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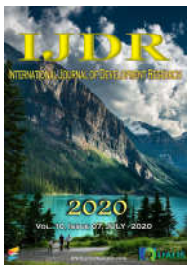

RESEARCH ARTICLE OPEN ACCESS

# PRODUCTION OF THERMOSTABLE FUNGAL AMYLASE AND ITS APPLICATION IN THE DISCOLORATION OF INDIGO CARMINE

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**ABSTRACT**

The indigo carmine dye is widely used within the textile industry, however its high toxicity for bringing several harms to humans and the environment. To minimize the consequences of its use, several methods have been studied, among them the degradation by biological agents has been of great interest. Amylases, which are hydrolytic enzymes commonly used in the textile industry, can be quite effective in the biodegradation of dyes. This study aims to produce amylases with broad stability and ability to discolor of dyes used in the industry. In the present study to optimize the production of amilase complex it was performed a central experimental design compound (23) with three central points and variables pH values, and concentration of starch. After it was characterized according to the effect of pH and temperature on the reaction and amylolytic stability. Thus, it was produced at 30ºC using starch 0.25% as an additive to the culture medium, a thermostable amylase, which hydrolyzes starch in a wide pH range (3.5-7.0) and high temperatures (50º and 60ºC). When analyzing the biodegradation of the indigo carmine dye, it showed high efficiency, decolorizing it about 92% in 48 hours.



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## INTRODUCTION

In recent years, because of strict government legislation and environmental regulation, remediation of dyestuff compounds is a deeply researched topic. The removal of textile dyes from colored industrial effluents is considered as most important for environment protection and sustainability. The most widely used dye in the textile industry is Indigo carmine (Indigo-5, 5′-disulfonic acid disodium salt). Besides using it as a textile dye, it can also be used as an additive in pharmaceuticals for medical diagnosis purposes (Huy*et al.*, 2020). This dye is also used routinely during urology and gynecology, aiming to locate ureteral orifices (Nguyen *et al*., 1998). Due to its high toxicity, indigo carmine can cause tumors, hypertension, disorders of the reproductive and nervous system, these consequences of use are well documented in the literature (Barka*et al.*, 2008). Dyes causes pollution in water bodies such as its colour affects photosynthesis and reduces water transparency, as its structure or tornado refractory and difficult degradation resisting basic treatment methods (Chaves *et al*., 2008; Crema *et al.* , 2019). Besidesaffects gas solubility in water as they interact with water molecule and interfere with the water solubility. The colour removal from industrial effluent is also of high concern as compared to other organic components. These dyes are hard to remove as they tend to disperse and become difficult to eradicate or even reduce levels of dye comparatively in the effluents (Muzaffar *et al.*, 2020).To minimize the damage caused by them, methods such as coagulation - flocculation, electrocoagulation, adsorption, ion exchange, irradiation, ultrasound, membrane filtration, chemical treatment, ozonation, electrochemical destruction and biological treatment are used to remove the dyes from the waters residuals. However, most of these methods are usually quite expensive and consume high amounts of chemicals and/or energy. Biological processes, due to their eco-friendly and cost-competitive benefits, are considered to be an alternative to the traditional physicochemical methods in dye effluents treatment (Wang *et al.*, 2017; Bento *et al.*, 2019).

Decolourization with biological means has gained great attention, and many researchers suggested several biotechnological approaches for combating the textile pollution, the biological degradation routes for recalcitrant and xenobiotic pollutant compound have been intensively researched, researchers have isolated and identified several fungi for dyes decolorization which are *Coriolus versicolor, Mycelia sterilia, Aspergillus fumigates, Phanerochaetechrysosporium Trametes versicolor* and *Aspergillus niger* (Ohmomo *et al.,* 1985, Knapp *et al.,* 1995; Libra *et al.,* 2003; Omar, 2016). Fungal decolourization has been shown to be of great importance, the fungi used for this purpose are capableof producing one or more extracellular, non-specific, non-selective enzymes which can able to degrade a wide range of xenobiotics. Enzymes can be utilized in various steps of textile processing, namely, desizing, scouring, bleaching, and biopolishing of cotton; degumming of silk; bleaching, and shrink-proofing of wool; biostoning (of denim); and for treatment of textile effluents (Choudhury, 2020). In the textile industry amylases are already used during the fabric manufacturing process, participating in the degreasing, and can also participate in the process of bleaching the fabric (Saravanan *et al.*, 2012; Vaidya *et al,* 2015; Saini, Saini e Dahiya, 2017). The International Union of Biochemistry (IUB) establies the categorisation of enzymes into six different classes, based on the mechanism of enzyme action. They are E.C 1 oxidorectases, E.C 2 transferases, E.C 3 hydrolases, E.C. 4 lyases, E.C. 5 isomerases, and E.C 6 ligases.

