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Article

Use of Residual Biomass from the Textile Industry as Carbon Source for Production of a Low-Molecular-Weight Xylanase from *Aspergillus oryzae*

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Abstract: Pretreated dirty cotton residue (PDCR) from the textile industry was used as an alternative carbon source for the submerged cultivation of *Aspergillus oryzae* and the production of xylanases. The filtered culture supernatant was fractionated by ultrafiltration followed by three chromatographic steps, which resulted in the isolation of a homogeneous low-molecular-weight xylanase (Xyl-O1) with a mass of 21.5 kDa as determined by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE) co-polymerized with 0.1% oat spelt xylan. Enzyme catalysis was the most efficient at 50 °C and pH 6.0. The K_m values ($mg \cdot mL^{-1}$) for the soluble fraction of oat spelt and birchwood xylans were 10.05 and 3.34, respectively. Xyl-O1 was more stable in the presence of 5,5-dithio-bis-(2-

nitrobenzoic acid) (DTNB), 1,4-dithiothreitol (DTT), L-cysteine or β-mercaptoethanol, which increased the rate of catalysis by 40%, 14%, 40% or 37%, respectively. The enzyme stability was improved at pH 7.0 in the presence of 20 mM L-cysteine, with the retention of nearly 100% of the activity after 6 h at 50 °C. Xyl-O1 catalyzed the cleavage of internal β-1,4 linkages of the soluble substrates containing D-xylose residues, with a maximum efficiency of 33% for the hydrolysis of birchwood xylan after 12 h of incubation. Identification of the hydrolysis products by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) indicated the predominance of the hydrolysis products X2-X6 during the first 12 h of incubation and the accumulation of higher xylooligomers after the elution of the last xylooligomer standard, xylohexaose.

Keywords: agro-industrial residues; hemicellulose; enzyme purification; xylanase; hydrolysis

1. Introduction

Agricultural and forestry residues, municipal solid waste, industrial processing residues and energy crops are readily available, low-cost sources of large quantities of holocellulose materials that can be used to obtain chemical feedstocks, biofuels, foods and feeds [1,2]. Dirty cotton residue is produced during cotton processing and when harvested cotton is brought to spinners where it is processed into yarn for weaving. The bales that are unsuitable for yarn processing comprise cotton powder, brittle fibers and large quantities of cotton plant waste, such as stalks, stems, leaves and seed hulls, and are referred to as dirty cotton residue. This dirty cotton residue is marketed to re-processing industries to produce new, second-quality cotton fibers and filter cotton powder [3].

Biotechnological use of lignocellulosic residues, including dirty cotton residue, requires the action of enzymes that degrade cellulose, hemicellulose and pectin [4,5]. The enzymes that catalyze these reactions are generally obtained from microorganisms that contain synergistic, multi-catalyst systems [6]. The utilization of abundant, low-cost agricultural by-products as substrates for xylanase production substantially reduces enzyme production costs [7].

β-Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) is a representative of an important group of endo-glycosyl hydrolases. It catalyzes the breakdown of β-1,4 glycosidic bonds in xylan, a linear polymer of β-D-xylopyranosyl units [8], to yield short xylooligomers [9]. Xylooligomers are further hydrolyzed into single xylose units by side-chain enzymes [10] that are essential for the efficient breakdown of the second most abundant natural polysaccharide in hardwoods and softwoods, [11]. The xylanases of microorganisms have a number of major biotechnological applications, including clarifying fruit juices and wines, food processing in combination with cellulases, improvement of the nutritional properties of agricultural silage, grain feed and poultry feed, degumming of plant fibers, and bio-pulping [7]. The use of low-molecular-weight xylanases in prebleaching of cellulose pulp has become an alternative approach in eliminating chlorine in bleaching and reducing chlorinated organic compounds in bleach plant effluents. It is worth stressing that their small size may represent an

advantage for the biobleaching application once the effects of enzymes on pulps depend largely on their penetration into the fibre wall of cellulose pulp.

Numerous reports describe the use of filamentous fungi, primarily those of the *Aspergillus* genus, in the production of xylanases. Among these fungi, *Aspergillus oryzae*, the so-called koji mold, has been used extensively in traditional Japanese fermentation products, such as sake (rice wine), shoyu (soy sauce), and miso (soybean paste), for more than 1000 years [12]. The long history of *A. oryzae* in the food fermentation industries prompted the United States Food and Drug Administration (FDA) to include *A. oryzae* in the list of Generally Recognized as Safe (GRAS) organisms [13]. In addition to its use in the manufacture of fermented foods, *A. oryzae* is an efficient producer of xylanases.

In light of the biotechnological importance of xylanases in plant cell wall degradation, this study aimed to use pretreated dirty cotton residue (PDCR) as a carbon source for *A. oryzae* cultivation and the subsequent production and purification of a low-molecular-weight xylanase. Some biochemical properties, such as pH, optimum temperature, thermal stability, and hydrolysis activity, are compared with other xylanolytic enzymes described in the literature. This report is the first on the purification and characterization of a xylanase produced by *A. oryzae* grown on textile industry residue.

2. Experimental Section

2.1. Feed Residue, Substrate and Chemicals

Dirty cotton residue was kindly supplied by Hantex (Textile Waste Co., Blumenau, Santa Catarina, Brazil). Microcrystalline cellulose (avicel) was obtained from Fluka (Seelze, Germany). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All the experiments described below were performed at least three times. The standard deviation of the enzyme assay experiments was less than 20% of the mean.

2.2. Residue Pretreatment

Dirty cotton residue was thoroughly washed with tap water and autoclaved at 121 °C for 2 h. After autoclaving, it was dried at 65 °C for 48 h and then ground to form a homogeneous blend. A fine powder was obtained and used as the substrate for the enzymatic hydrolysis experiments.

2.3. Organism and Enzyme Production

A. oryzae was isolated from the natural composting of textile wastes and was identified by the Laboratory of Food Microbiology, Lavras, Brazil. The fungus was maintained in PDA medium (2% potato broth, 2% dextrose and 2% agar). For xylanase production, an aliquot (5.0 mL) of spore suspension (10^8 spores/mL) was inoculated into Erlenmeyer flasks. The cultures were incubated at 28 °C with constant agitation at 120 rpm for six days in 500 mL liquid medium containing (*w*/*v*) 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.16% $(NH_4)_2SO_4$ at pH 7.0, with 1% (*w*/*v*) PDCR as the carbon source. The crude extract obtained from these cultures was centrifuged at 10,500 *g* for 10 min at 20 °C, filtered through a Büchner funnel with filter paper (Whatman No. 1), and stored at 4 °C.

