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 en estado sólido utilizando residuos 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 33°C, 70% humidity and 14 days of time. In the enzymatic characterization, the enzyme extract presented pH 5.0 and temperature 50°C, and α-amylase was thermostable up to 60°C, 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 de 122.88 Ug⁻¹, a 33°C, 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 *Kₘ* e *Vₘₐₓ* 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 a – amilase.

**Palavras-chave:** Bioprocesos; 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 33°C, 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 *Kₘ* e *Vₘₐₓ* 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 resíduos 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 40°C 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 121°C 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 to Freitas et al. (2017) in a 250 mL flask containing 5g of substrate, previously sterilized in a vertical autoclave at 121°C 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 30°C 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 (33°C, 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 (26°C to 40°C), 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

Thermostability was determined by incubating the enzyme at different temperatures (50, 60, 70, 80 and 90°C) 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, 0.15, 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 |
| ILby2L            | 353.07         | 1                  | 353.07        | 340.33  | 0.0029 |
| ILby2L            | 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 R² 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.
\[ \text{\(\alpha\)-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) \)} \]

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 \(\alpha\)-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.

These graphs of Figure 2 confirmed that, within the investigated experimental domain, all variables (time, humidity and temperature) influenced the production of \(\alpha\)-amylase. The enzyme production was initially slow; the specific activity of \(\alpha\)-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 33°C, 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 50°C and sodium acetate buffer solution pH 5 for α-amylase. The figures were obtained in STATISTICA v. 6.0.

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

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 45°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 50°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 MgSO4.7H2O, K2HPO4, KH2PO4, FeSO4.7H2O, potassium nitrate and starch at pH 6 and temperature 35°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 60°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.

The 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.](image)

Source: Authors.

The results indicated that the α-amylase enzyme is thermostable up to 60°C, 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.](image)

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 (FeCl₃), calcium chloride (CaCl₂), cobalt chloride (CoCl₂), potassium chloride (KCl), sodium chloride (NaCl), and sodium carbonate (Na₂CO₃) on α-amylase activity was studied at different concentrations. The salts that most influenced the activity of the enzyme were Na₂CO₃, CaCl₂, FeCl₃, and CoCl₂, which increased enzymatic activity. CaCl₂ increased it from the concentration of 0.1 M upwards, and FeCl₃ and CoCl₂ 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. Na₂CO₃ 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 penicilloides, 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 Ca²⁺ cation is a good stabilizer, but not efficient for activating the enzyme. When evaluating the influence of the Co²⁺ cation, this was a good activator (Dey & Banerjee, 2015).

This influence of Ca²⁺ and Co²⁺ 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 Km and Vmax 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.

The Km and Vmax 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 Vmax 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. Therefore, the identification and purification of such compounds is crucial.

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References

Adejuwon, O. A., Tsygankova, V. A., & Alonge, O. (2018). Effect of cultivation conditions on activity of α-amylase from a tropical strain *Aspergillus flavus* Link. J. Microbiol. Biotech. Food Sci. 7 (6), 571-575.

Asisien, E. T., & Igbinoso, I. H. (2019). Production, purification, and characterization of α-amylase from *Aspergillus niger*. *Aspergillus flavus* and *Penicillium expansum* using cassava peels as substrate. *Nig. J. Biotech.* 36 (2), 114-126.

Ali, I., Akbar, A., Anwar, M., Prasongsuk, S., Lotarakul, P., & Punnapayak, H. (2015). Purification and Characterization of a Polyextremophilic α-Amylase from an Obligate Halophilic *Aspergillus penicilloides* Isolate and Its Potential for Souse with Detergent. *BioMed Res. Int.*, 01-08.

Asrat, B., & Girma, A. (2018). Isolation, production and characterization of amylase enzyme using the isolate *Aspergillus niger* FAB-211. *Int. J. Biotechnol. Mol. Biol. Res.* 9(2), 7-14.

Cordeiro, L. Secco, R., Carneiro-Torres, D. S., Lima, L. R de, Caruzo, M. B. R., Berry, P., Riina, R., Silva, O. L. M., Silva, M. J da; & Sodê, R. C. (2015). *Croton* in Lista de Espécies da Flora do Brasil. Jardim Botânico do Rio de Janeiro.

Dey, T. B., & Banerjee, R. (2015). Purification, biochemical characterization and application of α-amylase produced by *Aspergillus oryzae* IFO-30103. *Biocatal. Agric. Biotechnol.* 4(1), 83-90.

El-Feky, R. M., Fattah, A. F. A. K. A., Gibriel, A. Y., & Farag, A. A. (2019). Optimization the parameter process of solid-state fermentation to produce the fungal α-amylase on agro-industrial by-products. *AUJAS, Ain Shams Univ. Special Issue*, 27(1), 441-456.

Freitas, S. L., Silva, T. M., Franco, M., Bonomo, P., & Freitas, J. S. de. (2017). Fermentação de farelo de mandioca, para obtenção de xilanase, a partir de um fungo endofítico. *Enciclopédia Biosfera. Centro Científico Conhecer*. 14(26), 995-1008.

Ghose, T. K. (1987). Measurement of cellulase activities. Pure & Appl. Chem. 59 (2), 257-268.

González, G. V., Torres, E. F., Aguilar, C. N., Gomes, S. J. R., Godínez, G. D., & Augur, C. (2002). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J.* 3643, 1-11.
Kanti, A., & Sudiana, I. M. (2018). Production of Phytase, Amylase and Cellulase by Aspergillus, Rhizopus and Neurospora on Mixed Rice Straw Powder and Soybean Curd Residue. JOP Conf. Ser. Earth Environ. Sci. 166.

