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Research Article

Xylan extraction of corn cob and enzymatic hydrolysis by xylanase from *Aspergillus labruscus* ITAL 22.223

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Abstract

The agriculture is one the most important economic activity in Brazil and the crop processing can generates great quantities of residues. The corn cob is an agro-residue that presents xylan. This polymer can be submitted to the enzymatic hydrolysis for obtainment of monomers to be fermented for obtainment of added value bioproducts asxylitol and ethanol. Accordingly, this study aimed the production of the xylanolytic enzyme by the fungus *Aspergillus labruscus* ITAL 22.223, and the enzyme application for the hydrolysis of the xylan extracted from the corn cob.Two xylan fractions were obtained, AST and BST, with yields of 10.8% (w/w) and 8.9% (w/w), respectively, using NaOH solution. The FTIR analysis revealed similar peaks as observed for arabinoxylans for both AST and BST xylans.The protein and phenol contents were 0.44 and 0.08 for AST, and 0.43 and 0.09 for BST, respectively. The xylanase produced by *A. labruscus* hydrolyzed both xylan fractions AST and BST, as well as its combination (AST + BST = ABST). The best temperature of activity varied from 45 to 75ºC according to the substrate used, and the T_{50} was 24h for 40-45ºC. The pH range of 3.5-5.5 was the best for the xylanase activity, with T_{50} of 1h for the pH range 3-5.Considering the commercial xylan as substrate and enzyme activity was sustained above 80% for all the time evaluated.The TLC profile revealed the presence of xylose, xylobiose and xylotriose as hydrolysis products from the ABST xylan. In conclusion, the extraction of the corn cob xylan was efficient, providing a substrate in which characteristics of temperature, pH and stability of the xylanase activityare attractive for the future application for obtainment of xylose that can be used in the production of xylitol and ethanol.

Keywords: corn cob; xylan; hydrolysis; fermentation; enzyme; *Aspergillus*.

Introduction

The global consume of different products around the world such as energy, food and pharmaceutical, among others, has intensified over the years. Brazil is

recognized as an important country with intense agricultural activity, producing high quantities of soybean, sugarcane, and corn. The 2021/22 Brazilian crop produced 128.5 million tons of soybean, 525 million tons of sugarcane and 110.9 million tons of corn[6, 7]. The processing of agro-products can generate residues that can be accumulated in theenvironment, causing severe problems as pollution and contamination [22]. The corn cob is anagro-residue from the corn processing with high added value, but little used.It is basically constituted by cellulose, pectin, lignin and hemicellulose, a polysaccharide characterized by water insolubility, but soluble in alkaline solution[28]. In general, the hemicellulose is constituted by repeated units of pentoses (D-Xylose and L-Arabinose), hexoses (D-Galactose, D-Glucose and D-Mannose) and uronic acids. In the specific case of corn cob, the xylans have chemical structure formed by 4-O-methyl-D-glucuronic acid, L-Arabinose and D-xylose at 2:7:1 proportion [28]. Oligomers of D-xylose are known as xylooligosaccharides (XOS)[19].

The hydrolysis of xylans is an important strategy to obtain fermentable sugar and XOS. For this purpose, hemicellulotic enzymes, as β-xylosidase (EC 3.2.1.37), endoxylanase (EC 3.2.1.8), α-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl-xylanesterases (EC 3.1.1.72) and p-coumaric estereases (EC 3.2.1.-), can be applied[16, 33]. These enzymes can be obtained from different sources, especially from microorganisms as filamentous fungi. The *Aspergillus* is an interesting genus with potential to produce and secrete cellulolytic and hemicellulolytic enzymes, such as *A. niger*, *A. foetidus*, *A. brasilienses*, *A. flavus*, *A. nidulans*, *A. terreus*, *A. tubingensis* and *A. labruscus*[2, 13, 15, 23, 30].

The enzymatic hydrolysis of corn cob, using fungal xylanases, allows the use of the sugars in the fermentative process to obtain bioproducts with high added value, as xylitol, used in the food and pharmaceutical industry, and ethanol. For xylitol production, the xylose can be fermented by yeasts as*Candida* species.For example, the *C. tropicalis* producedxylitol from the hydrolysates of agricultural residues asbanana leaves, chestnut shells, corncob, olive pruning, rice straw, sugarcane bagasse, water hyacinth leaves and cotton stalk[32]. According to this context, this manuscript describes the use of the corn cob xylan for the obtainment of xylose through enzymatic hydrolysis using xylanase produced by *A. labruscus* cultured under Solid State Fermentation using wheat bran as substrate.

Material and Methods

Extraction of the corn cob xylan and FTIR analysis

The corn cob, obtained through a small producer, was washed with distilled water for impurities removal and dried in air circulation oven at 100º C for 24h and, milled in knife mill type Willey from Solab® model SL-31 with 7 mesh strainers. The xylan extraction was conducted as described by Silva [28]. The crushed corn cob (CC) was added with distilled water at 1:32.5 (w/v) proportion, stirred for 18h at 24ºCandgauze filtered with aid of a vacuum pump. The filtrate was discarded and the retained material in the filter was added with 4% NaOH solution at 1:20 (w/v) proportion, stirred for 5h at 24ºCand submitted to the filtration process as described above. After filtration, glacial acetic acid (CH_3COOH) was added to the filtrate until reaching pH 7, and the precipitate formation was observed. The retained solid material on the filter was discarded.The solution was centrifuged at 9,681 x *g* for 10 min and the resulting pellet was named “xylan A” (AST). The supernatant was precipitated with addition of ethyl alcohol at 1:3 (v/v) proportion, left undisturbed for 15 min at 24°C and centrifuged at 9,681 x *g* for 10 min. The

resulting pellet was named "xylan B" (BST).

Xylanrich fractions extracted from corn cob and the"Birchwood" xylan (Sigma®), were analyzed using infrared absorption spectra by attenuated total reflection (ATR), with resolution of 4 cm^{-1}, time scan of 64 scans and wavelength from 4000 cm^{-1} to 400 cm^{-1}.

