



Production of xylanase by *Aspergillus* sp. ART500.1 on agroindustrial residues and its biochemical properties

Produção de xilanase por *Aspergillus* sp. ART 500.1 em resíduos agroindustriais e propriedades bioquímicas

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(Trabalho avaliado e selecionado pela Comissão do III CTOCTA)

The aim of this work was to evaluate the use of different agro-industrial residues for the production of xylanase by *Aspergillus* sp. ART 100.1, as well as analyzing the biochemical properties of the enzyme. Agroindustrial residues malt bagasse, pineapple crown, açai bagasse and soybean husk present in large quantities in the Tocantins region were used to evaluate the production of xylanase. Cultivation conditions for xylanase production were evaluated in submerged and solid-state cultivation. The highest production of xylanase in submerged cultivation was obtained using soybean husk residue (23.60 U/mL), while, for solid-state cultivation conditions, the highest production of xylanase was obtained with malt bagasse (110.00 U/g). The effect of additives to the culture medium was also evaluated, with the best result for the use of the xylose additive in the pineapple crown in solid-state cultivation. The enzyme produced in solid-state cultivation was characterized in terms of pH and temperature. The optimum activity pH was observed at 5.0 and for temperature at 55 °C. The xylanase was stable in a pH range between 4 and 5 and retained 50% of its activity at 45 °C after 110 minutes. The *Aspergillus* sp. ART 500.1 presents potential for the production of xylanase using agro-industrial residues, enabling the development of bioprocesses for the scaling of production.

Keywords: xylanase, agro-industrial residue, biochemical characterization.

O objetivo deste trabalho foi avaliar o uso de diferentes resíduos agroindustriais para a produção de xilanase por *Aspergillus* sp. ART 100.1, bem como analisar as propriedades bioquímicas da enzima. Os resíduos agroindustriais bagaço de malte, coroa de abacaxi, bagaço de açai e casca de soja presentes em grande quantidade na região do Tocantins foram utilizados para avaliar a produção de xilanase. As condições de cultivo para a produção de xilanase foram avaliadas em cultivos submersos e em estado sólido. A maior produção de xilanase nos cultivos submersos foi obtida utilizando o resíduo casca de soja (23,60 U/mL), enquanto, para as condições de cultivo em estado sólido, a maior produção de xilanase foi obtida com bagaço de malte (110,00 U/g). O efeito de aditivos ao meio de cultivo também foi avaliado, tendo o melhor resultado para o uso do aditivo xilose na coroa de abacaxi em cultivo em estado sólido. A enzima produzida em estado sólido foi caracterizada quanto ao pH e temperatura. O pH ótimo de atividade foi observado em 5,0 e para temperatura em 55 °C. A xilanase se mostrou estável numa faixa de pH entre 4 e 5 e reteve 50% da sua atividade a 45 °C após 110 minutos. A linhagem *Aspergillus* sp. ART 500.1 apresenta potencial para a produção de xilanase utilizando resíduos agroindustriais viabilizando o desenvolvimento de bioprocessos para o escalonamento da produção.

Palavras-chave: xilanase, resíduo agroindustrial, caracterização bioquímica.

1. INTRODUCTION

Some microorganisms produce enzymatic complexes that degrade hemicellulose, cellulose and lignin present in plant cell walls, releasing sugars with potential for applications in fermentation processes, generating products with high economic value [1]. Filamentous fungi are the main source of cellulases and hemicellulases [2]. Fungi have high extracellular release and

enzymatic yield, ensuring greater activity compared to bacteria or yeasts, being major producers of xylanase [3].

Enzymes are components of the metabolism of microorganisms responsible for catalyzing chemical reactions essential for cell maintenance, being useful in many applications such as biology, engineering, biotechnology and industry [4]. Xylanases are enzymes responsible for the hydrolysis of β -1,4 glycosidic bonds present in xylan. Xylan is a xylose polymer that is associated with other sugars forming glucuronoxylans, glucuronoarabinoxylans and glucomannans [5]. Xylanases can hydrolyze bonds inside and at the ends of the polymer, being called endoxylanases and exoxylanases [6].

