probably encode the maltose carrier proteins [35]. According to Kathiresan and Manivannan [36], the highest activity levels of α-amylase

produced by *Penicillium fellutanum* were obtained with the cultivation in a medium that was supplemented with maltose, followed by xylose and D-glucose serving as carbon sources. Thus, the results obtained by the cited researchers are similar to those observed with the amylase produced by *A. japonicus*. Giannesi et al. [37] found that the carbon sources maltose and starch induced α-glucosidase production by *Chaetomium thermophilum* var. *coprophilum* as well as sucrose inhibited the amylolytic enzyme production. In the present work, the growth and amylase production by *A. japonicus* in culture medium without carbon source (that was used as control) was also observed to prove that the fungus was using the carbon source as an inducer for enzyme production; fungus growth and enzyme production probably occurred due to the fact that there are salts, proteins and vitamins in the culture medium, derived from peptone, which can be utilized by the fungus. Thus, in this experiment, excellent enzyme production was verified when *A. japonicus* was grown in carbon sources that are used in the food industry; this was an interesting result from the industrial application point of view, because these sources are used in bread production and exert an excellent effect, inducing the synthesis of amylase by *A. japonicus*.

Table 4. Effect of carbon sources on amylase produced by *A. japonicus*

Carbon Sources	Extracellular Enzymes	
	Enzyme Activity	Proteins
Control	7.15 (±0.48)	0.06 (±0.02)
Amylopectin	34.59 (±1.15)	0.15 (±0.03)
Corn Starch	41.80 (±2.03)	0.12 (±0.02)
Glucose	44.04 (±1.13)	0.18 (±0.04)
Maltose	51.92 (±0.58)	0.36 (±0.06)
Pectin	16.20 (±0.74)	0.32 (±0.03)
Potato Amylose	31.44 (±1.57)	0.11 (±0.05)
Potato Starch	53.38 (±0.12)	0.32 (±0.07)
Raffinose	24.08 (±1.09)	0.12 (±0.02)
Rice Starch	49.40 (±.0.35)	0.15 (±0.01)
Starch Merck™	48.46 (±1.23)	0.22 (±0.03)
Starch Reagen®	49.72 (± 0.18)	0.41 (±0.08)

The mean standard deviations were ±0.88 for enzyme activity and ±0.04 for the protein.

3.8 Cultivation of the Fungus *A. Japonicus* in Fiber and Food Residues

In order to check the amylase production using fiber and food residues, *A. japonicus* was cultivated in Khanna liquid medium consisting of 1% cassava flour, rye flakes, oat flour, bark and orange bagasse, corn cob, corn mashed, wheat bran, glucose or peanut hulls, for 4 days, under static conditions, at 25ºC. It was verified that when grown on orange bark and bagasse, rye flakes and glucose, the extracellular amylase produced by *A. japonicas* hydrolysed the substrate starch, presenting on an average an activity of 38.82 U/mL. In contrast, the lower hydrolysis of this same substrate was observed with the amylase being produced on cassava flour, oat flour, corn cob, corn mashed, wheat bran and peanut hulls, which had, on an average, 7.14U/mL (Table 5).

The amylase produced by *A. japonicus* grown on orange bark and bagasse hydrolysed the starch with high levels (45.40 ± 1.13 U/mL) (Table 5). Facchini et al. [25] reported the same result in their study, where *A. japonicus* produced a good level of xylanase (31.59 ± 0.17 U/g) on orange bark, which corroborates the results obtained in this work. A similar result was also verified by Teixeira et al. [38], who used pectic enzymes to process “Cupuaçu” juice and diminished the amount of waste discharged using a pectinase produced by

Aspergillus japonicas during the ”Cupuaçu” juice processing. Thus, it was concluded that the extracellular amylase, produced by the thermotolerant *A. japonicus*, in medium which was supplemented with 1% of the tested carbon sources: starch Merck™, glucose, starch Reagen ®, rice starch, corn starch, maltose, potato starch, orange bark and bagasse has a great potential for the action of enzymes in industries, especially the food industry. This is because, currently, the supply of enzyme blends (xylanase, phospholipase, amylase and glucose oxidase) in conjunction with emulsifiers (polysorbate, DATEM) is a common practice which is used to correct the flour quality in mills that produce pre mixtures for bread, cakes, and cookies, mainly French bread, replacing the old chemical oxidants such as bromate and azodicarbonamide. Therefore, the enzyme application in cereals is becoming more promising nowadays, as well as the wheat milling and corn processing during flour production and use of by products (straws, brans) in biofuel production.

Table 5. Supplementation effect of industrial residues on the growth of *A. japonicus*, and the activity of the enzyme

Media	Extracellular Enzymes	
	Mean Enzyme Activity (U/mL)	Mean Protein (mg/mL)
Control	3.70 (±0.32)	0.06 (± 0.02)
Cassava Flour	3.96 (±0.12)	0.11 (± 0.05)
Corn Cob	10.20 (±0.41)	0.14 (± 0.03)
Corn Mashed	2.67 (±0.09)	0.09 (± 0.01)
Glucose	42.60 (±0.98)	0.23 (± 0.08)
Oat Flour	6.90 (±1.02)	0.18 (±0.06)
Orange bark and Bagasse	45.40 (±1.13)	0.20 (±0.07)
Peanut Hulls	7.90 (±0.54)	0.11(±0.05)
Rye Flakes	28.46 (±0.89)	0.16(±0.04)
Wheat Bran	14.69 (±0.65)	0.21(±0.02)

The mean standard deviation was ± 0.61 for enzyme activity and ± 0.04 for the protein.