2.4. Enzyme Assays

The xylanase activity was determined by mixing 25 μL of enzyme solution with 50 μL of oat spelt xylan (10 mg·mL^{-1}) in 50 mmol·L^{-1} sodium acetate buffer, pH 5.0, at 50 °C for 30 min, as described by Filho *et al.* [14]. The release of the reducing sugar was measured using the 3,5-dinitrosalicylic acid (DNS) method [15], and the xylanase activity was expressed as micromoles of reducing sugar released per min per milliliter (IU·mL^{-1}). The β-1,3-glucanase, β-1,4-glucanase and β-mannanase assays were performed under the same conditions as described above. The activity against filter paper was measured as described by Ghose [16]. β-Xylosidase, α-arabinofuranosidase, β-glucosidase and β-mannosidase activities were determined as reported elsewhere [17]. For the kinetics experiments, the soluble and insoluble fractions of oat spelt and birchwood xylans were used as substrates in a concentration range of 6.0–26.7 mg·mL^{-1}. The substrates were prepared as described by Filho *et al.* [14]. K_m and V_{max} were estimated from the Michaelis-Menten equation with a non-linear regression data analysis program (Enzfitter) [18]. The effect of temperature on Xyl-O1 activity was determined in the temperature range of 30 to 80 °C in 35 mmol·L^{-1} sodium acetate buffer, pH 5.0. To determine the effect of pH on Xyl-O1 activity at 50 °C, the Xyl-O1 activity in the pH range of 3.0 to 9.0 was determined. The following buffers were used for these experiments: 50 mmol·L^{-1} sodium acetate (pH 3.0–6.0), 50 mmol·L^{-1} sodium phosphate (pH 6.5–7.5), and 50 mmol·L^{-1} Tris-HCl (pH 7.0–9.0). All buffers, regardless of pH, were adjusted to the same ionic strength with NaCl [19]. Xyl-O1 stability was determined by pre-incubating the enzyme at pH 7.0 at either 50 or 55 °C. At various time points, aliquots were withdrawn, and the residual activity was measured in the presence of 25 mmol·L^{-1} sodium acetate, pH 6.0 (ionic strength adjusted with NaCl), at 50 °C. Purified xylanase activity was determined in the presence of the metal ions Ag^{+}, K^{+}, Na^{+}, Co^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+} at final concentrations of 1.0 and 10 mmol·L^{-1}. The effects of the reagents 1.0 mmol·L^{-1} N-bromosuccinimide (NBS), 2.0 mmol·L^{-1} 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) and 2,2-dithiodipyridine, 5.0 mmol·L^{-1} L-tryptophan, iodoacetamide, diethyl pyrocarbonate (DEPC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and β-mercaptoethanol, and 20 mmol·L^{-1} DTT (1,4-dithiothreitol), L-cysteine and sodium dodecyl sulfate (SDS) were also determined in the presence of 25 mmol·L^{-1} sodium acetate, pH 6.0 (ionic strength adjusted with NaCl), at 50 °C. Prior to these tests, the purified enzyme was added to either the ions or the reagents and pre-incubated for 20 min at 28 °C. The exception to this was the experiments with Mn^{+2}, which was tested at a final concentration of 20 mmol·L^{-1} and pre-incubated for 40 min. Appropriate controls were included for all the experiments, and the average values are reported as relative activity (%). The amino acids L-cysteine and L-tryptophan and the ion Mn^{2+} interfered with the quantification of the reducing sugars by the DNS assay by 20% and 40%, respectively, which were corrected to obtain the final value of xylanase activity.

2.5. Determination of Protein Concentration

Protein concentration was determined by the Bradford method [20] using a Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) and bovine serum albumin as the reference protein according to the manufacturer's instructions.

2.6. Enzyme Purification

A sample (110 mL) of filtered culture supernatant (FCS) was obtained after *A. oryzae* cultivation and was concentrated by ultrafiltration using an Amicon System with a 10 kDa cut-off point membrane (PM-10; Amicon Millipore Co., Billerica, MA, USA). The ultrafiltrate contained approximately 50% of the xylanolytic activity from the FCS and was subjected to lyophilization (Freeze Dryer Liobrás, Brazil) for 48 h. The lyophilized material was resuspended in 50 mmol·L^{-1} sodium phosphate buffer, pH 7.0, containing 150 mmol·L^{-1} NaCl and 0.02% NaN_3. The solution was then centrifuged at 10,500 *g* at 4.0 °C for 10 min. This supernatant was used in the subsequent chromatographic steps. Aliquots of 4.5 mL were fractionated by gel filtration chromatography using a Sephacryl S-100 column (45 × 2.6 cm) that had been pre-equilibrated with 50 mmol·L^{-1} sodium phosphate buffer, pH 7.0, containing 150 mmol·L^{-1} NaCl and 0.02% NaN_3. The enzyme sample was eluted at a flow rate of 20 mL·h^{-1}, and 3.0 mL fractions were collected and analyzed by absorbance at a wavelength of 280 nm. Fractions with xylanolytic activity were pooled, and a combined volume of 6.5 mL was re-subjected to gel filtration chromatography on a Sephadex G-50 column (65 × 2.6 cm) that was pre-equilibrated in the same buffer described above. Fractions were eluted with at a flow rate of 20 mL·h^{-1}, and 3.0 mL aliquots containing xylanase activity were collected and pooled. Finally, a volume of 20 mL that was obtained in the earlier fractionation was pooled and dialyzed at 4.0 °C overnight against 20 mmol·L^{-1} sodium phosphate buffer, pH 7.0, with 0.02% NaN_3. The dialyzed volume was subjected to ion exchange chromatography on a Q-Sepharose column (2.6 × 4.0 cm) and equilibrated with 20 mmol·L^{-1} sodium phosphate buffer, pH 7.0, containing 0.02% NaN_3. The column was washed with the same buffer and eluted with a linear gradient of NaCl (0–1.0 mol·L^{-1}). The fractions were eluted at a flow rate of 20 mL·h^{-1}, and 3.0 mL aliquots containing xylanolytic activity were collected and pooled for further characterization. All chromatographic steps were performed at room temperature.

