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PROSPECTION OF FILAMENTOUS FUNGI AND THE PRODUCTION OF AMYLASE BY *ASPERGILLUS* sp. M1.7.2

PROSPECÇÃO DE FUNGOS FILAMENTOSOS E PRODUÇÃO DE AMILASES POR *ASPERGILLUS* sp. M1.7.2

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ABSTRACT

Amylases are used in numerous industrial applications for converting starch into products of greater value. This work aimed to prospect filamentous fungi, analyze the morphological and physiological characteristics of the isolates; as well as to select an amylase producing fungus and to optimize the parameters for the cultivation of the microorganism and biochemically characterize the amylase. Among 21 filamentous fungi isolated in Janaúba, state of Minas Gerais, Brazil, the best amylase producer was selected for standardization of culture parameters and subsequent enzymatic characterization. Maximum activity was obtained in CP medium after six days of cultivation at 30 °C. Amylases produced by this fungus are stable to variations in pH and temperature, exhibited optimum activities at 65 °C and pH 6.0, and were significantly activated in the presence of 5 and 10 mm KH_2PO_4.

RESUMO

As amilases são utilizadas em inúmeras aplicações industriais para a conversão de amido em produtos de maior valor agregado. Esse trabalho objetivou prospectar fungos filamentosos, analisar as características morfológicas e fisiológicas dos isolados, assim como, selecionar fungo produtor de amilases e otimizar os parâmetros de cultivo do microrganismo e caracterizar bioquimicamente a amilase. Dos vinte e um fungos filamentosos isolados em Janaúba, Minas Gerais, Brasil, foi selecionado o melhor produtor de amilases para padronização dos parâmetros de cultivo e posterior caracterização enzimática. A máxima atividade foi obtida em meio CP, após seis dias de cultivo, a 30 °C. As amilases produzidas são estáveis ao pH e à temperatura; apresentaram maiores atividades a 65 °C em pH 6,0 sendo significativamente ativadas na presença de 5 e 10 mM de KH_2PO_4.

1. INTRODUCTION

Enzymes are biological catalysts used in the most diverse industrial sectors because they are capable of producing compounds of high value in an efficient, economical and sustainable manner (Oliveira et al., 2004; Mussatto et al., 2007; Pasin et al., 2014; Pasin et al., 2019). The enzymes occur naturally in all living beings, from the simplest to the most evolved organism, because the biological catalysts are indispensable to metabolism (Leadley, 1993; Oliveira, 2014; Rosado, 2013).

In industry, enzymes of microbial origin have stood out because microbes have a potential for production and secretion of a diversity of these catalysts (Benassi, 2012). Bacteria, yeasts and filamentous fungi have become the current focus of research for enzymes with a potential for industrial applications. Currently, the global market for industrial enzymes is well established; according to the study published by BCC Research (2017), it is estimated that the market value might reach US$ 6.3 billion in 2021.

Enzymes are separated into six classes according to the type of reaction they catalyze: transferases, lyases, isomerases, oxidoreductases, ligases and hydrolases. Approximately 80% of the enzymes used in industry correspond to hydrolases. Of this class, proteases and amylases lead the world production on an industrial scale (Nguyen et al., 2002; Coelho, 2008; Benassi, 2012; Rosado, 2013). The application of amylases is widespread in the most diverse industries and processes (Pandey et al., 2005; Wanderley et al., 2011; Lima et al., 2015), such as in starch saccharification, in textile, food and animal feed industries, in detergents, and in fermented drinks and distilleries. They also have a potential for application in the pharmaceutical and refined chemical industries (Benassi, 2012; Pandey et al., 2015), where they replace semi-integrally the chemical hydrolysis of starch.

Despite the variety and quantity of renewable raw materials and the knowledge for producing enzymes with large-scale biotechnological potential, enzyme technology is visibly lagging behind in Brazil. Studies reveal that Brazil imports most products that involve enzyme preparations, thereby demonstrating the fragility of our enzyme market in relation to global demand (Politzer et al., 2006, Pasin et al., 2014). Thus, the prospecting of microorganisms, the analysis and the optimization of the production of industrially important enzymes, such as amylases, are of paramount importance for the development of this sector in the country.

Enzymes with high catalytic activity at lower temperatures would generate energy savings in industrial processes (Carrasco, et al., 2017), and they are being widely searched. Thermostable enzymes, which can be applied to processes that must necessarily occur at elevated temperatures, and especially, enzymes that are stable at the various pHs utilized in industrial processes are being sought (Rabelo, 20017; Pasin et al., 2017).

This work sought to prospect filamentous fungi isolated from different samples collected in the city of Janaúba, in the north of the state of Minas Gerais, Brazil; to analyze the morphological and physiological characteristics of the isolates; to select an amylase-producing fungus; to optimize the physical-chemical parameters for the cultivation of the microorganism to improve the production of the enzyme; and, finally, to biochemically characterize the amylase produced by determining the optimum conditions for the enzymatic reaction, thermal and pH stability and analysis of the effect of adding ions to the reaction medium.

2. MATERIAL AND METHODS

2.1 Sampling, Isolation and Identification of Possible Fungus Genus

The sampling of the material for isolation of the micro-organisms was performed in the city of Janaúba, located in the north of the state of Minas Gerais, 15º 48' 09" S e 43º 18' 32" W. The material was collected aseptically. The three samples were collected: trunk bark (1), fruit skins (2) and decomposing foliage of *Licania tomentosa* (3). The samples were stored in glass flasks stoppered with hydrophobic cotton. The flasks were previously autoclaved at 120 ºC and 1.5 atm during 30 minutes.

