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Production and Characterization of Xylanase from *Aspergillus parasiticus* URM 5963 Isolated from Soil of Caatinga

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Research Article

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ABSTRACT

Filamentous fungi, *Aspergillus parasiticus* URM 5963 was biochemically selected among different species of *Aspergillus*, for xylanase production. The enzyme activity and specific activity of partially purified xylanase from *Aspergillus parasiticus* URM 5963 was 1,116 IU/ml and 6,245 IU/mL, respectively. Temperature and optimum pH for xylanase were found to be 50°C and 5.5, respectively. Of the many reported applications for xylanase, its use as a food supplement has played an important role for monogastric animals, because it can improve the utilisation of nutrients. In this work an effort has been made in order to increase available knowledge about a xylanase produced by submerged cultivation from *Aspergillus parasiticus* URM 5963 and partial characterization.

INTRODUCTION

Xylan, comprises of a homopolymeric backbone of β - 1,4 linked D-xylopyranose units and short chain branches of 0-acetyl,alfa-L- arabinofuranosyl, and alfa-D-glucuronyl residues. Due to its complex structure synergystic actions and interaction between many enzymes is required for its complete breakdown but endo-β-1,4-xylanase (-1,4-endoxylanases (1,4-β-D-xylan xylo-hydrolase, EC 3.2.1.8) serves the most important role in depolymeratation of xylan backbone [1,2]. Xylanase have gathered remarkable attention globally due to their unmatched role in modification of cereal-based food stuffs, bioleaching, bread baking [3,4]. Another rapidly emergency thrust is its use as a feed additive for monogastrics like poultry and pigs [5-7]. Thus increasing feed conversion, body weight gain and the yield of production [8,9]. Xylanases are produced by algae, protozoa, mollusks, crustaceans, insects, the seeds of terrestrial plants, bacteria and many fungal species belonging to the genus *Aspergillus*, are commonly found in nature [10]. However, the xylanase enzymes of filamentous fungi are potentially interesting for industrial use because they are produced in greater amounts compared to those of yeast and bacteria [11]; in addition, the use of filamentous fungi has many advantages, because these are recognized as "generally safe" and considered an excellent source of extracellular enzymes, mainly due to its high biochemical diversity [12]. These fungi can be isolated from natural resources such as soil, water, plants, among others, of different biomes, such as the Caatinga, which is exclusively Brazilian biome, little explored, and so with great biotechnological potential to be discovered.

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Therefore, the aim of this work was to produce and partially characterize the xylanase produced by *Aspergillus parasiticus* URM 5963, a filamentous fungus isolated from soil of Caatinga, in relation to their properties for industrial applications, especially industrial animal feed.

MATERIAL AND METHODS

Microorganism

Aspergillus parasiticus URM 5963 was obtained from the URM collection at the Department of Mycology, Federal University of Pernambuco, Brazil, and it was maintained in tubes containing potato dextrose agar (PDA). For sporulation, the fungus was grown in Petri dishes containing Czapek agar for 5 days at 28ºC.

Production in flasks

Xylanase was produced using Erlenmeyer flasks of 125 mL, containing yeast extract (0.5%), cassava bark (2%), (0,5%) dextrose and 50 mL water. The inoculum was prepared at a concentration of 10^6 spores mL^{-1}, and the fermentation was maintained at 26°C and 130 rpm for up to 72 h.

Xylanase pre-purification

Xylanase was pre-purified by acetone precipitation by addition of solvent to the crude extract at a ratio of 30% enzyme and 70% of acetone; the material was then centrifuged at 5,000 rpm for 20 minutes at 4°C. The precipitate was dissolved in 50 mM acetate buffer, pH 5. Subsequently, determinations of total protein and enzyme activity were measured and the precipitated enzyme was used for the other procedures.

Determination of the total protein content in the enzyme extract

The Bradford method was used to measure the total protein content [13]. The absorbance of the solutions was measured at 595 nm using a spectrophotometer (Biochrom Libra S6®, Cambridge, UK).

Enzyme assay

Xylanase activity was determined according to the method described by Bailey et al. [14], and the free sugar released was analyzed by the di-nitro-salicylic acid method, as described by Miller [15]. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugars (measured as respective monosaccharide) per minute by hydrolyzing the respective crude substrate under the specified assay conditions.