2.7. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% gels according to Laemmli [21], and the protein bands were visualized with silver stain [22]. The Low Molecular Weight (LMW) marker (GE Healthcare, Brazil) was used as the standard. Xyl-O1 activity was detected using a zymogram technique with 0.1% oat spelt xylan as the substrate [23]. In this procedure, a 12% gel was co-polymerized with oat spelt xylan solution and was stained for xylanase activity in a 0.1% Congo red solution for 30 min at room temperature. The gel was then washed with 1.0 mol·L^{-1} NaCl to remove any excess dye and fixed with 0.5% acetic acid.

2.8. Measurement of Substrate Specificity

The Xyl-O1 specificity for the type of glycosidic linkage and glycan moiety, and for soluble *versus* insoluble xylans, was investigated by monitoring the amount of reducing sugar released when the purified enzyme was incubated with a variety of polysaccharides (oat spelt xylan, birchwood xylan, beechwood xylan, 4-*O*-methyl-glucurono-D-xylan, CM-cellulose, pectin and mannan) at a final concentration of 6.67 mg·mL^{-1}. The assays were performed under the same conditions as described in

Section 2.4 for the xylanase assay. Other substrates, such as filter paper and avicel, were used as described in Section 2.4. For the experiments involving *p*-nitrophenylglycosides, the activities of β-xylosidase, α-arabinofuranosidase, β-glucosidase and β-mannosidase were determined by measuring the amount of *p*-nitrophenol (PNP) released. The assays were performed at 50 °C in 50 mmol·L^{-1} sodium acetate buffer, pH 6.0, with *p*-nitrophenyl-β-D-xyloside (PNPX), PNPA (*p*-nitrophenyl-α-L-arabinofuranoside), PNPG (*p*-nitrophenyl-β-D-glucoside) and PNPM (*p*-nitrophenyl-β-D-mannoside) as substrates [17]. The β-1,3-glucanase activity was measured by mixing 25 μL of enzyme solution with 25 μL of 1.0% laminarin as a substrate in 50 mmol·L^{-1} sodium acetate buffer, pH 6.0. The enzyme mixture was incubated at 45 °C for 30 min, and the amount of reducing sugar released was determined using the DNS method according to Noronha and Ulhoa [24].

2.9. Enzymatic Hydrolysis

Reaction mixtures containing 1/3 purified Xyl-O1 (6.0 μg·mL^{-1}) and ⅔ substrate solution (soluble or insoluble birchwood xylans (10 mg·mL^{-1}) prepared according to Filho *et al.* [14]) were incubated for 6, 12, 18 or 24 h at 28 °C with agitation at 120 rpm in a final reaction volume of 0.3 mL. Aliquots of 75 μL were removed at the specified intervals and analyzed for the amount of total reducing sugars. The xylooligosaccharides released during these intervals were identified and quantified with high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) as described previously by Ryan *et al.* [25]. In this process, 75 μL aliquots were boiled for 10 min at 100 °C, lyophilized and then dissolved in 500 μL Milli-Q water. The hydrolysis products were analyzed on a Dionex ICS-3000 Ion Chromatography DC System equipped with a CarboPac PA100 column and guard column with an operating flow rate of 0.25 mL·min^{-1} at 28 °C. Samples (10 μL) were injected using an AS40 auto sampler (Dionex Co.). The products were eluted for 70 min using 100 mmol·L^{-1} NaOH (50% solution, Dionex) and a gradient of 0–500 mmol·L^{-1} sodium acetate (Dionex). The column was re-equilibrated for 15 min with 100 mmol·L^{-1} NaOH solution between successive injections. Xylooligosaccharides were identified with an ED40 Electrochemical Detector (Dionex) and analyzed using Chromeleon 6.8 software (Dionex). Xylooligosaccharides that had a degree of polymerization from 2 to 6 D-xylose units (xylobiose, X2; xylotriose, X3; xylotetraose, X4; xylopentaose, X5; xylohexaose, X6; Megazyme Co., Wicklow, Ireland) and xylose (X1; 15 μg·mL^{-1}) were used as standards for the analysis of the reaction products.

3. Results and Discussion

3.1. Enzymatic Characterization of the FCS from Aspergillus oryzae *Grown on PDCR*

The enzymatic profiles of the FCS from *A. oryzae* grown for 6 days at 28 °C on PDCR are shown in Table 1. The xylanase and pectinase activity values under these culture conditions were 0.488 and 0.258 IU·mL^{-1}, respectively. The mannanase, CM-cellulase and FPase activities were all approximately 0.100 IU·mL^{-1}. The tests for avicelase activity revealed a residual enzymatic activity with a value close to zero. Based on the data in Table 1, it can be inferred that the use of PDCR as a carbon source for *A. oryzae* cultivation primarily induces hemicellulases, with an emphasis on xylanase production.

Our results are consistent with those of Siqueira *et al.* [3], who used agro-industrial residues such as sugarcane bagasse, banana stalks and dirty cotton for the cultivation of various fungi, including *A. oryzae*, which produced a large amount of xylanase. Other researchers have used lignocellulosic biomass from agro-industrial residues to produce cellulase and xylanase [26–31]. The use of lignocellulosic biomass waste for enzyme production would significantly reduce the cost of enzyme production because the productivities are comparable to those obtained with industrial carbon sources. According to Siqueira *et al.* [3], agro-industrial residues are cheap carbon sources for the growth of microorganisms, including filamentous fungi, and for the production of hemicellulases. The diversity of hemicellulases produced by *A. oryzae* may be related to the nature of the lignocellulosic biomass components. The untreated dirty cotton residue was subjected to bromatological analysis (results not shown), which indicated that this residue consisted of 56.7% total fibers and a holocellulose portion (the carbohydrate fraction of lignocellulose) of 75.5%. The analysis of individual polymers showed that the untreated dirty cotton residue was composed of 65.7% cellulose, 9.8% hemicellulose and 6.3% lignin. The cotton plant fragments were readily apparent in addition to the fibrous profile of this residue.

Table 1. Enzymatic activities of the filtered culture supernatant (FCS) from *Aspergillus oryzae* grown on pretreated dirty cotton residue (PDCR) for 6 days at 28 °C.