Protein and Phenol quantification

The protein quantification for the extracted xylans (AST and BST) and for the enzymatic extract was performed as described by Bradford[4] using BSA as standard. The protein concentration was expressed as mg of protein per mL of sample.The phenol quantification wasdone as described by Rao et al. [24] using analytical curve of gallic acid and phenol concentration was expressed as µg of phenol per mL of sample.

Microorganism,culture conditions and purified xylanase

The filamentous fungus *Aspergillus labruscus* ITAL 22.223 was maintained on potato dextrose agar (PDA) slants at 4ºC in a refrigerator until the use. The spore suspension was obtained by addition of 10 mL distilled sterile water and scraping the fungal growth with platinum handle. The spore count was done using the TC-20 automatic counter from Biorad®

The solid-state fermentation (SSF) cultures were prepared in 125 mL Enlenmeyer flasks containing 5 grams of wheat bran moistened with tap water at 1:2 (w/v) proportion.The culture media were autoclaved at 1.5 atm for 30 min at 120ºC and subsequently inoculated with spores suspension at 10^7 spores/mL. The cultures were kept in oven at 25ºC for 7 days for enzymatic extract production. The *Bacillus subtilis*purified xylanase was used only for comparative purposes in the hydrolysis of extracted xylans, ensuring that the method for xylan extraction was effective.

Obtainment of the enzymatic extract and precipitation procedure

After cultivation, the cultures were added with 50 mL ofcold distilled water (4ºC) and stirred in orbital shaker at 200 rpm for 30 min at 4ºC. Thereafter, the material was filtered in gauze and filter paper with aid of a vacuum pump,and the filtrate was used as source of xylanolitic enzyme and the retained material on the filter was discarded. The filtrate was saturated with 60% (w/v) of ammonium sulfate and kept under gentle agitation at 4ºC for 15h. Thereafter, the solution was subjected to centrifugation for 10 min at 9,681 x*g*. The pellet was suspended in distilled water and dialyzed against distilled water for 24h at 4ºC to recover the xylanase. The xylanase precipitated was used for xylan hydrolysis and of temperature and pH evaluation of activity.

Determination of the xylanase activity

The xylanase activity was determined by the method of reducing sugars [20]using the DNS, with modification, using 1% (w/v) commercial xylan (Birchwood, Sigma),1% and 5% (w/v) corn cob xylans (AST and BST) as substrates, separately, and 5% (w/v) of ABST (in proportion 1:1 of AST and BST) in 50mM sodium acetate buffer, pH 5.5, maintained in thermostatic bath at 55ºC for 5 min. After reaction, the absorbance was acquired at 540 nm. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 µmol of reducing sugar per minute under test conditions.

Evaluation of the products of hydrolysis using Thin Layer Chromatography (TLC)

The qualitative analysis of hydrolysis product of AST and BST xylans submitted to the enzymatic reaction in pH 5,0, temperature of 55ºC for 5 min, with *A. labruscus* xylanase was performed by thin layer chromatography (TLC), using as mobile phase, butanol:ethanol:destilled

water (5:3:2 v/v/v) solution. After the run, the spots were revealed spraying 0.2% orcinal in sulfuric acid:methanol (1:9 v/v) solution on the plate and maintaining at 100ºC until the spots appear. The 0.1 mg/mL standards used were xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose, glucose and cellobiose. The samples were enzymatic extract, commercial xylan, hydrolyzed commercial xylan, ABST xylan and hydrolyzed ABST xylan.

Influence of temperature and pH on enzyme activity

The influence of the temperature on enzyme activity was analyzed carried out the enzymatic reaction from 40ºC to 80ºC. The thermostability was assessed with the incubation of the xylanase samples in aqueous solution at different temperatures (45 to 65ºC) for different periods (20 min to 24h) and used for enzymatic reaction as described above.

Considering the influence of the pH on enzyme activity, the enzymatic reaction was carried out at different pH values (3 to 7.5) using 50mM phosphate citrate buffer, at optimal temperature [17]. For pH stability determination, xylanase samples were incubated at different pH values (3 to 7)50mM phosphate citrate buffer, at optimal temperature[17]. Aliquots were taken at 1h, 3h and 24h for enzymatic activity determination, with optimal temperature and pH.

Results and discussion

Extraction of corn cob xylan and FTIR characterization

The extraction procedure of the xylan from the corn cob with 7 mesh granulometry allowed the obtainment of the two xylan fractions, the AST with yield of 10.8% (w/w) and BST with yield of 8.8% (w/w) in relation to raw material.Using the same methodology, Silva et al. (1998) extracted xylan from corn cob (20 mesh granulometry) with yields of 4.94% (w/w) for xylan A and 14.84% (w/w) for xylan B in relation to raw material. The yield obtained for AST xylan was 2.1 times higher than that for xylan A, while for the BST xylan, it was 1.6 times lower than that observed for xylan B. Very small particles tend to form compact layer of dust, which makes it difficult for the solvent to penetrate [31], which explains the higher yields obtained when compared to study by Silva et al. (1998). Ebringerová [10] obtained total yield of 13.5% (w/w) considering both xylan fractions, value lower than that observed for AST and BST (19.7% w/w).

The Figure 1 depictures the FTIR spectra for both AST and BST xylans, and for the commercial xylan. In general, it was observed similarities among the peaks obtained for xylans extracted from the corn cob and birchwood xylan. An intense peak at 1039 cm^{-1}, observed for commercial xylan, refers to thevibration of the glycosidic ring, angular deformation C-O-H, axial deformation C-O and C-O-C (Fig. 1A), as observed for arabinoxylans [28]. Similar peak was observed for the spectra of the AST (1043 cm^{-1}) (Fig. 1B) and BST (1043 cm^{-1}) (Fig. 1C). It was also observed peaks around 1569 cm^{-1} and 1552 cm^{-1} for AST and BST xylans, respectively, which can be attributed to residual lignin in polysaccharide (axial deformation C=C of aromatic ring), which would be attached by physical or chemical bonds[28]. Broad band centers around 3500 cm^{-1} can be attributed to axial deformation O-H, while band at 1627 cm^{-1} (Fig. 1A), refers to H-O-H angular deformation, characteristic of water. The presence of bands near to 1415 cm^{-1} as presented (1397 cm^{-1} and 1403 cm^{-1}) refer to the angular deformation of CH_2, a characteristic of the cellulose[2]. The band at 896 cm^{-1}observed for the commercial xylan (Fig. 1A) refers to the CH deformation and the bands at 643 cm^{-1} and 646 cm^{-1} (Fig. 1B and C) refer to the OH angular deformation [28].

