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Purification and Physicochemical Characterization of a Novel Thermostable Xylanase Secreted by the Fungus *Myceliophthora heterothallica* F.2.1.4

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Abstract

Xylanases are enzymes that act in the depolymerization of xylan and that can be used in the food industry, the paper industry, and for bioenergy, among other uses. In this context, particular emphasis is devoted to xylooligosaccharides (XOS) that act as prebiotics, which, under the action of probiotic microorganisms, are capable of positively modifying the intestinal microbiota. In this sense, searching for microbial xylanases stands out as a sustainable strategy for the production of prebiotics. To date, there have been no reports in the literature regarding the purification of native xylanase from *Myceliophthora heterothallica* F.2.1.4. In this study, a xylanase from this fungus was purified and characterized. The xylanase, with 27 kDa, showed maximum activity at pH 4.5 and 65–70 °C. It maintained more than 80% of its residual activity when exposed to (i) temperatures between 30 and 60 °C for 1 h and (ii) pH 5–10 for 24 h at 4 and 25 °C. These high tolerances to different pH and different temperatures are important properties that add value to this enzyme. The hydrolysates of this enzyme on beechwood xylan, analyzed by HPAE-PAD, were mostly xylobiose (X2) and xylotriose (X3). Hydrolysates were also quantified, being retrieved from 234.2 mg xylooligosaccharides/g of hydrolyzed xylan for 12 h. According to the products obtained from the xylan hydrolysis and its tolerance properties of the enzyme, it has demonstrated potential for application production of xylooligosaccharides for use as prebiotics.

Keywords Fungal enzyme · Purification · Xylooligosaccharides · Xylanase

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Introduction

Endo-xylanases (endo-1,4-β-xylanases, EC.3.2.1.8) are enzymes responsible for the hydrolysis of β-1,4-glycosidic bonds and they belong to the glycoside hydrolase (GH) group. According to the CAZy database, xylanases are found in GH families 5, 8, 30, 43, 51, and 98, being mostly classified in families 10 and 11 [1]. They were first commercialized in the production of animal feed that, combined with other enzymes, provide the breakdown of arabinoxylan, generating improved digestibility. Later, they were being introduced into other areas, such as the food, pharmaceutical, textile, and paper industries [2]. Recently, researchers have searched for xylanases for producing xylooligosaccharides (XOS), which are oligomers of sugar made of units of xylose [3, 4]. The International Scientific Association for Probiotics and Prebiotics (ISAPP) defines a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" [5].

The XOS are considered prebiotics because they are not digested by the enzymes in the digestive system and have the ability to stimulate the growth of probiotic microorganisms, generating beneficial changes in intestinal microbiota, such as modulation of autochthonous microbiota, relief of the complications arising from intolerance to lactose, and prevention of antibiotic-associated diarrhea [6].

There are some challenges for producing XOS. Some substrates (raw materials) have different types of xylans, with varying substituent groups and different branching degrees (e.g., grasses xylan are different from wood xylan). Thus, it may occur that the enzyme is produced by a microorganism, but does not act properly on the substrate offered to it. Also, for the enzymatic production of XOS, it is necessary to use a xylanase that releases little or no xylose, since this monomer can act as an inhibitor in the hydrolysis process, decreasing the enzymatic efficiency [7, 8]. In addition, the xylan hydrolysis may require the use of high temperatures for a long period [9]. In this sense, a purified thermostable enzyme is especially necessary, either because it tolerates long reaction times or because, in its pure form, it can be ensured that other enzymes do not act on the substrate.

Xylanases can be produced by various organisms, such as bacteria, fungi, protozoans, marine algae, and insect. However, fungi are the best xylanase producer and the main commercial sources are filamentous fungi [10]. Also, tolerance to temperature is a property that may increase the use of the enzyme in productive processes [10]. In this context, particular emphasis has been devoted to thermophilic fungi that tend to produce enzymes with improved thermostability [11, 12].

Fungi of the genus *Myceliophthora* stand out for their ability to produce enzymes that can degrade lignocellulosic material, primarily by solid-state fermentation using lignocellulosic residue, such as wheat bran or sugarcane bagasse, as substrate [13]. Recent study evaluated the production of cellulolytic enzymes, like endoglucanases and β-glucosidase from the fungus *Myceliophthora heterothallica*, which it has showed to be a better producer of endoglucanases when using bagasse and wheat bran in solid-state cultivation [14]. The same fungus has been used to produce other enzymes of interest for industrial applications, such as xylanase and β-glucosidases through cultivation in a semi-solid state [15].

The purification of xylanases is an important step to eliminate contamination from other proteins and enzymes present, like cellulases, and also compounds derived from the substrate (mainly the insoluble ones such as wheat bran) used in the culture medium. This contamination

can interfere with analyses of activity, protein estimation, and physicochemical characteristics. The purification and physicochemical characterization (activity and stability at different temperatures and pH) of the purified xylanase provide knowledge about the structure and functional characteristics of the enzyme, in order to assess their potential for some uses. The purification process should focus on obtaining the highest possible yield, while maintaining the highest enzymatic activity and purity possible [10]. To date, to the best of our knowledge, no native xylanase has been purified from the thermophilic fungus *M. heterothallica*. In this context, the present work describes the purification and physicochemical characterization of a new xylanase from the fungus *M. heterothallica* F.2.1.4 produced by solid-state fermentation. Here, the enzyme was applied to hydrolysis of beechwood xylan, where it showed a profile of XOS recognized prebiotic importance.

