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α-amylase production obtained from *Aspergillus niger* ATCC 1004 by solid state fermentation using *Croton linearifolius* residues as substrate

Produção de α-amilase obtida de *Aspergillus niger* ATCC 1004 por fermentação em estado sólido utilizando resíduos de *Croton linearifolius* como substrato

Producción de α-amilasa obtenida de *Aspergillus niger* ATCC 1004 mediante fermentación em estado sólido utilizando resíduos de *Croton linearifolius* como sustrato

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Abstract
The objective of this work was to optimize the production and characterize α-amylase produced by *Aspergillus niger* through solid state fermentation, using leaf residues of *C. linearifolius* as substrate. For optimization, the incubation temperature, initial humidity and fermentation time were combined based on Doehlert experimental design. The highest productivity of the enzyme was 122.88 Ug^{-1}, at 33ºC, 70% humidity and 14 days of time. In the enzymatic characterization, the enzyme extract presented pH 5.0 and temperature 50ºC, and α-amylase was thermostable up to 60ºC, maintaining more than 90% of the activity. In evaluation of the effect of salt addition, sodium carbonate, calcium chloride, iron chloride, and cobalt chloride increased the enzymatic activity of α-amylase, while potassium and sodium from their chlorides served as enzyme inhibitors. The K_m and V_{max} values found were 0.04 mg/mL and 46.95 µmol/min/mL, respectively, indicating that the substrate has affinity for α-amylase. Therefore, the results demonstrate that the residues of *C. linearifolius* can be used as a substrate for *A. niger* in the production of enzymatic extracts, such as α-amylase.
Keywords: Bioprocesses; Enzyme kinetics; Optimization.

Resumo

O objetivo desse trabalho foi otimizar a produção e caracterizar a α-amilase produzida por *Aspergillus niger* através de fermentação em estado sólido, utilizando resíduos das folhas de *C.linearifolius* como substrato. Para otimização, a temperatura de incubação, umidade inicial e tempo de fermentação foram combinados com base no planejamento experimental Doehlert. A maior produtividade da enzima foi 122.88 Ug^{-1}, a 33ºC, 70% de umidade e 14 dias de tempo. Na caracterização enzimática, o extrato enzimático apresentou pH 5 e temperatura 50°C, e a α-amilase foi termoestável até 60°C, mantendo mais de 90% da atividade. Na avaliação do efeito de adição de sal, carbonato de sódio, cloreto de cálcio, cloreto de ferro, e cloreto de cobalto aumentaram a atividade da α – amilase, enquanto o potássio e o sódio de seus cloretos serviram como inibidores enzimáticos. Os valores de K_m e $V_{máx}$ encontrados foram 0.04 mg/mL e 46.95 µmol/min/mL, respectivamente, indicando que o substrato possui afinidade para α - amilase. Portanto, os resultados demonstram que os resíduos de *C.linearifolius* podem ser utilizados como substrato para *A.niger* na produção de extratos enzimáticos, como α – amilase.
Palavras-chave: Bioprocessos; Cinética enzimática; Otimização.

Resumen

El objetivo de este trabajo fue optimizar la producción y caracterizar la α-amilasa producida por *Aspergillus niger* mediante fermentación en estado sólido, utilizando residuos de las hojas de *C.linearifolius* como sustrato. Para la optimización, la temperatura de incubación, la humedad inicial y el tiempo de fermentación se combinaron según el diseño experimental de Doehlert. La mayor productividad enzimática fue de 122,88 Ug^{-1}, a 33ºC, 70% de humedad y 14 días de tiempo. En la caracterización enzimática, el extracto enzimático tuvo un pH de 5 y una temperatura de 50° C, y la α-amilasa fue termoestable hasta 60 ° C, manteniendo más de 90% de la actividad. Al evaluar el efecto de la adición de sal, el carbonato de sodio, el cloruro de calcio, el cloruro de hierro y el cloruro de cobalto aumentaron la actividad de la α-amilasa, mientras que el potasio y el sodio de sus cloruros sirvieron como inhibidores enzimáticos. Los valores de K_{my} V_{max} encontrados fueron 0.04 mg / mL y 46.95 µmol/min/mL, respectivamente, lo que indica que el sustrato tiene afinidad por la α-amilasa. Por lo tanto, los resultados demuestran que los residuos de *C.linearifolius* pueden usarse como sustrato para *A. niger* en la producción de extractos enzimáticos, como la α-amilasa.
Palabras clave: Bioprocesos; Cinética Enzimática; Optimización.

1. Introduction

The *Croton* genus is a native and non-endemic plant found in Brazil, with confirmed occurrence in the north, northeast, central-west, southeast, and south regions, represented by approximately 350 species (Cordeiro et al., 2015). The *Croton linearifolius* species, popularly known as "pepper canopy", has a larvicidal potential against the *Aedes aegypti* mosquito (Silva et al., 2014). Despite the importance at an ecological level and the potential of the species, studies on *C. linearifolius* are restricted (Silva et al., 2018), mainly for publications that indicate an application for plant residue, after extraction of essential oil.

