Isolation of high amylase-producing filamentous fungi from the caatinga: standardization of the culture conditions and amylase activity from *Penicillium* sp. L1

Abstract

Microbial amylases are commercially available and have almost completely replaced the chemical hydrolysis of starch in various industrial processes. Microbial amylases have a wide range of applications because they are more stable than amylases obtained from plants and animals. This work sought to isolate filamentous fungi from three different samples collected in Janaúba, in the north of the state of Minas Gerais, to select a filamentous fungus that produces amylases, to determine the best cultivation conditions for the selected fungus for amylase production, and to analyze the biochemical characteristics of the enzyme. After collecting the samples for the isolation of filamentous fungi, the macroscopic morphological characteristics of the fungi, the microculture for the determination of the possible genus and the effect of temperature on fungal growth were determined. The screening of the fungi was performed in a submerged medium to characterize the amylase-producing microorganism through the analysis of the physicochemical parameters of the medium with regard to the source of nitrogen, the type of salt solution, and the source of carbon, so as to optimize the production of amylase. The biochemical characterization of the enzyme was performed. The fungus *Penicillium* sp. (L1) was selected, and the greatest production of amylase was observed in submerged Carvalho-Peixoto (CP) medium associated with Wesson salts using yeast extract and starch as nitrogen and carbon sources, respectively, at 30 ºC, over a seven days period. The optimum temperature and pH of the assay were 60 ºC and 5.5, respectively. Thus, the northern region of Minas Gerais was an excellent source for the strains that exhibited a potential for the production of amylase for use in industry, indicating that the fungus *Penicillium* sp. (L1) and the amylase produced have a relevant potential for biotechnological applications.

Keywords: Filamentous fungus. Enzymatic production. Enzymes. Biotechnology. *Penicillium* sp. L1

1. Introduction

Research in the field of enzymology has stimulated the prospecting of promising enzyme-producing microorganisms that have applications in industry and analytical procedures [1,2]. The isolation and selection of microorganisms can facilitate the discovery of new enzymes

with desirable characteristics. Filamentous fungi are emphasized as good producers of amylases, which have advantages over chemical catalysts because they provide greater production, greater rate of production, better process control and high quality as clean technologies [3,4]. Among the genera of filamentous fungi described in the literature that are best known as major producers of amylases, the genus *Penicillium* stands out because it is able to grow in environments that contain a source of mineral salts and any source of carbon. Some species of *Penicillium* are of industrial importance because of the use of their amylases in food production [5]. Amylases are considered to be one of the most important families of enzymes for industry because they are used in starch saccharification processes, in textiles, food and feed, detergents, fermented beverages and distilleries [6,7]. In addition, they have a potential for application in the pharmaceutical and chemical industries and can also be applied to recycling and paper production [8,9]. Enzyme technology has increasingly been a promising tool for the generation of compounds with higher value, for obtaining high quality products and for environmental preservation [10,11]. Thus, the present study sought to isolate microorganisms that produce amylases, as well as to establish the culture medium and standardize the parameters for the cultivation of *Penicillium* sp. (L1). The culture medium, time of growth of the microorganism, salt solution, nitrogen source, initial pH of the culture medium and carbon source were studied to optimize the amylolytic production, in addition to biochemically characterizing the enzyme.

2. Materials and Methods

This work was conducted at the Biology Laboratory of the Institute of Engineering, Science and Technology (IECT), of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM), Janaúba, Minas Gerais, Brazil. The microorganisms were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under number A64AD93.

2.1 Sample collection, isolation of filamentous fungi and analysis of macroscopic morphological characteristics of the isolates

The filamentous fungi were isolated from three different samples, namely: (1) the seed of the plum tree, (2) the seed of the caja tree and (3) the legume pod from *Anadenanthera falcata*. All the samples were collected aseptically in the city of Janaúba, in the north of the state of Minas Gerais, Brazil.

The isolation of the filamentous fungi was achieved in a 4% (w/v) Quaker® oats and 2% (w/v) Bacteriological Agar solid culture medium [12] in sterile Petri dishes. The collected

material was inoculated and kept in a bacteriological incubator at 30 ºC during the course of four days. The growth of filamentous fungi in the plates was determined every 24 hours.

As the specimens grew, the isolation of these organisms was performed in another Petri dish containing the same initial culture medium [12], and they were maintained under the same conditions. After isolation, the macroscopic morphological characteristics of the fungi, such as color, border, texture, background and absence or presence of pigmentation in the culture medium, were analyzed.

2.2 Microculture of isolated filamentous fungi and maintenance of strains

The microculture was performed according to the technique of Ridel [13] for the analysis of the microscopic characteristics of the isolates and possible identification at the genus level. The slides of the filamentous fungi were observed by optical microscopy at a 100-fold magnification.