Effect of pH on enzyme activity and stability

The optimum pH for the enzymatic activity of xylanase was determined using different buffers at 50 mM: acetate buffer (pH 4.2–6.0), phosphate buffer (pH 6.0–7.0) and Tris-HCl buffer (pH 6.5–8.0). The pH stability was determined by incubating the reaction mixture in the same buffers. Aliquots were taken at 0, 60, 120 and 180 minutes to determine the xylanase activity at each time point. Enzyme activity was measured as described previously.

Effect of temperature on enzyme activity and stability

Optimum temperature was determined by measuring the xylanase activity of the crude extract at different temperatures in the range of 30-80°C. To determine heat stability, the enzyme was subjected to same temperatures of optimum temperature. Aliquots were taken at 0, 60, 120 and 180 minutes to determine their specific activities. Samples from each time point were subjected to enzyme activity measured as described previously.

Effect of the activation or inhibition of metal ions on xylanase activity

The following ions were tested at concentrations of 5 and 10 mM: Cu^{2+}, Mn^{2+}, Na+, Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, K^+ and Na^+. The degree of inhibition or activation of xylanase was evaluated by subjecting the enzyme extract to the ions and inhibitors for 30 minutes, followed by measurement of xylanase activity.

Kinetic parameters

For kinetic parameters, five different concentrations of beech wood xylan (4-12 $g.L^{-1}$) with constant enzyme concentration were prepared. Assays were performed under standard assay. The kinetic parameters (apparent K_m and V_{max}) were calculated were determined from a Lineweaver–Burk plot.

Effect of simulated upper monogastric digestive tract conditions on xylanase activity

Simulations of monogastric digestion were determined *in vitro*, as described by Boyce and Walsh [16]. Intestinal digestion of the test compound was simulated by adding trypsin and 1% taurocholic acid for 4 h.

Polyacrylamide gel electrophoresis and zymogram

Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [17], using gel

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concentrations of 4.9% and 15.4% to achieve separation. Samples were generated in the fermentation bioreactor in conditions that produced the best yield and fermentation at concentrations of 100 and 200 mg.mL^{-1} protein. The molecular mass of the bands was determined using LabImage 1D software (Loccus Biotechnology, Brazil). The zymogram was performed in a polyacrylamide gel (15.4%), according to the method described by Mattéotti et al. [18].

Statistical analysis

Statistica Version 7.0 (Statsoft, Tulsa, OK) software was used for graphical analyses of the data. All experiments were performed in triplicate, and the results are expressed as the mean ± standard deviation (SD). Effects of the variables and the significance of regression coefficients were determined by Student's t test ($p<0.05$) [19].

RESULTS AND DISCUSSION

The xylanase activity from *Aspergillus parasiticus* URM 5963 was investigated at different pH values using birchwood xylan as substrate. Our results show that enzyme remains active at a pH range varying from 4.2 to 8.0 with a maximal activity at pH 5.0 **(Figure 1).** The effect of pH on the stability of xylanase was studied. Xylanase production from *A. parasiticus* URM 5963 increased significantly with increasing the time, the residual activity maximum value (163,4%) was obtained at after 180 min-incubation at pH 6.0 **(Figure 2).**