Amylases are enzymes that hidrolyse the glycosidic linkages in starch, and are thus categorised in the class of E.C 3 hydrolases. Amylases can be categorised into endo- and exo-amylases as well as 3 classes including α-, β-, and γ- amylases, catalysing the hydrolysis of α-1,4 and α-1,6-glycosidic bonds in starch, yielding a variety of disaccharides and monosacchrides (Souza *et al.*, 2010; Silva *et al.*, 2017; Chen*et al.*, 2013; Lim *et al.*, 2020). The α-amylase (α-1,4-glucan-4-glucanohydrolase) can be found in microorganisms, plants and higher organisms. The α-amylase belongs to a family of endo-amylases that catalyses the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1–4) glycosidic bonds. Neither terminal glucose residues nor **α**-1,6-linkages can be cleaved by **α**-amylase (Silva *et al.*, 2017; Chen*et al*., 2013). The end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins, which constitute a mixture of maltose, maltotriose, and branched oligosaccharides of 6–8 glucose units that contain both **α**-1,4 and **α**-1,6 linkages (Van der Maarel*et al*., 2002; Whitcomb, 2007) . Others amylolytic enzymes participate in the process of starch breakdown, but the contribution of α-amylase is the mos important for the initiation of this process. There have been identified three types of amylase including α-Amylase, β – Amylase, and γ – Amylase. Alpha-amylase is an industrial enzyme (EC 3.2.1.1.), which cleaves internal alpha 1-4 glycosidic bands of starch and other polysaccharides to produce several products such as glucose and maltose. It belongs to the family of GH13 (most of them), GH57, GH119, and GH126 and is one of the most widely used commercial enzyme (Chen*et al.*, 2013; Silva *et al*., 2017). They are present in plants, animals, and microorganisms and have extensive applications in medicine, textiles, fermentation, and the food industry (Sudan *et al.*, 2018). Regarding the high rate of proliferation and growth, microorganisms are the primary source of amylases producing a high volume of the enzyme, filamentous fungi are microorganisms secrete large amounts of protein in culture medium, have been used for the industrial production of a wide variety of native products, such as antibiotic , organic acid and commercial enzymes (Saranrajand Stella, 2013; Singh *et al.*, 2014). In this context, aiming to reduce environmental and human health damage, the present study aims to produce amylolytic enzyme extract capable of discoloring the indigo carmine dye, thus minimizing the adverse effects on its industriais use.

## MATERIALS AND METHODS

**Selection of amylase-producing fungi:** Initial selection for the production of amylase for the use of forty-seven filamentous fungi previously inspected by the research group of the Laboratory of Environmental and Industrial Microbiology at the Federal University of Pernambuco. The fungi were seeded in petri dishes containing Sabouraud agar (SAB) and incubated at 30ºC for a period of 5 days. Then, the stationary submerged fermentation was carried out in 125ml capacity Erlenmeyer flasks containing 30 ml of modified Czapek medium with addition of 1% (w/v) starch. Three blocks of agar obtained from the culture previously performed in SAB were added to the bottles and incubated for 48h at 30ºC, in stationary condition. After this period, the fungal biomass and the liquid containing the enzyme of interest were separated using centrifuge (10,000 rpm for 7min).

**Determination of amylase activity:** Amylase quantification was determined according to Caraway (1959) modified. Thus, 60µL of enzymatic extract was added to 100 µL of solution 1 (0.4 g / L starch in 50mM sodium phosphate buffer pH 7.0), after incubated at 40ºC for a period of 30min. Then 100 µL of Solution 2 (potassium iodate 16.7 mmol / L; potassium iodide 271 mmol / L; hydrochloric acid 112 mmol / L) was added, after 5 minutes a reading on a spectrophotometer was performed using the 660nm wavelength. The negative control performed by replacing the enzymatic extract with deionized distilled water. For negative control, water was used to replace the test sample / enzymatic extract.

Amylase activity (AA) was calculated according to the following formula:

$$AA = ((Ac-At) / Ac) * 40$$

Ac: Absorbance of negative control
At: Absorbance of the test sample

**Determination of total proteins:** The determination of total proteins was carried out as described by Bradford (1976).

**Production Optimization**

**Effect of the incubation period:** The kinetics of enzymatic production was carried out in stationary submerged fermentation with the two fungi that showed greater amylase activity in the initial selection described above. These microorganisms were seeded in petri dishes containing sabouraud agar and incubated for a period of 7 days at 30ºC. Then, 15 blocks of agar obtained from this plate were added to the sabouraud broth supplemented with 1% (w/v) starch, which was incubated under horizontal agitation (130rpm) for 48 hours, a procedure called pre-inoculum. To evaluate the best enzyme production time, 3 mL of the pre-inoculum to

30mL of modified Czapek medium added 1%(w/v) starch, and incubated at 30ºC in stationary condition, a test sample is taken each and amylase quantification is performed every 24h.