Enzymatic activity	$IU \cdot mL^{-1}$
Xylanase	0.488 ± 0.011
Pectinase	0.258 ± 0.048
Mannanase	0.098 ± 0.006
CM-cellulase	0.095 ± 0.004
FPase	0.081 ± 0.015
Avicelase	0.009 ± 0.001

3.2. Xyl-O1 Purification

Xyl-O1 was purified by ultrafiltration through a PM-10 ultrafiltration membrane, gel filtration chromatography on Sephacryl S-100 and Sephadex G-50 and ion exchange chromatography on Q-Sepharose. In the first step, a xylanase activity of only 0.294 $IU \cdot mL^{-1}$ was detected in the ultrafiltrate. Because this ultrafiltrate had low protein concentration, the solution was lyophilized prior to chromatography. The chromatographic profiles from the gel filtration columns (results not shown) initially showed that the enzyme sample subjected to chromatography on a Sephacryl S-100 column eluted in two peaks that had xylanase activity. The second peak was selected because it contained a xylanase activity that was approximately 3.5 times higher than the first peak and corresponded to the fractions with the greatest absorbance at 280 nm. The pooled fractions were applied onto a Sephadex G-50 column. The xylanase activity was eluted off this column in a single peak with a low absorbance at 280 nm. An additional peak was eluted with a greater absorbance at 280 nm and an unidentified colored substance. As shown in Figure 1, fractionation on a Q-Sepharose column revealed that Xyl-O1 eluted before the salt gradient at pH 7.0. According to Wong *et al.* [32], there is a conserved relationship between molecular weight and p*I* when xylanases from different species are compared. Low-molecular-weight xylanases tend to be basic, whereas high molecular weight is associated with a

more acidic p*I*. An exception to this relationship is the *Schizophyllum commune* xylanase (21 kDa; p*I* 4.5); however, the relationship is conserved even in yeast-like fungi that lack multiple xylanases, e.g., *Cryptococcus* sp.

Figure 1. Ion exchange chromatography of Xyl-O1 on Q-Sepharose. Absorbance at 280 nm is represented by closed diamonds [—◆—], xylanase activity is represented by thin Xs [- ✳ -], and the linear gradient (0–1.0 mol·L^{-1} NaCl in equilibration buffer) is represented by a solid line [▬▬].

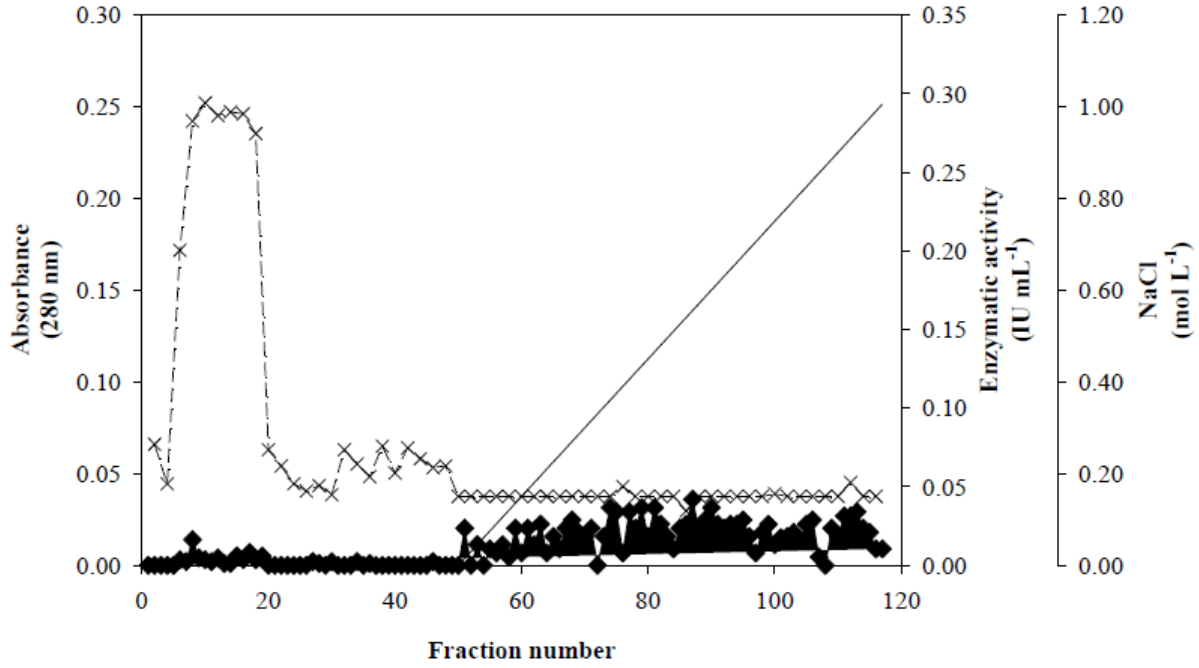


Table 2. Summary of purification of Xyl-O1.

Purification steps	Total volume (mL)	Total protein (mg)	Total activity (IU)	Specific activity (IU·mg^{-1}) *	Purification (fold)	Yield (%)
Filtered culture supernatant	110.00	5.830	53.350	9.151	1.000	100.00
Ultrafiltrate	100.00	1.500	29.400	19.600	2.142	55.11
Lyophilization	4.50	0.828	1.647	1.989	0.217	3.08
Sephacryl S-100 (fractions 58–64)	21.00	0.441	7.833	17.762	1.941	14.68
Sephadex G-50 (fractions 71–84)	40.00	0.080	9.880	123.500	13.496	18.52
Q-Sepharose FF (fractions 7–18)	36.00	0.036	8.928	248.000	27.101	16.74

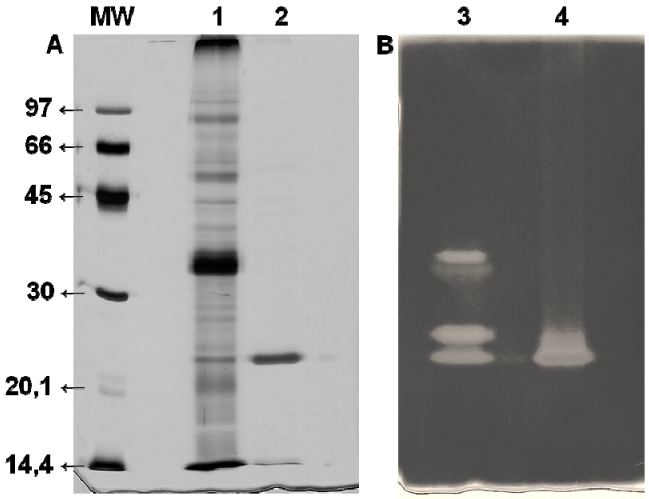
* Specific activity (IU·mg^{-1}) was determined with soluble oat spelt xylan as substrate.