Despite being an experimental route, the reuse of residues in fermentation processes with emphasis on agro-industrial residues has been an option for its application at an industrial level, aimed at producing a natural substance, reducing production costs, providing a final destination for this type of substance that until then is only discarded in the environment, and adding value to the devalued raw material (Panesar, Kaur & Panesar, 2015).

Agro-industrial residues have a significant amount of nutrients and bioactive compounds in their composition, they can be a source of carbohydrates, minerals, and proteins, making them an option for the growth of microorganisms, through fermentative processes (Sadh, Duhan & Duhan, 2018).

In this context, there are two main types of fermentation processes: solid state fermentation (SSF) and submerged fermentation (SF). SSF is a technique in which the growth of microorganisms takes place inside moist porous particles, and the liquid contained in the solid matrix must be kept in water activity values, which ensures cell growth and metabolism; not exceeding the maximum water holding capacity in the matrix (Pandey, 2003). SF is a technique in which excess water is present in the culture medium, making it more homogeneous (González et al., 2002). In this type of process, a microorganism is introduced into the liquid medium as an inoculum, remaining in fermenters with agitation and aeration, pH correction, and controlled temperature (Reguly, 2000).

Regarding the production of enzymes, according to Ravindran et al. (2018), SSF is higher than SF, as it is more similar to the natural conditions for the growth of fungi, and there is a higher yield regarding the production of enzymes. We need to emphasize that the production and yield of enzymes can also be influenced by the temperature of the culture medium, source of carbon and nitrogen (Shruthi, Achur & Boramuthi, 2020).

The filamentous fungus *Aspergillus niger* is part of a group of microorganisms that has biotechnological applications, especially as an enzyme producer (Reilly et al., 2018). Among the enzymes produced by the *Aspergillus* genus, there is α-amylase. The α-amylase is capable of breaking glycosidic bonds (α1-4) between glucose units, promoting hydrolysis of glycogen and starch (Nelson & Cox, 2014). Thus, it can be applied in different sectors, such as the pharmaceutical, textile, and food industries, such as in the manufacture of beer, in which it is necessary to promote the hydrolysis of starch (Onofre et al., 2016).

Thus, this work aimed at the production and characterization of the α-amylase enzyme through SSF by *A. niger*, using leaf residues of *C. linearifolius* from essential oil extraction as a substrate.

2. Methodology

2.1 Substrate for Fermentation

Leaves of *C. linearifolius* were collected in the National Forest of Contendas do Sincorá (FLONA), municipality of Contendas do Sincorá, Bahia, Brazil. Exsiccates were deposited in the herbarium of the Feira de Santana State University (UEFS), Bahia, under the registration HUEFS 146620.

2.2 Extraction of Essential Oils

The plant material was dried in an air circulation oven (model SL 101 SOLAB) at a temperature of 40ºC for a period of 12 hours. Then, the leaves were manually shredded and subjected to extraction of essential oil by the hydrodistillation technique, using a Clevenger extractor (model SL 76 SOLAB) at the Natural Products Research Laboratory - LAPRON, State University of Southwest Bahia - UESB.

After essential oil extraction, leaf residues were dried in an air circulation oven (model SL 101 SOLAB) at a temperature of 40°C for 12 hours and ground through a Wiley type knife mill (ACB LABOR) to a particle size of approximately 2 mm.

2.3 Obtaining the Microorganism

The fungus used in this study was *A. niger* ATCC 1004, obtained from the collection of microorganisms from the National Institute for Health Control and Quality (INCQS) 40018, Lot 068840018, provided by the Oswaldo Cruz Foundation (Fiocruz, Manguinhos, Rj, Brazil). The collection is kept in the Laboratory of Reuse of Agroindustrial Waste (LABRA) and preserved in silica.

2.4 Preparation of Inoculum for Solid State Fermentation

The microorganism *A. niger* was inoculated into a 250 ml conical flask at 30°C in HIMEDIA PDA (Potato Dextrose Agar) culture medium. After the incubation period, the spores were recovered in sterile distilled water solution containing 0.01% Tween 80 solution (VETEC) previously sterilized in a vertical autoclave (Prismatec) at 121ºC for 15 minutes. The suspension was collected in a flask and a 0.1 mL aliquot was removed and diluted in an assay tube for spore counting in a Neubauer chamber, observed under a binocular microscope (BIOVAL). The spore concentration used as inoculum was 10^7 spores/gram (Santos et al., 2011).

2.5 Solid State Fermentation

2.5.1 Fermentation Profile

Fermentation was carried out according Freitas et al. (2017) in a 250 mL flask containing 5g of substrate, previously sterilized in a vertical autoclave at 121ºC for 15 minutes. For cooling, the material was placed in a biological safety cabinet under the incidence of ultraviolet light (Vertical Laminar Flow - Filterflux). Then, the amount of 10^7 spores/gram of substrate and sterile distilled water were added until the desired humidity was obtained (Tung, Miyata & Iwahori, 2004). Fermentations were conducted in a greenhouse (SL 101 SOLAB) at 30ºC and 75% humidity. Enzymatic activity was determined for 15 days, every 24 hours.