**Experimental planning:** The following steps to optimize amylase production were carried out with the fungus that demonstrated better production capacity in less time.Thesefact observed during the analysis of the effect of the incubation period, the data obtained in it were used in the evaluations following. The experimental design was based on Statistica 7.0 (StatSoft Co., USA), assays were carried out according to a factorial experimental design $2^3$ with three central point ($2^n$ where $n$ is the number of independent variables), totalizing 11 experiments. The variables studied were initial pH, temperature and astarch concentration submitted for the analysis in the design, experiments are given in Table 1.

**Table 1. Experimental conditions for experimental planning**

| Variable | Parameter | CodedLevel | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| $X_1$ | pH | 4.2 | 6.0 | 7.8 |
| $X_2$ | Temperature, °C | 25 | 30 | 35 |
| $X_3$ | Starch concentration % (w/v) | 1.1 | 2.0 | 2.9 |

All statistical analysis were performed by the software Statistica 7.0 for factorial design analysis assuming $p < 0.05$ (95% of significance), considering the variables and their linear interactions.

**Individual evaluation of the influence of incubation temperature and substrate concentration on the production of fungal amylase:** To evaluate the effect of the incubation temperature during the production of amylase, sowing, pre-inoculum and inoculum were performed as described in the item effect of the incubation period. The data obtained after realizing this same item were used to define the time that the fermentation remained in incubation. The test temperature range used was 25-40ºC, with a variation of 5ºC. As for the effect of varying the substrate concentration, modified Czapek medium was used, to which soluble starch was added in different concentrations (0.25%, 0.5%, 1%, 1.5%, 2.0%).

**Enzymaticcharacterization:** In the enzymatic characterization, 5 parameters were evaluated: optimal H, optimal temperature and reaction substrate, thermal stability and at stability at different pHs. To perform it, an enzymatic extract produced using data evidenced in the production optimization process was used.

**Effect of pH and temperature on the enzymatic reaction:** To evaluate the effect of pH on the reaction, the reaction substrates were prepared using 1% (w/v) starch solutions at different pHs within the range 3.5-8.0. For this preparation, sodium acetate(pH 3.5-5.5) and sodium phosphate(pH 6.0-8.0) buffers were used in a concentration of 50mM. After obtaining this data, tests were carried out to analyze the best temperature at which the amylase under study would react with the substrate. For this analysis, the enzymatic extract was added to a 1% (w/v) starch solution prepared at the pH that stood out the most in the previous analysis. In sequence, the mixture was subjected to different temperatures in the range 30-80ºC for 30 min. Finally, the following steps of the protocol already described in determining amylase activity were performed.After obtaining the best reaction parameters, these were used for amylase quantification in the dosages following.

**Thermal stability and stability at different pHs:** To analyze the stability of the enzyme when exposed to different pHs, it was put in contact with McIlvaine buffer solutions with pH ranging from 0.5 in a range from 4 to 8, in the ratio 1: 1 (v: v), for a period after 24h, then this mixture was used as an enzyme extract during the quantification of amylase. As for the thermal stability analysis, the enzymatic extract was exposed to 35ºC, 50ºC and 60ºC, aliquots being removed at the following times 5, 10, 20, 30 and 60 min and after these they went through the amylase quantification process.

**Discoloration of indigo carmine:** The amylase-producing fungus was sown on a plate containing saboraud agar medium and incubated at 30ºC for 7 days. Like this 10 blocks of agar obtained from this culture were inoculated at 100mL of Saboraud broth supplemented with 1% starch, which was kept at 30ºC under horizontal stirring of 130rpm. After, 40mL of this was added to 400 mL modified Czapek medium with addition of starch using the parameters obtained in the production optimization item. After incubation, the fermentate was centrifuged at 10,000 rpm for 6 minutes, then the supernatant was separated from the fungal biomass, to then be tested for its dye discoloration capacity. For this evaluation of the discoloration capacity of indigo carmine, the biomass was resuspended with sterile distilled water in the proportion 1: 1 (w:v) and fractionated (6 x 30mL), the same fractionation was carried out with the supernatant containing the amylase under study. To each fraction, of resuspended biomass or supernatant, 30ml indigo carmine solution (25ppm) was added. Then the vials containing the samples were kept at room temperature and aliquots removed after 24h and 48h. These were centrifuged at 10,000 rpm for 8 minutes and thus read on the supernatant spectrophotometer. For this, a range of 200 to 900nm was used, with a variation of 5nm between each reading. The negative control was prepared to measure the absorbance peak of the dye, in which 30 ml of sterile deionized distilled water was added to the dye and kept under the same experimental conditions mentioned above. Subsequently, the absorbances were read in the same range as the test sample.
The discoloration index was calculated according to the formula below:

% discoloration = (A control - A test) / A control x 100

A control = Absorbance of negative control
A test = Absorbance of the test sample

Note: For the calculation, the value obtained at the wavelength that represents the absorbance peak of the dye was used.