As noted in Table 2, the xylanolytic activity remaining in the ultrafiltrate portion after lyophilization was considerably recovered during fractionation using gel filtration and ion exchange chromatography. In addition, Table 2 also shows Xyl-O1 purification and yield values of 27.1% and 16.7%, respectively. These values are consistent with other studies involving xylanase purification in

which the recovery was low [33–36]. The ultrafiltration procedure retained most of the β-xylanase activity in the concentrate. The absence of other isoforms or isoenzymes of xylanase in the ultrafiltrate portion, which act synergistically in the hydrolysis of the substrate, may have underestimated the purification and yield values during the final fractionation step [14].

However, our fractionation method, beginning with protein concentration by lyophilization, resulted in higher purification and yield compared to other fractionation methods that are initiated by precipitation with $(NH_4)_2SO_4$ (results not shown). However, the total xylanase activity of the ultrafiltrate (29.400 IU) was significantly reduced to 1.647 IU after the lyophilization step. This finding suggests the presence of xylanase inhibitors that were produced as a result of PDCR degradation and that were also concentrated during the lyophilization step. According to Panagiotou and Olsson [37], the lignin derivatives (furans, phenols and low-molecular-weight acids) that are found in the hydrolysate of pretreated wheat straw inactivated both a mixture of commercial enzymes and a crude enzyme sample from *Penicillium brasilianum* IBT 20888 during the enzymatic digestion of filter paper and xylan. The purification procedures provided an apparently homogeneous preparation of xylanase activity from *A. oryzae*. An increase in the specific activity from 9.15 $IU \cdot mg^{-1}$ in the FCS to 248 $IU \cdot mg^{-1}$ at the end of chromatography was observed. This is an approximately 27-fold increase and confirms the efficiency of the Xyl-O1 purification. The apparent purity of the enzyme was demonstrated by SDS-PAGE because under denaturing conditions, the gel showed a single band (Figure 2).

Figure 2. SDS-PAGE (12%) of purified Xyl-O1. The gel was stained with (**A**) silver nitrate or (**B**) 0.1% Congo red. MW—molecular weight marker (phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)); Lane 1—FCS from *Aspergillus oryzae* (10 µg); Lane 2—Purified Xyl-O1 (5.0 µg); Lane 3—FCS from *A. oryzae*; Lane 4—Purified Xyl-O1.



The molecular weight of the purified Xyl-O1 was 21.5 kDa, as estimated by the relative migration of the standard markers. A low-molecular-weight xylanase can be useful in pulp bleaching, since smaller enzymes can penetrate further the fiber wall structure and alter more efficiently pulp properties. The molecular weight of Xyl-O1 was also estimated by gel filtration chromatography on

Sephadex G-50; however, the value was less than 10 kDa (result not shown), suggesting that the enzyme interacted with the matrix. The same phenomenon has been described for β-xylosidase from *Trichoderma harzianum* strains [17] and for a low-molecular-weight xylanase from *A. fumigatus* Fresenius [34]. According to Poutanen [35], the reason for the lower apparent molecular weight that is obtained by gel filtration could be the retarded migration of the enzyme in the gel matrix.

3.3. Enzyme Characterization

The xylanase activities of the FCS and purified Xyl-O1 were greatest in the temperature range of 45–60 °C and the pH range of 5.5 to 6.0. The two samples were stable in the temperature range of 45–60 °C, with xylanase activity greater than 70%. The greatest FCS activity was observed at 55 °C, and the greatest Xyl-O1 activity was at 50 °C. The two enzyme samples presented the best activity at pH 6.0 in sodium acetate buffer. The activity of the two samples was greater than 90% in the pH range of 5.5 to 6.0. Other studies involving the purification and characterization of endo-β-1,4-xylanases from filamentous fungi have indicated that 50 °C is the most effective temperature for enzyme activity [33,38,39]. Our results are consistent with Kimura *et al.* [40], who found that the greatest activity for a xylanase, XynG2, that was isolated from a genomic library of *A. oryzae* KBN616 was at pH 6.0. However, its greatest enzymatic activity was at 58 °C, and it displayed a different Km value (5.1 $mg \cdot mL^{-1}$) on birchwood as the substrate. The K_m values ($mg \cdot mL^{-1}$) of Xyl-O1 on a soluble fraction of oat spelt and birchwood xylans were 10.05 and 3.34, respectively. The apparent K_m values indicated that the purified Xyl-O1 had a preference for birchwood xylan as a substrate. Birchwood xylan contains 90% xylose, whereas oat spelt xylan contains 75% xylose, 10% arabinose and 15% glucose residues. The preference of Xyl-O1 for substrates containing a higher proportion of D-xylose residues is a relevant property that can be used in the future targeting of this enzyme for alternative methods of cellulose pulp biobleaching.

The Xyl-O1 enzyme activity was reduced to 86 and 8% in the presence of Hg^{2+} at concentrations of 1.0 and 10 $mmol \cdot L^{-1}$, respectively. The same inhibitory effect was observed for Ag^{+} at a concentration of 10 $mmol \cdot L^{-1}$. The inhibitory effects of Hg^{2+} and Ag^{+} on xylanase activity are known [7,25,41,42]. According to Sandrim *et al.* [41], the addition of Hg^{2+} drastically inhibits xylanolytic activity, suggesting the existence of thiol groups in the catalytic site of the enzyme. In general, purified Xyl-O1 remained stable in the presence of most of the ions that we tested. However, Mn^{2+}, which is commonly referred to as an activator of xylanase activity, showed an inhibitory effect at a concentration of 10 $mmol \cdot L^{-1}$ and promoted a 10% reduction in activity compared to the control. The same inhibitory effect was observed when the enzyme was pre-incubated for 40 min at 28 °C with the same concentration of Mn^{2+}. Conversely, Carmona *et al.* [43] and Teixeira *et al.* [39] reported an activating effect of 10 $mmol \cdot L^{-1}$ Mn^{2+} on xylanase from *A. versicolor* and *A. awamori* 2B.361 U2/1. Here, purified Xyl-O1 in the presence of amino acid-modifying reagents and amino acids remained relatively stable or was activated by certain reagents (Table 3). The enzyme retained 74% of its xylanolytic activity in the presence of 20 $mmol \cdot L^{-1}$ SDS.