Materials and Methods

Microorganisms

The thermophilic fungus *M. heterothallica* F.2.1.4, isolated from poultry litter [16], belongs to the culture bank of the Laboratory of Biochemistry and Applied Microbiology, *Instituto de Biociências Letras e Ciências Exatas* (*IBILCE*), *Universidade Estadual Paulista* (*UNESP*), São José do Rio Preto, São Paulo, Brazil. It was inoculated in Petri dishes with oat medium (2%) and agar (1%) and was incubated at 45 °C for 2 days until complete growth. Afterwards, the dishes were kept at room temperature until further use.

Enzyme Production

Endo-β-1,4-xilanases were produced by solid-state fermentation (SSF) using wheat bran as a substrate. Five grams of wheat bran was added to 10 mL of saline solution composed of 0.1% NH_4NO_3, $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$. The flasks were autoclaved for 20 min at 121 °C and the medium was then inoculated with four mycelial disks (1 mycelial disk = 0.5 cm^2) from a standardized fungal growth for 48 h in oat medium at 45 °C. The flasks of fermentative medium were kept for 2 days at 45 °C. After, 50 mL of water was added, the media were homogenized, filtered, and centrifuged at 8.000×g for 20 min at 4 °C.

Enzyme Activity

The xylanolytic activity was evaluated as described by Bailey, Biely, and Poutanen [17] with volume size adapted for smaller volumes. The reaction mixture was composed of 90 μL of 1% (*w/v*) beechwood xylan in a 0.1 M sodium acetate buffer (pH 5.0) to which 10 μL of enzyme solution was added, and incubated at 45 °C for 10 min. Then, 100 μL of 3,5-Dinitrosalicylic acid (DNS) was added to stop the reaction and the reducing sugars were quantified at 540 nm according to Miller [18]. The activity was calculated using a standard curve of xylose. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of xylose/mL/min under the assay conditions cited.

The ability to hydrolyze other substrates was evaluated in the presence of 1% (*w/v*) Avicel, 1% (*w/v*) carboxymethyl cellulose, 4 mM of 4-nitrophenyl α-L-arabinofuranoside (pNPA), 4 mM of 4-nitrophenyl β-D-glucopyranoside (pNPG), and 4 mM of 4-nitrophenyl β-D-

xylopyranoside (pNPX). For cellulolytic activity, DNS was used to interrupt the reaction and reducing sugars were quantified at 540 nm according to Miller [18]. For the other substrates, 2 M sodium carbonate was used to quench the enzymatic reaction and the quantification of p-nitrophenol was performed at 410 nm. The analyses were performed at pH 4.5 (acetate buffer, 0.1 M) and 65 °C.

Purification of Xylanase

A crude extract (500 mL) was precipitated with ethanol 96 °GL in a proportion of 1:3 (v/v) overnight at − 20 °C. After that, the solution was centrifuged at 3500×g for 10 min at 4 °C and the precipitate resuspended in 20 mL of sodium phosphate buffer, 50 mM, pH 6.0. The xylanase purification was performed with two steps of chromatography: gel filtration (Sephadex® G-75) (GE Healthcare) and anion-exchange (Resource™ Q) (GE Healthcare). First, a Sephadex® G-75 resin column (2.5/100 cm) was equilibrated with 5 column volumes using a sodium phosphate buffer (50 mM) with 50 mM NaCl (pH 6.0). After the column equilibration, 2 mL of concentrated extract from the SSF was applied to the column. A flow rate of 0.5 mL/min was established and fraction volumes of 4.5 mL were collected.

The fractions that had xylanolytic activity were submitted to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, fractions with similar electrophoretic profiles were pooled and dialyzed using a 6 to 8 kDa cutoff membrane in sodium phosphate buffer (50 mM), pH 6.0.

Desalted samples were subjected to anion-exchange chromatography using a Resource™ Q resin column (6 mL) coupled to an ÄKTApurifier (GE Healthcare). The resin column was equilibrated with 5 column volumes using buffer A (sodium phosphate buffer, 50 mM, pH 6.0). Buffer B (sodium phosphate buffer, 50 mM, pH 6.0 + 0.4 M NaCl) was used for protein elution in a NaCl linear gradient. Protein elution was detected at 280 nm and a flow rate of 1 mL/min with fraction volume of 2.0 mL was established. Fractions exhibiting xylanolytic activity were then analyzed by 10% SDS-PAGE and silver nitrate gel staining was performed according to the methods of See and Jackowski [19].

Peptide Analysis by Mass Spectrometry

This analysis was done at the *Faculdade de Ciências Farmacêuticas - Universidade de São Paulo/USP*, São Paulo, Brazil. Initially, the band of the pure enzyme was visualized by 10% SDS-PAGE using Coomassie blue G-250. This protein band was excised from the gel and treated with a solution of 50% (v/v) acetonitrile in 0.1 M NH$_4$HCO$_3$, pH 7.8 [20–22].

After that, the sample was dehydrated using 100% acetonitrile and subjected to tryptic digestion in a reaction using 20 μL of a 20 ng/μL stock of modified trypsin (T6567 - Sigma-Aldrich) in 0.1 M NH$_4$HCO$_3$ (pH 7.8) for 20 h at 37 °C. The tryptic reaction was stopped with 5 μL of formic acid. Then, the peptides were applied to a ZipTip column (C18; Tip Size: P10; Millipore) previously equilibrated with 0.2% formic acid. In this methodology, the peptides were eluted with a 60% methanol:5% formic acid solution [20–22].