## RESULTS AND DISCUSSION

**Selection of amylase-producing fungi:** Initial selection of amylase-producing fungiInitial selection of amylase-producing fungiInitial selection of amylase-producing fungiInitial selection of amylase-producing fungi Amylases are significant enzymes employed in the starch processing industries for the hydrolysis of polysaccharides. Microbial amylases meet industrial demands; a large number of them are available Amylases are one of the essential groups of enzymes used in a wide range of industries and represent almost 25% of the total

sale of enzymes. Used in various sectors, such as food, detergent, paper and textiles, this enzyme is used to remove starch-based stains in the detergent industry and in the food industry, its use of amylase is liquefaction and saccharification manufacture of corn syrups. Amylolytic enzyme activity is present in several microorganisms. In the present study of the forty-seven filamentous fungi studied, 34% showed the production of amylases, with fungi 75 and 48 presenting the highest amylolytic activities with values corresponding to 39.53 ± 0.43 U / dL and 39.15 ± 0.10 U / dL respectively and with specific activity values corresponding to 5.77 ± 0.006 U / mg protein for fungus 75 and 2.49 ± 0.03 U / mg protein for fungus 48, these data are present in Figure 1. Griebeler *et al*. (2015) working with 180 fungi detected a percentage of 12.2% amylase producers. Souza *et al*. (2008) showed higher rates, 40% of the 60 fungi studied, showed production capacity. Pasin *et al*. (2020) and Khokhar *et al*. (2011) found that all strains were able to use starch as a carbon source.

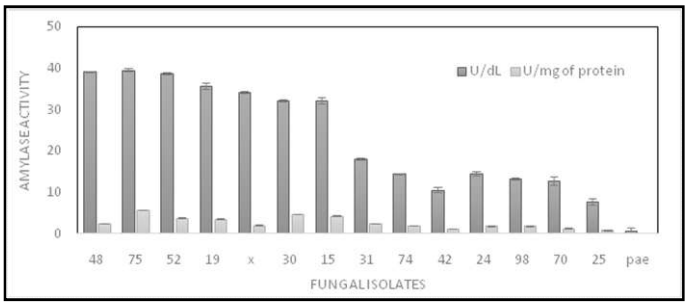


**Figure 1. Determination of the extracellular activities em U/dL and specific activity (U/mg of protein) of amylolytic fungi**

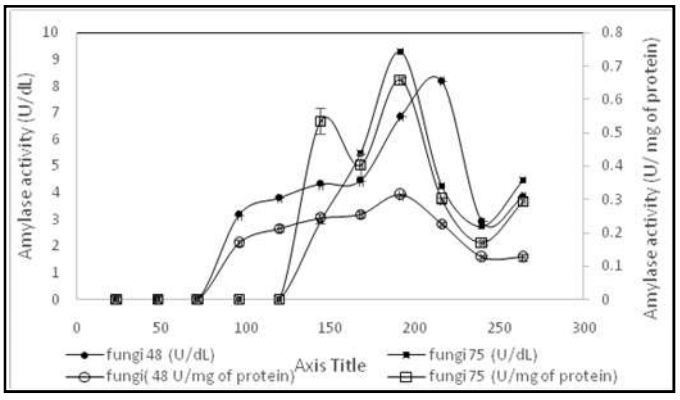


**Figure 2. Time course of extracelluar amylase production by fungi 48 and fungi 75. The fungus was incubated in cazapek medium modified liquid médium with 1% starch as carbon source at 30°C and pH 7.0.**

**Production Optimization:** The study of amylase production in U / dL and in specific activity in relation to the fermentation time for the two fungal strains (48 and 75) selected as the best producers in the previous stage of the referred work is illustrated in Figure 2. Under conditions of stationary fermentation, initial pH 7.0 and temperature of 30 ° C, the fungal strain 48 showed a peak of amylase production with 8.19 ± 0.01 U / dL in 216 hours of fermentation and a peak of specific activity of values corresponding to 0.34 ± 0.01 U / mg of protein with 192 hours of fermentation. However, the fungal strain 75 stood out, presenting greater amylolytic production with values corresponding to 9.29 ± 0.13 U / dL and 0.65 ± 0.01 U / mg of protein with 192 hours of fermentation. Adejuwon (2011) evaluating the ability of Penicillium species isolated from apple to produce dextrinizing amylase, observed that it showed its highest production after 9 days (216h) of incubation at 25ºC. Marques and collaborators (2018), using the fungus Trichoderma harzianum to produce α-amylase, obtained, even in less incubation time (48h), a peak of specific amylase production (0.41 U / mg protein) significantly lower than the present study. However, when compared to the present study, it presented relatively less specific activity (63%). Pasin *et al*. (2020) studying the production of amylases by Aspergillus carbonarius in Khan liquid medium with 1% starch as a carbon source at 30 ° C and pH 6.0, detected a peak production with 96 hours of cultivation.