The reaction mixture containing 5.0 $mmol \cdot L^{-1}$ L-tryptophan did not have increased enzymatic activity. However, for compounds containing thiol groups, such as L-cysteine, DTT and β-mercaptoethanol, the xylanase activity increased by 40, 14 and 37%, respectively. The enzyme was

stable in the presence of NBS and EDC reagents at concentrations of 1.0 and 5.0 mmol·L^{-1}, respectively. However, the enzyme activity was completely inhibited by NBS at a concentration of 10 mmol·L^{-1} (result not shown). Teixeira *et al.* [39] also reported the inhibition of xylanase from *A. awamori* by NBS at the same concentration. NBS is an effective inhibitor of xylanases [44,45] and promotes the oxidation of tryptophan residues that are involved in enzymatic catalysis. Xyl-O1 was slightly inhibited by the alkylating reagent iodoacetamide, indicating the need for thiol groups in catalysis. DEPC and DTP reduced the Xyl-O1 activity by 15 and 12%, respectively (Table 3).

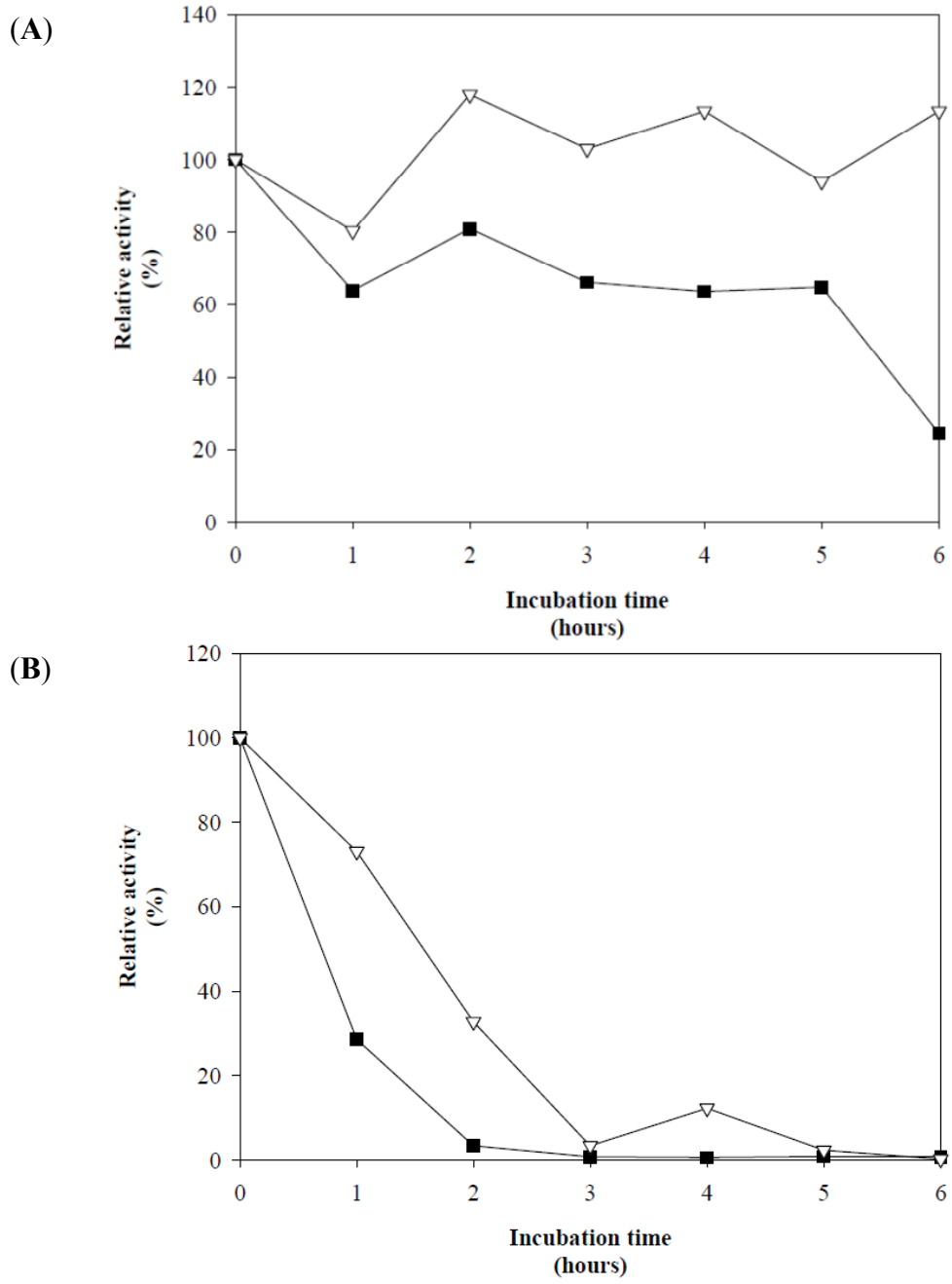
Table 3. Effect of modifying agents and amino acids on the xylanolytic activity of purified Xyl-O1.