The materials were transported aseptically to the Biology Laboratory of the Institute of Engineering, Science and Technology of the Janaúba campus of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM). Each sample was placed in a Petri dish containing from 15 to 20 mL of Emerson sterile culture medium (1941) with the aid of an autoclaved forceps to allow the microorganisms present in the samples to develop. Subsequently, the plates containing the material were placed in a bacteriological oven at 30 °C for four days. The growth of new microorganisms was analyzed every 24 hours. As the specimens grew, point isolation was performed in the center of a new culture plate containing the same medium and maintained at 30 °C

The isolation was accomplished according to the observation of important characteristics of the fungal somatic and reproductive structures, such as colony colors, pigmentation and texture (Domsch et al., 2007). The microculture was performed to identify the strains obtained at the genus level (Lacaz et al., 1991). The analysis of macroscopic characteristics, such as the conidiophore facilitated further identification of fungi, as previously described by Pasin et al. (2019).

The analysis of the growth of fungi with respect to temperature was performed for 48 hours in a Potato-Dextrose-Agar (BDA) culture medium from 35 ºC to 50 °C, with intervals of 5 °C. The growth halo was measured, and the fungal growth rate in centimeter per hour (mm/h) was calculated. The strains were maintained in solid Emerson medium (1941) in inclined test tubes. Transplants were performed periodically, kept at 30 °C in a bacteriological oven for seven days and, subsequently, stored at 4 °C. The fungus samples were also kept at 4 ºC on silica gel, according to the method of Michelin (2009).

2.2 Selection of Potential Amylase-Producing Fungi

The analysis of potential amylolytic fungi was qualitatively performed with all the fungi isolated by cultivating them in a solid medium BDA at 35 ºC. The halo of enzymatic degradation was revealed through the addition of a Lugol solution.

Four of the best enzyme producers were grown in CP submerged culture medium (Peixoto et al., 2003) at pH 6.5, using Dinâmica® soluble starch as a carbon source at a concentration of 1.5 and the inoculum was standardized at 1.1 x 10^7 spores. The medium was previously autoclaved and incubated at 30 °C in a bacteriological oven without stirring for three days. The media were vacuum filtered, and the crude enzyme extract obtained was used to quantify the amylolytic activity using the same conditions under which they were grown.

2.3 Fungus Inoculum in Culture Medium

The inoculum process consisted of scraping the fungus maintenance tubes and diluting the spores in approximately 10 mL of sterile distilled water, and then transferring one milliliter of this suspension, 1.1 x 10^7 spores, to a 125 mL Erlenmeyer flask containing 25 mL of the submerged medium.

2.4 Obtaining Mycelial Mass and Enzyme Extract

The mycelial mass was obtained through vacuum filtration through a Büchner funnel containing 12.5 cm diameter Unifil® filter paper. After drying, at room temperature, the mycelial mass was weighed on an analytical balance. The filtrates containing the extracellular enzymes were subjected to measurement of pH, volume and determination of the amylolytic activity.

2.5 Dosage of Amylolytic Activity

Amylolytic activity was determined by quantifying the reducing sugars formed during the enzymatic reaction using 3',5'-dinitrosalicylic acid (DNS), as suggested by Miller (1959). The enzyme-catalyzed reaction consisted of the incubation of 1000 εL of the 1% (w/v) starch substrate in 100 mM sodium acetate buffer, pH 5.0, together with 1000 εL of the crude extracellular extract, in a water bath at 55 ºC for 5 minutes. After the reaction, aliquots of 400 εL were removed and added to tubes containing 400 εL of DNS. A 400 εL aliquot was removed from the reaction mixture immediately after adding the enzyme extract to the substrate. This aliquot was transferred to a tube containing 400 εL of DNS reagent, and this sample was used to control the reaction (zero time). Subsequently, the tubes were boiled for 5 minutes and, after cooling, 4000 εL of distilled water was added. Readings were taken at 540 nm in a Femto® spectrophotometer and compared with that corresponding to the reaction time zero.

The method was previously standardized using glucose (0.1ï 1.0 mg/mL). The unit of activity (U) was defined as the amount of enzyme that hydrolyzes one εmol of soluble starch per minute under the test conditions. Total activity (total U) = εmol/mL x volume of filtrate.

2.6 Definition of the Culture Medium for the Fungus Cultivation and Production of Amylases

The original culture media used in this work should be composed as follows: Khanna medium - 5 mL of mineral solution 20-fold concentrated, constituted (g/100 mL) of 2 NH_4NO_3, 1.3 KH_2PO_4, 0.362 $MgSO_4.7H_2O$, 0.098 KCl, 0.007 $ZnSO_4.H_2O$, 0.0138 $MnSO_4.H_2O$, 0.0066 $FeCl_3.6H_2O$, 0.0062 $CuSO_4.5H_2O$ and 0.1 yeast extract (Khanna et al., 1995); SR medium - 5 mL of mineral solution 50-fold concentrated, constituted (g/100 mL) of 0.3 KH_2PO_4, 0.23 $MgSO_4.7H_2O$ and 1 $NH_4H_2PO_4$, 0.02 yeast extract and 0.45 peptone (Rizzatti et al., 2001); Czapek medium - (g/100 mL) 0.3 $NaNO_3$, 0.1 KH_2PO_4, 0.5 $MgSO_4.7H_2O$, 0.5 KCl, 0.001 $FeSO_4.7H_2O$ (Wiseman, 1975) and CP medium - (g/100 mL) 0.03 KH_2PO_4, 0.05 $MgSO_4.7H_2O$ and 0.8 yeast extract (Peixoto et al., 2003).

Different culture media were tested to select a favorable medium for the production of amylases by *Aspergillus* sp. M1.7.2. The media tested were Khanna (Khanna et al., 1995) modified by the removal of the NH_4NO_3, KCl, $ZnSO_4$, $MnSO_4$, and $FeCl_3$ salts and the addition of $ZnCl_2$ and $ZnCl_3$; SR (Rizzatti et al., 2001) modified by removing $NH_4H_2PO_4$; Czapek (Wiseman, 1975) modified by removing the $NaNO_3$, KCl, and $Fe_2(SO_4)_3$ salts; and the CP medium (Peixoto et al., 2003). Soluble starch was used in all the tested media as a carbon source. After preparation, all the submerged media had their pHôs adjusted to 6.5. Subsequently, the fungus inoculum was performed, and the cultures were incubated in a bacteriological incubator at 30 °C for three days. After the incubation, the cultures were filtered, and the amylolytic activity was determined. The medium in which the highest activity was observed was chosen for the subsequent determinations.