**Experimental planning:** As mentioned, the investigation of the substrate concentration, temperature and initial pH of production of the amilotitic complex excreted by the fungus 75, and the relationship between these variables were validated by a factorial design and the complete experimental matrix is present in Table 2, together with yield of amylase in U / mL.According to the experimental conditions, the highest enzyme value (0.217 U / mL) was detected at pH 4.2, 35ºC and a concentration of 1.1% starch (m / v) for 192 h of incubation whereas in experiments 2 and 6 there was no it was possible to detect amilotitic activity. From the experimental results with the enzymatic dosages, effects of the three factors (pH, temperature and concentration of the inducing substrate) were also analyzed in the Statistica 7.0 program through a $2^3$ experimental design and were submitted to ANOVA (Table 3) and analysis of regression. Analyzing the Pareto graph shown in figure 3, it was possible to detect that only the temperature significantly interfered in the production, the other parameters and the associations between the variables did not show any significant influence during the production of amylases excreted by the fungus 75 under the conditions in that experiment. In this way it was possible to propose a model for enzymatic production, in its reduced form (Equation 1).

$$\hat{y} = 0.070 - 0.039b_1 + 0.062b_2 - 0.021b_3 + 0.010b_1b_2 - 0.0163b_1b_2 - 0.007b_1b_3 - 0.014b_2b_3 \quad (1)$$

The analysis of variance (ANOVA) revealed that the regression was significant at the 95% confidence level, in the tests performed the equation of the line showing an adjustment with correlation coefficient (R-sqr) of = 0.8691 and MS Residual = 0.0019222.The data obtained in this work corroborate those of Freitas (2014), when studying amylases of filamentous fungus S. racemosum, detected that the modification of the initial pH of the fermentation did not provide a significant difference in the production of the enzyme. Akassou and Groleau (2018) evaluating the production of α-amylase by T. thermophilus through experimental design found that the substrate concentration and temperature significantly influenced. In addition to the authors already mentioned and the study exposed here, others have demonstrated that temperature is a variable of great importance and has a significant influence on the production of amylase. Among these authors is Almeida and his collaborators (2017) presented 30ºC as the best temperature for the production of amylase by filamentous fungus, when there was an increase in this variable, there was a decay of amylase activity.

**Evaluation of the Influence of Incubation Temperature and substrate concentration in the production of fungal amylase:** After carrying out the experimental planning where it was observed that the temperature is the only variable studied that significantly influences the enzymatic production,

**Table 3. Analysis of variance (ANOVA) for the amylase production by fungi 75**

| Factor | Effect | Std.Err. | t(4) | P | -95,% Cnf.Limt | +95,% Cnf.Limt | Coeff. | Std.Err. Coeff. |
|---|---|---|---|---|---|---|---|---|
| Mean/Interc. | 0.0707 | 0.0131 | 5.3655 | 0.0058 | 0.0341 | 0.1073 | 0.0707 | 0.0132 |
| pH | -0.0782 | 0.0309 | -2.5312 | 0.0645 | -0.1640 | 0.0076 | -0.0391 | 0.0154 |
| temperature | 0.1247 | 0.0309 | 4.0353 | 0.0156 | 0.0389 | 0.2106 | 0.0623 | 0.0154 |
| starch | -0.0417 | 0.0309 | -1.3505 | 0.2482 | -0.1275 | 0.0440 | -0.0208 | 0.0154 |
| 1 by 2 | -0.0327 | 0.0309 | -1.0593 | 0.3491 | -0.1185 | 0.0530 | -0.0163 | 0.0154 |
| 1 by 3 | -0.0142 | 0.0309 | -0.4609 | 0.6687 | -0,1000 | 0.0715 | -0.0071 | 0.0154 |
| 2 by 3 | -0.0288 | 0.0309 | -0.9138 | 0.4125 | -0.1141 | 0.0576 | -0.0141 | 0.0154 |

it was then performed the evaluation of this factor in a univariable way. This step aimed to find the optimal production temperature of the amylolytic enzyme excreted by the fungus 75 at pH 7.0 and 1% starch without stirring. The results obtained corroborate with the analysis of the previous item showing that the temperature exerts a great influence of this on the enzymatic production, this occurring mainly at 30ºC. Detecting amylase activity of 29.43 ± 0.37 U / dL, with a specific activity of 2.89 ± 0.03 U / mg of protein (Figure 4). The maximum production of α-amylase by filamentous fungus at this temperature was also described by Erdal and Taskin (2010) where the fungus Penicillium expansum MT-1 was used to generate this enzyme through solid state fermentation, which was also shown in this study to decay this when decreasing or increasing the temperature by 5ºCObafemi *et al*. (2018) when studying an enzyme produced by Aspergillus niger at 25ºC showed amylase activity similar to that of this study, however when the specific activity was analyzed, it was shown to be about 3 times lower. Sakthi *et al* (2012) studying the production of alpha amylase by Aspergillus niger using cassava as a substrate showed better production when the fungus was incubated at 40ºC, for a period of 72h. Observing the need to evaluate the minimum amount of substrate necessary to produce amylase without influencing the final result of production, a study of the influence of the substrate concentration on the production of the enzyme was carried out unilaterally.