Modifying agents or amino acids	**Activity (IU·mL^{-1})**	**Relative activity (%)**	**Concentration (mmol·L^{-1})**
H_2O control	0.262 ± 0.021	100.00	---
4% Ethanol control	0.285 ± 0.026	100.00	---
NBS	0.285 ± 0.079	108.42	1
DTNB *	0.399 ± 0.025	140.02	2
DTT	0.300 ± 0.037	114.35	20
L-Cysteine	0.367 ± 0.076	140.04	20
H_2O control	0.290 ± 0.029	100.00	---
4% Ethanol control	0.322 ± 0.006	100.00	---
DTP *	0.285 ± 0.025	88.43	2
L-Tryptophan	0.285 ± 0.012	98.13	5
Iodoacetamide	0.255 ± 0.046	87.71	5
DEPC	0.248 ± 0.037	85.32	5
EDC	0.291 ± 0.046	100.08	5
β-Mercaptoethanol	0.397 ± 0.026	136.68	5
H_2O control	0.306 ± 0.031	100.00	---
SDS	0.225 ± 0.025	73.56	20

***** These compounds were diluted in 4% ethanol. Enzymatic activity: 0.320 ± 0.014 IU·mL^{-1}.

The assays investigating the effect of incubation time on Xyl-O1 activity at pH 7.0 revealed that at 50 °C, the enzyme remained active for 5.0 h, retaining 60% of its activity. Conversely, at 55 °C, the activity decreased to less than 30% within the first hour of incubation. The thermostability of Xyl-O1 at 50 °C was greatly increased in the presence of 20 mmol·L^{-1} L-cysteine, and the activity remained close to 100% for an incubation period of 6.0 h (Figure 3(A)). At 55 °C, the xylanase activity was enhanced in the presence of L-cysteine and remained stable for 1.0 h, with the catalytic activity remaining greater than 75% (Figure 3(B)).

Figure 3. Effect of 20 mmol·L^{-1} L-cysteine on the thermostability of purified Xyl-O1 at pH 7.0 and (**A**) 50 °C or (**B**) 55 °C [▽]. The control without L-cysteine [■].



The purified Xyl-O1 efficiently hydrolyzed the substrates that contained D-xylose residues, especially the soluble fraction of oat spelt and the birchwood and beechwood xylans (Table 4). These results suggest that Xyl-O1 is a potential candidate for the pulp bleaching processes, whereas the application of xylanases in the pulp and paper industry requires a cellulase-free activity [8,11,41,46]. However, the enzyme activity was significantly reduced in the insoluble fractions of the same substrates, especially for oat spelt xylan, which had a relative activity of less than 1.0%. The enzyme also showed a hydrolysis efficiency of 49.87% when tested with 4-*O*-methyl-glucurono-D-xylan as the substrate. The relative activities for the birchwood and beechwood xylans were 85 and 84.4%, respectively (Table 4). The residual activity was less than 10% for filter paper, CM-cellulose,

laminarin, mannan and pectin, indicating that these substrates might have been contaminated with pentosan, which is susceptible to hydrolysis [14]. Furthermore, Xyl-O1 was completely inactive against the avicel and *p*-nitrophenylglycoside substrates (Table 4).

Table 4. Substrate specificity of purified *Aspergillus oryzae* Xyl-O1.

Substrate	Main chain linkage	Activity (IU·mL^{-1})	Purified Xyl-O1 (% RA) [a]
Oat spelt xylan (S) [b]	β-1,4	0.659 ± 0.005	100.00
Oat spelt xylan (I) [c]	β-1,4	0.003 ± 0.004	0.39
Birchwood xylan (S)	β-1,4	0.560 ± 0.011	85.03
Birchwood xylan (I)	β-1,4	0.228 ± 0.025	34.60
Beechwood xylan (S)	β-1,4	0.556 ± 0.030	84.38
Beechwood xylan (I)	β-1,4	0.024 ± 0.030	3.58
4-*O*-methyl-glucurono-D-xylan (I)	β-1,4	0.328 ± 0.028	49.87
Filter paper	β-1,4	0.010 ± 0.007	1.46
CM-cellulose	β-1,4	0.045 ± 0.004	6.82
Avicel	β-1,4	0.000 ± 0.002	0.00
Laminarin	β-1,3	0.039 ± 0.018	5.93
Pectin	β-1,4	0.024 ± 0.019	3.62
Mannan	β-1,4	0.002 ± 0.003	0.37
PNPX	PNP-β-1,4	0.000 ± 0.000	0.00
PNPA	PNP-α-1,4	0.000 ± 0.000	0.00
PNPG	PNP-β-1,4	0.000 ± 0.000	0.00
PNPM	PNP-β-1,4	0.000 ± 0.000	0.00

[a] Relative activity to oat spelt xylan; [b] (S): Soluble fractions; [c] (I): Insoluble fractions.

The specificity of Xyl-O1 was determined by analyzing the hydrolysis products of the soluble and insoluble birchwood xylan using the DNS method and HPLC on a Dionex system. The results presented in Table 5 show that the enzyme was more active on the soluble fraction, with a maximum hydrolysis of approximately 33% obtained after 12 h of incubation. A similar effect was observed with the insoluble fraction, but the hydrolysis reached only 17.5%. These results are consistent with the observations of Ryan *et al.* [25], who also found higher rates of hydrolysis for soluble xylan. The preference of Xyl-O1 for the soluble fraction of the substrate may indicate a catalytic efficiency for the branched sites of *O*-acetyl-(4-*O*-methyl-D-glucurono) in the structure of the birchwood xylan. According to Coughlan and colleagues, certain constituents in the structure of the xylan act as binding sites for the catalytic activity of some xylanases [11]. The residues that comprise these sites in the branching structure were partially removed during the preparative alkaline and acid extractions that were performed on the soluble and insoluble fractions of the substrate. However, as shown in Table 5, we observed that 18– and 24 h hydrolysis periods did not increase the amount of reducing sugar that was released. It is possible that Xyl-O1-promoted hydrolysis is more efficient after 12 h and represents a plateau of activity that remains constant for longer incubations.

Table 5. Hydrolysis of soluble or insoluble birchwood xylan catalyzed by purified Xyl-O1.

	Soluble		Insoluble	
Hydrolysis time (h)	Specific reducing sugar (mg) *	Hydrolysis (%)	Specific reducing sugar (mg) *	Hydrolysis (%)
0	0.000 ± 0.000	0.00	0.031 ± 0.006	1.55
6	0.434 ± 0.011	21.72	0.209 ± 0.036	10.47
12	0.667 ± 0.029	33.35	0.351 ± 0.057	17.54
18	0.554 ± 0.025	27.69	0.345 ± 0.039	17.23
24	0.554 ± 0.025	27.70	0.350 ± 0.037	17.50

Reaction mixtures (final volume of 0.3 mL) containing ⅓ purified Xyl-O1 (6.0 μg·mL^{-1}) and ⅔ substrate solution with soluble or insoluble birchwood xylans (10 mg·mL^{-1}) were incubated at 28 °C with agitation at 120 rpm. * The amount of the reducing sugar released was estimated by the DNS method.

