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Application of an endo-xylanase from *Aspergillus japonicus* in the fruit juice clarification and fruit peel waste hydrolysis

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ABSTRACT

The endo-xylanase from *Aspergillus japonicus* (UFMS 48.136) was purified in a single step using carboximethyl-cellulose chromatographic column and applied in fruit juice clarification process and fruit peel waste hydrolysis. This purification procedure resulted in 38.9-fold purification of endo-xylanase with 83.3% final yield. MALDI-TOF analysis confirmed the molecular mass of 32 kDa. The optimal purified endo-xylanase activity was at a range of pH from 5.0 to 6.0 and from 50 to 60 °C, retaining more than 70% of its activity at all pH studied (3.0–8.0) for 24 h at room temperature. The *A. japonicus* endo-xylanolytic activity stimulation curve was assayed in the presence of different birchwood xylan concentrations (ranging from 0.02 to 0.5% w/v) and the endo-xylanase activity presented a Vmax of 467.4 ± 30.38 μmol/min/mg, with a km of 2.59 ± 0.17 mg/mL, a kcat of 253.95 ± 16.51 s^{-1} and a kcat/km value of 98.05 ± 4.41 mL s^{-1} mg^{-1}. The endo-xylanase was activated by Mn^{2+} (34.5%) and inhibited by Cu^{2+} (56.9%). The endo-xylanase was activated by β-mercaptoethanol, Triton X-100, Tween-20, Tween-80 and ferulic acid. In the clarification assay, endo-xylanase successfully clarified the juices of mango (51.11%), banana (9.99%) and tangerine (8.54%). Furthermore, the enzyme also hydrolysed all fruit peel wastes that were tested. In summary, *A. japonicus* endo-xylanase showed potential for applications in fruit juice clarification and in the treatment of fruit peel wastes, and it is a good candidate for the food industry due to its wide pH stability under acidic conditions.

1. Introduction

Xylan is a major structural component of plant cell walls and its degradation requires the action of several enzymes, among which endo-xylanases (EC 3.2.1.8) play a key role (Polizeli et al., 2005; Fortkamp and Knob, 2014). Xylanases occur widely in bacteria, yeasts and fungi. Many reports on xylanases from *Aspergillus* sp., *Trichoderma* sp., *Bacillus* sp., *Streptomyces* sp. and other microorganisms are available (Sharma, 2017). Filamentous fungi are particularly useful producers of xylanases from an industrial point of view because they excrete larger amounts of xylanolytic enzymes into the medium than yeast or bacteria (Polizeli et al., 2005). Microbial xylanases have fascinated researchers because of their potential applications in various industrial processes, being potentially employed in the production of hydrolysate from agro-industrial wastes, as a food additive in poultry, and increasing animal feed digestibility, thereby improving both weight gain and feed conversion efficiency (Polizeli et al., 2005; Amita et al., 2006). Redgwell et al. (2001) reported a constant decrease in the viscosity of wheat flour batter using crude xylanase. Other industrial applications include the use of xylanases as kraft pulp bleaching agents (de Alencar Guimarães et al., 2013; Guimarães et al., 2013; Silva et al., 2015a; Walia et al., 2017; Wu et al., 2018), in orange peel hydrolysis (Uday et al., 2017) and in the clarification of wines and juices (Dhiman et al., 2011; Rosmine et al., 2017; Sharma, 2017). In the food industry, the main desirable biochemical properties for xylanases are optimal activity and high stability at acidic pH values (Polizeli et al., 2005). When xylanases are used along with amylases, pectinases and cellulases, they improve juice production yield by liquefying the fruit, reducing its viscosity,