2.5.2 Obtaining Crude Enzyme Extract

After the end of the fermentation time, 25 mL of sterile distilled water were added to each assay for solubilization of α-amylase. This suspension remained under orbital shaking in a shaker incubator (SOLAB) at 30°C, 160 rpm for 30 minutes. The suspended solids were then removed by mechanical pressing through gauze to separate the solid from the enzyme extract. The filtrate was collected in a Falcon tube and centrifuged at 6000 rpm for 15 minutes in a centrifuge (TECNAL) to concentrate the crude enzyme extract (CEE).

2.5.3 Determination of α-amylase Activity

Amylase activity was determined as described by Okolo et al. (1995), with adaptations. The reaction mixture was made up of 1% (w/v) soluble starch (dissolved in 100 mM sodium acetate buffer, pH 5.0). The released reducing sugars were stimulated using the 3.5 dinitrosalicylic acid (DNS) method as described by Miller (1959). Reaction assays were conducted in test tubes with 0.25 mL of 1% (w/v) soluble starch solution, and 0.35 mL of crude enzyme extract. The blank assay contained 0.35 mL of enzyme extract and 0.25 mL of sodium acetate buffer solution, pH 5. All samples were incubated in a water bath (Cientec CT-266) at 50°C for 15 minutes. The reaction was stopped with addition of 0.6 mL of DNS. Then, the tubes were submerged in boiling water (SOLAB) at 100°C for 5 minutes. Then 6.0 mL of distilled water was added. Absorbance was measured at 540 nm using a spectrophotometer (BEL Photonics SP 2000 UV), a methodology adapted from Ghose (1987). One unit of enzymatic activity releases 1 µmol of reducing sugar per gram of extract per minute (Lineweaver & Burk, 1934).

2.5.4 Statistical Analysis

The optimization of experimental variables (temperature, humidity and time) was delineated using a Doehlert matrix, with thirteen different experimental conditions. Each proposed combination was performed in triplicate, with central point values (33ºC, 70% and 14 days) to allow the estimation of error. The variables (temperature, humidity and time) were studied at different levels (-1, 0 and 1) and chosen based on the results of the planning performed, where the temperature ranged from (26ºC to 40ºC), the humidity from (60 % to 80%), and the time between (7, 14, and 21 days).

Based on an analysis of variance (ANOVA), the goodness of fit of the generated models was evaluated by the Fisher test (F test), by means of significance of regression, lack of fit and multiple determination coefficient. The effects were considered significant when $p < 0.05$. The relationship between the independent variables was analyzed using the response surface methodology (RSM) to find an optimal enzyme production point. The STATÍSTICA Software v. 6.0 was used for data analysis and graphing.

2.6 Enzymatic Characterization of α-amylase

2.6.1 Determination of Stability Before pH Variation

The pH was assessed using 100 mM sodium acetate buffer solution to pH (3, 4, 5, 6 and 7). Three repetitions were used in each assay. The activity tests were carried out according to item 2.5.3. The experimental data were plotted and adjusted in components and the statistical significance of each of the terms was evaluated by analysis of variance (ANOVA) in the STATÍSTICA v. 6.0.

2.6.2 Thermostability

Thermalstability was determined by incubating the enzyme at different temperatures (50, 60, 70, 80 and 90ºC) each for 10, 20 and 30 minutes. The activity tests were carried out according to item 2.5.3. The crude extract was subjected to each temperature in capped test tubes, and immediately put in ice baths to analyze further activity.

2.6.3 Effect of Salt Addition

The effect of the presence of salts (ferric chloride, calcium chloride, cobalt chloride, potassium chloride, sodium chloride, sodium carbonate) was evaluated at different concentrations (0.05, 0.10, 0.15, 0.20 and 0.25 M). The activity tests were carried out according to item 2.5.3. Each salt was diluted separately in distilled water and then homogenized in a 25 mL volumetric flask.

2.6.4 Estimation of Kinetic Parameters

Kinetic parameters for the Michaelis-Menten model (K_m and V_{max}) were estimated using starch-containing crude enzyme extract. The analyses were made by five starch solutions at different concentrations (0.05, 0.10, 015, 0.20 and 0.25 M). The K_m constant (mg/mL) and V_{max} (µmol/min/mL) were estimated by the Lineweaver-Burk regression method (Lineweaver & Burk, 1934).

3. Results and Discussion

The chemical characterization of the residues of leaves of *C. linearifolius* confirmed a high fiber content. According to El-Feky et al. (2019) a high fiber content in the residue indicates its possibility of being used in microbial processes, with the objective of producing α-amylase. The compositions (%, g/100g of dry mass) obtained were: Crude protein = 13.68, Ether extract = 1.07, Mineral material = 10.35, Neutral detergent fiber = 64.03, Acid detergent fiber = 51.55, Lignin = 25.03, Cellulose = 0.37, Hemicellulose = 12.47.