Protein and phenol quantification of the xylans

The protein and phenol composition of the extracted and commercial xylansare shown in Table 1. The concentration of proteins in commercial xylan is lower than that observed for the extracted xylans, what can be justified by the degree of purity of commercial xylan compared to the AST and BST xylans. However, the degree of contamination by proteins in the extracted xylans can be considered low, corroborating the study of Melo-Silveira [18], which described composition of 0.4% of protein in the xylan extracted from corn cobs. The phenol concentration was low in all xylans analyzed, but 8-9folds higher in the xylans extracted from corn cob. Melo-Silveira [18] determined the concentration of total phenols in extracted xylan from corn cob as 0.01%.

Xylans	Protein (%)	Phenol (%)
birchwood	0.018*	0.014*
AST	0.40*	0.080*
BST	0.43*	0.090*

*standard deviation < 0,0001

Table 1. Protein and phenol composition of the commercial xylan and for the extracted (AST and BST) xylans from corn cob.

Enzymatic hydrolysisof the xylans

The filamentous fungus *A. labruscus* is an interesting producer of xylanases, as previously reported by Maestrello and Guimarães[16]. According to this, the potential of this xylanase to hydrolyze the corn cob xylans (AST and BST) was evaluated, as presented in the Figure 2. The *A. labruscus*xylanase in the crude extract obtained from theSSF cultivation using with wheat,hydrolyzed both AST and BST xylans, and its mixture (ABST) as well. At 1% (w/v) substrate, the high activity was obtained using BST xylan, differing from that observed for the use of 5% (w/v) substrate with similar activity for both AST and BST xylans. However, the best enzymatic activity was obtained using the mixture of both xylans (ABST) as substrate.The reduced activity verified for AST xylan (1%, w/v) probably can be explained by the presence of sodium acetate residue formed during the neutralization process [31]. Salt residues can modify the enzymatic activity. Ions can interact with the amino acid residues in the active site of the enzyme, influencing the catalytic reaction, modifying its structure and functionality. The cofactors ions are those that enzyme need to perform its catalytic function, undergoing positive modulation. However, there are ions that can inhibit catalytic function, undergoing negative modulation [9, 12, 16].

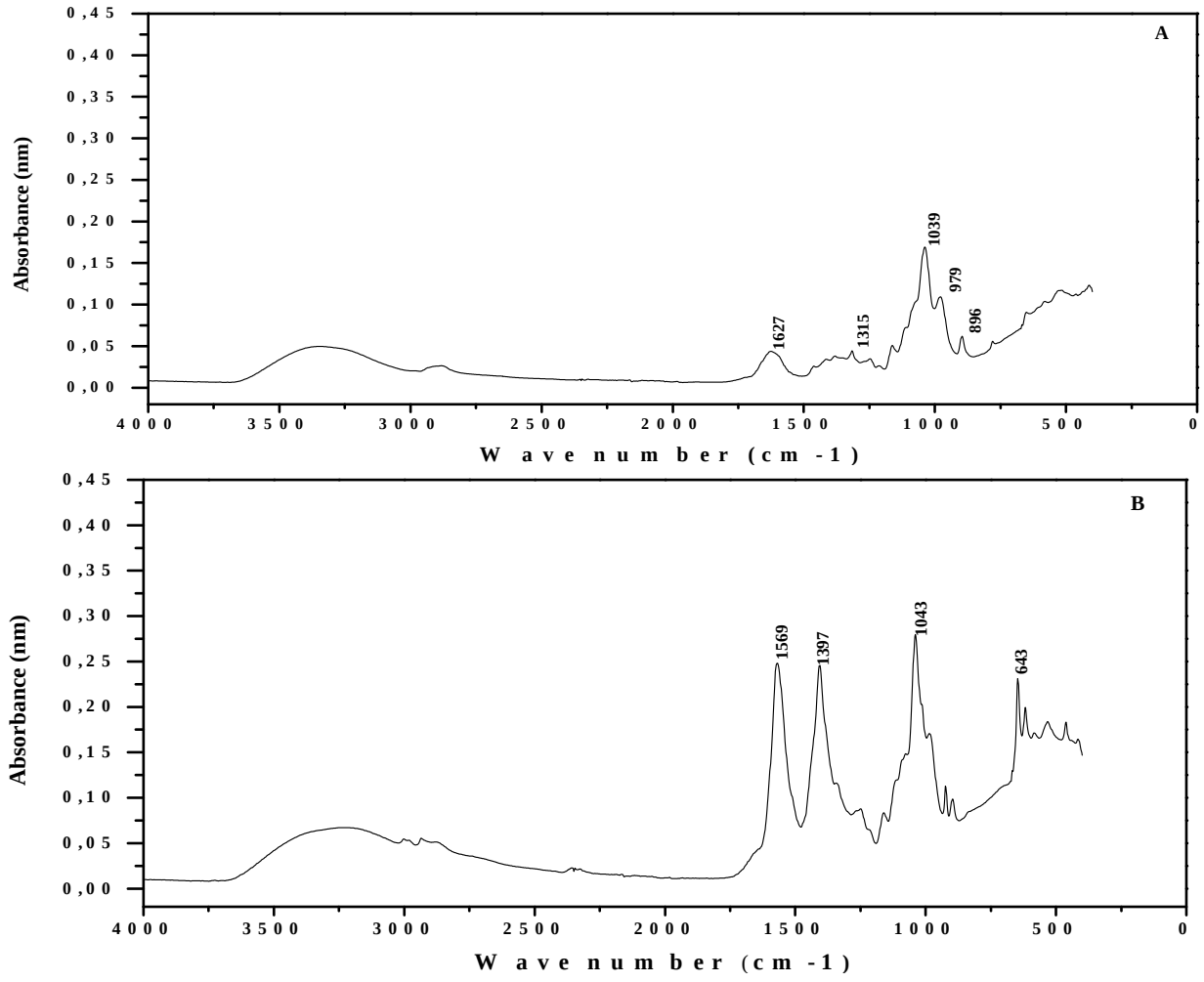
Considering the hydrolysis of the commercial and ABST xylans by the *A. labruscus* xylanases and *Bacillus subtilis*purified xylanases (Figure 2, insert), the former presented a hydrolytic potential of 71% for ABST compared to the commercial substrate, while for the latter this index was 83%. The hydrolysis values observed for both xylanases were similar. In spite of this, the difference can be justified by the purity degree of the enzymes since it was used pure *B. subtilis* xylanase and unpurified *A. labruscus* xylanase. In terms of xylanase activity, the enzyme produced by *A. labruscus* presented hydrolytic potential (23.1 U/g) close to those found in the literature for the same fungal genus, as reported by Zúñiga [34] for *A. niger* xylanase with activity of 23 U/g produced using wheat bran. The xylanase activities of 60 U/g and 32.7 U/g were observed for the enzymes produced by *A. oryzae*[33] and *A fumigatus* [26],respectively, when cultured in presence of wheat bran.