The microorganisms were maintained in the laboratory in a solid oat medium [12]. These media were kept in tilted test tubes, previously autoclaved at 120 ºC and 1.5 atm for 30 minutes. Transplants were performed periodically, initially growing in a bacteriological oven at 30 ºC, and subsequently stored in a refrigerator at 4 ºC. The strains were also kept on silica gel, where a spore suspension was prepared in a sterile environment from 2 mL of powdered milk solution (200 $g.L^{-1}$ of distilled water). Approximately 1 mL of this suspension was added to test tubes (10 x 150 mm) containing 7 g of 4 - 8 mm silica gel and stirred. These tubes were sealed and stored at 4 ºC.

2.3 Analysis of the effect of temperature on the growth of filamentous fungi in solid culture medium and determination of the qualitative production of amylases by the isolates

The microorganisms were transplanted in the center of a solid Potato-Dextrose-Agar (BDA) culture medium in a Petri dish and kept in a bacteriological oven for 48 hours at temperatures that varied from 30 ºC to 50 ºC, with intervals of 5 ºC. After the growing time had elapsed, the radius of the fungal halo was measured, and the growth rate was calculated in centimeters per hour ($cm.h^{-1}$). The qualitative production of amylases by the filamentous fungi was analyzed by adding 5 mL of Lugol solution, and the enzyme halo was measured in centimeters. In addition, fungi were grown at 50 ºC; after having remained at 30 ºC for 24 hours, they were maintained at 50 ºC for 24, 48, 72 and 96 hours.

2.4 Inoculum in submerged culture medium

The cultures of the filamentous fungi in the tubes were suspended with 5 mL of sterile distilled water, and a 1 mL aliquot of the spore suspension containing 123 x 10^4 spores.mL^{-1} was inoculated in a 125-mL Erlenmeyer flask, containing 25 mL of submerged culture medium.

2.5 Selection of the amylase-producing microorganism in submerged culture medium

The isolated filamentous fungi were grown in 25 mL of Liliane-Tamires-Silva (LTS) submerged culture medium in a 125-mL Erlenmeyer flask, proposed by the author [14], and composed of 1.0 g soluble starch as a carbon source; 0.10 g of yeast extract as a nitrogen source; 5 mL of salt solution composed of 0.362% (w/v) $MgSO_4$, 1.30% (w/v) KH_2PO_4, 0.007% (m/v) $ZnCL_2$, 0.066% (m/v) $FeCl_3$, and 0.00062% (m/v) $CuSO_4$ in 100 mL of distilled water.

After preparing the culture and autoclaving the media, 1 mL of spore suspension was inoculated, and the culture was kept in a bacteriological incubator at 30 ºC for four days. After cultivation, the mycelial mass and the soluble crude extract containing the amylases were separated, and the activities were determined by the saccharification method.

2.6 Obtaining mycelial mass and crude soluble enzyme extract

After the incubation of the microorganism, the mycelial mass was obtained by separation of the culture medium through vacuum filtration through a Büchner funnel with filter paper. The mycelium was weighed on a semi-analytical balance, whereas the pH, volume (mL) and amylolytic activity were measured in the filtrates containing extracellular enzymes.

2.7 Quantification of enzymatic activity by the saccharification method

The determination of the enzymatic activity was achieved according to the method of Miller [15]. The quantity of reducing sugars formed during the incubation of 1000 µL of crude enzymatic extract with 1000 µL of the soluble starch substrate at a concentration of 1% (w/v) in 100 mM sodium acetate buffer, pH 5.0, at 60 ºC for 5 minutes was measured. After the reaction time, 400 µL aliquots were added to tubes containing 400 µL of 3,5-dinitrosalicylic acid (DNS) reagent. The control of the reaction was represented by the mixture of the enzyme with the substrate at zero incubation. An aliquot of 400 µL was removed from the reaction mixture, and after rapid homogenization, it was transferred to a tube containing 400 µL of DNS reagent. Subsequently, the tubes were boiled for 5 minutes, and, after cooling, 4 mL of distilled water was added. Readings were taken at 540 nm on a Femto® spectrophotometer. The method was previously standardized using a standard glucose curve (0.1 to 1.0 mg.mL^{-1}).

The enzyme activity was represented by $U.mL^{-1}$, defined as the amount of enzyme that hydrolyzed one µmol of substrate per minute per mL under the test conditions. Total activity (total U) = $\mu mol.mL^{-1}$ x volume of the filtrate.

2.8 Evaluation of different submerged culture media for greater production of amylases by the selected microorganism

The inoculation of 1 mL of the spore suspension of the isolated filamentous fungus previously selected for study was performed in different previously autoclaved, submerged culture media containing starch as a carbon source: Segato-Rizzatti (SR) without $NH_4H_2PO_4$ [16], Czapeck without $NaNO_3$ [17], Liliane-Tamires-Silva (LTS) [14] and Carvalho-Peixoto (CP) [18]. The media were maintained at 30 ºC for four days under steady growth conditions. After the incubation, the mycelial mass and the soluble crude extract containing the amylases were separated, and the activities were determined by the saccharification method.