2.7 Determination of the Salt Solution in the Fungus Cultivation for the Production of Amylases

After determining the submerged culture medium, the concentration of the salt solution was changed to 0.05% (m/v) Wesson Salts, CP Salts, Wesson Salts with CP Salts and modified SR salts solution. Subsequently, the fungus inoculum was performed, and the cultures were incubated in a bacteriological incubator at 30 °C for three days. After the incubation, the cultures were filtered, and the amylolytic activity was determined.

2.8 Determination of the Time for Cultivation of *Aspergillus* sp. M1.7.2

The fungus was incubated at 30 °C and pH 6.5, in CP submerged culture medium (Peixoto et al., 2003) containing the predetermined salt solution and 1.5% starch as a carbon source. The cultures were incubated in a bacteriological incubator from one to nine days under static conditions. A triplicate was removed every 24 hours, the cultures were filtered, and the extracellular amylolytic activity was determined.

2.9 Determination of the Nitrogen Source of the Culture Medium

In a standardized environment, the nitrogen source was varied using 0.8% (w/v) casein peptone, 0.8% (w/v) yeast extract and 0.04% (w/v) yeast extract plus 0.04% (w/v) of casein peptone. Subsequently, the fungus inoculum was performed, and the cultures were incubated in a bacteriological incubator at 30 °C for six days. After the incubation, the cultures were filtered, and the amylolytic activity was determined.

2.10 Determination of the Initial pH of the Medium and the Concentration of the Fungus Inoculum

The fungus under study was incubated in CP submerged medium at various initial pHs: 4.0, 4.5, 5.0, 5.5, and 6.0. The incubation was accomplished at 30 °C, stationary in a bacteriological oven**.** Concomitantly, the effect of spore concentration in the inoculum was verified for the greatest production of enzyme. That is, 1 mL of spore solution was inoculated at all pHs in cultures at the following concentrations: 1.1 x 10^5 5.6 x 10^4, 1.0 x 10^4 and 5.0 x 10^3 of spores/mL.

2.11 Effect of Carbon Source on Amylolytic Production

Ten different carbon sources were analyzed in the culture medium of *Aspergillus* sp. M1.7.2 at a concentration of 1.5% (w/v). These sources were soluble starch (Dinâmica®), glucose (Isofar®), *Musa* spp. ñPrata-Anãò genomic group AAB pulp and peel, English potato pulp and peel (*Solanum tuberosum*), cassava pulp and peel (*Manihot esculenta*), corn starch (Maizena®) and ground corn (*Zea mays*).

2.12 Effect of pH and Temperature on Enzyme Activity

The ideal conditions of pH and temperature of the enzymatic assay were evaluated by determining amylase activity at temperatures ranging from 40 °C to 75 °C, at 5 °C intervals, using 100 mM sodium acetate buffers for the pH 4.5; 5.0 and 5.5; and 100 mM sodium phosphate for pH 6.0, 6.5, 7.0 and 7.5.

2.13 Stability of Amylases with Respect to Temperature

The temperature stability of the enzymes was evaluated by incubating the enzyme for 30, 60, 90 and 120 minutes (in the absence of substrate) at temperatures from 50 °C to 70 °C, with intervals of 5 °C. Subsequently, the enzymatic activity of each sample was determined under ideal conditions of pH and temperature.

2.14 Stability of Amylases at pH

The variation of enzyme stability with pH was evaluated by incubating the enzyme extract in 100 mM sodium acetate buffer (pH 4.5; 5.0 and 5.5) and 100 mM sodium phosphate buffer (pH 6.0; 6.5 and 7.0) for 30, 60, 90 and 120 minutes in an ice bath. The enzymatic activity of the samples was determined in 200 mM sodium phosphate buffer pH 6.0 at 65 °C.

2.15 Effect of Ions on Enzyme Activity

To study the effect of ions on amylolytic activity, 5 and 10 mM solutions (final concentrations) of $CuSO_4.5H_2O$, NH_4Cl, $CoCl_2.6H_2O$, KCl, NaCl, $MgCl_2.6H_2O$, $ZnCl_2$, $AgNO_3$, KH_2PO_4 and EDTA were incubated with the enzyme and the substrate in a water bath under ideal conditions.

2.16 Statistical Analysis

All the experiments were performed independently and in triplicate. The results presented are the average of the values obtained. The standard deviations were calculated using Excel 2007.

3. RESULTS AND DISCUSSION

3.1 Sampling, Isolation and Identification of Fungi

Twenty-one filamentous fungi were isolated and identified. M1.1, M1.2, M1.3, M1.4, M1.5, M1.6, M1.7.1, M1.7.2, M1.8, M1.9, M1.10, M1.11, M1.12 were isolated from the tree bark. The M2.1.1, M2.1.2, M2.2, M2.3, M2.4, M2.5 microorganisms were obtained from the fruit peels, and the M3.1, M3.2 organisms were isolated from the decomposing leaf.

The colors of the colonies varied between shades of green, black, white and blue, some with pigmentation secretion. All the microorganisms had a smooth bottom, with the exception of M3.2. Four possible genera were identified. *Aspergillus* was the most common, with fourteen strains being identified, followed by the *Penicillium* genus, which was found in two of the three materials collected, with four strains being identified. Only one *Mucor* and one *Neurospora* organism were identified. One fungus did not have its genus recognized, as is shown in Table 1.