The thin layer chromatography (TLC) profile obtained for the hydrolysis products of the commercial and ABST xylans (Figure 3) revealed the presence of xylose, xylobiose and xylotriose for both. However, for the commercial xylan, xylotetraose, xylopentaose and xylohexaose were also

observed. Some intermediate spots were also observed for the ABST hydrolysis, what can correspond to the arabinoxylan. The presence of xylose is an important fact to be highlighted because this saccharide can be applied for the future fermentation aiming the production of xylitol.

Partial characterization of xylanase activity using different xylans as substrates

The influences of the temperature and pH on enzymatic activity of the *A. labruscus* xylanase using commercial xylan as substrate are presented in the Figure 5. The best enzyme activity was achieved at a temperature range from 55ºC to 75ºC (Fig. 4A), corroborating the observation of the Maestrello and Guimarães [16] for xylanase not precepted. Similarly, the enzyme produced by*A niveus*cultivated in SSF in the presence of wheat bran showed optimal activity from 55ºC to 65ºC[3], while for the *A. niger*xylanase, 55ºC was the best temperature for its activity[11], similar to that observed for the *A. versicolor* enzymewhen cultivated in Vogel medium supplemented with wheat bran[8]. The *A. labruscus* xylanase was stable at 45ºC for 120 min, with reduction of 45% of the activity with superior periods of incubation. At 50ºC the half-life(T_{50}) was 120 min. Considering the temperatures of 55-65ºC, the T_{50} was decreased to 18-20 min (Fig. 4C). The *A. casielus* xylanase was stable at 45ºC, losing only 18% of its activity after 90 min of incubation [14]. The enzyme produced by *A. fumigates* SK1 was stable at 40ºC, maintaining 80% of the enzymatic activity for 120 min. On the other hand, the *A. awamori* xylanase was stable between 30ºC and 40ºC, but above 90% of activity was maintained for 240 min [1].



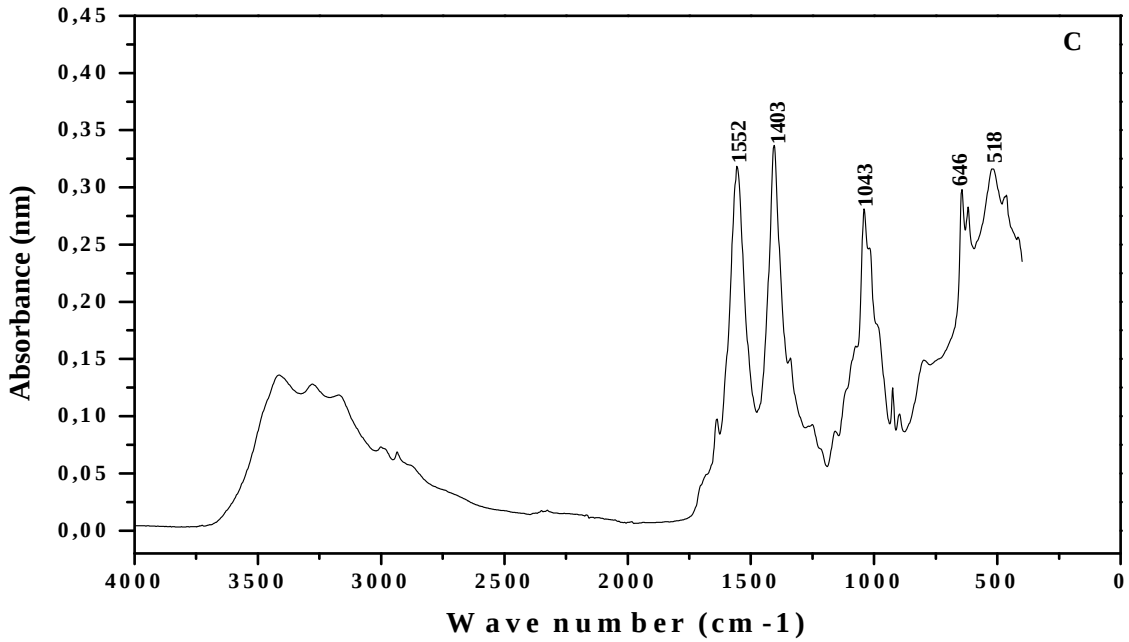


Figure 1. FTIR spectra for the birchwood xylan (A) and for the AST (B) and BST (C) xylans extracted from corn cob.