2.9 Evaluation of the salt solution of the culture medium and the incubation time of the isolated filamentous fungus to determine the greatest amylolytic activity

Different salt solutions of the previously established submerged culture medium were analyzed, these being: (1) only CP salts (0.03% (w/v) of KH_2PO_4 and 0.05% (w/v) of $MgSO_4$); (2) only 0.05% (w/v) of Wesson salts; and (3) CP salts plus 0.05% (w/v) of Wesson salts. After inoculation of the isolated filamentous fungus, the media were kept in a bacteriological oven at 30 ºC for ten days. The mycelial mass was separated every 24 hours, and the activity of the soluble crude extract containing the amylases was determined by the saccharification method.

2.10 Evaluation of different nitrogen sources in the submerged culture medium for the production of amylase by the selected filamentous fungus

The fungus was inoculated in a previously determined submerged medium containing the solution of salts that resulted in the greatest amylolytic activity and starch as a carbon source and using the following organic nitrogen sources: (1) 0.48% (m/v) of yeast extract (control), (2) 0.48% (w/v) of peptone, and (3) 0.24% (w/v) of yeast extract plus 0.24% (w/v) of peptone. The media were maintained in a bacteriological incubator at 30 ºC for seven days. The mycelial mass and the soluble crude extract containing the amylases were separated, and the activities were determined by the saccharification method.

2.11 Evaluation of the initial pH of the submerged culture medium for the production of amylases by the isolated filamentous fungus

The culture medium was prepared by varying the initial culture pH from 4.5 to 7.0 with intervals of 0.5. After inoculation of the fungus, the media were maintained at 30 ºC for seven days. To adjust the pH of the media, 1 M solutions of hydrochloric acid and sodium hydroxide were used.

2.12 Evaluation of different sources of carbon in the submerged environment to increase the production of amylases

To assess the best carbon source, the fungus was grown in a submerged medium containing the following carbon sources: starch, glucose, fructose, maltose, lactose, sucrose, corn starch (Maizena®), oatmeal, powdered milk, and fresh sugarcane bagasse. The culture medium without a carbon source was used for the control. The media were kept in a bacteriological incubator under static conditions at 30 ºC, for seven days. The mycelial mass and the soluble crude extract containing the amylases were separated, and the activities were determined by the saccharification method.

2.13 Effect of temperature and pH on enzyme activity

The enzyme activity as a function of pH and temperature was evaluated in the temperature range of 40 ºC to 75 ºC, using intervals of 5 ºC, and at pHs of 4.5, 5.0, 5.5, 6.0 and 7.0. The 1% (w/v) starch substrate in 0.1 M sodium acetate buffer was used for determining the enzyme activity for each temperature at pHs of 4.5, 5.0 and 5.5. The 0.1 M sodium phosphate buffer was employed at pHs 6.0 and 7.0.

2.14 Stability of the enzyme at different temperatures and pH

The temperature stability of the enzyme was evaluated by incubating the enzyme for 30, 60, 90 and 120 minutes at temperatures of 55 ºC to 80 ºC, with intervals of 5 ºC. The enzymatic activity of each sample was then determined under the ideal conditions of pH and temperature. The pH stability was determined in 100 mM sodium acetate buffer for pH 4.5, 5.0, 5.5; 100 mM sodium phosphate buffer for pH 6.0 and 7.0. The enzyme-buffer mixture was kept in an ice bath for periods of 30 to 120 min with intervals of 30 min; a 500 μL aliquot was added to the starch substrate in 200 mM sodium acetate buffer under ideal conditions of pH and temperature.

2.15 Reproducibility of the results and Statistical analysis

Quantitative data were presented as the mean ± standard deviation of three independent experiments. The statistical program used was SISVAR [19]. The data were submitted to analysis of variance and the means were compared by the Tukey test to the level of ($P < 0.05$) among the groups.

3. Results and Discussion

3.1 Isolation, analysis of macroscopic morphological characteristics of filamentous fungi and determination of the possible genus of the isolates

Three different filamentous fungi were isolated from the samples collected, these being denominated L1, L2 and L3 (Figure 1). The microorganism L1 was collected from the seed of the plum tree, and the fungi identified as L2 and L3 were obtained from the *Anadenanthera falcata* legume pod. No fungus was isolated from the seed of the caja tree sample, probably because it was a dry sample with no moisture when it was collected.

The fungus with a green color, white edges, wrinkled background, powdery texture and the presence of yellowish pigmentation was identified as L1 (Figure 1A and Table 1). The microorganism with a white color, smooth background, velvety texture and the presence of pink pigmentation was identified as L2 (Figure 1B and Table 1), and the fungus that had a gray color with a black and white border, smooth background, velvety texture and the presence of gray pigmentation in the culture medium was identified as L3 (Figure 1C and Table 1). The L1 fungus, according to the optical microscopic image, is possibly of the *Penicillium* genus (Figure 2A); the L2 fungus is possibly a *Paecilomyces* (Figure 2B), and the filamentous fungus L3 is possibly of a *Mucor* genus (Figure 2C).