4. CONCLUSION

This work demonstrated the importance of prospecting filamentous fungus from the environment and the standardization of physical and chemical conditions for its growth and amylase production. The amylolytic system is preferentially produced by microorganisms, and amylase has important and wide industrial applications. The use of industrial waste, which contains sufficient residual quantities of nutrients and carbon sources is one of the most viable alternatives. Agro-industrial wastes are alternative substrates for amylase production, and their use for this purpose helps in solving the pollution problems that are caused by the disposal of these wastes into the environment. Among all tested fungi, *A. japonicus* exhibited the best results. It was observed that it is a thermotolerant microorganism that grows to approximately 40ºC and produces high levels of amylase. The fungus cultivation and amylase production were standardized in this work. Nutritional sources with more complex composition acted as an inductor of amylase production. The data obtained from *A. japonicus* amylase suggest a possible ability for biotechnological application, as it degraded alternative carbon sources that are commonly considered waste by the industries. These results, on a large scale can be used in food industries for the production of glucose from the residues by the application of amylase produced by *A. japonicus.*

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Gurung N, Ray S, Bose S, Rai V. A broader view: microbial enzymes and their relevance industries, medicine, and beyond. Biomed. Res. Int. 2013;329121.
2. Pandey A, Webb C, Soccol CR, Larroche C. Enzyme Technology. New Delhi: Asiatech Publishers; 2005.
3. Kumar D, Muthukumar M, Garg N. Kinetics of fungal extracellular alpha-amylase from *Fusarium solani* immobilized in calcium alginate beads. J. Environ. Biol. 2012;33:1021-1025.
4. Ichinose S, Tanaka M, Shintani T, Gomi K. Improved α-amylase production by *Aspergillus oryzae* after a double deletion of genes involved in carbon catabolite repression. Appl. Microbiol. Biotechnol. 2014;98:335-343.
5. Varalakshmi KN, Kumudini BS, Nandini BN, Solomon J, Suhas R, Mahesh B, Kavitha AP. Production and characterization of alpha-amylase from *Aspergillus niger* JGI 24 isolated in Bangalore. Pol. J. Microbiol. 2009;58:29-36.
6. Kar S, Ray RC, Mohapatra UB. Alpha-amylase production by *Streptomyces erumpens* MTCC 7317 in solid state fermentation using response surface methodology (RSM). Pol. J. Microbiol. 2008;57:289-296.
7. Mariani DD, Lorda G, Balatti AP. Influencia del amaranto en la producción de α-amilasa empleando *Aspergillus niger* NRRL 3112. Rev. Arg. Microbiol. 2000;32:185-189.
8. Ribeiro J. MDIC lança estudo que busca atrair investimentos para setor de alimentação de animais de produção. Accessed October, 31st, 2013. Available: http://www.mdic.gov.br/sitio/interna/noticia.php?area=2&noticia=12146. Brazil
9. Khanna P, Sundari SS, Kumar NJ. Production, isolation and partial purification of xylanase from *Aspergillus* sp. World J. Microbiol. Biotechnol. 1995;11:242-243.
10. Facchini FDA, Vici AC, Benassi VM, Freitas LAP, Reis RA, Jorge JA, Terenzi HF, Polizeli MLTM. Optimization of fibrolytic enzyme production by *Aspergillus japonicus* C 03 with potential application in ruminant feed and their effects on tropical forages hydrolysis. Bioprocess Biosys. Eng. 2011;34:1027-1038.
11. Maller A, Damasio ARL, Silva TM, Jorge JA, Terenzi HF, Polizeli MLTM. Potential application in animal feed of phytase produced from agro-industrial residues by *Aspergillus japonicus*. J. Biotechnol.2010;150:514-514.
12. Emerson R. An experimental study of the life cycles and taxonomy of *Allomyces.* Lloydia. 1941;4:77-144.
13. Bradford MM. A rapid and sensitive for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. A. Biochem.1976;72:248-254.

14. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem.1959;31:426-428.
15. Peixoto SC. Jorge JA, Terenzi HF, Polizeli MLTM. *Rhizopus microsporus* var. *rhizopodiformis:* a thermotolerant fungus with potential for production of thermostable amylases. Int. Microbiol. 2003;6:269-273.
16. Rizzatti ACS, Jorge JA,Terenzi HF, Rechia CGV, Polizeli MLTM. Purification and properties of a thermostable extracellular β-D-xylosidase produced by thermotolerant A*spergillus phoenicis*. J. Ind. Microbiol. Biotechnol. 2001;26:156-160.
17. Adams PR. Mycelial amylase activities of thermophilic species of *Rhizomucor, Humicola* and *Papulaspora*. Mycopatol. 1990;112:35-37.
18. Vogel HF. Distribution of lysine pathway among fungi: evolutionary implications. Am. Nat. 1964;98:435-446.
19. Oyewole OA, Oyeleke SB, Dauda BEN, Emiade S. Production of amylase and protease enzymes by *Aspergillus niger* and *Penicillium frequentans* isolated from Abattoir Effluent. Microbiol. J. 2011;1:174-180.
20. Madigan M, Martinki JM, Parker J. Brock Biology of microorganisms. P. Hall. 2003; 10ª Ed.
21. Rosling A, Lindahl BD, Taylor AF, Finlay RD. Mycelial growth and substrate acidification of ectomycorrhizal fungi in response to different minerals. FEMS Microbiol. Ecol. 2004;47:31-37.
22. Andersen MR, Lehmann L, Nielsen J. Systemic analysis of the response of *Aspergillus niger* to ambient pH. Genome Biol. 2009;10:R47.
23. Okoth S, Nyongesa B, Ayugi V, Kang'ethe E, Korhonen H, Joutsjoki V. Toxigenic potential of Aspergillus species occurring on maize kernels from two agro-ecological zones in Kenya. Toxins (Basel). 2012;4:991-1007.
24. Spadaro D, Patharajan S, Lorè A, Garibaldi A, Gullino ML. Ochratoxigenic black species of *Aspergilli* in grape fruits of northern Italy identified by an improved PCR RFLP procedure. Toxins (Basel). 2012;4:42-54.
25. Facchini FDA, Vici AC, Reis VRA, Jorge JA, Terenzi HF, Reis RA, Polizeli, MLTM. Production of fibrolytic enzymes by *Aspergillus japonicus* C03 using agro-industrial residues with potential application as additives in animal feed. Bioprocess Biosys. Eng. 2011;34:347-355.
26. Aguirre-Ezkauriatza EJ, Galarza-González MG, Uribe-Bujanda AI, Ríos-Licea M, López-Pacheco F, Hernández-Brenes CM, Alvarez MM. Effect of mixing during fermentation in yogurt manufacturing. J Dairy Sci. 2008;91:4454-4465.
27. Michelin M, Ruller R, Ward RJ, Moraes LAB, Jorge JA, Terenzi HF, Polizeli MLTM. Purification and biochemical characterization of a thermostable extracellular glucoamylase produced by thermotolerant fungus *Paecilomyces variotii*. J. Ind. Microbiol. & Biotechnol. 2008;35:17-25.
28. Maktouf S, Kamoun A, Moulis C, Remaud-Simeon M, Ghribi D, Chaabouni SE. A new raw-starch-digesting α-amylase: production under solid-state fermentation on crude millet and biochemical characterization. J. Microbiol. Biotechnol. 2013;23:489-498.
29. Oyeleke SB, Oduwole AA. Production of amylase by bacteria isolated from a cassava dumpsite in minna, Nigerstate, Nigeria. Afr. J. Microbiol. Res. 2009;3:143-146.
30. Oyeleke SB, Egwin EC, Auta SH. Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production. J. Microbiol. Antimicrob. 2010;2:2141-2307.
31. Oyeleke SB, Oyewole OA, Egwim, EC. Production of protease and amylase from *Bacillus subtilis* and *Aspergillus niger* using *Parkia biglobossa* (Africa Locust Beans) as substrate in solid state fermentation. Adv. Life Sc. 2011;1:49-53.
32. Teixeira MFS, Filho JLL, Durán N. Carbon sources effect on pectinase production from *Aspergillus japonicus 586.* Braz. J. Microbiol. 2000;31:286-290.

33. Wakiyama M, Yoshihara K, Hayashi S, Ohta K. Purification and properties of an extracellular α-xylosidase from *Aspergillus japonicus* and sequence analysis of the encoding gene. J. Biosc. Bioeng. 2008;106:398–404.
34. Binupriya AR, Sathishkumar M, Swaminathan K, Jeong ES, Yun SE, Pattabi S. Biosorption of metal ions from aqueous solution and electroplating industry wastewater by *Aspergillus japonicus:* phytotoxicity studies. Bull. Environ. Contam. Toxicol. 2006;77:219–227.
35. Needleman RB, Kaback DB, Dubin RA, Perkins EL, Rosenberg NG, Sutherland KA, Forrest DB, Michels CA. MAL6 of *Saccharomyces*: a complex genetic locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. 1984;81:2811-2815.
36. Kathiresan K, Manivannan S. α-amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. Afr. J. Biotechnol. 2006;5:829-832.
37. Giannesi GC, Polizeli MLTM, Terenzi HF, Jorge JA. A novel α-glucosidase from *Chaetomium thermophilum* var. *coprophilum* that converts maltose into trehalose: Purification and partial characterisation of the enzyme. Process Biochem. 2006;41:1729-1735.
38. Teixeira MF, Andrade JS, Fernandes OC, Durán N, de Lima Filho JL. Quality attributes of cupuaçu juice in response to treatment with crude enzyme extract produced by *Aspergillus japonicus* 586. Enzyme Res. 2011;2011:494813.

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