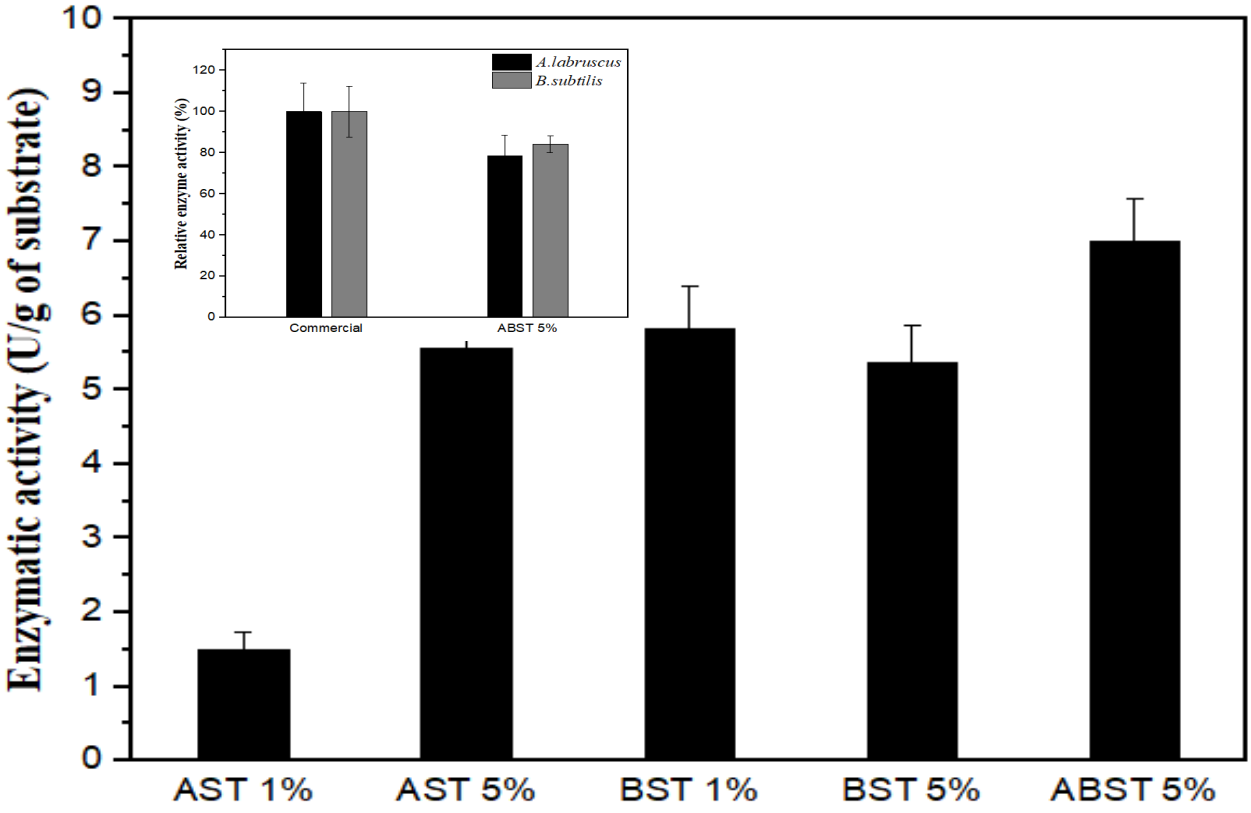


Figure 2. Hydrolysis of the corn cob xylans (AST and BST) separately and for the mixture (ABST) at different proportions (1% and 5%, w/v) using the *A. labruscus* xylanase. Insert: Comparative analysis of the enzymatic activity of the xylanases from *A. labruscus* and *B. subtilis* using commercial and ABST xylans.
Relative enzyme activities of the xylanase produced by *A. labruscus* and *B. subtilis* using commercial xylan (1% w/v) and extracted xylans (1% w/v) from the corn cob. The 100% correspond to 23.15 ± 3.18 U/g of substrate for*A. labruscus*and 3.44 ± 0.42 U/mLfor*B. subtilis* enzymes.

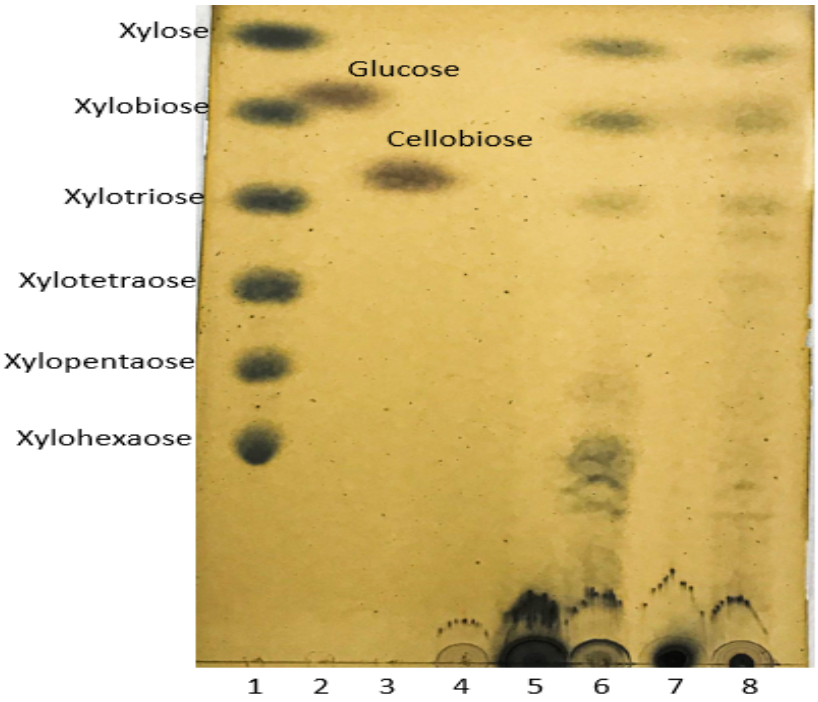
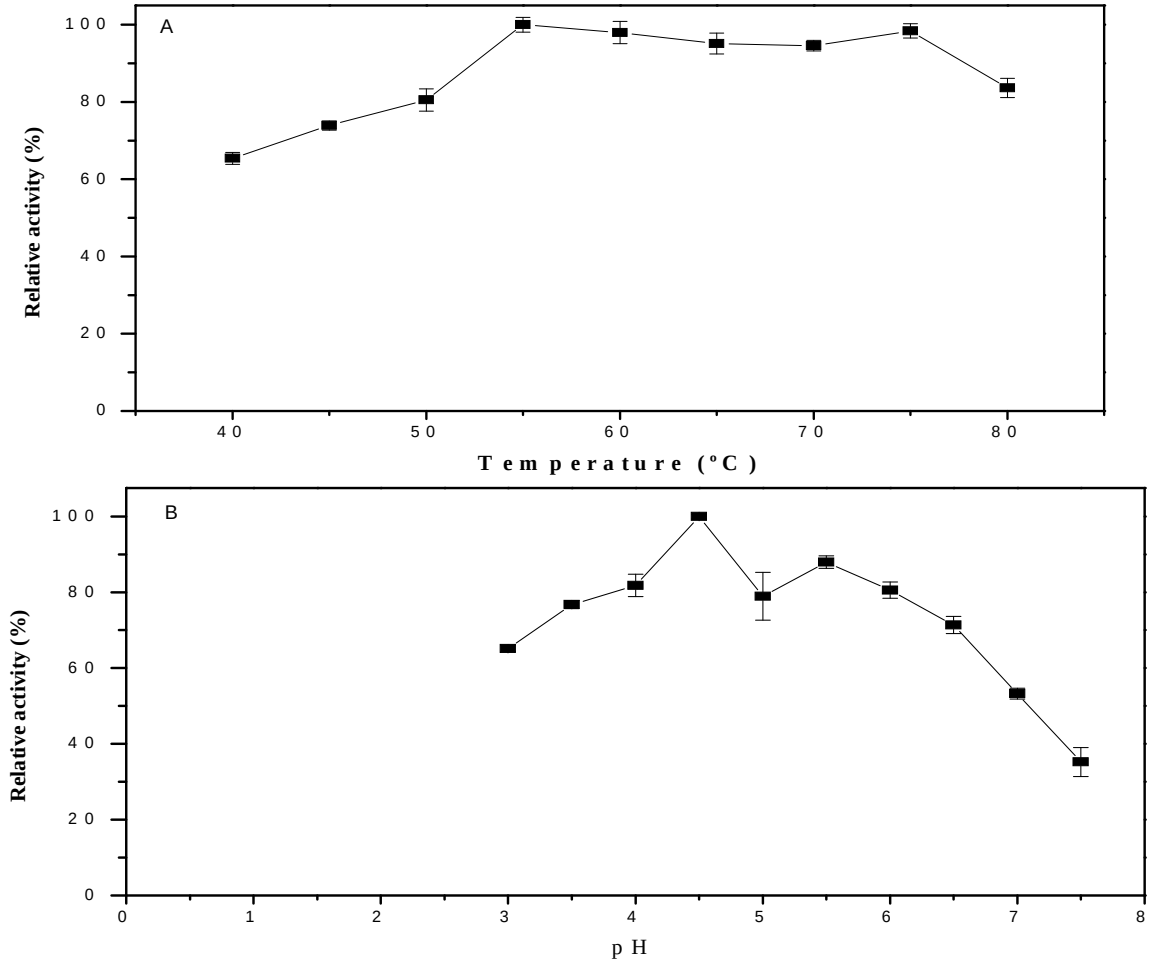