The results obtained regarding the collected genus were similar to those found by several authors. The predominantly isolated fungi found in sampling made in various parts of the country were cosmopolitan *Aspergillus* and *Penicillium* (Cavalcanti et al., 2006; Gomes, 2007; Benassi et al., 2014; Almeida, 2015; Griebeler, et al., 2015, Pasin et al., 2020).

Table 1 - Analysis of macroscopic morphological characteristics of isolated filamentous fungi and possible genus of the microorganisms.

Code	Color	Texture	Pigmentation	Genus
M1.1	Green	Powdery	Absence	*Aspergillus*
M1.2	Black	Powdery	Absence	*Aspergillus*
M1.3	Green	Powdery	Absence	*Aspergillus*
M1.4	Green	Velvety	Absence	*Penicillium*
M1.5	White	Cotton	Presence	-
M1.6	Black	Powdery	Absence	*Aspergillus*
M1.7.1	Green	Powdery	Absence	*Aspergillus*
M1.7.2	Green	Powdery	Absence	*Aspergillus*
M1.8	Green	Furfuraceous	Presence	*Penicillium*
M1.9	Black	Cotton	Absence	*Neurospora*
M1.10	Blue	Velvety	Absence	*Penicillium*
M1.11	Green	Powdery	Absence	*Aspergillus*
M1.12	Black	Powdery	Absence	*Aspergillus*
M2.1.1	Black	Powdery	Absence	*Aspergillus*
M2.1.2	Black	Powdery	Absence	*Aspergillus*
M2.2	Green	Velvety	Presence	*Penicillium*
M2.3	Black	Powdery	Absence	*Aspergillus*
M2.4	Black	Powdery	Absence	*Aspergillus*
M2.5	Black	Powdery	Absence	*Aspergillus*
M3.1	White	Cotton	Absence	*Mucor*
M3.2	Black	Powdery	Presence	*Aspergillus*

Pelczar et al. (1997) divided the organisms into three classes, according to the ideal growth temperature: psychrophiles (temperatures below 20 °C), mesophiles (between 25 and 40 °C) and thermophiles (temperatures above 40 °C)**.** Mesophiles form the group that involves a large portion of the microorganisms. Although they exhibit excellent growth at moderate temperatures, some of them can grow at higher temperatures. These are called thermotolerants.

After the analysis of growth at different temperatures, it was concluded that all the isolated microorganisms are mesophiles because they grew more rapidly at 35 °C, as can be seen in Table 2. Despite this observation, some fungi were able to develop at higher temperatures, including thirteen fungi (M1.1, M1.3, M1.6, M1.7.1, M1.7.2, M1.8, M1.11, M1.12, M2.1.1, M2.1.2, M2.4, M2.5, M3.2) that grew at 40 °C, seven (M1.7.1, M1.7.2, M1.8, M1.11, M2.1.1, M2 .1.2, M2.4) that grew at 45 °C, and finally three microorganisms (M1.7.1, M1.8, M2.1.1) that grew at 50 °C. These filamentous fungi were classified as thermotolerant. These studies corroborate the results obtained by Pasin et al. (2014), Benassi et al. (2014) and Pasin et al. (2020).

Table 2 - Growth rate (mm/h) of filamentous fungi isolated at different temperatures.

	Growth rate (mm/h) at			
Code	**35 °C**	**40 °C**	**45 °C**	**50 °C**
M1.1	0.29	0.08	-	-
M1.2	0.17	-	-	-
M1.3	0.29	0.17	-	-
M1.4	0.06	-	-	-
M1.5	0.17	-	-	-
M1.6	0.35	0.18	-	-
M1.7.1	0.24	0.12	0.11	0.10
M1.7.2	0.33	0.14	0.12	-
M1.8	0.35	0.35	0.17	0.06
M1.9	0.06	-	-	-
M1.10	0.08	-	-	-
M1.11	0.28	0.12	0.04	-
M1.12	0.19	0.10	-	-
M2.1.1	0.31	0.28	0.04	0.02
M2.1.2	0.34	0.19	0.02	-
M2.2	0.06	-	-	-
M2.3	0.19	-	-	-
M2.4	0.25	0.19	0.02	-
M2.5	0.23	0.08	-	-
M3.1	1.11	-	-	-
M3.2	0.21	0.05	-	-

Fungi that are resistant to high temperatures are interesting from a biotechnological point of view because they are capable of producing enzymes with high thermal stability. However, a low heat tolerance does not imply that the organism does not produce thermostable enzymes; the low temperature tolerance is frequently due to the complex membrane systems (Gomes et al., 2007).

3.2 Selection of Filamentous Amylase-Producing Fungi

On the basis of the results of the qualitative assessment of amylases produced by fungi, four microorganisms were chosen, namely: *Aspergillus* sp. M1.3, *Aspergillus* sp. M1.7.2, *Penicillium* sp. M1.8 and *Aspergillus* sp. M1.11 for cultivation in submerged CP and analysis of amylolytic activity. As can be seen in Figure 1, the highest amylase activity was observed for *Aspergillus* sp. M1.7.2, which was 4118 total U, followed by microorganisms M1.3 and M1.11 with enzymatic activities close to 296 and 274 total U, respectively, representing approximately 7% of the activity of isolate M1.7.2. Very low amylolytic activity, only 5.27 U total, was observed for the only strain belonging to the genus *Penicillium* (M1.8).

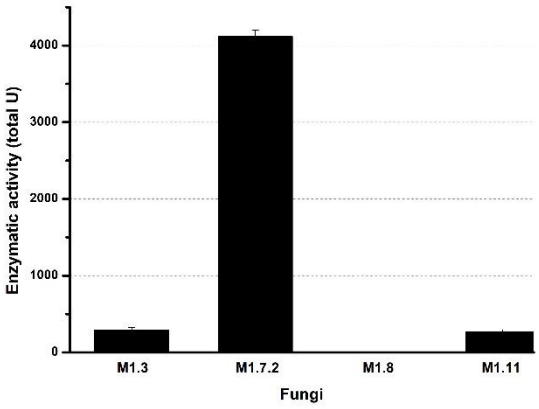

Figure 1 - Selection of the isolated filamentous fungi that produced amylases in a submerged CP culture medium.