The results obtained for α-amylase (U/g) production under the Doehlert matrix conditions can be seen in Table 1. The response values are the average result of the triplicate performed in the enzymatic activity assays.

Table 1 - Optimization of α-amylase (U/g) production by *Aspergillus niger* ATCC 1004, using *Croton linearifolius* by Doehlert matrix with the independent variables: time (t) in days, humidity (U) in% and temperature (T) in ºC.

Exp.	x1	x2	x3	t(d)	U (%)	T (ºC)	α-amylase (U/g)
1	0	0	0	14	70	33	122.88±0.07
2	0	0	0	14	70	33	122.74±0.04
3	0	0	0	14	70	33	121.05±0.05
4	0	-1	0	14	60	33	75.25±0.09
5	0	0	1	14	70	40	36.27±0.16
6	0	1	0	14	80	33	76.15±0.06
7	0	0	-1	14	70	26	20.98±0.13
8	0.707	-0.5	-0.5	21	65	29.5	48.17±0.10
9	0.707	-0.5	0.5	21	65	36.5	47.75±0.17
10	0.707	0.5	0.5	21	75	36.5	71.39±0.08
11	0.707	0.5	-0.5	21	75	29.5	74.19±0.16
12	-0.707	-0.5	-0.5	7	65	29.5	56.78±0.22
13	-0.707	-0.5	0.5	7	65	36.5	61.40±0.25
14	-0.707	0.5	0.5	7	75	36.5	72.14±0.21
15	-0.707	0.5	-0.5	7	75	29.5	42.42±0.08

Exp: Experiment; x1: variable 1; x2: variable 2; x3: variable; t(d): Time in days; U (%): Humidity in percentage.
Source: Authors.

The initial enzymatic activity of α-amylase produced by *A. niger* was 44.05 ± 1.5 U/g, in 13 days of fermentation. The results showed that from the Doehlert matrix, and considering the optimization of the enzyme production, there was enzymatic production in all experimental conditions. The α-amylase activities ranged from 20.98 to 122.88 U/g. The condition of test 1 resulted in the highest production of α-amylase, observing independent variables of time, humidity, and temperature, showing 14 days, 70%, and 33ºC, respectively.

It was initially observed that the fungus *A. niger* was able to synthesize α-amylase in an optimal time of 14 days. This long fermentation period between the microorganism and the substrate is due to the fibrous nature of *C. linearifolius*, which may have caused an increase in the time of enzymatic secretion.

The experimental values found were used to elaborate statistical adjustments in order to generate significant models, according to the Table 2.

Table 2 - Analysis of Variance (ANOVA) for α-amylase produced by *Aspergillus niger* ATCC 1004 grown on *C. linearifolius* residues, with the independent variables of time in days, humidity in percent and temperature in ºC, considering the coefficient of determination (R2) for one p-value of 0.005.

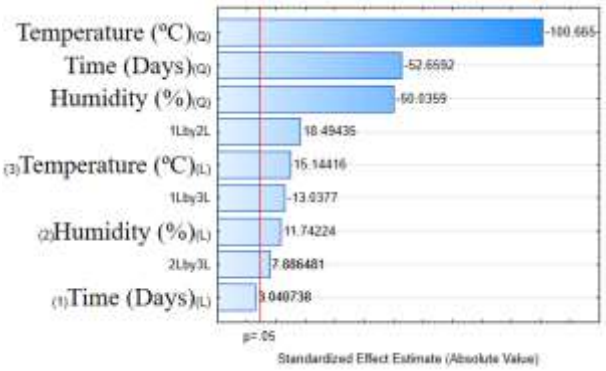
Source	Sum of squares	Degrees of freedom	Square Medium	F	P
(1) Time (L)	9.93	1	9.93	9.57	0.0905
Time (Q)	2880.23	1	2880.23	2776.30	0.0004
(2) Humidity (L)	143.88	1	143.88	138.69	0.0071
Humidity (Q)	2597.12	1	2597.12	2503.41	0.0004
(3)Temperature (L)	236.75	1	236.75	228.21	0.0043
Temperature (Q)	10512.40	1	10512.40	10133.09	0.0001
1Lby2L	353.07	1	353.07	340.33	0.0029
1Lby2L	174.97	1	174.97	168.66	0.0059
2 Lby3L	65.40	1	65.40	63.04	0.0155
Lack of fit	218.63	3	72.88	70.25	0.0141
Pure Error	2.07	2	1.04		0.0043
Total	13797.10	14			0.0001

Source: Authors.

As presented in Table 2, it was observed that the mathematical model was statistically significant ($p < 0.05$) for α-amylase, and the R^2 of 98% indicated that the model was well adjusted to the experimental results. The lack of adjustment was not significant.

The statistical significance of the quadratic and linear terms and their interaction is presented in the Pareto Diagram (Figure 1).