The identification and quantitative analysis of the soluble xylooligosaccharides that were released seem to indicate that the enzyme catalyzes the random cleavage of internal glycosidic linkages in the soluble and insoluble fractions of the birchwood xylan (Table 6). A similar activity has been described for other enzymes, including the endo-β-1,4-xylanases produced by *Penicillium capsulatum* [25], *Cephalosporium* sp. RYM-202 strain [38], *A. fumigatus* Fresenius [34] and *Acrophialophora nainiana* [33]. As shown in Table 6, the hydrolysis products X2-X6 predominated during the first 12 h of incubation. During the subsequent 12 h, either the X4-X6 xylooligomers predominated, or it was not possible to identify the products, as shown for the insoluble fraction after 24 h (Table 6). The decreased identification or quantification of the hydrolysis products by HPAEC-PAD indicated the accumulation of higher xylooligomers after the elution of the xylohexaose standard primarily during incubation periods of 18 and 24 h (results not shown). This suggests that the purified Xyl-O1 is involved in transglycosylation reactions at certain times. In this case, the chromatographic profile showed irregular peaks presenting more intense electrochemical signals and longer retention times than those of established xylooligomers patterns. Because only xylooligosaccharides containing six or fewer xylose units could be detected with the selected chromatographic parameters, we postulated that the products with a higher degree of polymerization (DP) could not be identified by the absence of xylooligomer patterns with longer retention times. With regard to the highest hydrolysis percentage obtained with the DNS method, we hypothesize that the identification of the hydrolysis products by their retention times results in greater specificity. Conversely, the detection of reducing sugars by the DNS method, even using xylose as reference, would not distinguish between the reducing ends of the xylose or more complex xylooligomers. Indeed, the effect of transglycosylation could explain the constant rate of hydrolysis obtained by the DNS method after 12 h of incubation (Table 5). As reported by Biely and Vranská [47], the transfer of glycosyl groups is a well-known activity of glycosidases and glycanases. Consistent with the findings of these authors, the enzyme acts on oligosaccharides or aryl glycosides (e.g., invertase, β-galactosidase, *A. niger* β-glucosidase) to catalyze the formation of various positional isomers of the oligosaccharides. These positional isomers retain the configuration of the glycosidic linkage without specificity for the saccharide acceptor molecule and one particular linkage. For endo-acting glycanases, such as lysozyme, α-amylases and cellulases, the specificity is evident, and the same type of glycosidic linkage in the saccharide acceptors is formed from the oligosaccharide glycosyl donors that were cleaved previously. In addition, the xylanase produced by

Anoxybacillus flavithermus BC [48] catalyzes this transglycosylation activity. In other studies, endo-1,4-β-xylanases and β-xylosidases have been used for the synthesis of specific oligosaccharides [49,50] to obtain a cellulose-xylan polymer hybrid [51]. Our results suggest that the transglycosylation reaction catalyzed by Xyl-O1 can occur after a period of effective hydrolysis, in this case, after a 12 h incubation at 28 °C. However, specific testing of the transglycosylation reaction is required to confirm this hypothesis of Xyl-O1-promoted catalysis. Table 6 shows the general profile of hydrolysis over the 6.0–24 h incubation period, confirming the predominance of X2 because of the action of Xyl-O1 on the soluble and insoluble fractions of the substrate. For the insoluble and soluble fractions, Xyl-O1 showed a hydrolysis profile with a more relaxed specificity, allowing for the quantification of the products X1 to X6 within six hours of incubation. During this incubation period, xylobiose was the main hydrolysis product released through the activity of the enzyme (Table 6).

Table 6. Identification and quantitative analysis by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) of the xylooligosaccharides released during the hydrolysis reactions catalyzed by purified Xyl-O1.

Xylooligosaccharide products *	Hydrolysis (%) Soluble Fraction				Hydrolysis (%) Insoluble Fraction			
	Incubation time (h)				Incubation time (h)			
	6	12	18	24	6	12	18	24
X1	0.005	0.000	0.000	0.000	0.015	0.000	0.000	0.000
X2	0.178	0.280	0.000	0.000	0.245	0.000	0.000	0.000
X3	0.078	0.000	0.008	0.000	0.083	0.047	0.000	0.000
X4	0.022	0.000	0.045	0.016	0.102	0.063	0.027	0.000
X5	0.031	0.000	0.005	0.005	0.066	0.055	0.005	0.000
X6	0.033	0.000	0.005	0.000	0.052	0.022	0.010	0.000

Reaction mixtures (final volume of 0.3 mL) containing ⅓ purified Xyl-O1 (6.0 μg·mL^{-1}) and ⅔ substrate solution with soluble or insoluble birchwood xylans (10 mg·mL^{-1}) were incubated at 28 °C with agitation at 120 rpm. ***** Xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6).

The reduced percentage of X1, especially during the incubation period of 6 h, suggests that Xyl-O1 has an endo-acting mechanism, whereas the predominant hydrolysis product released by Xyl-O1 was X2, corresponding to 0.458% in the range of 6 to 12 h for the soluble fraction and 0.245% for the insoluble fraction after 12 h of incubation (Table 6). Additionally, the total hydrolysis percentages obtained by the release of X4, X5 and X6 from the insoluble fraction were 0.192, 0.126 and 0.084%, respectively, in the range of 6 to 18 h. This result shows that the purified Xyl-O1 presents catalytic flexibility by acting on both soluble and insoluble substrates.

4. Conclusions

The use of agro-industrial PDCR as a carbon source for the submerged cultivation of *A. oryzae* is a viable and inexpensive alternative for the production of holocellulose-degrading enzymes, mainly xylanases. A low-molecular-weight xylanase was purified and characterized using standard assays. Xyl-O1 was optimally active at 50 °C and pH 6.0. It seems to belong to the group of xylanases with an

endo-acting mechanism. This xylanase hydrolyzed preferentially xylan as the substrate, being an attractive enzyme for potential future applications in the pulp and paper industries. The lignocellulosic biomass contained in the agro-industrial residues, such as PDCR, may enable the efficient production of enzymes at a low cost, thereby reducing the financial support necessary to apply these catalysts to biotechnology.

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