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stabilizing the fruit pulp and increasing the recovery of aromas. Low-viscosity juices with high clarity are more attractive to consumers and are more nutritive. Increases in clarity and decreases in viscosity of fruit juices using xylanases have been reported by *Bacillus stearothermophilus* (Dhiman et al., 2011), *Bacillus pumilus* SV-85S (Nagar et al., 2012) and *Streptomyces* sp. (Rosmine et al., 2017). Additionally, xylooligosaccharides obtained by xylan enzymatic hydrolysis are important ingredients for the development of novel functional foods due to their prebiotic effects and association with the reduction of blood glucose and cholesterol, a reduction of pro-carcinogenic enzymes, an enhancement of mineral absorption and stimulation of the immune system (Samanta et al., 2015). Endo-xylanases also play a key role in the production of high-value xylooligosaccharides as prebiotics derived from agricultural by-products and other low-cost raw material (Linares-Pastén et al., 2018). The aim of this study was to purify an endo-xylanase produced from wheat bran in a single step and to characterize and employ the enzyme in the clarification of fruit pulps and hydrolysis of fruit peel wastes.

2. Materials and methods

2.1. Microorganism, growth conditions and enzyme production parameters

The filamentous fungus *A. japonicus* (UFMS 48.136) was collected and isolated from the Natural Heritage Private Reserve (RPPN) soil of the Universidade Federal de Mato Grosso do Sul (UFMS), Campo Grande (MS)-Brazil. The identification of the fungus was performed using morphological characteristics by the UFMS mycology technician MSc Clarice Rossato Marchetti. The species was deposited in the UFMS mycology collection under number UFMS 48.136. It was cultivated on Potato-Dextrose-Agar (Himedia, IND) and preserved in silica gel. The *A. japonicus* endo-xylanase production was started using conidia that were suspended in sterile distilled water; the concentration was adjusted to 1×10^6 spores/mL, and a volume of 1 mL of this suspension was used as inoculum. The optimal fermentation conditions, such as carbon source and growth time (stationary or agitated) that would maximize endo-xylanase production were previously determined by OFAT (one-factor-at-a-time) methodology (Guimarães et al., 2013). Cultures were prepared in Erlenmeyer flasks (500 mL) containing 100 mL of liquid medium (Rizzatti et al., 2001) with 1% $NH_4H_2PO_4$; 0.3% KH_2PO_4; 0.24% $MgSO_4{\cdot}7H_2O$; 0.45% yeast extract; 0.02% peptone and 1% wheat bran as carbon source (w/v), maintained at 30 °C for 96 h under static conditions. The medium was subsequently vacuum-filtered using filter paper (Whatman n° 1) and the filtrate was used as a crude extract source rich in endo-xylanolytic activity.

2.2. Endo-xylanase assay and protein determination

Endo-xylanase activity was determined by incubating enzyme samples with 1% (w/v) birchwood xylan (Sigma-Aldrich, USA) at 50 °C in McIlvaine buffer, containing a mixture of 0.1 M citric acid (10.3 mL) and 0.2 M disodium hydrogen phosphate (9.7 mL) to obtain pH 5.0 (McIlvaine, 1921). At suitable intervals, the reaction was interrupted with 3, 5-dinitrosalicylic acid (DNS) reagent (Merck, DEU) and the amount of released reducing sugars was quantified by the methodology according to Miller (1959) using a standard curve of xylose. One unit of activity (U) was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugars per minute. Protein (crude extract and purified enzyme) was determined by the Lowry method (Lowry et al., 1951) using the bovine serum albumin as a standard. The specific activity was expressed by the relation between enzyme activity and protein content (U/mg protein).

2.3. Purification of endo-xylanase

The crude extract rich in endo-xylanase was dialysed overnight in 50 mM sodium acetate buffer (pH 4.5) and applied to a CM-cellulose chromatography column (2.0 × 6.0 cm) that was pre-equilibrated with the same buffer. The column was then washed with 150 mL of this buffer, and a volume of 300 mL of a 0–1 M NaCl gradient was applied to elute the proteins that were adsorbed on the resin. The protein fractions collected from the chromatography column were detected by reading the absorbance at 280 nm, and the fractions showing high endo-xylanase activity were pooled and dialysed against deionized water for 24 h. The sample was then used for purity analysis, biochemical characterization, fruit juice clarification and hydrolysis of fruit peel wastes.