Figure 1 - Pareto diagram showing the significance of the variables under SSF in the adjusted model for α-amylase production by *A. niger* (ATCC 1004), with residues of *C. linearifolius.* The figures were obtained in STATISTICA v 6.0.



(Q) = Quadratic, (L) = Linear. Source: Authors.

The Pareto diagram with the confidence level of 95%, represented by the red vertical line indicates that only the time in the linear model was not significant. All variables in the quadratic model were significant, especially at the temperature for α-amylase production.

Given the optimization for the production of α-amylase, the maximum value obtained (128.88 U/g), exceeds that presented by El-Feky et al. (2019), in which the enzymatic activity of amylase was 3.49 U/g produced by the same fungus species, but in another type of substrate, in this case, sugarcane bagasse. The value obtained is also greater than in Santana et al. (2012), in which the enzyme activity of amylase was 24.98 U/g produced by *A. niger*, using cocoa bran as a substrate.

The maximum value obtained for α-amylase in this study is close to the value found by Kanti and Sudiana (2018) which was 141.85 U/g, using *A. niger* as an enzyme producer and medium with culture medium consisting of rice straw powder and soy curd in the proportion of 30:70 w/w. Comparing these data with other studies, they are lower than the value found by Mukherjee et al. (2019) when citing an initial production of 176.30 U/g of α-amylase using *A. niger* RPB7 and potato peel as a substrate.

While Santana et al. (2012) evaluated temperature, humidity, and time for the production of α-amylase, with cocoa bran as a substrate, they identified significance only for moisture (65%) in the production of the enzyme. When using forage palm as a substrate, moisture was not significant in enzyme production, but time (24 hours) and temperature (35° C).

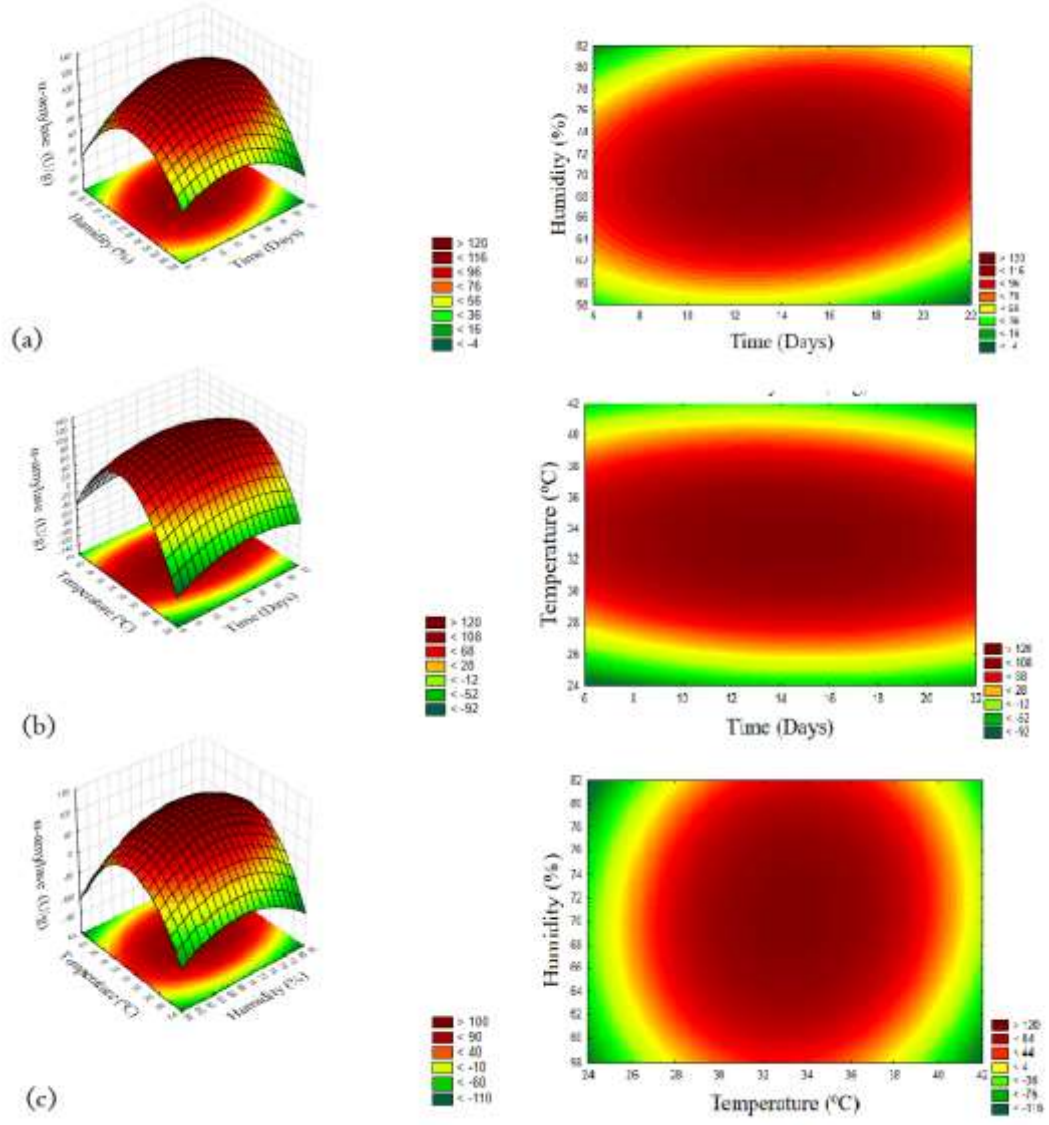
All of these results indicate that *A. niger* can produce α-amylase under different substrates and that the independent variables have a significant influence on the production of the enzyme. Together with this information, our study showed that both time, humidity, and temperature were significant in the fermentation process for enzymatic production.