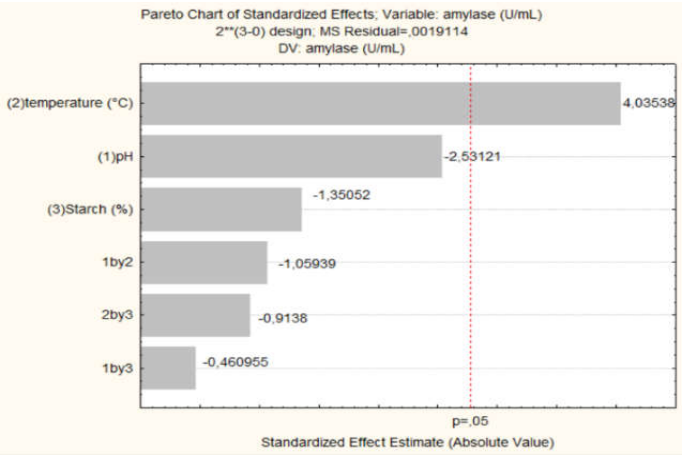


**Figure 3. Pareto chart showing the significance of variables pH (1), temperature (2) and concentration of the starch % (w/v) (3) in the amylase production by fungi 75**

It was then found that the fungus produced a greater amount of enzyme when subjected to low concentrations of substrate in the medium (0.25%), 36.63 ± 0.88 U / dL, as well as when the evaluation of specific amylase activity (2.86 ± 0.06 U / mg of protein), this being the maximum amylase activity obtained when finalizing the production optimization (Figure 5). Pereira and collaborators (2017) evaluated the production of amylase by *Aspergillus niger* obtaining greater production when the concentration of substrate in the medium was 2%, this more than eight times higher than that presented in this work. The study published in 2016 by Cunha and his collaborators showed a specific activity value significantly lower than that presented in figure 5, approximately twenty times. The greatest production occurred when or authors used culture medium composed of 70% substrate (residues from the soybean harvest), thus obtaining specific amylase activity 0.14 U / mg of protein.Wandeley *et al*. (2004), demonstrates that the maximum amylase activity of its crude enzymatic extract produced by C. flavus was 0.25 U / mL, which is below that shown as a product of the fungus studied in this work (0.36 U/mL).

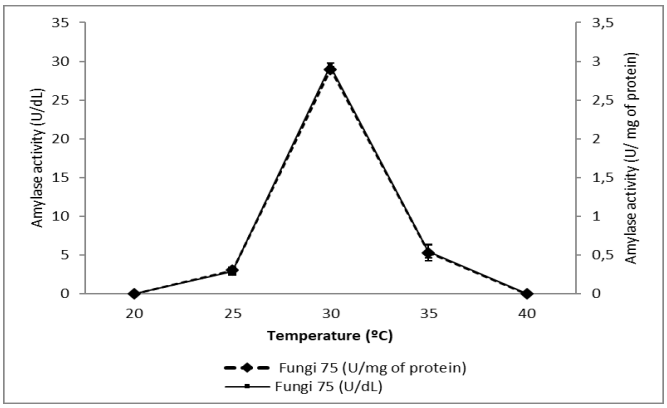


**Figure 4. Effect of initial pH on amylase production by Fungi 75**

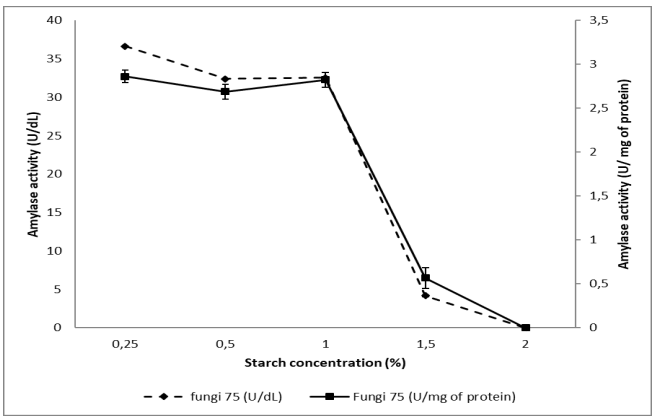


**Figure 5. Effect of different starch concentrations for amylase production by Fungi 75**

**Enzyme Characterization:** The amylase excreted by filamentous fungi 75 was cultivated at the optimum conditions of production defined in the previous steps of this study and partially characterized, showing a maximum activity at the optimum pH of 5.5 (figure6a). Optimal activity was observed at 50 °C, the former presented more than 85% of the activity