2.4. SDS-PAGE of crude extract and purified endo-xylanase

The crude extract and the purified endo-xylanase were evaluated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis as described by Laemmli (1970) to verify homogeneity and the endo-xylanase molecular mass. Protein bands (17 μg) were visualized by a silver stain kit (Sigma-Aldrich, USA). Prestained protein marker (Kaleidoscope, Bio-Rad) was used.

2.5. MALDI-TOF mass spectrometry

The CM-cellulose chromatographic fraction containing endo-xylanase was desalted and concentrated with acetone (1:1) and then applied to a C18 Zip-Tip (Millipore), according to the manufacturer's instructions, prior to MALDI-TOF analysis. The peptide mass fingerprint was obtained after in-gel digestion with trypsin (Promega) (Jiménez et al., 2001). The samples were mixed 1:1 with the indicated matrices (Sigma-Aldrich), and spectra were acquired in positive mode on an Autoflex III Smartbeam (Bruker Daltonics). Mass calibrants ranging from 757.3 to 66.430 Da (Sigma-Aldrich) were used for external calibration, and spectra were processed with Flex Analysis 3.3 software (Bruker Daltonics) and the MASCOT website (Matrix Science).

2.6. Genomic DNA extraction, PCR and sequencing

Mycelia of 3 days of culture was collected by filtration and homogenized in liquid nitrogen. Next, genomic DNA was extracted from the mycelia using a Wizard Genomic DNA Purification Kit (Promega-USA) according to the manufacturer's instructions. PCR was used to amplify the internal transcribed spacer regions ITS1-5.8S-ITS2 (ITS1 and 4) of the rRNA gene cluster using two primer pairs: ITS1 (5' –TCCGTAGGTGAACCTGCGG– 3′) and ITS4 (5' –TCCTCCGCTTATTGATATGC– 3′) (Druzhinina et al., 2005). The PCR amplification of the ITS regions was performed according to Druzhinina et al. (2005) and Raja et al. (2017). Amplified product sequencing was performed by Sanger technology in a 3500 Genetic Analyzer sequencer (Applied Biosystems).

2.7. Characterization of the purified endo-xylanase

2.7.1. Optimal pH and temperature

The optimal pH of the purified endo-xylanase was determined at 50 °C using McIlvaine buffer incubating the enzyme at different pH ranging from 3 to 8. The endo-xylanase optimal temperature was measured by incubating the reaction mixtures at different temperatures (40–80 °C) with McIlvaine buffer at pH 5.0. The relative activity was calculated as a percentage of the maximal activity.

2.7.2. Stabilities under different temperature and pH

The stability under different temperatures were assessed by incubating for 6.0 h the endo-xylanase by *A. japonicus* in McIlvaine buffer pH 5.0 (in the absence of substrate) under temperatures of 40, 45, 50 and

Table 1
Endo-xylanase purification from *Aspergillus japonicus* crude extract.

Step	Volume (mL)	Total Activity (total units)	Protein (total mg)	Specific activity (U/mg protein)	Yield (%)	Purification fold
Crude Extract	300	2700	234	11.5	100	1
CM-Cellulose	300	2250	5	448.8	83.3	38.9

55 °C. Aliquots were taken at intervals from 10 to 240 min and subjected to determination of endo-xylanase activity. The pH stability was determined by incubating for 24 h the purified endo-xylanase by *A. japonicus*, in appropriate McIlvaine buffer, under different pH (from 3.0 to 8.0) at room temperature. Aliquots were taken at 1, 2, 4 and 24 h intervals and the endo-xylanase activity was determined. The assays were as described in item 2.2 and these results were expressed as relative activity, which is calculated as a percentage of the maximal activity.

2.7.3. Substrate specificity and hydrolysis products analysis in thin layer chromatography

Endo-xylanase specificity was verified by assaying the activity against 1% (w/v) birchwood xylan, carboxymethyl cellulose (CMC), avicel and starch prepared in McIlvaine buffer pH 5.0 at 50 °C, and endo-xylanase activity was measured as described in item 2.2. Thin layer chromatography (TLC) was performed using a solvent system containing ethyl acetate: acetic acid: formic acid: water (9:3:1:4 per vol.). The compounds formed by the endo-xylanase action on 1% (w/v) birchwood xylan substrate were detected by spraying the TLC with a solution of H_2SO_4 and methanol (1:9 per vol.) that contained 0.2% orcinol, followed by heating at 100 °C.