The results of the microscopic identification of the filamentous fungi indicated the possible genera *Penicillium*, *Paecilomyces* and *Mucor*. These results were obtained from the analysis of the morphological characteristics of the spores and conidiophores of the isolated strains. The isolate L1 was classified as *Penicillium* sp. because the conidium of its spore producing structure looks like a brush [20]. The fungus L2 received the classification of *Paecilomyces* sp. because its conidiophores are branched into irregular groups and the conidia are separated from the phialides in the form of a chain [21]. Finally, the L3 was identified as a possible *Mucor* sp. for having fruiting bodies formed only by a small mass of spores on a stem, simple and very small [22].

Microorganisms, mainly filamentous fungi, are of great importance for the environment. Thus, the three organisms isolated were a result of the habitats present in the Brazilian

caatinga and the enrichment conditions of the medium, emphasizing that the culture medium contained oat flour, a complex and rich source for the development of organisms.

3.2 Analysis of the effect of temperature on the growth of isolated filamentous fungi

To better understand the physiological characteristics of the isolated filamentous fungi and to verify the tolerance of the organisms to different temperatures, the temperatures at which the greatest growth of the isolated filamentous fungi occurred were determined. The greatest growth of L1 was 30 ºC, with a growth rate of 0.038 $cm.h^{-1}$. No significant growth was seen at temperatures from 35 ºC to 50 ºC. An increase of 5 ºC in the temperature (30 ºC to 35 ºC) caused a decrease in the growth rate of the organism to about 5% of that observed at 30 ºC (Table 2).

The microorganism *Paecilomyces* sp. (L2) achieved its greatest growth at 30 ºC with a growth rate of 0.029 $cm.h^{-1}$, whereas the growth of the fungus at 35 ºC represented only 23% of the development achieved at the lowest temperature analyzed. A decrease in the growth rate at the other temperatures was observed, and no growth was observed at 50 ºC (Table 2). The *Mucor* sp. (L3) fungus achieved its greatest growth (0.019 $cm.h^{-1}$) at 30 ºC. At temperatures of 40 ºC and 45 ºC, a growth rate of 0.002 $cm.h^{-1}$ was maintained, and no growth was observed at 50 ºC (Table 2).

Among the three filamentous fungi, the highest growth rate was observed with the fungus identified as *Penicillium* sp., all the isolates were mesophilic organisms that exhibited the highest growth rates at 30 ºC. In addition, an increase of only 5 ºC in the temperature resulted in a decrease of more than 50% in the growth rate of the microorganisms (Table 2).

Because the isolated filamentous fungi did not grow at 50 ºC, the microorganisms were cultivated for 24 hours at 30 ºC, a temperature that, according to Table 2, favored the greatest development of the isolates. The plates containing the fungi were then cultivated at 50 ºC for periods of 24 hours, 48 hours, 72 hours and 96 hours. An average growth rate of 0.11 $cm.h^{-1}$ was observed for L1, which is a value higher than that presented in Table 2, when the same microorganism was cultivated only at 50 ºC for 48 hours (Table 3).

An average growth rate of 0.037 $cm.h^{-1}$ was observed for L2 (Table 3). This rate was much higher than those found for the growth of this fungus at any other temperature analyzed in Table 2. However, when the isolated fungus identified as L3 was analyzed, it did not grow at 50 ºC even after being kept for 24 hours at 30 ºC, thereby demonstrating that this fungus cannot grow at high temperatures (Table 3).

The microorganisms L1 and L2 produced the enzyme of interest; the largest amylolytic

production occurred at 30 ºC, and a decrease of more than 40% in the production of amylases occurred with increasing temperature (Table 4). The isolated fungus L1 produced a smaller enzymatic halo at 30 ºC than that of the isolated fungus L2. However, it maintained this production even with an increase in the temperature to 50 ºC. The L3 isolate produced a smaller enzymatic halo than those of the other two filamentous fungi.

3.3 Screening of the isolated filamentous fungus for the largest production of amylases

It was observed that L1 was the greatest producer of amylase, with a total activity of 6.80 U, followed by the microorganism L2, with a total activity of 1.17 U, and L3, for which no amylolytic activity was observed under the assay conditions (Table 5). The fungus L1 was selected for the remaining experiments.

In experiments performed by Griebeler et al. [23] with 180 isolated strains, activity for all the evaluated enzymes, including amylase, was observed with two of the strains. *Penicillium* sp. and *Paecelomyces* sp. produced the largest quantity of amylolytic enzymes. In studies by Schuber et al. [24], the filamentous fungi selected as good producers of the enzyme *β*-galactosidase were identified as *Penicillium* and *Aspergillus*, and the greatest enzyme production capacity was observed with *Penicillium*. These data corroborate the results obtained in the present study, which highlighted the genus *Penicillium* sp. as a producer of amylolytic enzymes.