The production of enzymes is a market in evidence thanks to an increasingly significant increase in their use in various industrial processes. However, the high production cost associated with the steps of purification, formulation and high price of the substrate, still limits the consolidation of this market [7]. Thus, alternatives for enzyme production and cost reduction are intensively studied to replace some synthetic substrates with low value products such as agro-industrial residues, adding value and reducing the environmental impact of this residue on the environment [8]. In this context, the objective of this work was to analyze agro-industrial residues as a carbon source, in solid and submerged crops, using a strain of *Aspergillus* sp. ART 500.1 isolated from the fruit of Araticum (*Annona crassiflora*) and using an additive (xylose) in order to improve its cultivation conditions and characterize the biochemical properties of the enzyme produced.

2. MATERIALS AND METHODS

The experiments were conducted at the Laboratory of Biotechnology, Analysis of Food and Products (LABAP) of Habite – Incubator of Biotechnology Companies at the Federal University of Tocantins, Campus de Gurupi, located in the southwest region of the state of Tocantins.

2.1 Microorganism and inoculum

Aspergillus sp. ART 500.1 was isolated from Araticum fruits in spontaneous fermentation. The stock culture was maintained and stored using the Castellani method at 4 °C. Peaks were periodically performed in slant tubes containing potato dextrose agar medium and incubated at 28 °C. After 5 days of cultivation, the *Aspergillus* sp. ART 500.1 was kept in slanted tubes and the spores were suspended in 0.85% saline solution at a concentration of 10^7 spores/mL. The cultures were added with 1 mL of this suspension in liquid and solid culture media.

2.2 Agro-industrial residues

Four agro-industrial residues were selected to be used as a carbon source: pineapple crown, malt bagasse, açaí bagasse and soybean husk. The residues were in an oven at 70 °C for 48 hours, then crushed in a knife mill (particle size = 1 mm).

2.3 Culture media preparation

For the submerged culture medium, the experiments were carried out in Erlenmeyer flasks (125 mL) containing 20 mL of Mandel and Weber medium [9]: KH_2PO_4 2.0 g/L; $(\text{NH}_4)_2\text{SO}_4$ 1.4 g/L; urea 0.3 g/L; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.3 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g/L; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5.0 mg/L; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 1.6 mg/L; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4 mg/L; CoCl_2 2.0 mg/L; yeast extract 1.0 g/L and peptone 1.0 g/L. Submerged cultures were supplemented with 0.2 g of each carbon source. The pH was adjusted to 6.0 and after inoculation, the culture remained incubated at 28 °C, 180 rpm for 72 hours. For the solid-state cultivation, 10 g of each carbon source were weighed and 10 mL of Mandel and Weber medium [9] were added, pH adjusted to 6 in autoclavable bags (12x14 cm). The bags were

sealed with staples at the ends, after inoculation the culture remained in the greenhouse at 28 °C for 120 hours.

2.4 Effect of glucose and xylose on xylanase production

The effect of xylose or glucose addition on xylanase production was analyzed by adding 1% (w/w). The cultivations were carried out in autoclavable bags containing 10 g of pineapple crown, soybean husk or barley spent grain and moistened with Mandel and Weber medium [9] with pH adjusted to 6.0. Cultivations were kept at 28 °C. Controls were carried out without the addition of additives. The cultures were maintained for 168 hours with samples taken every 24 hours and carried out in triplicate.

2.5 Crude extract

The clarification of the enzymatic extract was carried out by filtration process using Mucelini cloths cut to a diameter of 12.5 cm, placed in a Büchner funnel coupled to the kitassate and vacuum pump. The filtrate was centrifuged for 10 minutes at 10,000 g at 4 °C. The supernatant was collected and used for enzyme activity analysis.