Almeida (2015), Silva (2009) and Facchini (2010) demonstrated the superiority of *Aspergillus* filamentous fungi, compared to other genera, in the production of enzymes. Almeida (2015) reports that the amylolytic activity achieved by *Aspergillus brasiliensis* under identical conditions corresponded to 24.36% of the activity shown by the filamentous fungus *Aspergillus* sp. M1.11 and about 2.1% of the activity of *Aspergillus* sp. M1.7.2. Silva (2009) observed a considerably greater amylolytic activity for *Aspergillus niveus* than that of *Aspergillus* sp. M1.3 and M1.11. However, it corresponded to only two-fifths of the activity of *Aspergillus* sp. M1.7.2. Under these circumstances, *Aspergillus* sp. M1.7.2 was selected to optimize the production of amylases.

3.3 Definition of the culture medium and the salt solution for cultivation of the selected fungus

In the laboratory, the growth of microorganisms is achieved by inoculation in culture medium, which can be complex or simple, and this medium must meet the nutritional needs of microorganisms, providing them with nitrogen sources, essential inorganic ions, water and, mainly, a carbon source. It is worth mentioning that the nutrients offered have the power to induce or inhibit enzymatic production and, consequently, the growth of the fungus (Borzani et al., 2001).

Very different reactions were observed for the four media tested, thus demonstrating the importance they have on the production of enzymes. A high amylolytic activity was observed when the *Aspergillus* sp. M1.7.2 was cultivated in the CP medium (4229.88 U total), follow to the modified SR medium

(722.43 U total) and modified Khanna (321.10 U total) and the very low activity of the Czapek medium (15.16 Total U) (Table 3).

Table 3 – Determination of the culture medium for amylolytic production by the fungus *Aspergillus* sp. M1.7.2.

Medium	Activity (total U)
CP	4229.78 ± 2.78
Modified SR	722.43 ± 2.24
Modified Khanna	321.10 ± 2.79
Modified Czapeck	15.16 ± 2.45

The medium with the best performance for the microorganism was that in which the yeast extract, at a concentration of 0.8% (m/v), was the only source of nitrogen. The salts added to the medium were dipotassium phosphate (0.03%) and hydrated magnesium sulfate (0.05%); all the other media contained these two salts in greater concentrations and also contained other salts. This fact represents a greater savings in the cost of producing enzymes.

Almeida (2015) tested the Khanna, SR and CP media without any modifications. For *Aspergillus brasiliensis*, the greatest production of amylases was observed under static conditions in SR, CP and Khanna media, respectively, where the variation in activity between the two best media was very small, considering the difference between the compositions. The same author found a greater enzyme production with stirring in CP, SR and Khanna media, respectively, for *Rhizopus oryzae.* Facchini (2010) verified the low cellulolytic induction in Czapek culture medium for *Aspergillus terreus* and *Aspergillus japonicus* fungi. Silva (2009) found that the best medium for the production of amylases was Khanna, followed by the SR medium. In addition, he found that the Czapek medium was irrelevant in the production of amylases by *Aspergillus niveus*.

Borzani et al. (2001) explained that sources of only carbon and nitrogen are often insufficient and require the addition of inorganic compounds. These compounds are often required in very low amounts. Sulfur, phosphorus, magnesium and potassium ions are important, and they are essential for good microbial growth.

The solution of modified CP and SR salts is formed by dipotassium phosphate and hydrated magnesium sulfate, compounds which have been described as essential by the authors. According to Corrêa (2006), the Wesson salt contained copper sulfate heptahydrate, calcium carbonate, tricalcium phosphate, potassium iodide, ferric phosphate, potassium and aluminum sulfate, magnesium sulfate, sodium chloride, manganese sulfate, potassium chloride, monopotassium phosphate and sodium fluoride. The essential compounds mentioned by the author for the production of enzymes were present among the salts more than once.

Among the salt solutions tested, the highest enzymatic activity, 5076.6 total U, was observed for the CP medium after three days at 35 ºC. The second highest enzyme activity was obtained using the salt solution containing CP medium and Wesson salts, 3086.6 total U. In a medium in which the SR salt solution was used, a total activity of 2366 total U was observed, and only 373.4 total U were observed in the medium containing Wesson salts. Again, the results obtained indicate greater savings for the production of amylases by *Aspergillus* sp. M1.7.2 (Table 4).

Table 4 – Determination of salt solution to the culture medium for amylolytic production by the fungus *Aspergillus* sp. M1.7.2.

Salt solution	Activity (total U)
CP	5076.60 ± 2.48
CP + Wesson	3086.62 ± 2.35
SR	2366.51 ± 2.12
Wesson	373.40 ± 1.98

3.4 Determination of the cultivation time of *Aspergillus* sp. M1.7.2 for amylolytic enzyme production

The ideal cultivation time varies with the fungus species and growing conditions. In the test performed, a rapid increase in amylolytic activity was observed over time until the sixth day of cultivation, slowly decreasing after this period, and an activity of 8098 total U was verified after six days at 30 ºC without stirring (Figure 2).

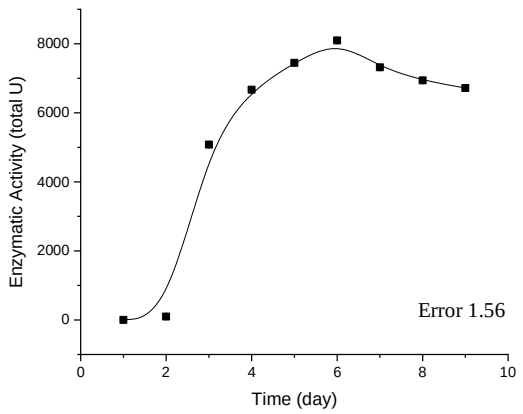

Figure 2 - Determination of the Time for Cultivation of *Aspergillus* sp. M1.7.2.