3.4 Determination of the submerged culture medium, the salt solution and the cultivation time of the isolated *Penicillium* sp. L1 for amylase production

To optimize the production of amylolytic enzymes by the fungus *Penicillium* sp., different variables were evaluated in the culture of the microorganism. Not all of the media analyzed resulted in a satisfactory induction of the enzyme. A total amylolytic activity of 6.63 U was observed with the LTS medium. The use of the modified Czapeck medium resulted in a total activity of 1.05 U, whereas the use of the modified SR medium did not lead to an induction of amylase production. The study of fungus cultivated in CP medium resulted in an average total activity of 9.79 U, this medium being the standard for fungal growth and enzyme production (Figure 3).

The influence of salts on the cultivation of the fungus was also evaluated, and the greatest amylolytic activity was observed when the microorganism was cultured in submerged CP medium with Wesson salts after seven days of cultivation, yielding an activity of 40.95 total U. This result was followed by that obtained with the medium containing only CP salts after five days of cultivation and medium containing only Wesson salts after seven days of

cultivation (Figure 4).

In the work performed by Almeida [25], the submerged culture media CP, SR and Khanna were tested with the fungus *Rhizopus oryzae* to obtain the greatest production of the enzyme. The author observed that the best result for the production of amylases was obtained with the CP medium. Almeida [26] analyzed the amylolytic activity of different culture media for the fungus *Mucor* sp. and obtained the highest enzymatic activity in the submerged medium CP, data that corroborate the present work.

It is known that several parameters stimulate the production of amylases, such as the type of substrate and physicochemical factors related to the growth of the microorganism [27]. The salts of the culture medium can also have a great influence on the production of amylolytic enzymes. Guimarães et al. [28] analyzed the influence of different compounds on the enzyme activity produced by *A. ochraceus* and found that there was an increase in amylase production in the presence of $MgSO_4.H_2O$. Bhatti et al. [29] observed that the addition of KCl, $MgSO_4.H_2O$ and other compounds led to an increase in the production of the invertase produced by *F. solani*. Their results with salts in the medium corroborate those analyzed in this study because the CP medium contains $MgSO_4$. However, the fungus L1 was induced to produce amylases when the medium was supplemented with Wesson salts, which, according to Corrêa [30], contains copper sulfate heptahydrate, calcium carbonate, tricalcium phosphate, potassium iodide, ferric phosphate, potassium aluminum sulphate, magnesium sulfate, sodium chloride, manganese sulfate, potassium chloride, potassium monophosphate and sodium fluoride, these being important factors for the growth and production of enzymes by the fungus.

The time for growth of the fungus is also a crucial factor during the standardization of enzyme production. Freitas et al. [31] observed a greater amylase production within three days of cultivation. This period was the shortest period for cultivation, and the highest activities were still observed. This result differs from the results presented here, in which the period of seven days was observed to be the best period for amylase production by L1 in CP culture medium containing the salts of the medium plus Wesson salts at 30 ºC, under static conditions.

3.5 Influence of nitrogen source and initial pH on the amylase production by *Penicillium* sp. L1

The next step of this work was to evaluate the influence of different sources of nitrogen and the initial pH of the culture medium on the production of amylase by the fungus L1. Yeast extract was used as control and it was observed to be the best nitrogen source for enzyme

production by this enzyme, which reached a total enzymatic activity of 39.88 U. The second-best source of nitrogen for the production of the enzyme was peptone, for which a total activity of 14.38 U was observed. The third best source of nitrogen was yeast extract plus peptone, the use of which resulted in a total activity of 7.45 U (Table 6).

When the influence of the initial pH of the culture medium on the activity of amylases was evaluated, a considerable production of enzyme by the fungus in the pH range of 4.5 to 7.0 was observed, and the greatest amylolytic activity (46.78 total U) was observed at pH 5.5. This value represents an increase of 65% over the activity observed at pH 5.0 (Figure 5).

It is known that the nitrogen source has a great effect on both the growth of the fungus and its production of enzymes [32, 33]. Facchini [34] mentioned that an excellent growth of the fungus under study was observed using organic sources of nitrogen such as peptone, yeast extract and urea at pH 5.5. As noted by Veron [35], yeast extract proved to be the best source of nitrogen for the production of amylases by the fungus *Talaromyces tratchyspermus* T10-5 at pH 5.0. However, Pasin et al. [36] observed that the use of yeast extract and peptone did not significantly alter the amylolytic activity of the fungus *A. japonicus* when grown at pH 6.0. These results differ from the present study where the use of yeast extract at pH 5.5 induced greater amylolytic activity by the fungus L1 than the use of peptone or peptone plus yeast extract.

3.6 Different carbon sources for amylase production by *Penicillium* sp. (L1)

The carbon sources that yielded the greatest production of amylases when compared to the medium without a carbon source were soluble starch (47.69 total U), oatmeal (32.49 total U) and corn starch (22.97 total U) (Table 7). When Silva [37] evaluated the production of amylase in the presence of different carbon sources, he found that ground corn, oat flour, rice straw and corn starch were the best sources of carbon for inducing amylolytic activity. Peixoto et al. [18] evaluated the production of amylases by *R. microsporus* var. rhizopodiformis in the presence of several sources of carbon, including glucose, maltose, soluble starch, wheat bran and sugarcane bagasse, and the highest yields of amylase were obtained with soluble starch. These results corroborate those of the present study, in which the highest activities were observed when the fungus L1 was grown with soluble starch as a carbon source.