2.6 Xylanolytic activity assay

Xylanase activity was performed using a 1% (w/v) xylan solution in McIlvaine pH 5.0 buffer. Four hundred milliliters of this solution were added to test tubes and kept for 5 minutes in a water bath at 50 °C. To start the reaction, 0.4 mL of the enzymatic extract was added, 0.2 mL being removed at time 0 (control), 5 and 10 min, adding to tubes containing 0.2 mL of DNS at the end of each time. Tubes with DNS were boiled in boiling water for 5 min. It was allowed to cool, and 2.0 mL of ultrapure water was added and analyzed in a spectrophotometer at 540 nm. The presence of reducing sugars was evaluated according to Miller [10]. One unit of enzyme was determined as the amount of enzyme needed to release 1 µmol of xylose per milliliter per minute.

2.7. Biochemical properties

2.7.1 Effect of pH and temperature on xylanolytic activity

The effect of pH on the enzymatic activity was performed in different reaction media containing 1% xylan (w/v) with pH values ranging from 3.0 to 8.0 with 0.5 intervals.

The effect of temperature on enzymatic activity was analyzed at different temperatures from 25 to 75 °C.

2.7.2 Thermal and pH stability

The stability of the enzyme at different pH was performed in McIlvaine buffers with a pH of 3 to 8, with an interval of 0.5. Enzyme was diluted 1:2 (v/v) for each buffer. The experiment was kept at 25 °C for 24 hours and enzyme activity was analyzed in the initial and final time.

The thermal stability of the enzyme was performed at different temperatures without the presence of substrate. The enzyme was diluted in buffer and incubated in a water bath at temperatures 45, 50, 55 and 60 °C. Samples were collected at a time interval of 0, 5, 10, 15, 20, 30, 40, 60, 90 and 120 minutes and placed in an ice bath. The xylanolytic activity was performed using the extract collected at each different time interval, using optimum activity temperature and pH.

3. RESULTS AND DISCUSSION

3.1. Xylanase production in submerged and solid-state cultivations

Figure 1 shows the results for extracellular xylanase production by *Aspergillus* sp. ART 500.1 in submerged and solid-state cultivations, using agro-industrial residues. In submerged cultivation, the highest level of xylanase production was observed with soybean husks (23.60 U/mL), followed by açai bagasse (16.00 U/mL) with orbital agitation. Pineapple crown and barley spent grain produced 5.60 U/mL of xylanase, respectively (Figure 1A).