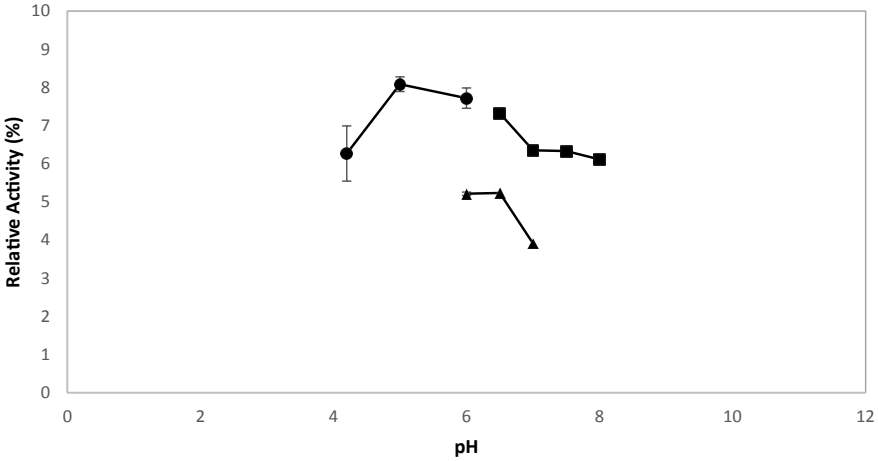

Figure 1. Curve of the optimum pH of the xylanase produced by *Aspergillus parasiticus* URM 5963. (●) acetate - Na buffer (pH 4.2. 5.0 and 6.0), (▲) fosfate - Na buffer (pH 6.0, 6.5 and 7.0) and (■) Tris-HCl (pH 6.5, 7.0, 7.5 and 8.0).

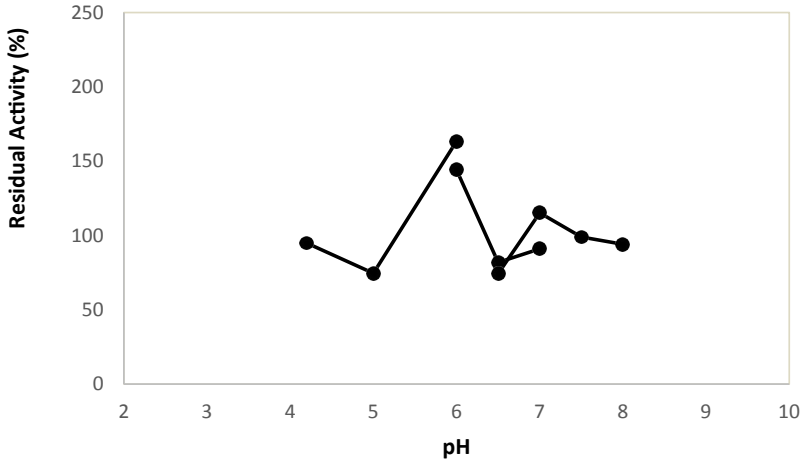

Figure 2. pH-stability curve of the xylanase produced by *Aspergillus tamarii* URM 4634 after 180 minutes. (●) acetate - Na buffer (pH 4.2, 5.0 and 6.0), (▲) fosfate - Na buffer (pH 6.0, 6.5 and 7.0) and (■) Tris-HCl (pH 6.5, 7.0, 7.5 and 8, 0).

The xylanase activity was also determined at different temperatures under standard assay conditions **(Figure 3).** The results show that the xilanase activity increased with increasing the temperature to reach its maximum value at 30–60°C thereafter fell sharply at 70°C and 80°C. The thermostability was determined by measuring the residual activity after incubation of the pure enzyme at various temperatures. The enzymes retained its full activity after 180 min-incubation at 50°C.

The majority of fungal xylanases reported to date are optimally active in acid pH (pH 4.5–6.5) or neutral pH range [20-22]. Usually, the optimal temperatures for most xylanases from fungi are typically between 45 and 60°C [23-26].

The effect of metal ions on enzyme activity is shown in **Table 1.** At trial using ion concentration to 5 mM, only Ca^{2+} ion did not affect the enzymatic activity. Silva et al. [21], working with xylanase produced by *Aspergillus tamarii* URM 4634, reported that

the Ca^{2+} ion did not affect the enzyme activity at 1 mM (101.1%), however when analyzed at 5 mM, the residual activity was 93.1 ± 0.07% to 6.9% inhibition. The 10 mM ions which most affected the enzymatic activity were Fe^{3+} and Mg^{2+}, in which the enzyme retained 85.52 ± 0.16 and 86.93 ± 0.28 residual activities, respectively. The highest value found for inhibition in all assays (5 and 10 mM) was 15.74%, in which the enzyme remained 84.26 ± 0.00% residual activity. Working with *Aspergillus niger* US368, Hmida-Sayari et al. [19] found to inhibition of 20 ± 0.03% when subjected to the enzyme Mn^{2+} ion. This result shows that the xylanase from *Aspergillus parasiticus* 5963 URM has potential use in diets for monogastric animals, especially with regard to mineral supplementation treatment is carried out in animal production.