3.7 Effect of pH and temperature on the activity of amylase produced by *Penicillium* sp. L1

There was a significant effect of pH on the activity of amylases at different reaction

temperatures, where a small increase in enzymatic activity was obtained by varying the pH from 4.5 to 7.0 at temperatures that varied from 40 ºC to 50 ºC. However, an optimum activity (62.87 total U) was observed when the temperature was increased from 55 °C to 60 °C, especially at pH 5.5. However, the denaturation of the enzyme was observed at a temperature of 65 °C, as was verified by the lack of enzymatic activity (Figure 6).

Among the biochemical parameters, pH and reaction temperature play a major role in determining optimum enzyme activity. Although the most favorable pH for the development of fungi is between 5 and 7, many enzymes produced can tolerate or even prefer extreme values of pH and temperature. These types of results are very interesting from the point of view of biotechnological and industrial applications because the enzymes have a wide range of action and can be used in processes with different pH and temperatures.

Negi and Banerjee [38] characterized the amylase from *Aspergillus awamori* Nakazawa and reported that the enzyme had an optimum temperature of 70 ºC at pH 4.0, values that disagree with those observed in this work. However, Michelin et al. [39] evaluated the optimum temperature of an amylase produced by *Paecilomyces variotti* and observed that the best reaction temperature was 60 °C. Ayansina et al. [40] found that the optimum temperature for an amylase produced by *Aspergillus niger* was 70 ºC at pH 6.0. Oliveira et al. [41] studied the amylases of *Penicillium* sp., and the optimum activity of these enzymes was observed at 55 ºC and pH 6.5. These data differ from the present study, in which an optimum temperature of 60 ºC at pH 5.5 for *Penicillium* sp. was observed.

3.8 Stability of the amylase produced by *Penicillium* sp. L1 at different temperatures and pHs

Regarding the stability of amylases at different temperatures, the enzyme maintained about 80% of its activity after 60 minutes at temperatures of 55 ºC to 75 ºC, and more than 50% of its activity after 90 minutes at all the temperatures studied, with the exception of 50 ºC (Figure 7). This result is fundamental when seeking the application of the enzyme in industrial processes.

Very interesting results were observed for the stability of the enzyme at different pHs from an industrial point of view. Excellent stability was observed at pH 5.5; the amylase maintained an activity of 60% after 120 minutes.

Gupta et al. [42] mentioned that α-amylases are rarely stable at temperatures above 70 ºC. Michelin et al. [39] evaluated the enzymes produced by *Paecilomyces variotii*, which were very stable at 50 ºC and 55 ºC, as was observed for the fungus L1 in the present study. In addition, Pacheco et al. [43] described a lipase from *Trametes hirsuta* that exhibited great

stability at pH 7.0, values that disagree with those observed in our study. Benassi et al. [44] observed a high stability of the amylase produced by *A. phoenicis* when subjected to pHs that varied from 3.0 to 6.0, results that corroborate the present work. The stability of amylase from L1 at different pHs shows that the enzyme under study can withstand extreme reaction conditions over a long period. Therefore, these results indicate that the enzyme is an exceptional enzyme that might be able to act in several industrial processes, such as the production of food, juices, and textiles.

4. Conclusion

This work is of great scientific interest because of the potential of the fungus *Penicillium* sp. (L1) for the breakdown of starch and formation of reducing sugars that could be fermented when grown in a submerged CP medium containing its own salts or with added Wesson salts, with yeast extract as a source of nitrogen, initial pH of the culture medium at 5.5 and soluble starch as a carbon source. The amylases produced had an expressive range of stability to variations in temperature and pH, as well as favorable values for optimum temperature and pH, important characteristics for the use of these enzymes in several industrial processes.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Table 1. Analysis of macroscopic morphological characteristics from isolated filamentous fungi grown in solid culture medium with oatmeal, at 30ºC.

Fungi	Color	Backgroun(	Texture	Pigmentation
L1	Green with White border	Wrinkled	Powdery	Present
L2	White	Smooth	Velvety	Present
L3	Gray with White and Black	Smooth	Velvety edge	Present

Table 2. Growth rate, in centimeters per hours, of isolated filamentous fungi at different temperatures in Potato-Dextrose-Agar solid culture medium.

Fungi	**Growth rate ($cm.h^{-1}$)**				
	30 ºC	**35 ºC**	**40 ºC**	**45ºC**	**50 ºC**
Penicillium sp. L1	0.038±0.002	0.009±0.000	0.004±0.000	0.002±0.000	0.002 ±0.000
Paecilomyces sp. L2	0.029±0.000	0.007±0.000	0.002±0.000	0.002 ±0.000	0
Mucor sp. L3	0.019±0.002	0.004±0.000	0.002±0.000	0.002 ±0.000	0

Table 3. Growth rate of filamentous fungi after 24 hours at 30 ºC and, subsequently, at 50 ºC for different times in a solid Potato-Dextrose-Agar culture medium.