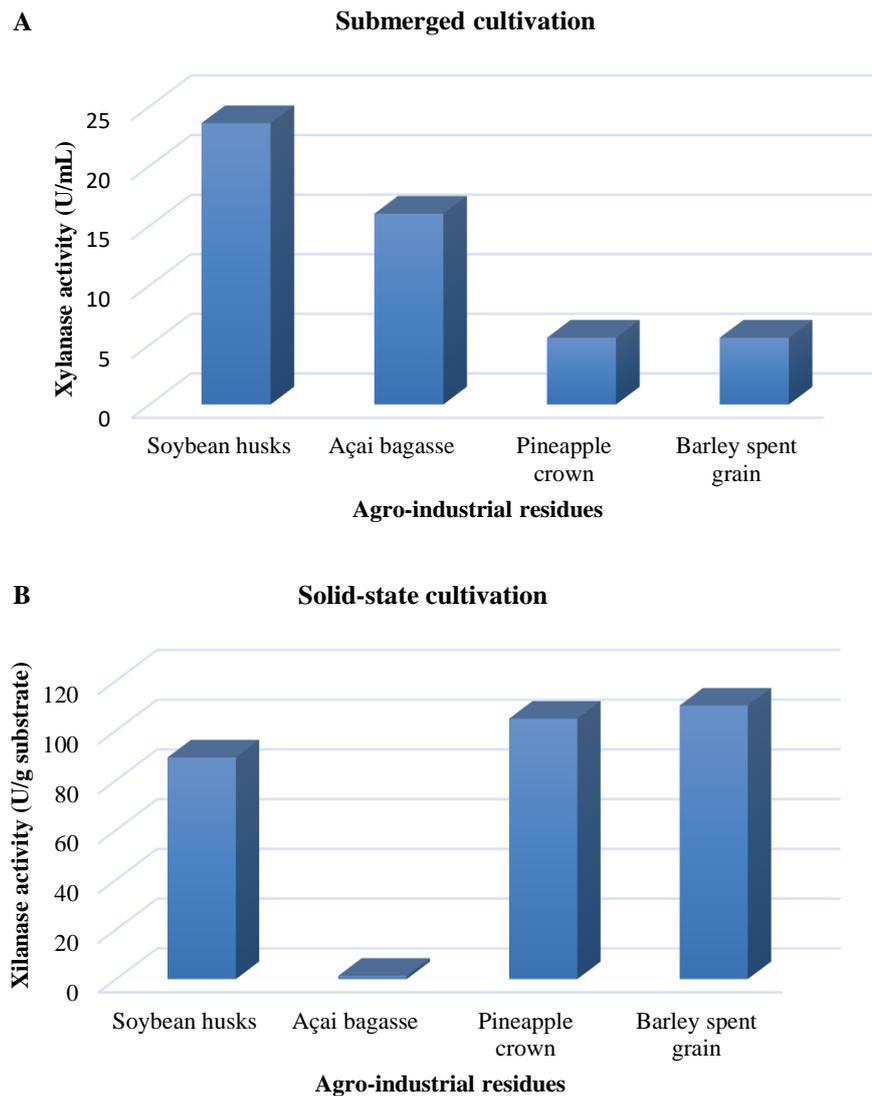


Figure 1. Xylanase production by *Aspergillus* sp. ART 500.1 in submerged cultivation (A) and solid-state cultivation (B). Experimental conditions: submerged cultivation performed for 72 hours, at 28 °C, 180 rpm; solid-state cultivation carried out at 28 °C, 50% humidity for 72 hours.

The use of agro-industrial residues for the production of enzymes has been concentrated in the production of xylanase using corn cob, citrus pectin, beet pulp, wheat straw and wheat bran have shown to have the potential capacity to induce the xylanolytic and hemicellulolytic complexes for the enzyme production [11]. Submerged crops represent the vast majority of bioprocesses for enzyme production. The optimization of xylanase production by *Aspergillus foetidus* was

achieved using soybean hulls after seven days of submerged cultivation (9.72 U/mL) [12]. Javed et al. (2019) [13] studied the production of xylanase by *Aspergillus niger* KIBGE-IB36 using residues of corn cob, wheat bran, rice husk, orange peel, pomegranate peel and potato starch in submerged crops. The xylanase activity was considered maximum (3071 U/mg) when 1% wheat bran was used as substrate. Xylanase production was not observed in the presence of potato starch.

Solid-state cultivation, in many studies, show better yields in enzyme production when compared to submerged cultivation. In this study, solid-state cultivation showed higher levels of productivity for xylanase production by *Aspergillus* sp. ART500.1. The highest activities were observed with barley spent grain (110.00 U/g), pineapple crown (104.70 U/g), soybean husks (89.10 U/g). Açai bagasse had the lowest xylanase production (1.30 U/g) (Figure 1B). Singh et al. (2021) [14] evaluated the production of xylanase by *Aspergillus flavus* in solid-state cultivation with different agro-industrial substrates. In this work, the highest production of xylanase was observed with pretreated rice husks. Kronbauer et al. (2007) [15] observed that the production of xylanase with barley spent grain in solid-state cultures by *Aspergillus casingii* was 92.20 U/g after 96 hours.

Enzyme induction in submerged cultivation is highly influenced by different parameters such as carbon sources, temperature, pH and operational parameters such as incubation times, agitation. In this work, the results indicate that the carbon source and the cultivation system are the main factors influencing the production of xylanase. Considering the highest yields of xylanase in solid-state cultivations, the other experiments were carried out using soybean husk, pineapple crown and malt bagasse.