A Equation 1 presents the mathematical model proposed to determine the specific activity of α-amylase.

$$\alpha\text{-amylase activity } (Ug^{-1}) = 122.22 - 23.40\ (T^2) + 3.85\ (T) - 11.63\ (U^2) + 3.00\ (U) - 27.93\ (Tp^2) + 2.85\ (T) * (U) - 4.68\ (T) * (Tp) + 6.64\ (U) * (Tp) \quad \text{Eq. (1)}$$

Response surface graphs and contour curve are shown in Figure 2, illustrating the effect of the variables and their interactions.

Figure 2 - Response surface graphs and contour curve for α-amylase activity in relation to the variables: (a) Humidity x Time, (b) Temperature x Time, (c) Temperature x Humidity. The figures were obtained in STATISTICA v. 6.0.



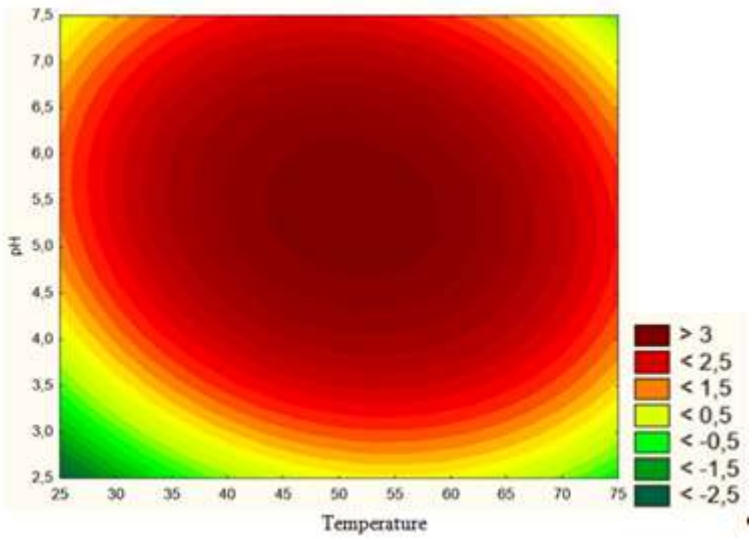
Source: Authors.

These graphs of Figure 2 confirmed that, within the investigated experimental domain, all variables (time, humidity and temperature) influenced the production of α-amylase. The enzyme production was initially slow; the specific activity of α-amylase obtained an optimal value of 122.88 Ug^{-1} over a period of 14 days of fermentation. Upon reaching the peak, the enzymatic activity decreased due to nutrient depletion. The utilization of *C. linearifolius* leaf

residue under the conditions of the central point experiments resulted in a higher production of α-amylase, with variables (temperature, humidity and time) at 33ºC, 70% and 14 days, respectively.

The effects of pH and temperature on α-amylase activity were analyzed based on the construction of optimal pH and temperature graphs (Figure 3).

Figure 3 - Response surface with the variables of temperature 50ºC and sodium acetate buffer solution pH 5 for α-amylase. The figures were obtained in STATISTICA v. 6.0.



Source: Authors.

The data indicated that the best enzyme activity occurred between pH 5.0 and 6.0 at temperatures between 40ºC and 60ºC. It is noteworthy that the optimal value for enzymatic activity occurred at pH 5.0 and temperature 50ºC.

In a similar study for the production of α-amylase, from *A. niger*, using potato peel as a substrate, when studying the effect of pH, they found an optimum pH equal to 6 and an optimum temperature of 40° C (Mahmood et al., 2018). In another study, when evaluating the effect of optimum pH and temperature for α-amylase activity produced by *A. niger*, using cassava peel as a substrate, they found an optimum pH for enzyme activity at 4.5, and a temperature of 45° C (Aisien & Igbinosa (2019).

On the effect of pH and temperature on the activity of the α-amylase enzyme produced by *Aspergillus niger* FAB-211, using a culture medium containing maltose and yeast extract Asrat and Girma (2018) identified a maximum activity at pH 6 and a temperature of 45º C.