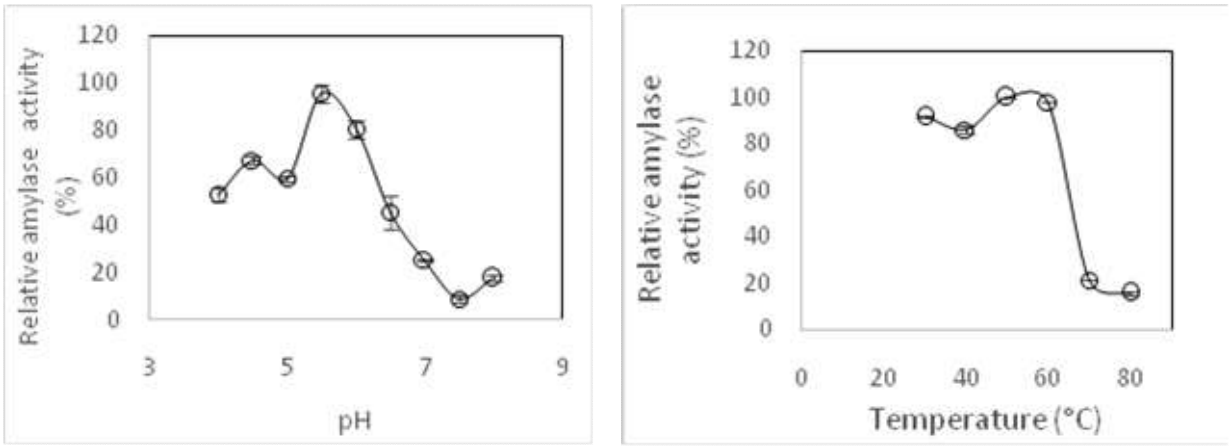


**Figure 6. Influence of pH (a) and temperature (b) on the activity of amylase from filamentous fungi 75**

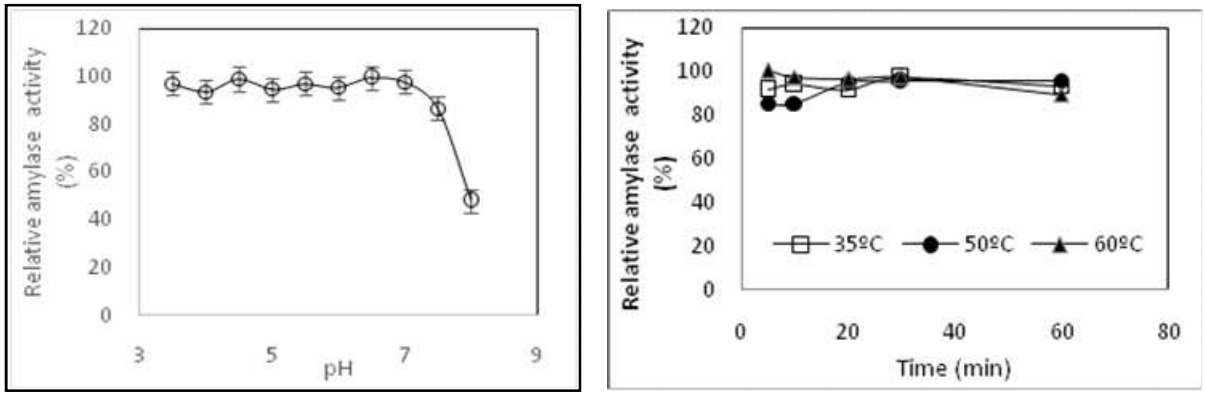


**Figure 7. Stability on different pH (a) and thermal stability (b) of amylase from filamentous fungi 75**

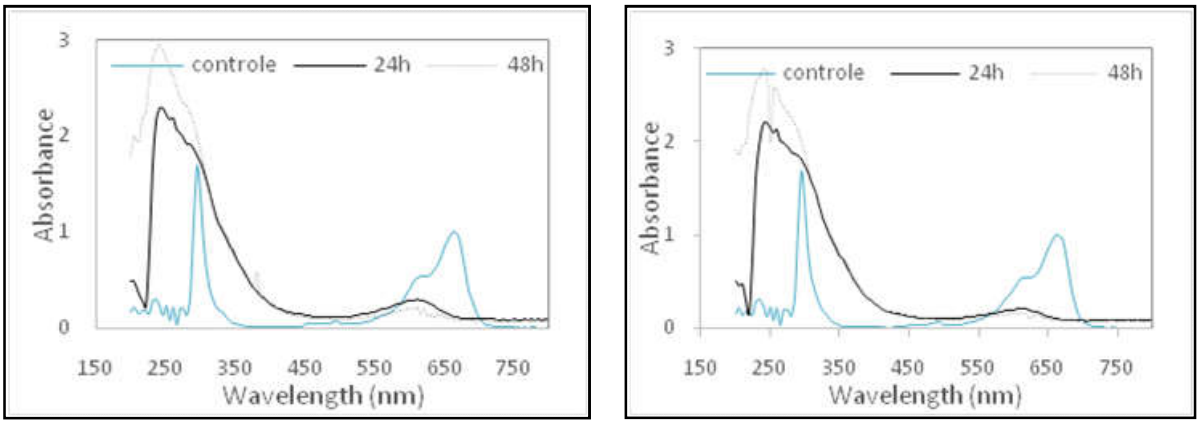


**Figure 8. Spectrophotometer scan of indigo Carmine and its treatments. (a) Treatment with fungal biomass (b) treatment with supernatant containing studied enzyme**

between 30-60 °C (figure 6b). In this study, the amylase from the filamentous fungi 75 was highly stable between pH 3.5-7.5 (figure 7a) and presented more than 80% of the activity at 35, 50 and 60 °Cfor up to 60 minutes (Figure 7b). Ferreira and collaborators (2014) demonstrated similar results, where crude enzymatic extract produced by Rhizopus orizae maintained similar activity in the pH 3-6 range, showing a decrease from pH 6.5. Bernardes *et al*. (2014), when characterizing amylase produced by Rhizomucormiehei, reported that the same reacts better when at pH 5.0, it declines substantially when at pH 5.5. This proved to be more stable at acid pH (4.5), being considered stable over a wide range of pH 3.5-6.0. Corroborating this study, Bhanja Dey and Banerjee (2015) shows that the α-amylase produced by Aspergillus oryzae is stable when exposed to pH 5.5, but when it is subjected to pHs that are more alkaline or more acidic than this, it if unstable.. Nwagu and Okolo (2011) studying the amylase production by Aspergillus fumigatus observed that the fungus showed amylase production at 60ºC, corroborating this work. As well as stability at 35º, 50º and 60ºC for a period of 60 min.Mitidieri *et al*. (2006) showed better reaction temperatures of 50-55ºC for amylase produced by A. niger, maintaining its amylolytic activity above 60% when subjected to 53ºC for a period of 60min. AtolagbeOluwabunmi *et al*. (2019) showed that amylase activity of the enzyme excreted by a strain of Aspergilluniger was extremely dependent on two variables. The first was temperature, where amylase presents its best activity at 30ºC, thus corroborating this work. The second variable that significantly influenced the enzymatic activity

was the reaction pH, where the enzyme d did not show the stability in acidic pHs exposed in the present study, but still it was more effective in acidic pH.