3.2 Effect of glucose and xylose

Aspergillus sp. ART 500.1 cultivations were carried out in solid-state with the addition of glucose or xylose for 168 hours (Figure 2). The addition of glucose in the culture media of soybean husk and barley spent grain reached the maximum xylanase production of 32.50 U/g substrate and 31.20 U/g substrate, respectively. In pineapple crown cultivation, the addition of glucose induced the production of 225.00 U/g substrate after 168 hours of cultivation. The addition of xylose to the culture media had distinct effects on enzyme production. In pineapple crown cultivation, the addition of xylose promoted the production of 272.30 U/g substrate after 144 hours of cultivation, causing repression in xylanase production. The addition of xylose in pineapple crown induced the production of 271.20 U/g xylanase substrate after 144 hours, while the control cultures produced 236.80 U/g substrate after 120 hours. The results for soybean husk show that the addition of xylose had no effect on the production of xylanase, with the highest values observed being 99.50 U/g substrate for control and 95.60 U/g substrate for cultivations with addition of xylose. However, in cultures with barley spent grain, xylose had a similar repressive effect on xylanase production to cultures with the addition of glucose with the maximum observed enzyme production of 28.90 U/g substrate after 144 hours of cultivation. In this substrate, the highest production of xylanase was observed in the control cultures after 120 hours (108.00 U/g substrate).

Faria et al. (2019) [16] evaluated the production of xylanase by strains of *Moesziomyces* spp. using xylose, xylan and barley bagasse. In this work, the evaluated strains were able to grow and produce xylanolytic enzymes using all substrates tested, showing robust and versatile enzyme production systems when induced by barley bagasse, xylose or xylan. The presence of glucose in the pretreated barley bagasse did not harm enzyme production, probably because this sugar was first consumed during the initial growth phase. The xylanase production by *Colletotrichum graminicola* on solid substrates showed that the supplementation of wheat bran with 1% ground corncob slightly increased the xylanase production. Most of the other supplemental carbon sources tested, including xylan, had no effect, while xylose inhibited about 33% of enzyme production [17].