For application to a mass spectrometer, the eluted samples were concentrated, resuspended in 0.1% formic acid, and injected into an ESI-IT (Electrospray Ionization - Ion Trap) (Amazon Speed/Bruker). Data analysis was performed using the MASCOT search engine (version 2.5)

with the following search parameters: trypsin hydrolysis, maximal number of one missed cleavage, and methionine oxidation as a variable modification [20–22].

Total Protein Quantification

Protein quantification was performed according to Noble and Bailey [23] and Noble [24].

Physicochemical Characteristics

Effect of pH and Temperature on Enzymatic Activity

Optimum pH: xylanolytic activity was measured by using 1% (w/v) beechwood xylan (Sigma-Aldrich) in 0.1 M buffers with pH ranging from 3 to 10, the buffers being citrate (pH 3.0), acetate (pH 3.5 to 5.5), Mes (pH 6.0 and 6.5), Hepes (pH 7.0 and 7.5), Bicine (pH 8.0 to 9.0), and Caps (pH 9.5 and 10.0). Optimum temperature: enzymatic activity was determined using 1% (w/v) beechwood xylan at optimum pH ranging from 30 to 85 °C. pH stability: the enzyme was pre-incubated without the substrate in 0.1 M at different pH values (3.0 to 10.0) for 24 h at 4 °C and 25 °C. Thereafter, all activity was performed under optimum conditions of pH and temperature. Thermostability: the enzyme was pre-incubated, without substrate, for 60 min in the temperature range between 30 and 85 °C. Subsequently, the residual enzymatic activity was measured under optimal pH and temperature conditions.

Effect of Metallic Ions and Different Compounds on Enzymatic Activity

The effect of metallic ions ($AlCl_3$, $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, $MnCl_2$, $MgCl_2$, $NiCl_2$, and $ZnCl_2$) and chemical agents (Triton X-100, SDS, DMSO, DTT, and EDTA) on enzyme activity was evaluated at a concentration of 5 mM. The influence of organic solvents (acetone, ethanol, and methanol) at concentrations 20, 30, and 50% (v/v) was also investigated in this study. The enzyme activity was performed at the optimum conditions of pH and temperature.

Kinetic Assays

The kinetic parameters K_m and V_{max} were investigated under optimum conditions of the enzyme activity through the variation in the concentration (1–20 mg/mL) of beechwood xylan (Sigma-Aldrich). The values of K_m and V_{max} were obtained through non-linear regression as Michaelis-Menten kinetics model using the GraphPad Prism (version 5.0) software (GraphPad Software, La Jolla, CA, USA). Additionally, a Lineweaver-Burk plot was also performed.

Hydrolysis of Xylan from Beechwood

The hydrolysis of 3% (w/v) beechwood xylan (Sigma-Aldrich) was performed in 0.1 M sodium acetate buffer (pH 5.0) at 40 °C using purified enzyme from *M. heterothallica* (60 U/g beechwood xylan). The enzyme reaction was conducted for 12 h under agitation at 180 rpm and, at the end of the hydrolysis, the samples were subjected to a boiling bath for 5 min and then filtered and centrifuged at 2500×g for 10 min.

High-Performance Anion-Exchange Chromatography Coupled with Pulsed Amperometric Detection (HPAEC-PAD)

The analysis of hydrolysates was carried out in a Dionex ICS-3000 Ion Chromatography System (Thermo Fisher Scientific, Waltham, USA). The XOS determination was performed using a CarboPac PA200 column (3 mm × 250 mm) and a CarboPac PA200 guard column (3 mm × 50 mm) at 30 °C using a flow (0.5 mL/min) for 17 min. The identification and quantification of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and xylohexaose (X6) were performed using commercial standards (Megazyme, for XOS; Sigma-Aldrich, for xylose).

Statistical Analysis

The averages of the data were compared using the statistical variance (ANOVA, $p < 0.05$) using GraphPad Prism (version 5.0) software.

Results and Discussion

Enzyme Purification

Purification of xylanase secreted by the fungus *M. heterothallica* was carried out in two chromatographic steps, namely gel filtration chromatography (Sephadex® G-75) and ion-exchange chromatography (Resource™ Q) (Table 1).

In the first step, the xylanase was eluted from Sephadex® G-75 resin showing a purification of about 10 times and a recovery of approximately 6%. Then, the enzyme was applied to Resource™ Q column. At the end, the yield was 3.52% and a purification fold of approximately 31 times. This final yield is higher than that reported by McPhillips et al. [25], in the purification of xylanase from *Remersonia thermophila* CBS 540.69, who gained 1.37% final recovery of that enzyme using the same chromatographic steps.

Analyzing the chromatogram of Sephadex® G-75 resin (Fig. 1a) shows the importance of this chromatographic step as being fundamental to a pre-purification of the crude extract. Figure 1a shows six defined peaks for protein and enzyme activity over the wide range of fractions collected. We detected that the fractions identified as "A" (88–89) were better pre-purified as indicated by 10% SDS-PAGE, and they were, therefore, used in the next step. The other fractions, although demonstrating greater enzyme activity, were less pre-purified than the selected fractions, which justifies the choice of fractions 88–89.