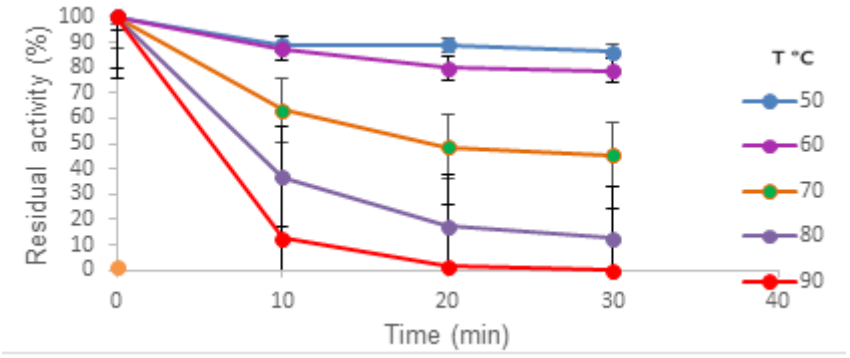
This same pH value for better enzyme activity was also found when α-amylase was produced by other fungi of the *Aspergillus* genus. When checking the effect of pH on the α-amylase activity produced by *Aspergillus oryzae*, using residual water with starch, Shah et al. (2014) found a maximum activity for the enzyme at pH 6 and a temperature of 50º C. While Adejuwon, Tsygankova and Alonge (2018) found a maximum activity of α-amylase produced by *Aspergillus Flavus* Link, with a nutritive culture medium composed of MgSO4.7H20, K2HPO4, KH2PO4, FeSO4.7H2O, potassium nitrate and starch at pH 6 and temperature 35º C.

Therefore, there is a variation in the optimum pH and the optimum activity temperature of the α-amylase produced. However, its best activity occurs at acid pH in a temperature range between 35 and 60º C. Also, we observed that the

Aspergillus genus manages to produce α-amylase in different agro-industrial residues or from nutritive culture media containing salts and starch, but that regardless of them, the crude, partially purified or purified enzyme extract maintains its activity optimal in pH and temperature values very close in the different related studies.

O effect of thermostability of α-amylase produced by *A. niger* is described in the Figure 4.

Figure 4 - Effect of thermostability of α-amylase produced by *A. niger*.



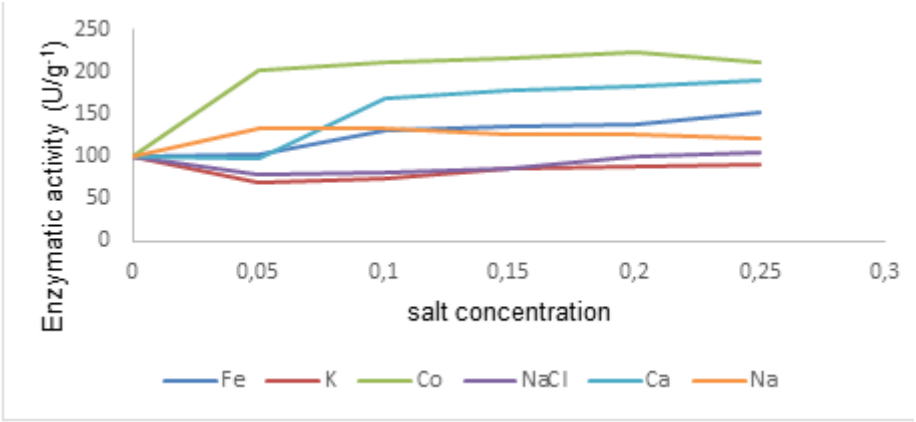
Source: Authors.

The results indicated that the α-amylase enzyme is thermostable up to 60ºC, as it maintained over 90% of activity. In contrast, a study that evaluated the thermostability of α-amylase produced by *Aspergillus niger* found 100% activity when the enzymes (native and mutant) remained at 50° C. After 15 minutes at 55° C, there was a partial loss of activity, with a sharp decline when they remained at a temperature between 60 and 80° C, for 10 minutes (Shafique & Shafique, 2017).

Santos et al. (2020) found a similar result when studying the thermostability of α-amylase produced by *Rhizopus microsporus var. oligosporus*; where the enzyme was exposed to a temperature equal to or greater than 60° C, showing a reduction in its activity when its temperature was increased.

The Figure 5 demonstrates the effect of salt influence on the activity of α-amylase produced by *A. niger*.

Figure 5 - Effect of salt influence on the activity of α-amylase produced by *A. niger*.



Source: Authors.

Thus, the enzyme produced in this study has thermostability at a temperature higher than other studies, this is interesting because it expands its application in processes that are carried out at an elevated temperature.