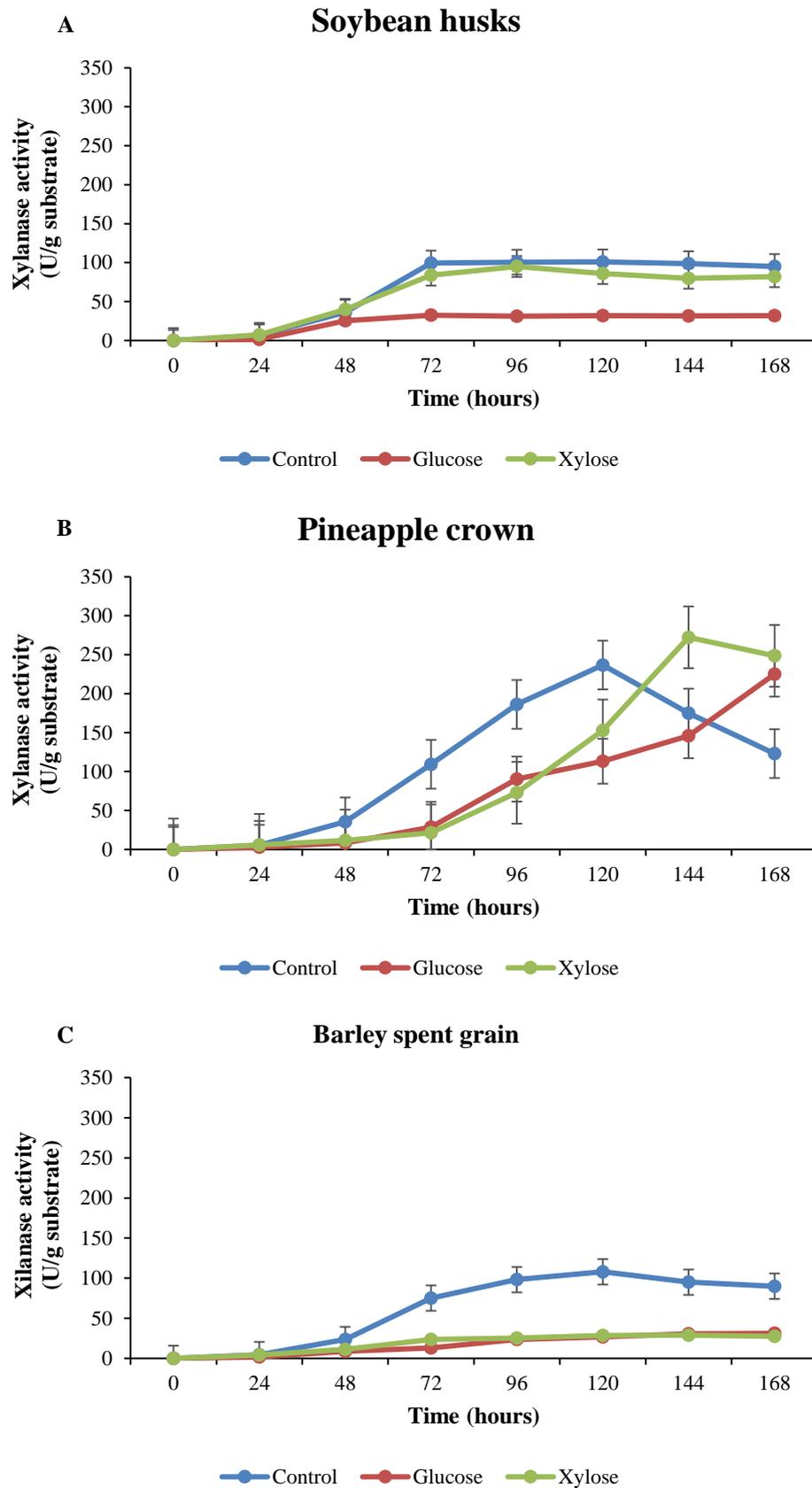


Figure 2. Effect of addition of glucose and xylose on xylanase production by *Aspergillus* sp. ART 500.1 in solid state crops using (A) soybean husk, (B) pineapple crown and (C) malt bagasse. Growing conditions: solid-state cultivation was maintained at 28 °C, 50% humidity.

3.3 Biochemical properties of xylanase

3.3.1 Effect of pH and temperature on enzyme activity

The biochemical properties of an enzyme, such as pH and optimum activity temperature, are important operational information for the application of enzymes in industrial processes. The xylanase produced by *Aspergillus* sp. ART 500.1 in solid state cultivation showed optimal activity at pH 5.0 (100%). Above pH 5.0, a decrease in activity was observed up to pH 8.0 (7.63%) (Figure 3). The xylanase produced by *Penicillium roqueforti* ATCC 10110 showed maximum activity at pH 3.0, but with similar values at pH 4.0 and pH 5.0 [18]. According to the authors, the favorable behavior of enzymes in acidic pH ranges may be the result of a positive unfolding on the protein's amino acid residues, resulting in increased exposure of the enzyme's active site and the occurrence of the reaction [18]. Krisana et al. (2005) [19] observed optimal pH 5.0 for the *A. niger* BCC14405 strain.