The second step (Fig. 1b) exhibited two peaks, corresponding to proteins not adsorbed (peak I) and adsorbed (peak II) onto the resin. It is possible to note that the enzyme does not

Table 1 Purification summary of xylanase secreted by *M. heterothallica*

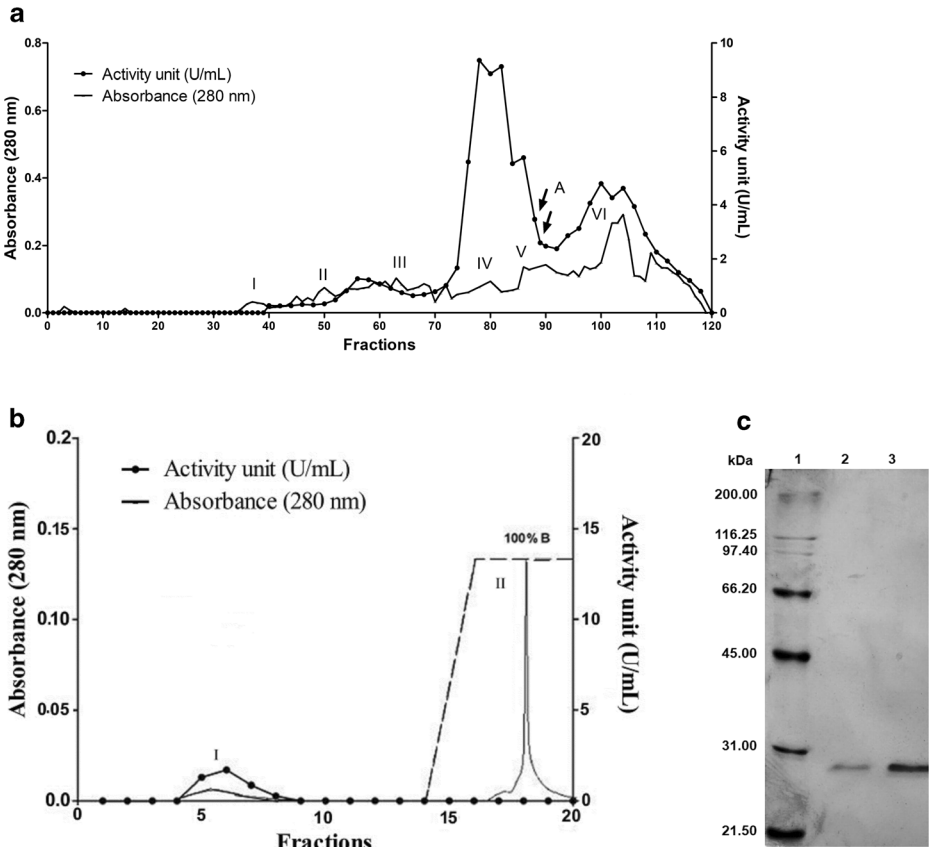| Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude extract	10,900	210.0	52	1.00	100
Sephadex® G-75	645	1.19	542	10.44	5.92
Resource™ Q	384	0.24	1600	30.83	3.52

Fig. 1 a Sephadex® G-75: I to VI protein peaks, selected fractions 88 and 89. b Resource™ Q 6 mL: proteins not adsorbed (peak I) and adsorbed (peak II). c 10% SDS-PAGE: line 1 (marker), line 2 (fraction 6), line 3 (fraction 7)

adsorb onto the resin. Visualization on 10% SDS-PAGE showed that fractions 6 and 7 contain pure xylanase with estimated molecular mass to be 27 kDa (Fig. 1c).

Based on the molecular mass of the purified enzyme, it can be inferred that it does not belong to the GH10 family, since enzymes belonging to this family feature larger molecular weight, approximately 40 kDa [26]. Some xylanases GH11 produced by thermophilic fungi presented similar molecular weight to the ones found in this study, such as the xylanases from *Malbranchea flava* (25.2 kDa) [27] and *Rhizomucor miehei* (27 kDa) [28].

Peptide Sequence of the Enzyme

Analysis of the protein fragments by mass spectrometry exhibited one peptide sequence identified with other microbial glycoside hydrolases (Table 2). We found the protein fragment LRLADDNWSYR which exhibited 100% identity with the protein fragment from the hypothetical protein from *M. thermophila* (*Thermothelomyces thermophila* ATCC 42464), and similarity with other peptide sequences from fungal glycoside hydrolases: GH family 43 from *M. thermophila* (*T. thermophila* ATCC 42464) and xylanase from *Aspergillus oryzae*; and the

Table 2 Peptide sequence of the enzyme obtained by tryptic digestion and analysis by mass spectrometry

Microorganism	Peptide sequence	Enzyme
M. heterothallica	**LRLADDNWSYR**	Xylanase—present paper
M. thermophila (*Thermothelomyces thermophila* ATCC 42464)	**LRLADDNWSYR** (XP_003667363.1)	Hypothetical protein
M. thermophila (*T. thermophila* ATCC 42464)	A**RLADD**MRQFA (XP_003666322.1)	GH family 43
Aspergillus oryzae	EA**LADD**G-**SYR** (AFP43760.1)	Xylanase precursor
Bacteroidales bacterium KA00344	RV**LSDDNW**RTG (WP_066264617.1)	Xylanase

bacterial xylanase from *Bacteroidales bacterium* KA00344. However, no identity with xylanases from *M. heterothallica* was seen, which suggests this is a new enzyme of this fungus described for the first time. So far, there have been no reports in the literature about purified xylanase referring to this fungus.

pH and Temperature on the Activity and Stability of the Xylanase

The prospection of new enzymes for industrial application mostly searches for tolerance to a wide range of pH and temperatures. Figure 2 shows the results of the effect of temperature (Fig. 2a) and pH (Fig. 2b) on enzyme activity of xylanase. The pure enzyme showed the highest activity at pH 4.5 (optimal pH) and, in the pH range from 3 to 6.5, it maintained more than 50% of its relative activity. As to the optimum temperature, the highest activity was obtained at 65 and at 70 °C, showing no significant difference between them ($p < 0.05$). This corroborates the work of Basit et al. [29], in which the optimum temperature obtained for recombinant GH11 family xylanase from *M. thermophila*, belonging to the same genus of *M. heterothallica*, was moderately lower (60 °C) than that of the present work. However, the optimum pH for enzyme activity differed significantly, being 6.0 and 7.0. A similar result regarding optimum temperature to xylanolytic activity was described with *Remersonia thermophila* CBS 540.69 [22]. Souza et al. [30] reported that a xylanase from fungus *Thermoascus aurantiacus* expressed in *E. coli* showed optimum values of xylanolytic activity at pH 5.0 and a temperature of 65 °C.