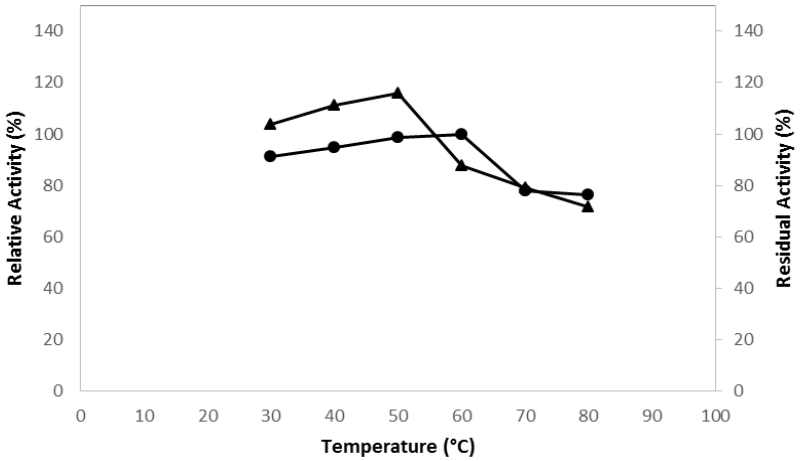

Figure 3. Curve of the optimum temperature of xylanase produced by *Aspergillus tamarii* URM 4634 (●). Curve temperature stability of the xylanase produced by *Aspergillus tamarii* URM 4634 after 180 minutes (▲).

Table 1. Effect of the activation or inhibition of metal ions on xylanase activity.

Metal ions	(5 mM)	Residual Activity (%) Inhibition (%)	(10 mM)	Inhibition (%)
*Controle+	100	---	100	---
Fe^{3+}	98,52 ± 0.04	1.48	85.52 ± 0.16	14.48
Zn^{2+}	90.09 ± 0.16	9.91	99.02 ± 0.04	0.98
Mg^{2+}	84.26 ± 0.00	15.74	86.93 ± 0.28	13.07
K^{+}	93.67 ± 0.01	6.33	100.14 ± 0.07	0
Na^{+}	92.69 ± 0.06	7.31	96.91 ± 0.15	3.09
Mn^{2+}	99.71 ± 0.08	0.29	188.93 ± 0.00	0
Ca^{2+}	107.73 ± 0.13	0	89.18 ± 0.19	10.82

The kinetic constants of xylanase produced by *Aspergillus parasiticus* URM 5963 were 1.5 $g.L^{-1}$ (K_m) and 9.19 U mL^{-1} (V_{max}); according this values, when compared with other fungal species K_m, is noted that this xylanase can be considered more specific for substrate utilized once the value K_m is lower: *Aspergillus niger* GH10 (2.43 $g.L^{-1}$) (Chantasingh et al.) [27] and *Aspergillus niger* DSM 1957 (25 $g.L^{-1}$) (He et al.) but less specific than xylanase produced by *Aspergillus tamarii* URM 4634 (0.464) (Silva et al.). The V_{max} value for xylanse from *Aspergillus parasiticus* URM 5963 (9.19 $U.mL^{-1}$) was lower than that obtained for *Aspergillus niger* DSM 1957 (5000 µmol/min/mg) (He et al.) and *Aspergillus tamarii* URM 4634 (63.39 U mL^{-1}) [21,28].

The xylanase produced in this study behaved positively in the presence of digestive enzymes from monogastric animals *in vitro*. The lowest enzyme activity was approximately 37.75% in gastric conditions (pepsin+HCl), and its activity was above 6% in response to intestinal activity (63.5% at pancreatin and bile extract assay and 81.7% at trypsin and taurocholic acid). When complete digestion was simulated, the xylanase activity was 194.7%, the better result. The xylanase produced by *Aspergillus tamarii* URM 4634 [21] obtained a better performance in gastric conditions with 65% of enzyme activity, but in the instestinal and complete digestion its performance was lowest (above 70% and 106%, respectively) than xylanase produced by *Aspergillus parasiticus* URM 5963.The xylanase produced by the same microrganism (*Aspergillus tamarii* URM 4634) but in different conditions was more sensitive to the conditions of monogastric digestion getting lower activities in the simulation (pepsin-22%; pancreatin+bile extract 29%; tripsin+taurocholic acid–44.53%) [29]. These results suggest that the enzyme retains its activity or is potentiated in the presence of digestive enzymes from monogastric animals, and *in vivo*, the presence of digestive components can improve the performance of the enzyme [16].

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