Lineweaver, H., & Burk, D. (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56, 658-666.

Malmood, S., Shahid, M. G., Irfan, M., Nadeem, M., & Syed, Q. (2018). Partial Characterization of α-amylase Produced from Aspergillus niger using Potato Peel as Substrate. Punjab Univ. J. Zoo. 33(1), 22-27.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31(3), 426-428.

Mukherjee, R., Tanmay, P., Soren, J.P., Halder, S. K., Mondal, K. C., Pati, B. R., & Das Mohapatra, P. K. (2019). Acidophilic α-Amylase Production from Aspergillus niger RBP7 Using Potato Peel as Substrate: A Waste to Value Added Approach. Waste Biomass. Valor. 10, 851-863.

Nelson, D. L., & Cox, M. M. (2014). Princípios de Bioquímica de Lehninger. (6a ed.). Editora Artmed.

Okolo, B. N., Ezeguog, L. I., & Mba, C. N. (1995). Production of raw starch digestive amylase by Aspergillus niger grown on native starch sources. J. Sci. Food Agric. 69, 109-115.

Onofre, S. B., Abatti, D., Refosco, D., Tessara, A. A., Onofre, J. A. B., & Tessaro, A. B. (2016). Characterization of α-amylase produced by the endophytic strain of penicillium digitatum in solid state fermentation (SSF) and submerged fermentation. Afr. J. Biotechnol. 5 (28), 1511-1519.

Pandey, A. (2003). Solid state fermentation. Biochem. Eng. J. 13(23), 81-84.

Panesar, R., Kaur, S., & Panesar, P. S. (2015). Production of microbial pigments utilizing agro-industrial waste: a review. Curr. Opin. Food Sci. 1, 70-76.

Ravindran, R., Hassan, S. S., Williams, G. A., & Jaaiswal, A. K. (2018). A Review on Bioconversion of Agro-Industrial Wastes to Industrially Important Enzymes. Bioengineering. 5 (4), 1-20.

Reilly, C. M., Kim, J., Lynn, J., Simmons, B. A., Gladden, J. M., Magnuson, J. K., & Baker, S. (2018). Forward genetics screen coupled with whole-genome resequencing identifies novel gene targets for improving heterologous enzyme production in Aspergillus niger. Appl. Microbiol. Biotechnol. 102(4), 1797-1807.

Reguly, J. C. (2000). Biotecnologia dos processos fermentativos: produção de enzimas e engenharia das fermentações. Pelotas: Editora Universitária.

Sadh, P. K., Duhan, S., & Duhan, J. S. (2018). Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresour. Bioprocess. 5(1), 1-15.

Shah, I. J., Gami, P. N., Shukla, R. M., & Acharya, D. K. (2014). Optimization for α-amylase production by Aspergillus oryzae using submerged fermentation technology. Basic Res. J. Microbiol. 1(4), 01-10.

Shafique, S., & Shafique, S. (2017). Partial purification and characterization of α-amylase from hyperactive mutants of Aspergillus niger for economic enzyme production and starch hydrolysis. Int.J.Biol. Biotech., 14 (2), 159-167.

Santana, L. S. M. de, Gonçalves, Z. S., & Franco, M. (2012). A produção de amilase a partir da fermentação em estado sólido do farelo de cacau. Enciclopédia Biosfera. Centro Cientifico Conhecer, 8 (14), 1981-1982.

Santos, I. R., Mendes, T. P. S., Miranda, A. C. A., Costa, D. N., Figueroa, G. M., Soares, V. D. M., Valasques Jr, G. L., & Cedro, P. E. P. (2020). Production and characterization of amylase obtained from Rhizopus microsporus var. oligosporus. Research, Society and Development, 9(7), 1-13.

Santos, J. C., Leal, I. R., Cortez, J. S. A., Fernandes, G. W. & Tabarelli, M. (2011). Caatinga: The scientific negligence experienced by a dry tropical forest. Trop. Conserv. Sci. 4 (3), 276-286.

Santos, P. S. dos, Solidade, L. S., Souza, J. G. B., Lima, G. S., Braga Jr, A. C. R., Assis, F. G. V de, & Leal, P. L. (2018). Fermentação em estado sólido em resíduos agroindustriais para produção de enzimas: uma revisão sistemática. JCEC. 4 (2).

Shruthi, B. R., Achur, R. N. H., & Boramuthu, T. N. (2020). Optimized Solid State Fermentation Medium Enhances the Multi-enzymes Production from Penicillium citrinum and Aspergillus clavatus. Curr. Microbiol. 77, 2192 – 2206.

Silva, S. L. C., Gaalberto, S. A., Carvalho, K. S., & Fries, D. D. (2014). Avaliação da atividade larvicida de extratos obtidos do caule de Croton linearifolius Mull. Arg. (Euphorbiaceae) sobre larvas de Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae). Biotemas. 27(2), 79-85.

Silva, T. S. S., Freitas, J. S., Santos, E. S. L. dos, Cardoso, T. S., & Cerqueira-Silva, C. B. M. (2018). Caracterização e seleção de marcadores moleculares em Croton linearifolius Mull. Arg. como subsídio para estudos genéticos. Multi-Science Journal. 1 (10), 4-8.

Tung, Q. T., Miyata, N., & Iwahori, K. Growth of Aspergillus oryzae during treatment of cassava starch processing wastewater with high content of suspended solids. J. Biosci. Bioeng. 97(5), 329-335.