Regarding the influence of pH on stability (Fig. 3a), the enzyme showed wide-ranging pH tolerance, especially over pH 5–10, in which range activity superior to 85% was observed at both temperature incubation (4 and 25 °C). These characteristics are promising for some industrial applications. Alkaline-tolerant and thermostable enzymes are required for use in the paper industry and, in the food industry, acid-tolerant enzymes are fundamental for application in fruit juice and wine making [2].

The significant stability at different pH is very important, especially to facilitate working conditions for obtaining of pure enzyme, since some methodologies, such as dialysis and chromatography, often require long incubation times in buffer solutions. Lower results were found for two isolated fungal xylanase from the same genus, in which both showed retention of between 50 and 75% of the maximum activity at pH in the range 5–10 [29].

In some biotechnological processes involving the release or uptake of protons, for example, steam explosion and liquid hot water pretreatments, there is the cleavage of O-acetyl bonds and uronic acid substitutions from hemicellulose to generate acetic acid and other organic acids [31], which, in turn, can change the pH. Therefore, a robust enzyme, stable over a broad range
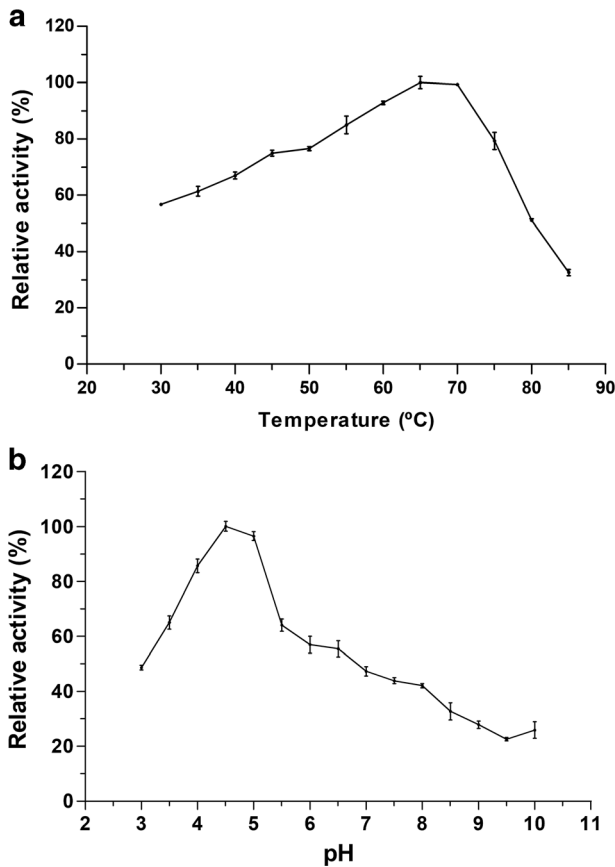
Fig. 2 Influence of **a** temperature and **b** pH on xylanase activity. The activity was determined by using 1% (*w/v*) beechwood xylan as a substrate

of pH, is highly desirable to avoid loss of the enzyme during the enzymatic hydrolysis process. For the pulp bleaching application, the xylanase must tolerate high temperature and alkaline pH, which are the conditions of the reaction medium, after the alkaline cooking of the pulp [32].

Another important feature of this enzyme is its thermostability (Fig. 3b). The purified enzyme was stable to temperature variations (up to 60 °C), showing 92% of relative activity when exposed for 1 h to 55 °C. Similar result was found by Liu et al. [4]. However, in that case, the enzyme was subjected to treatment for 30 min. Purified fungal xylanase from *Remersonia thermophila* exhibited a significant reduction of activity when exposed to different pH ranges in shorter times up to 60 min [25].

The advantages of reactions that occur at high temperatures are well known, because the solubility of the reagents and products are increased, the viscosity is low, and the mass transfer rate is higher. This results in a significant reduction of reaction time which, in turn, results in process cost savings. In this sense, stability and activity at high temperatures is a highly desirable feature when looking for enzymes for industrial use. In general, the degradation of vegetable fiber requires a long reaction time. When the enzymes are used at lower temperatures, microbial contamination is more probable. This problem can be overcome when the
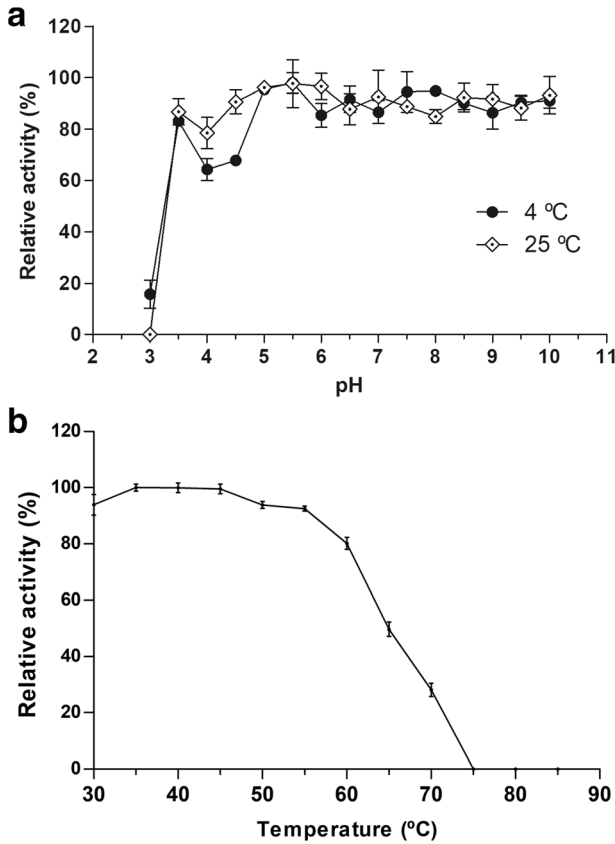
Fig. 3 Effect of **a** pH and **b** temperature on xylanase stability. The activity was determined using 1% (w/v) beechwood xylan as substrate