According to Santos (2008), fermentation by filamentous fungi occurs in two distinct morphological phases, which are the tropophase, the stage where the growth of the microorganism (from the germination of the spores to the formation of the mycelium) occurs, and the idiophase stage, in which product formation occurs. The cultivation time of a fungus directly and significantly influences the production of the enzyme. If incubated for a short period of time, the enzyme activity can be much lower than its maximum activity because it will be in the tropophase or vegetative phase. Likewise, incubation for prolonged periods can deplete nutrients and cause a decrease in enzyme activity.

Silva (2009) observed that the highest amylase activity occurred on the third day of cultivation. The author justified his result by proposing that, at the end of the exponential growth phase when the sugars in the medium were not easily assimilated, the fungal growth decreased to guarantee survival,

and it was induced to produce a large amount of enzymes, enough to degrade many molecules of starch to meet its nutritional needs.

Pasin (2015) observed that the greatest production of amylase by *Aspergillus japonicus* occurred after four days of cultivation without stirring, a result similar to those obtained in our work. It also occurred after three days when stirred. However, the greatest activity occurred under static conditions. Nwagu and Okolo (2011a) found that the fifth day of cultivation at 30 °C was the best time for the *Fusarium sp*. fungus. A similar result was obtained by Almeida (2015), who, in his research, performed the day test on two filamentous fungi *Aspergillus brasiliensis* and *Rhizopus oryzae*, and the ideal time for each was five and four days, respectively. Paris et al. (2012) found that the best growth time for the production of amylases, proteases and lipases by *Aspergillus niger* was on the sixth day.

3.5 Determination of the nitrogen source of the submerged culture medium for the production of amylase

Among the nitrogen sources tested, the lowest production of amylases was obtained with the casein peptone, which yielded an activity of 3995 total U. Again, the result led to the continuation of the use of yeast extract, the original nitrogenous component of the CP medium, whose use yielded a total enzymatic activity was 8425 U, which is about 47.7% greater than any other nitrogen source (Table 5).

Table 4 – Determination of nitrogen source to the culture medium for amylolytic production by the fungus *Aspergillus* sp. M1.7.2.

Nitrogen sources	Activity (total U)
Yeast Extract	8425.65 ± 1.58
Casein Peptone	3995.78 ± 1.22
Yeast extract + Casein Peptone	4049.24 ± 1.74

Nitrogen is essential in the synthesis of amino acids, nitrogenous bases present in nucleic acids, vitamins and glucosamine, a component of chitin. Both natural and synthetic media must have at least one source of nitrogen to enable the production of enzymes by the fungus and, consequently, growth (Veron, 2016).

Yeast extract was also defined as the best source of nitrogen to support the production of amylases by the fungus *Talaromyces trachyspermus* T10-5, as observed by Veron (2016). Nwagu and Okolo (2011b) cultivated *Aspergillus fumigatus* in medium containing several organic sources of nitrogen. The best source was yeast extract, this source being about twice as good as the casein peptone. Nwagu and Okolo (2011a) also cultivated *Fusarium sp*. in culture media with various sources of nitrogen, such as amino acids, yeast extract, casein peptone and soybean meal. The soybean meal was the source that best induced amylolytic activity; the activity obtained in the presence of the peptone was slightly greater than that obtained when using the yeast extract.

3.6 Determination of the inoculum concentration and the initial pH of the medium

The highest concentration of spores in the inoculum of the medium was the most favorable with regard to enzymatic activity. Proportionality was noted, where the decrease in the quantity of spores lead to a decrease in amylolytic activity (Figure 3).

The initial pH of the culture medium favorable for the growth of the fungus and production of amylase was 5.5, a result similar to that observed by Pasin (2015). However, that author observed acidification of the medium during cultivation, a process opposite that observed in the present work.

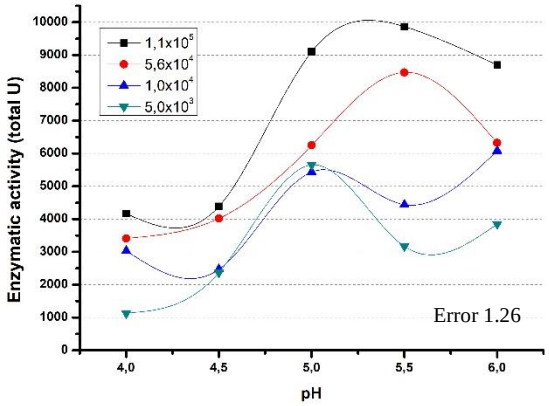

Figure 3 - Amylolytic activity of *Aspergillus* sp. M1.7.2 as a function of the initial pH of the culture medium and the concentration of spores in the inoculum.

Ming Chu et al. (1992) explained that changes in the pH of the culture medium during the growth of the fungus are related to the consumption of the substrate. The medium becomes more basic when organic nitrogen (amino acids and peptides) is being incorporated or more acid when ammonium ions are consumed. Dutton et al. (1996) also suggested that the acidification of the culture medium can be caused by the production of acids, such as oxalic acid, which increases the availability of metal ions that favor amylase activity because it is a chelating agent.

Freitas et al. (2014) observed a small influence of the initial pH of the medium on the final amylolytic activity; that is, the production of amylases was not inhibited by the pH of the medium in *Fusarium sp.*. Sales et al. (2010) analyzed the effect of the initial spore concentration on the production of several cellulases by the fungus *Aspergillus aculeatus*, and they observed that the highest concentration of spores favored the production of four of the five enzymes tested.

In the experiments carried out by Spier (2005) with the *Aspergillus niger*, an inverse effect was observed in the production of amylases by increasing the concentration of inoculum; that is, a larger number of spores resulted in a decrease in enzymatic activity. These results were obtained by solid state fermentation. Silva (2009) observed that the ideal concentration for *Aspergillus niveus* was 5 x 10^5 spores/mL, an intermediate concentration among those tested by the author and close to the ideal concentration verified in this study.