Figure 3. Thin Layer Chromatography (TLC) from hydrolysis of commercial and ABST xylans. Standards of xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose (1), Glucose (2) and Cellobiose (3); crude extract (4); commercial xylan (5); commercial xylan hidydolyzed (6); ABST xylan (7) and ABST xylan hydrolyzed (8).



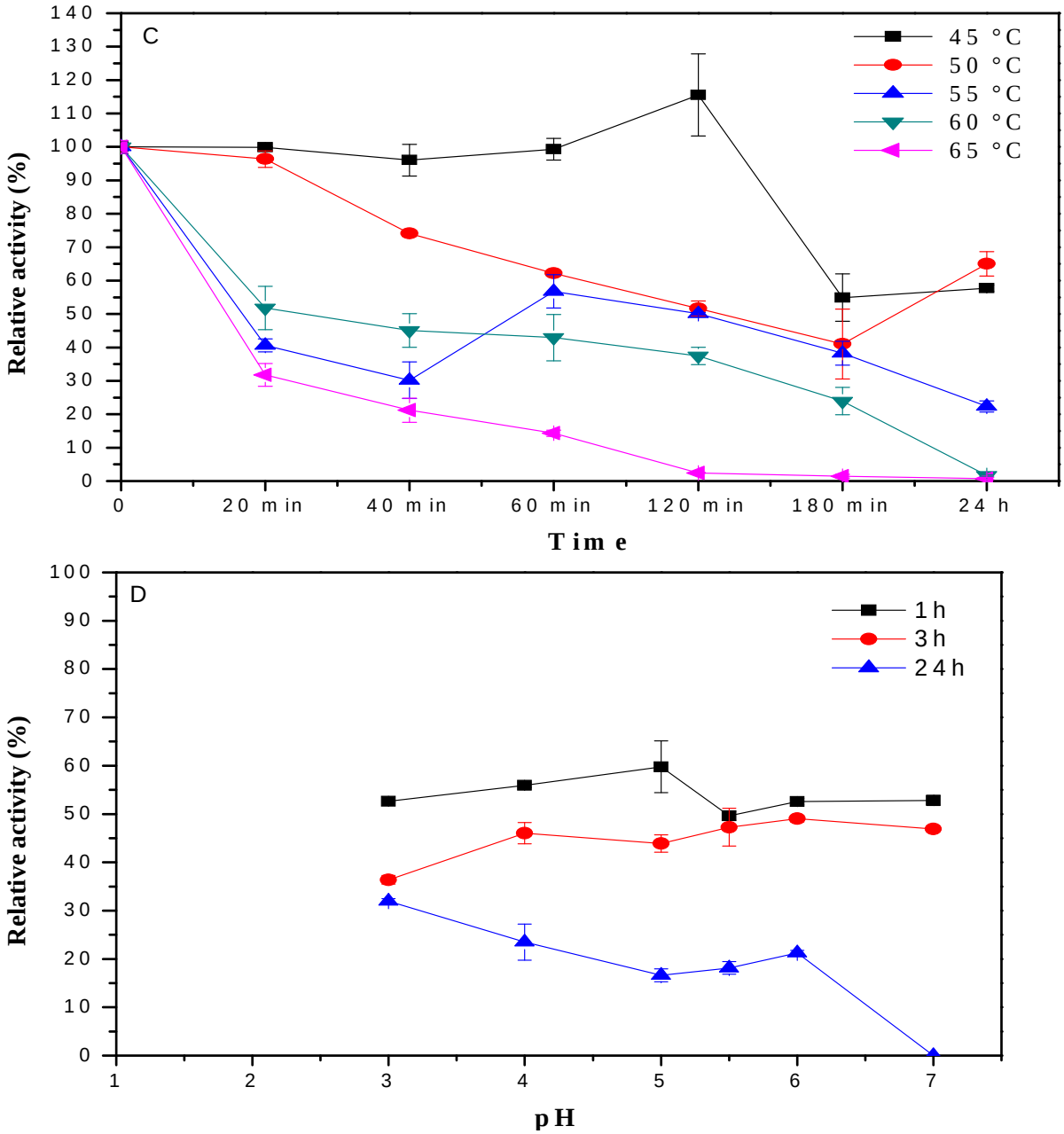
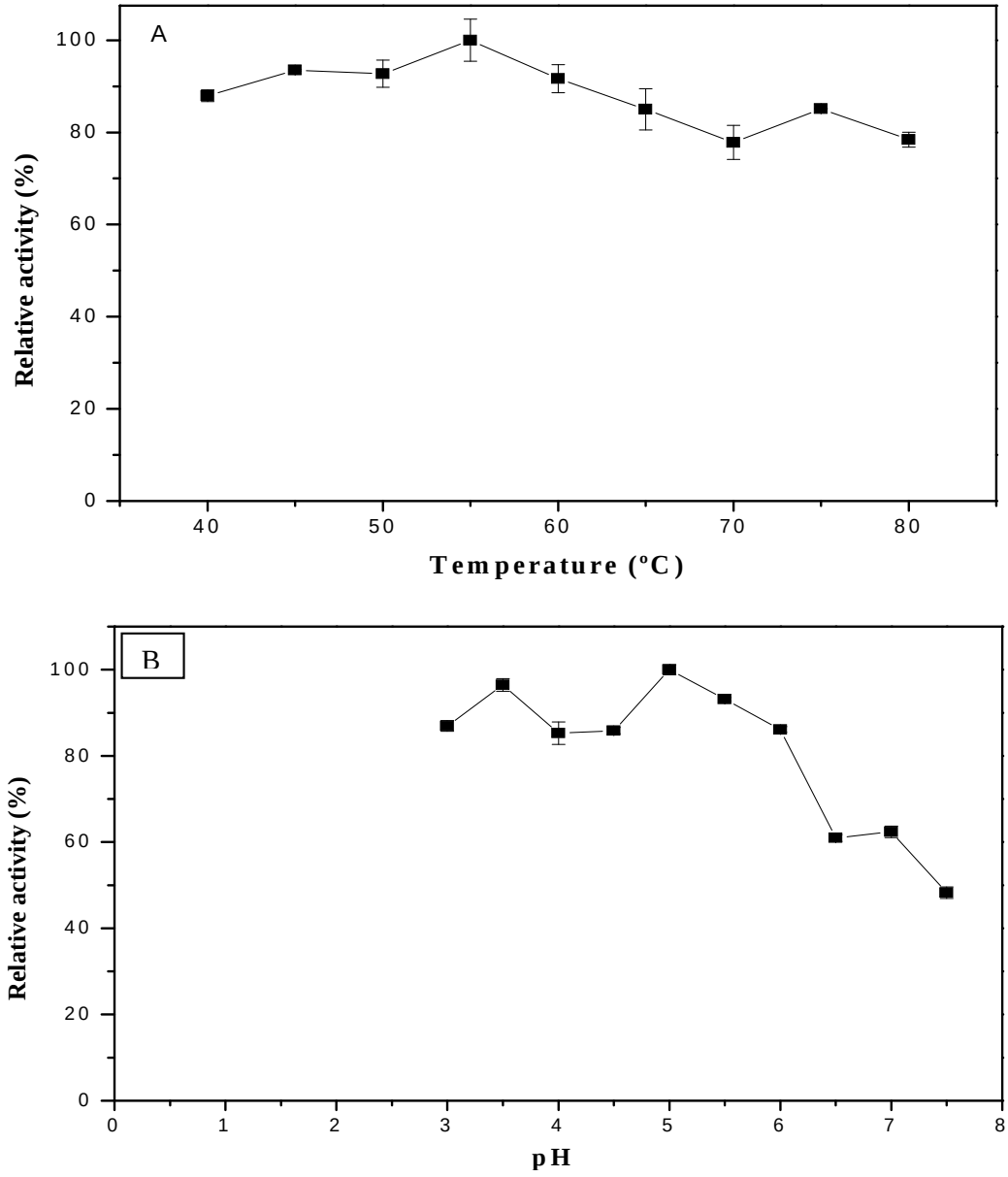


Figure 4. Influence of the temperature (A) and pH (B), thermostability (C) and pH stability (D) for the activity of the *A. labruscus* xylanase, using commercial xylan as substrate. The 100% correspond to 91.76 ± 1.76 U/g of substrate for A and C, 324.80 ± 1.93 for B and 190± 14.68 U/g of substrate for D, without enzyme incubation.

Regarding the influence of pH onenzyme's activity, it can be noted that the best performance was obtained at pH 4.5 (Fig. 4B), but another peak at pH 5.5 was also observed, as reported by Maestrello and Guimarães [16] for xylanase not precepted.These observations indicate the possibility of the existence of xylanase isoforms since several xylanases act synergistically for the full hydrolysis of the xylan [21]. The same was also reported by Moreira [21], where two xylanases of *A. terreus* showed best activity at pH 6.0 and pH 5.0.The best pH value for the activity of the *A. caespitosus*xylanase was 5.5 [25]. For the xylanase produced by *A. niveus*the best performance was obtained at pH 5.0-5.5, and 5.5-6.0 for the *A. niger*xylanase [3].When dealing pH stability (Fig. 4D), the enzyme activity was maintainedaround 50%

for all pH tested during the 1h of incubation. With 3h of incubation, the enzyme maintained its activity above 35% for all pH values. When incubated for 24h, the activity was maintained between 10% and 30% for the pH range from 3 to 6, but no activity was verified at pH 7. The *A. awamori* IOC-3914 xylanase was stable at pH 5-7 for up to 3h. According to Sandrim[25], the most xylanase from different *Aspergillus* species are stable between pH 4 and 6.