depolymerization of cellulose and hemicellulose reaction occurs at higher temperatures. In this scenario, thermophilic enzymes are better [33].

Considering the above comments, it can be stated that *Myceliophthora heterothallica* F.2.1.4 xylanase presented important characteristics from the point of view of industrial application, both in relation to temperature (exhibiting greater activity at 65/70 °C) and its thermostability (maintaining more than 80% of the residual activity when exposed for 1 h at temperatures between 30 and 60 °C).

Effect of Different Compounds on Xylanase Activity

Among the compounds evaluated (Fig. 4), SDS provided greater negative modulation of enzyme activity. At a concentration of 5 mM, xylanase activity was significantly different compared to the control, keeping only 28.7% of its catalytic performance. The reduction in enzyme activity occurs because SDS is an anionic surfactant able to destabilize existing interactions in the protein structure, causing changes in enzyme folding [34]. According to Silva et al. [21], the electrostatic attraction between the charges of the enzyme and SDS facilitates interaction of the hydrophobic portion of the surfactant with the hydrophobic enzyme center, favoring the formation of micelles, which increase the denaturation of the

Fig. 4 Effect of 5 mM of different chemical compounds on xylanase activity. The values are expressed as mean ($n = 3$) ± standard deviation. The asterisks represent significant differences ($p < 0.05$)
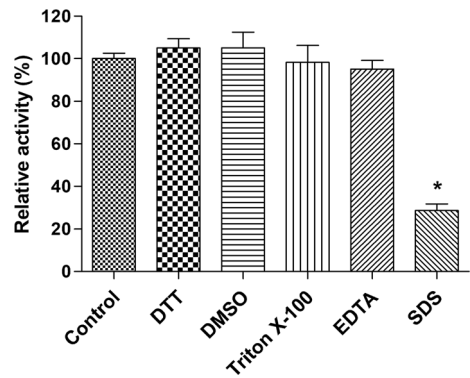
enzyme. Triton X-100, another surfactant but non-ionic, did not influence the activity of the enzyme.

EDTA showed no significant modulatory effect on the enzyme, so it is assumed to be independent of metals as cofactor. This was also shown for fungal xylanases from *M. thermophila* expressed in *Pichia pastoris* [29].

The other compounds, DL-Dithiothreitol (DTT) and dimethyl sulfoxide (DMSO), did not influence the activity of the enzyme, showing that, in the evaluated concentration, the enzyme does not suffer breakage of disulfide bonds (effect of DTT) and is able to remain functional at low concentrations of the organic solvent DMSO. Some studies have been using DMSO as an agent for xylan extraction from the biomass [15], resulting in a water-soluble form of xylan which retains the acetyl groups present in the native state. From our results with this compound, it may be interesting to investigate the combination of our xylanase and DMSO for the bleaching of brown pulp in the bleaching process, since the enzyme does not undergo any inhibitory effect by this reagent.

Regarding the other solvents evaluated (acetone, ethanol, and methanol), Fig. 5 shows that the enzyme was stable in up to 20% acetone and relatively stable in this concentration of methanol and ethanol, retaining 71.2% and 62.1% of activity, respectively. At higher
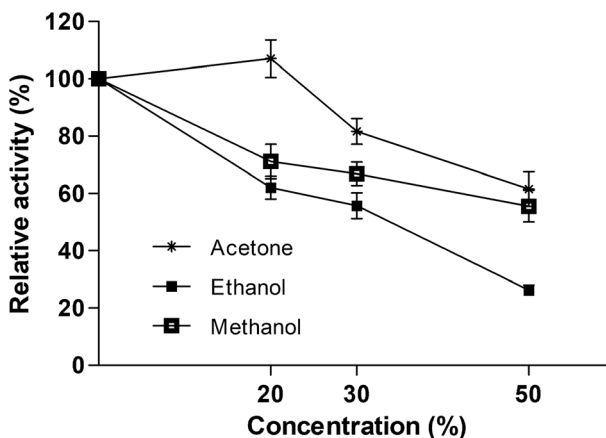

Fig. 5 Effect of different solvents on xylanase activity. The values are expressed as mean ($n = 3$) ± standard deviation

concentrations, the enzyme became less active as the concentration of solvents increased and increasingly sensitive to ethanol. This greater inactivation of the enzyme by ethanol is due to the fact that it is less polar than methanol. Al-Darkazali et al. [35] reported that xylanase was slightly inhibited by the solvents acetone, ethanol, methanol, and 1-propanol, at 5 M concentration, showing the same inhibition profile as the present study.