3.7 Effect of variation of carbon source on enzyme activity

The best source of carbon among those tested was the English potato (*Solanum tuberosum*) pulp. The total amylolytic activity obtained was 9,538.32 U, an activity slightly higher than that obtained with soluble starch, which was 8551.93 total U.

The other sources also induced high activities, such as potato skins, cassava and cassava skins, corn and bananas, as is shown in Figure 4. All the sources contributed to high amylase secretion, except for glucose. This source is described in the literature as a classic catabolic repressor, which acts on the regulation of gene expression at transcriptional levels (Carlson, 1987; Benassi et al., 2012).

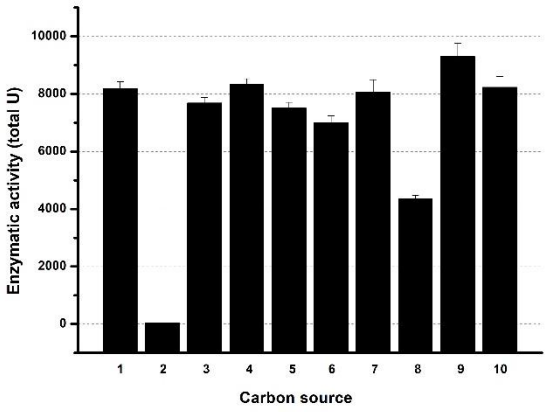

Figure 4 - Amylolytic activity of *Aspergillus* sp. M1.7.2 as a function of the carbon source of the submerged culture medium.

Note: (1) soluble starch; (2) D-glucose; (3) corn starch; (4) corn; (5) cassava; (6) cassava skins; (7) banana; (8) banana skins; (9) potato and (10) potato skins.

The high cost associated with the production of enzymes is a major obstacle to their application in industrial processes. The carbon sources usually used in fermentations are the elements that most contribute to this cost (Pandey et al., 1999). The production of amylases is commonly induced by the presence of starch (or some of its hydrolysis products, such as maltose), either in soluble or native form (present in seeds, tubers, among others). Currently, several studies searching for economically viable carbon sources, such as agro-industrial waste, are in progress. The use of these compounds as a carbon source for the cultivation of microorganisms for the purpose of enzyme production significantly reduces the cost of obtaining the enzyme, in addition to contributing to the reduction of environmental impacts (Gonçalves, 2016).

Nwagu and Okolo (2011b) tested the effects of a diversity of starches and sugars on enzyme activity produced by *Aspergillus fumigatus*, and they obtained good results for corn, potato and cassava starch. The catabolism induced by these carbon sources (native starch) was better than that obtained with soluble starch and the various sugars tested. Benassi et al. (2012) explained that the agro-industrial residues used were probably hydrolyzed in the culture medium to maltooligosaccharides by constitutive amylases. These can act as true inducers of gene expression. Despite the fact that the result obtained with the potato starch was superior to that observed with soluble starch, it was decided to continue using the latter as a standard carbon source for cultivation of the isolated microorganism M1.7.2.

3.8 Effect of pH and temperature on the activity of amylases produced by *Aspergillus* sp. M1.7.2

The amylolytic activity of the enzyme extract from *Aspergillus* sp. M1.7.2 was maximum at 65 °C and pH 6.0 (9447.9 total U). High enzymatic activities were obtained at all the pH and temperature ranges analyzed. Activities greater than 70% of the maximum activity were obtained at pH 5.0 and 6.5 and temperatures of 45 ºC to 70 ° C, giving these enzymes applicability in various industrial processes. The findings of this experiment are in agreement with those of other amylases described in the literature. Silva et al. (2000) and Pasin et al. (2017) found an optimal activity at 65 °C and pH 5.0.for the amylases produced by *Aspergillus fumigatus* and the purified glucoamylases of *Aspergillus japonicus*, respectively.

Ali et al. (2014) and Nwagu and Okolo (2011b) observed that the amylases produced *by Aspergillus gracilis* and *Aspergillus fumigatus* possessed greater activity at 60 °C and an ideal pH of 5.5. The amylases produced by *Paecilomyces variotti,* as described by Michelin et al. (2010), exhibited greater catalytic power at 60 °C and pH 4.0. High activities were also obtained at pH 4.5 and 5.0, as was observed for the amylases of *Aspergillus* sp. M1.7.2. Carrasco et al. (2017) observed greater catalytic activities for Ŭ-glycosidase secreted by the yeast *Dioszegia fristingensis* at pH 5.5 - 6.5. According to Oliveira, Watanabe, and Rodrigues (2011), the optimal activity of the amylases of *Penicillium sp.* was obtained at 55 °C and pH 6.5. For the thermotolerant *Fusarium sp.* cultivated by Nwagu and Okolo (2011a), the best conditions were pH 6.5 and 50 °C.

3.9 Enzyme Thermostability

A high stability at 50 °C was observed for the enzymes, with a half-life greater than 120 minutes. At 55 °C, an activity greater than 50% was observed for more than 60 minutes. After 120 minutes at this temperature, the enzyme still retained about 30% of its activity. However, the half-life was less than 30 minutes above 60 °C; 98.89% of the activity was lost when exposed for 120 minutes at this temperature, as is shown in Figure 5.

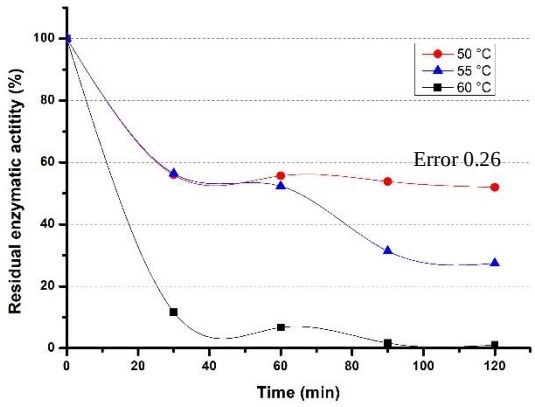

Figure 5 - Variation of the stability of the enzyme with temperature and reaction times.