Fungi	**Growth rate ($cm.h^{-1}$) - 30 ºC for 24 h plus**			
	50 ºC - 24h	**50 ºC - 48h**	**50 ºC - 72h**	**50 ºC - 96h**
Penicillium sp. L1	0.230 ±0.003	0.130 ±0.000	0.100 ±0.001	0.100 ±0.000
Paecilomyces sp. L2	0.400 ±0.001	0.400 ±0.001	0.360 ±0.000	0.330 ±0.000
Mucor sp. L3	0	0	0	0

Table 4. Amylolytic halo of isolated filamentous fungi grown at different temperatures in solid Potato-Dextrose-Agar culture medium.

Fungi	**Enzymatic halo (cm)**				
	30 ºC	**35 ºC**	**40 ºC**	**45 ºC**	**50 ºC**
Penicillium sp. L1	0.860 ±0.004	0.500 ±0.002	0.500 ±0.002	0.500 ±0.002	0.500 ±0.002
Paecilomyces sp. L2	1.380 ±0.002	0.330 ±0.003	0.300 ±0.000	0.300 ±0.000	0.000 ±0.000
Mucor sp. L3	0.060 ±0.002	0.023 ±0.002	0	0	0

Table 5. Screening of amylase producing filamentous fungi.

Fungi	Micelial mass (g)	Final pH of the crude extracellular extract	Amylolitic activity (U total)
Penicillium sp. L1	0.280	5.82	6.800 ±0.011
Paecilomyces sp. L2	1.704	6.46	1.170 ±0.000
Mucor sp. L3	2.809	6.92	0

Table 6. Cultivation of *Penicillium* sp. L1 in the medium containing different nitrogen sources.

Nitrogen source	Micelial mass (g)	Final pH of the crude extracellular extract	Activity (U total)
Yeast extract	0.2649	7.47	45.88 ±0.120
Peptone	0.3440	3.94	14.38 ±0.134
Yeast extract + Peptone	0.2590	4.21	7.45 ±0.080

Table 7. Production of amylases by *Penicillium* sp. L1 in CP medium with Wesson salts and yeast extract as a nitrogen source supplemented with different carbon sources for seven days at 30 °C.

Carbon sources	Amylolitic activity (U total)
No carbon source	0.50 ±0.00
Corn starch	22.97 ±0.00
Fructose	8.87 ±0.00
Glucose	2.32 ±0.00
Lactose	10.94 ±0.00
Maltose	5.70 ±0.00
Oat Quaker®	32.49 ±0.00
Powder Milk	6.51 ±0.00
Starch	47.69 ±0.00
Sucrose	15.94 ±0.00
Sugarcane Straw	0.00

Figure captions

Figure 1. Representative image of the isolated fungi. (A) L1, (B) L2 and (C) L3.

Figure 2. Optical microscopy of isolated filamentous fungi. (A) *Penicillium* sp. L1; (B) *Paecilomyces* sp. L2 and (C) *Mucor* sp. L3.

Figure 3. Determination of the submerged culture medium for growth of the isolated filamentous fungus and amylase production.

Figure 4. Effect of different inorganic salts and the growth time of *Penicillium* sp. L1 for amylase production.

Figure 5. Effect of the initial pH of the CP submerged culture medium on the amylase production by the isolated fungus *Penicillium* sp. L1.

Figure 6. Effect of temperature and pH on the enzymatic reaction.

Figure 7. Stability of amylases produced by the Fungus *Penicillium* sp. L1 at different temperatures.

Figure 1.

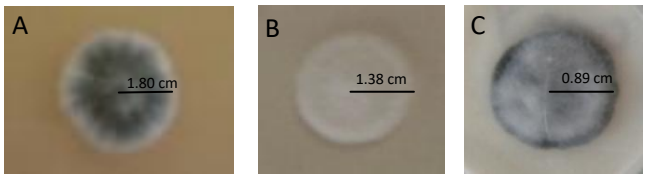
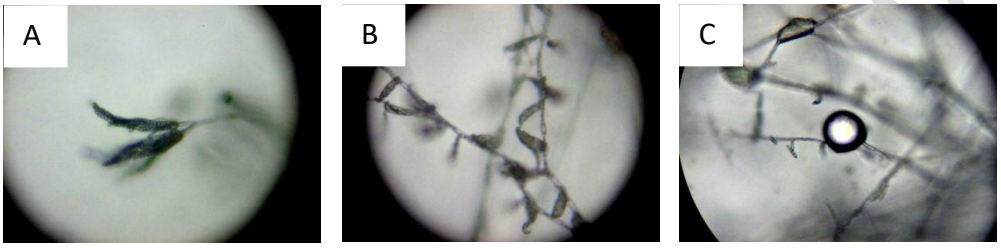


Figure 2.