In enzymatic technology, some methodologies require the use of organic solvents, for example, in enzyme concentration processes. In this work, we used the ethanol for the concentration of crude enzyme. Additionally, in studies of characterization of an enzyme, it is always important to know the chemical agents capable of modulating the enzymatic activity, so that under enzyme application conditions, it is possible to maximize enzyme activity, as well as minimize other compounds that may decrease enzyme activity.

Modulator Effect of Metallic Ions on Xylanase Activity

The effect of ions on activation or inhibition of the enzyme may be due to chemical interactions between the protein structure and the ion forming complexes or it may also be a physical effect due the ability of an ion to modify the structure of the water, thus influencing the hydration environment of the enzyme. These effects are dependent on the pH of the medium (PI of the enzyme) and the concentration of the agent used [36].

Among the metal ions evaluated (Fig. 6), none of them had the ability to positively modulate the enzyme. Negative modulation was observed in the presence of certain ions, as seen with Cu^{2+}, Fe^{3+}, and Al^{3+}, which caused about from 30 to 60% loss of activity. The highest decrease in activity was detected in the presence of Cu^{2+}, which reduced enzyme activity to about 40%. Cu^{2+} is a heavy metal ion and strongly binds to sulfhydryl groups of proteins, which can destabilize the molecular structure and, thus, causing loss of enzymatic activity [37]. The activity of xylanase can be influenced negatively in the presence of Cu^{2+}, according to previous research [25, 27, 29]. The ions can interact with protein and modify their molecular arrangement or they can interact with important residues of the active site and
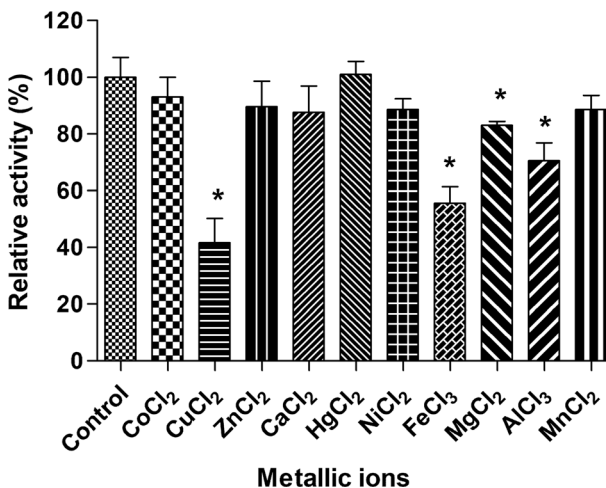

Fig. 6 Effect of different ions on the activity of xylanase. The values are expressed as mean ($n = 3$) ± standard deviation. The asterisks represent significant differences ($p < 0.05$)

negatively modulate the enzyme activity [20–22]. Ding et al. [38] reported an inhibitory effect of Fe^{3+} on the xylanase activity of *Pichia stipitis*, which corroborates the present work.

The negative modulation effect of the Hg^{2+} ion, reported in the literature as a potent inhibitor [38], was not seen in this study. This result, however, is in accordance with other studies that also reported not having observed the inhibition effect of mercury on xylanase [39].

According to these results, it appears that the enzyme does not need any of the ions evaluated here to improve its hydrolysis on xylan. Additionally, for future application studies, it is imperative to avoid the presence of Cu^{2+}, Fe^{3+}, and Al^{3+} ions in the reaction mixture.

Kinetic Assays and Substrate Specificity

In the concentration range of the analyzed substrate, which was from 1 to 20 mg/mL, the purified xylanase had behavior compatible with the Michaelis-Menten kinetics profile.

Observing the enzyme saturation curve (Michaelis-Menten curve) and Lineweaver-Burk plot, we found similar results. The values of K_m and V_{max} for the Michaelis-Menten curve were 2.46 ± 0.26 mg mL^{-1} and 18.64 ± 0.48 µmol min^{-1} mg^{-1}, respectively (Fig. 7a), while the K_m and V_{max} to Lineweaver-Burk plot were 2.7 ± 0.28 mg mL^{-1} and 19 ± 1 µmol min^{-1} mg^{-1}, respectively (Fig. 7b). The V_{max}/K_m values are very similar between the Michaelis-Menten
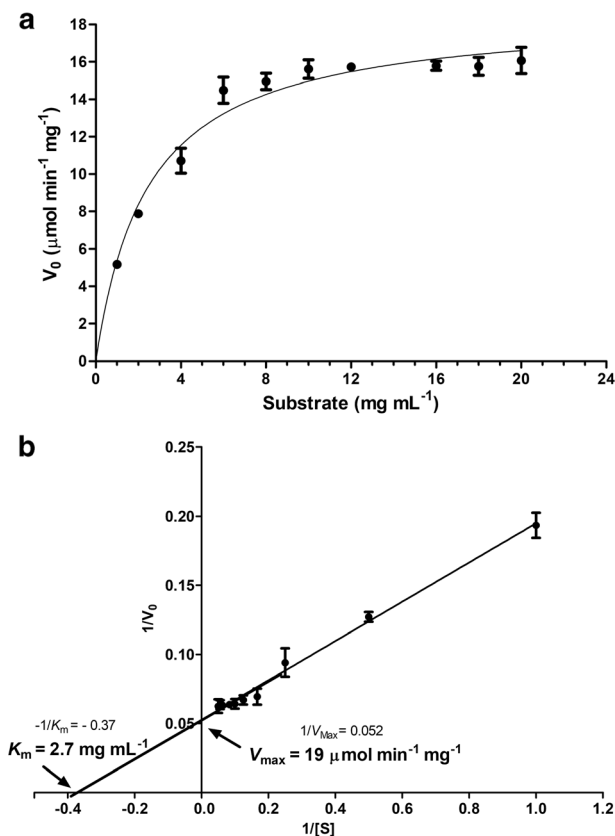

Fig. 7 a Michaelis-Menten curve. b Lineweaver-Burk plot

curve (7.5) and Lineweaver-Burk plot (7) analyses. This reinforces the fidelity of the results pointed out here.