The influence of the temperature and pH on the enzyme activity was also analyzed using the ABST xylan as substrate (Fig.5). The best temperature condition for the enzymatic reaction was observed from 45ºC to 65ºC, as also observed for the use of birchwood xylan. Regarding the thermostability (Fig.5C), the T_{50} was 24 h at 45, 50 and 55ºC. At 60ºC, the activity was maintained above 40% for 180 min and when evaluated at 65ºC, the activity was maintained at 40% for 60 min. The thermostability of the enzyme in the presence of ABST xylan was lower when compared to that obtained in the presence of commercial xylan (Fig.5C).



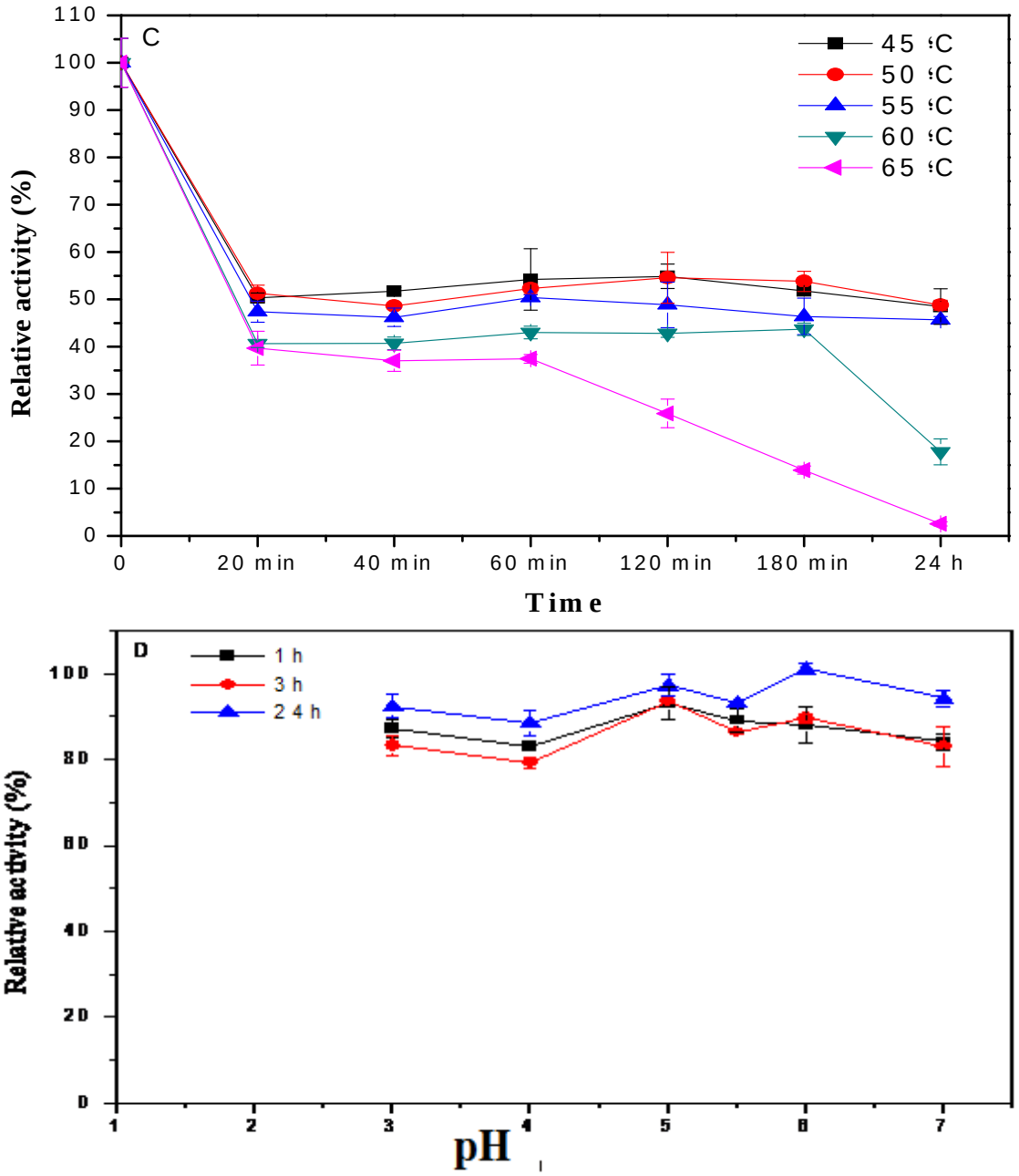


Figure 5. Influence of thetemperature (A) and pH (B), thermostability (C) and stability to pH (D) for the activity of the *A. labruscus* xylanase, using ABST xylan as substrate. The 100% represents 25.75 ± 1.17 U/g of substrate for A, 52.20 ± 0.61 U/g of substrate for B and 57.05 ± 2.26 U/g of substrate for C and D.

The high enzymatic activity of *A. labruscus* xylanase in presence of ABST as substrate was obtained at pH 5. However,anothersecondary enzymatic peak can be noted at pH 3.5, corroborating the results obtained with commercial xylan that indicate the possibility of at least two xylanase isoforms. The results concerning the temperature and pH ranges for *A. labruscus* enzyme agree with the data reported by Sandrim[25]for xylanase performance.For the pH stability analysis (Fig.5D), the enzyme activity was maintained above 80% throughout the incubation period for all analyzed pH values, differing from that observed for the commercial xylan (Fig. 5), Finally, the enzyme showed best stability in the presence of ABST when compared to presence of commercial xylan.

Conclusion

The xylan of corn cob was efficiently extracted using a simple methodology with good yields. The FTIR analyzes indicated that the xylan extraction method caused dragging of characteristic components of the lignocellulosic material, such as lignin and cellulose, which do not interfere in the future use of xylans.Regarding the enzymatic activities, the ABST substrate was the best for the enzyme activity. The *Aspergillus labruscus* xylanase presented interesting characteristics concerning the temperature and pH of activity and stability, favoring its future application in diverse industrial areas as for obtainment of xylose to be used in the fermentative process for the production of xylitol and ethanol.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Attenuated total reflection (ATR)
“xylan A” (AST)
“xylan B” (BST)
Xylooligosaccharides (XOS)
Thin Layer Chromatography (TLC)
Fourier Transform Infrared Spectroscopy (FTIR)

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