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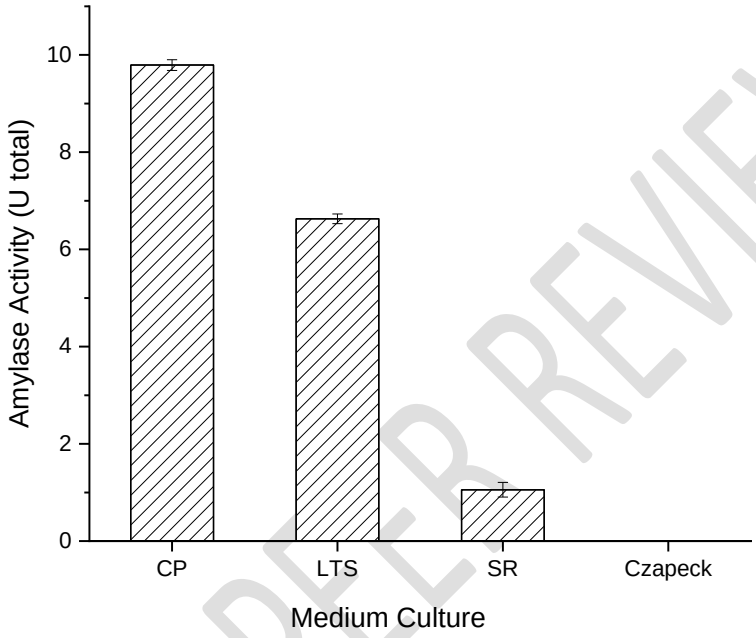
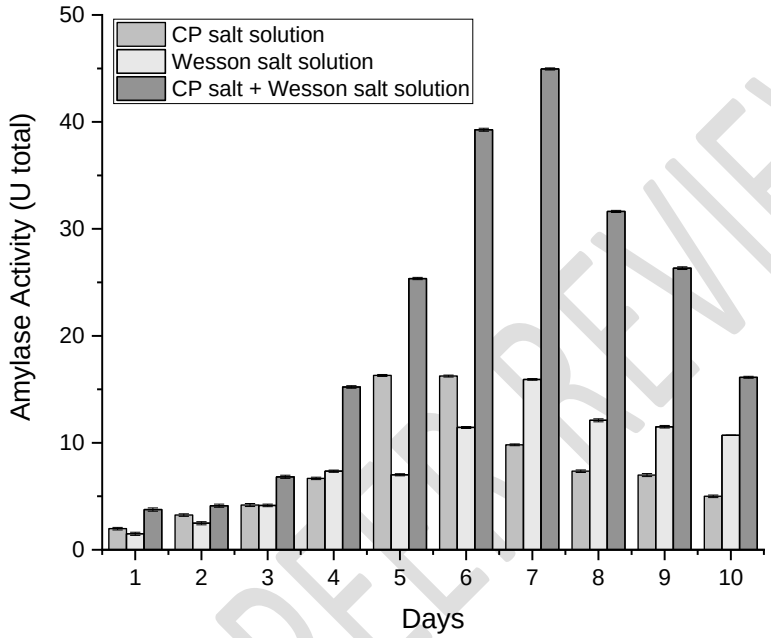


Carvalho-Peixoto (CP) médium
Liliane-Tamires-Silva (LTS) medium
Segato Rizzatti (SR) médium
Czapeck medium

Figure 4.



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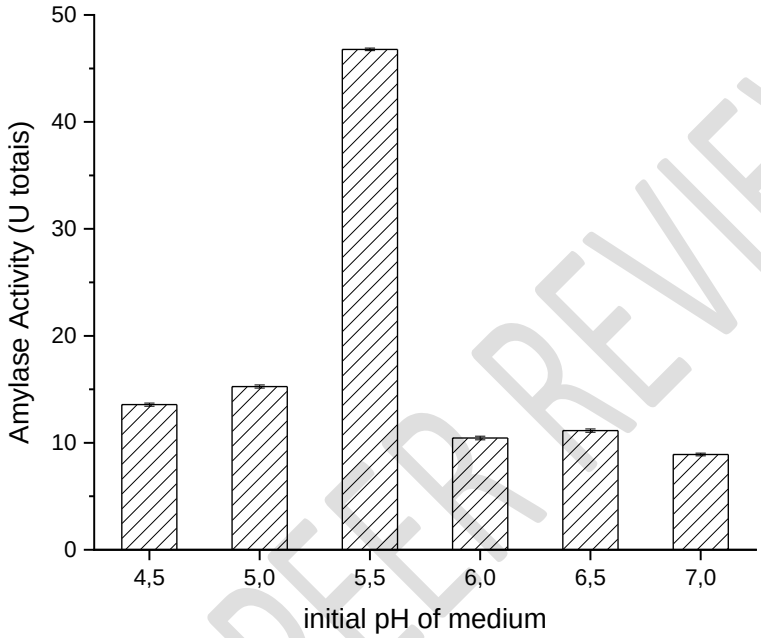

Figure 6.

Figure 7.