The value of K_m is within the range for fungal xylanases, that is, from 0.14 to 14 mg mL^{-1} [3]. A similar value of K_m (2.477 mg mL^{-1}) was found for *Remersonia thermophila* xylanase [25]. Basit et al. [29] obtained higher values of K_m (8.80 mg mL^{-1} and 5.67 mg mL^{-1}) for two *M. thermophila* endo-β-1,4-xylanases. Because the purified enzyme has a low K_m value, it can be inferred that it has high specificity in relation to the substrate, which favors the XOS production.

Different substrates were evaluated for their susceptibility to enzyme hydrolysis. The xylanase showed activity only in xylan (Table 3). The enzyme described in this work was not able to hydrolyze other evaluated substrates. This strengthens the understanding that this is an endo-xylanase. Endo-xylanases can release xylose [40, 41], especially when exposed to long reaction times, as described in this study, where xylan hydrolysis was performed for 12 h, and xylobiose, xylotriose, and higher XOS as reaction products [40, 42–46].

Application of Xylanase for Xylooligosaccharide Production

The products of beechwood xylan hydrolysis by purified xylanase were identified and quantified by HPAE-PAD (Fig. 8). According to Fig. 8, the main products released in the hydrolysis were xylobiose and xylotriose and, in smaller amounts, xylose, xylotetraose, xylopentaose, and xylohexaose. Hoffmam et al. [47] also obtained xylobiose as major products of the hydrolysis of beechwood xylan by the action of chimeric enzyme (GH11). The characteristics of the purified enzyme, as well as the profile of products released in the hydrolysis of xylan, characterize the purified enzyme as an endo-xylanase belonging to the GH11 family [47, 48].

The total hydrolysis and XOS yields were obtained by the ratio of the total sugars after hydrolysis and total xylan used in the tests, being 30.4% of which 26% of the total obtained sugar was XOS. After 12 h of hydrolysis, 234.2 mg XOS/g beechwood xylan was released, with approximately 50% of the total xylobiose production (115.9 ± 4.52 mg/g xylan) being released.

The purified enzyme has a mode of action that allows the production of xylooligosaccharides of interest for application in functional foods, since XOS with lower degrees of polymerization, such as xylobiose and xylotriose, are those that are assimilated more quickly by probiotic microorganisms [49].

This study emphasizes the importance of obtaining this enzyme in its pure form in order to know its specificity in producing the XOS of interest. With the crude enzyme, this would not

Table 3 Specificity in relation to different substrates. Values are expressed as mean ($n = 3$) ± standard deviation

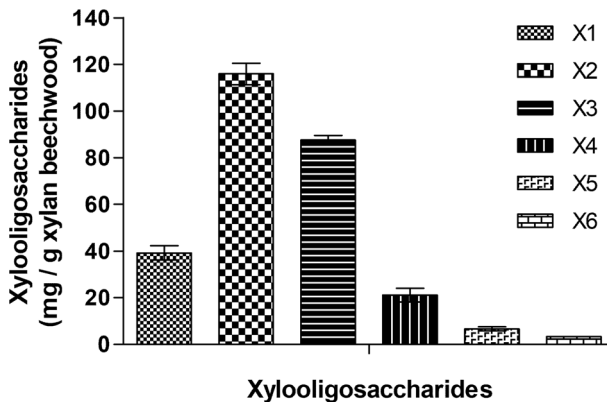| Substrate	Enzymatic activity (U/mL)	Standard deviation
Avicel	0	0
Carboxymethyl cellulose	0	0
Beechwood xylan	2.1	0.04
pNPA	0	0
pNPG	0	0
pNPX	0	0

Fig. 8 Enzymatic hydrolysis of beechwood xylan with purified xylanase after 12 h. The released xylooligomers were detected and quantified by HPAE-PAD methods. The substrate concentration was 3% (*w/v*), using 60 U/g at 40 °C

be possible, considering the possible interference of the constituents of the crude enzyme. So, the use of a crude enzyme could lead to a non-specific release of XOS. Another strong argument is based on the versatility of the fungus *M. heterothallica* F.2.1.4 in secreting proteolytic enzymes, whose action on the crude extract has been recognized as detrimental for degrading lignocellulolytic enzymes [50, 51]. Unlike the crude extract, the pure enzyme shows its specificity as demonstrated in this work, with high production of X2 and X3 and it is free of the action of peptidases.

Conclusion

In this study, a thermostable xylanase from the fungus *M. heterothallica* F.2.1.4 was purified and characterized. This enzyme presents advantages in relation to its high specificity to the substrate, its thermostability, and wide pH stability, exhibiting characteristics that enable its use in enzymatic saccharification processes of xylan for the production of XOS. In this sense, xylanases capable of hydrolyzing xylan to release xylobiose and xylotriose as major end products are greatly needed. Furthermore, to the best of our knowledge, no native xylanase has been purified from the thermophilic fungus *M. heterothallica*, which suggests this study was dealing with a new enzyme and that favors for future assays for application of XOS.

Compliance with Ethical Standards

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