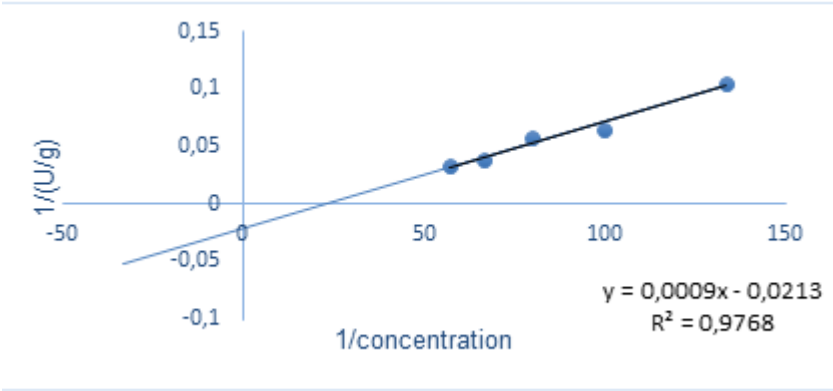
The influence of ferric chloride ($FeCl_3$), calcium chloride ($CaCl_2$), cobalt chloride ($CoCl_2$), potassium chloride (KCl), sodium chloride (NaCl), and sodium carbonate (Na_2CO_3) on α-amylase activity was studied at different concentrations. The salts that most influenced the activity of the enzyme were Na_2CO_3, $CaCl_2$, $FeCl_3$, and $CoCl_2$, which increased enzymatic activity. $CaCl_2$ increased it from the concentration of 0.1 M upwards, and $FeCl_3$ and $CoCl_2$ from 0.05 M upwards. In turn, potassium and sodium cations from their chlorides served as enzyme inhibitors, decreasing enzyme activity. Potassium at all concentrations and sodium chloride up to the concentration of 0.2 M showed a slight increase at the concentration of 0.25 M. Na_2CO_3 slightly increased enzyme activity at all concentrations. These results are described in Figure 5.

When evaluating the effect of salts on an α-amylase produced by *Aspergillus penicillioides*, Ali et al. (2015) noted that in the presence of CaCl2, it provided a slight increase in its activity, since when adding ZnCl2, FeCl2 or EDTA the activity of the enzyme was shown to be inhibited. In another study when evaluating the effect of salts on an α-amylase produced by *Aspergillus oryzae* IFO-30103, the authors identified that in the presence of CaCl2 the enzyme was able to maintain its activity in 61.9%, since the Ca^{+2} cation is a good stabilizer, but not efficient for activating the enzyme. When evaluating the influence of the Co^{+2} cation, this was a good activator (Dey & Banerjee, 2015).

This influence of Ca^{+2} and Co^{+2} was also found in this study, showing that even though it is a crude enzymatic extract, it has a behavior similar to the partially purified or even purified enzyme mentioned by the authors.

The values of K_m and V_{max} obtained from the Lineweaver and Burk (1934) graph are represented in Figure 6.

Figure 6 - Lineweaver-Burk graph demonstrating the effect of substrate concentration on the amylase activity of enzymatic extract of residues of *C. linearifolius* after extraction of essential oil.



Source: Authors.

The K_m and V_{max} values were 0.04 mg/mL and 46.95 µmol/min/mL, respectively. Kinetic parameters of amylase were estimated using the linearization method, in which the substrate is starch. Considering that Km is a constant value and V_{max} depends on the amount of enzyme used for its determination, the values were good because the enzyme amylase has a greater affinity for the substrate, demonstrating its viability.

When optimizing the production of α-amylase by *Aspergillus oryzae*, Shah et al. (2014) found a Km equal to 1.4 mg/mL and a Vmax of 37,037 µmol/min/mL. The Km value can vary according to the enzyme and even if they are considered different substrates of the same enzyme (Nelson & Cox, 2014), this may justify this difference between the value of this study and the authors above. The proximity between the Vmax values can occur, as this value is estimated considering that all the binding sites are occupied, no longer depending on the amount of substrate (Nelson & Cox, 2014).

4. Conclusion

With these results, we can state that the leaves of *C. linearifolius*, until then considered a residue and discarded without purpose in the environment, after extraction of essential oil can be used as a substrate for the production of enzymes through bioprocesses. *Aspergillus niger* is considered an enzyme-producing fungus, using different culture media. The adaptation by the microorganism to the culture medium was confirmed when it managed to grow and produce α-amylase using only residues of *C. linearifolius*, possibly because the leaves of the plant are a source of starch, stimulating the production of this enzyme.

Thus, optimizing the conditions of cultivation in a fermentative process is fundamental to increase the production of the desired enzyme, especially when aiming at an industrial scale application, especially due to the low cost associated with the residue that until then has no specific purpose. Another relevant issue is to characterize the enzyme to know its properties and check the best conditions for its maximum activity. Although the characterization of the enzyme in this study was carried out with the crude extract, we could notice that it presented characteristics similar to α - amylases produced in other fermentation processes with the genus *Aspergillus* partially or purified.

In this sense, we believe that this study is a pioneer in the use of this residue for the production of α-amylase through solid-state fermentation, starting a new path for the study of *C. linearifolius* that until then has been studied from the genetic and biodiversity point of view. For future work we suggest the study of biological activities of the enzymatic extract of amylase after extraction of essential oil to indicate the presence of bioactive compounds of pharmacological and/or industrial interest. Thefore, the identification and purification of such compounds is crucial.

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