A high stability at 50 °C and 55 °C was observed for the enzymes produced by *Paecilomyces variotii*. Their activities were considerably lower at 60 °C (Michelin et al., 2010). The Ŭ-amylase produced by *Syncephalastrum racemosum* was stable at 55 °C and 60 °C (Freitas et al., 2014). The amylases produced by *Aspergillus niger* exhibited greater resistance at 60 °C, with a half-life 40 minutes at this temperature (Pereira, 2015). The catalytic activity of the Ŭ-glucosidases produced by *Dioszegia fristingensis* decreased by 70% after two hours of incubation at 50 °C (Carrasco et al., 2017).

3.10 pH stability

The amylases possessed a high stability to variations in pH, losing less than 30% of their activity after 120 minutes of incubation at all the pHs tested. The enzyme was activated at pHs 4.5; 5.0; 6.0; 6.5 and 7.0 when exposed for 30 minutes (Figure 6). Similar stability has been described by Pasin et al. (2017) for glucoamylases produced by *A. japonicus*. These enzymes retained more than 80% of their activity during an hour of incubation. Benassi et al. (2014) also observed a high stability between pH 3.0 and 6.0 for the amylases produced by *A. phoenicis*.

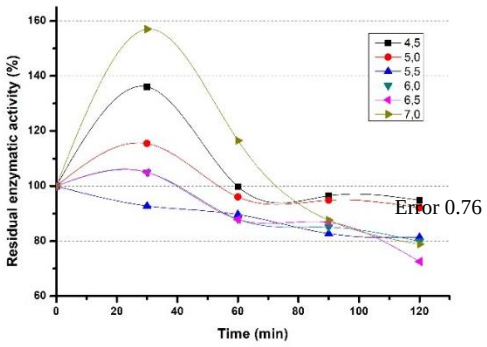

Figure 6 - Variation of the stability of the enzyme with pH and reaction times.

3.11 Effect of ions on enzyme activity

The enzymes produced by *Aspergillus* sp. M1.7.2 were active in the presence of NaCl, NH_4Cl, $MgCl_2$ and KH_2PO_4 at concentrations of 5 and 10 mM. The greatest activity was observed at a concentration of 10 mM. The activation by 10 mM KCl, 5 mM NaCl and $MgCl_2$ at both concentrations was not very significant. However, activation by NH_4Cl was greater; the activity of the enzyme extract increased by up to 20%. KH_2PO_4 stood out because an increase in enzyme activity by more than 50 and 60% at concentrations of 5 and 10 mM, respectively, was observed. When the concentrations of the other compounds tested, such as $ZnCl_2$, $AgNO_3$, $CuSO_4$, $CoCl_2$ and EDTA, were increased, a decrease in enzymatic activity was observed (Figure 6).

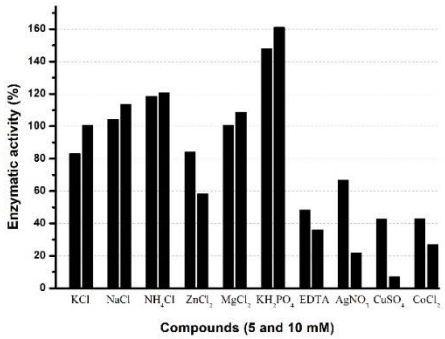

Figure 7 - Effect of ions and EDTA on enzyme activity.

Metal ions are important during enzymatic synthesis because they are incorporated into amylases and contribute to the conformation and catalytic activity of these enzymes (Nwuagu et al., 2011a). The work of Ezeji et al. (2005) presented similar results regarding the activities against Co^{2+} and Zn^{2+} ions. Pires et al. (2012) observed that the presence of EDTA significantly reduced the activity of the enzyme, as well as suggesting that the enzymes used are dependent on Ca^{2+} ions. For the enzymes studied by those authors, the addition of $CuSO_4$ to the reaction was observed to significantly inhibit the activity of amylases. Their results were similar to those observed in the present work.

However, Nwagu and Okolo (2011b) observed that the amylases produced by *Aspergillus fumigatus* were activated by Cu^{2+} and Zn^{2+} ions at a concentration of 2 mM, results that are inconsistent with those obtained in this work. Pasin et al. (2017) verified enzymatic activation by the KH_2PO_4 salt. However, more significant activation was verified in the presence of $MnCl_2$; they observed the same effect as that observed for KH_2PO_4 regarding the enzyme activity in the present work, with an increase in the activity by about 50 and 60% at the concentrations of 5 and 10 mM, respectively. This salt repressed the enzymatic activity of the fungus *Paecilomyces variotii* by almost 70%, as verified by Michelin et al. (2010); an opposite effect was also observed regarding the use of $CoCl_2$, which substantially activated the amylases of *P. variotii* and deactivated those of *Aspergillus* sp. M1.7.2.

4. Conclusions

There is a diversity of microorganisms to be isolated, many of them with a potential for industrial application, such as *Aspergillus* sp. M1.7, which stands out as a potential amylase-producing organism with desirable characteristics for applications in biotechnological processes. The cultivation of the microorganism was standardized under ideal conditions for enzyme production and throughout the standardization. A large amount of amylase secretion by the fungus was observed, thus dispensing with extracellular enzyme extraction steps. As for their physicochemical characteristics, a similarity with the enzymes described in the literature was observed; however, with more desirable characteristics, as well as temperature stability and a wide range of pH stability. The amylases produced also exhibited excellent activation, especially with KH_2PO_4, noting that the inclusion of NH_4Cl also caused an increase in enzyme activity. Thus, a great potential for biotechnological and industrial use in the future was demonstrated.

Acknowledgment

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