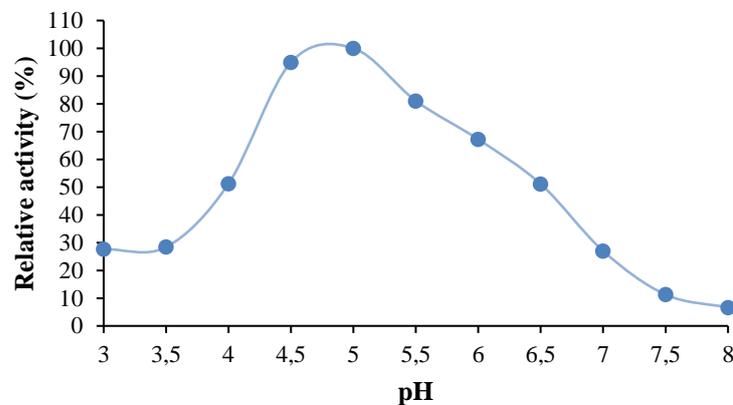


Figure 3. Optimal pH of xylanase produced by *Aspergillus* sp. ART 500.1 on solid-state cultivation.

The optimum temperature of the enzyme produced by *Aspergillus* sp. ART 500.1 is shown in Figure 4. Xylanase showed optimal activity between temperatures of 55 °C and 60 °C (100%). Above this temperature there was a strong reduction in enzymatic activity promoted by the denaturation of the enzyme. Temperatures below 55 °C showed a milder reduction, reaching 50% of the enzymatic activity at 25 °C. Similar results were observed by Souza et al. (2018) [18]. The xylanase activity produced by *P. roqueforti* ATCC10110 gradually increased with increasing reaction temperature, reaching a maximum value of 60 °C. Subsequently, there was a decrease in enzymatic activity, however, not exceeding 40% even at maximum temperature.

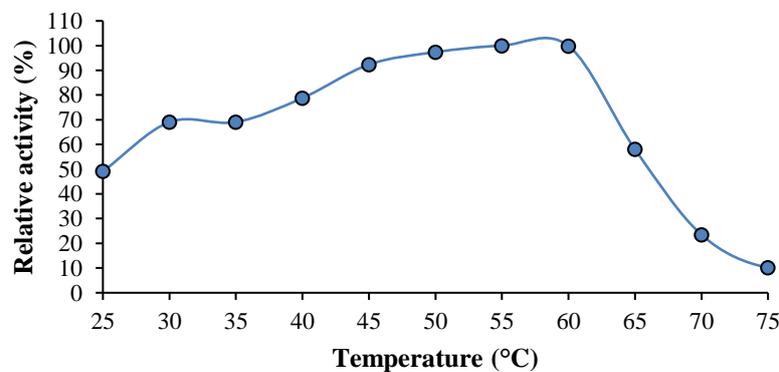


Figure 4. Optimal temperature of xylanase produced by *Aspergillus* sp. ART 500.1 on solid-state cultivation.

3.3.2 Thermal stability and pH

Xylanase from *Aspergillus* sp. ART 500.1 showed greater stability between pH 3.0 and 4.5 (~100%) (Figure 5). Above these pH values the activity gradually reduced until reaching 25% at pH 8. The pH stability study is essential for the characterization of an enzyme before it can be commercially exploited. Xylanase from *Penicillium glabrum* was stable between pH 2.5-5.0 (80%) [20]. Results similar to those obtained in this work were found by Souza et al. (2018) [18]. The xylanase produced by *P. roqueforti* ATCC 10110 showed stability above 70% between pH 3.0 and 5.0 after 5.0 h at 50 °C and at pH 6.0 there was a decrease of 46%. After 3.0 h at pH 7.0 and 8.0, significant reductions in activity until enzyme inactivation were observed.