**Discoloration of indigo carmine:** The scanning spectrum from 200 to 900 nm obtained in the decolorization tests of the indigo carmine dye (25ppm), treated with 15 g of biomass or 50% of the amylolytic complex excreted by the optimized filamentous fungus F75 and characterized as described in the previous steps of that work , and incubated for a period of 24h and 48h at 25 ° C, as well as the negative control of the experiment are shown in figure 8, in which it is possible to detect that at the wavelength 610 nm a high rate of discoloration using both treatments.Using the decolorization treatment with fungal biomass, he obtained 88.61% of discoloration after 24h of treatment and 91.73% after 48h of treatment. In the second treatment, using the amylolytic complex, he detected a discoloration value corresponding to 90.07% after 24h of exposure, an index that increased to 91.54% after 48h of treatment, a result similar to that found using filamentous fungus F75 biomass as bleaching agent, showing no significant difference. Almeida *et al*. (2012), when studying the degradation of the indigo carmine dye using a fungus of the genus Penicillium, showed a 60% dye degradation index obtained after 5 days of treatment. Almeida, Miranda and Gomes (2018) evaluating the degradation process of the same dye by a fungus also of the genus Penicilium obtained 58.12% of discoloration with 48h of treatment and only after 120h indexes above 70%. The two studies cited used fungal biomass as a decolorizing agent, however they presented degradation below that observed in the present study when using only the supernatant containing the studied enzyme. However Silva and collaborators (2015) studied the discoloration process of the Indigo Carmine dye present in textile effluent under the influence of ammoniacal nitrogen with reactors, using the fungus Aspergillus niger AN 400, these obtained better discoloration rates when the external source of nitrogen favored the production of enzymes by the fungi and thus presented a maximum discoloration of 97%, in contrast, a high study time (86 days) was observed. By analyzing the data exposed here, it was possible to observe the high efficiency of the bleaching agents tested, with a discoloration rate above 85% in the first 24h of treatment and 90% after 48h. Thus, both fungal biomass and amylolytic extract become interesting for the discoloration of indigo carmine not only because it is a bioprocess and considered "eco-friendly" but also because of its efficiency in a short period of time.

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