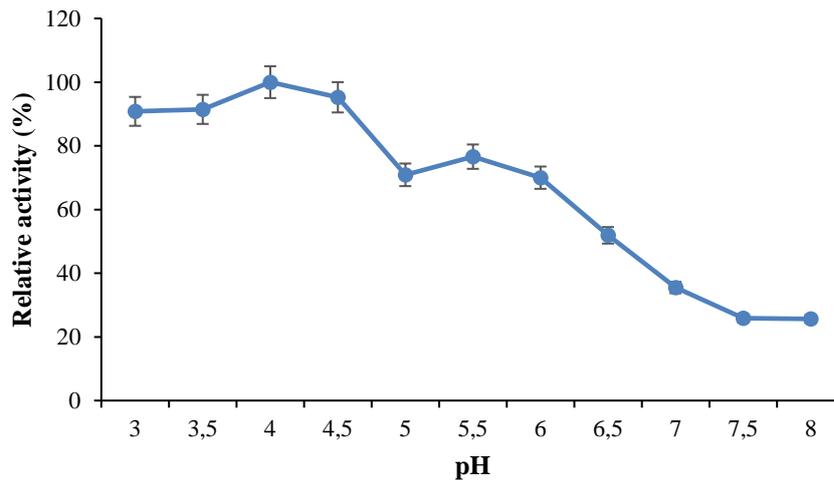


Figure 5. pH stability of xylanase produced by *Aspergillus* sp. ART 500.1 on solid-state cultivation.

Regarding the enzymatic thermostability shown in Figure 6, the enzyme was more stable at a temperature of 45 °C, maintaining the relative activity at 90% for 30 min. After this period, the relative activity dropped to 76% in 90 min, in 105 min the relative activity dropped to 50%, showing that the xylanase remains stable up to the time of approximately 90 min at a temperature of 45 °C. For the temperature at 55 °C, in 5 min it showed a relative activity of 40%, remaining so for up to 20 min. For temperatures of 60 °C and 65 °C the relative activities were 30% with 5 minutes, being inappropriate temperatures for applications as they do not guarantee the stability of the enzyme. Similar results were obtained for xylanase from *A. foetidus* where the enzyme showed high stability at intermediate temperatures and no activity was observed at 75 °C [12].

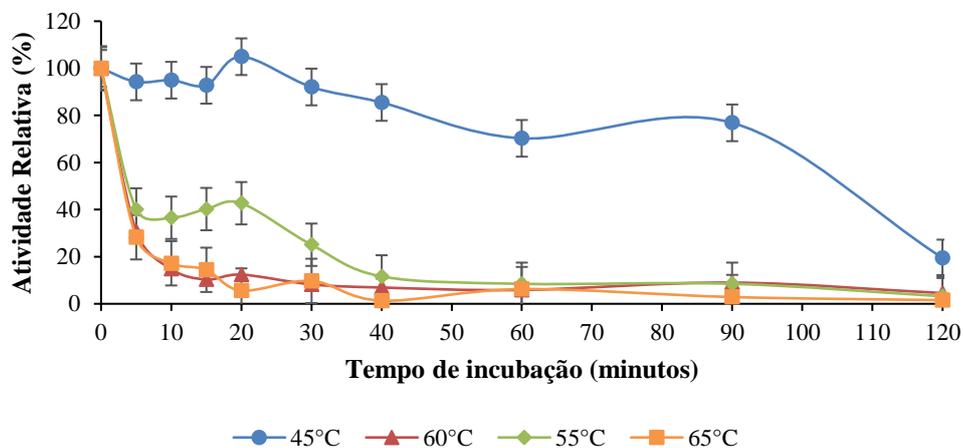


Figure 6. Thermostability of xylanase produced by *Aspergillus* sp. ART 500.1 on solid-state cultivation.

4. CONCLUSION

The production of xylanase by *Aspergillus* sp. ART 500.1 evaluated on different agro-industrial substrates was more efficient with solid-state soybean husks in submerged crops. Agro-industrial residues supplementation showed that pineapple crown supplemented with xylose increased xylanase production after 144 hours of cultivation, while glucose had a repressive effect on enzyme production. The enzyme produced in solid-state cultivation had an optimal pH between 5.0-5.5 and an optimal activity between 55 and 60 °C. The enzyme showed stability at a temperature of 45 °C and a pH stability range from 3.0 to 4.5. The production of xylanase by *Aspergillus* sp. ART 500.1 in different agro-industrial residues presents potential for the exploration of new alternatives for the production of enzymes and their scaling for production in pilot scales.

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