2.7.4. Effect of metal ions, ethylenediaminetetraacetic acid (EDTA) and various substances on endo-xylanase activity

The relative activity of the purified endo-xylanase was evaluated in the presence of the ions Ba^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, Fe^{3+}, K^{2+}, Mg^{2+}, Mn^{2+}, NH^{4+}, Zn^{2+} and EDTA, in concentrations of 1 and 5 mM, under the conditions established in item 2.2. The effect of various substances on the endo-xylanase activity was measured by incubating the enzyme samples at 25 °C for 30 min with phenolic compounds (vanillin, ferulic acid, cinnamic acid, 4-hydroxybenzoic acid, coumaric acid, tannic acid) at 1 mg/mL final concentration. Additionally, 10% detergents (Triton X-100, Tween-20, Tween-80, SDS), 10% organic solvents (acetonitrile, butanol, acetone, isopropanol, methanol, DMSO, ethanol) and reducing agents (β-mercaptoethanol 0.01 and 0.1 M) were tested.

2.7.5. Kinetic parameter determination

The kinetic parameters, Michaelis-Menten constant (Km, mg/mL) and the maximum reaction rate (Vmax, μmol/min/mg), were calculated using the software SigrafW, which fits the experimental data to the Hill equation by nonlinear regression (Leone et al., 2005). The *A. japonicus* endo-xylanolytic activity stimulation curve was assayed in different birchwood xylan concentrations (ranging from 0.02 to 0.5% w/v). The kinetic experiments were repeated in triplicate, using three different homogenates, and each enzymatic assay was performed in duplicate. The kinetic parameters are presented as the mean ± SD of the values calculated for the three replicates (n = 3).

2.7.6. Fruit juice clarification and hydrolysis of fruit peel wastes by endo-xylanase of A. japonicus

The fruits used for the clarification assays were pineapple, banana, mango, orange, tangerine, nectarine, peach and tangelo. All fruits were acquired from markets and were washed, peeled and macerated using a blender to obtain the pulp. The pulp and enzyme (1:1) were incubated at 55 °C for 4 h. After this period the samples were boiled for 5 min for enzyme inactivation and centrifuged at 21,000 g for 15 min. The supernatant (juice) was used for determining juice clarity by recording transmittance at 650 nm, taking distilled water as the blank. For each

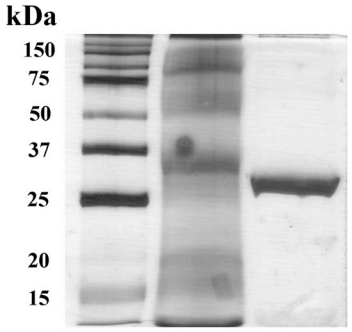

Fig. 1. Purified xylanase SDS-PAGE (12%). Line 1: Molecular mass marker; Line 2: Crude extract; Line 3: Purified enzyme. Protein bands were visualized by a silver stain kit (Sigma-Aldrich, USA).

fruit pulp, a control was carried out using the fruit pulp and distilled water (1:1 v/v). Clarification was calculated as follows:

$$\% \text{ Clarification} = \frac{Tt - Tc}{Tc} \text{ x } 100$$

(Tt = transmittance of test; Tc = transmittance of control), as described by Rosmine et al. (2017). The eight peel wastes obtained from the fruits were dried, milled and weighed and used to determine the biomass hydrolytic potential of the purified endo-xylanase. The separate fruit peels (5 mg) were incubated with the enzyme for 15 min and the amount of released reducing sugars was determined by the methodology according to Miller (1959).

3. Results and discussion

3.1. Purification and identification of the endo-xylanase from A. japonicus and its genomic identification

Aspergillus japonicus is considered a non-pathogenic fungus in the food industry, as reported in the literature (O'Toole et al., 2006), and is widely used as a producer of enzymes of industrial interest like pectinase, cellulase and xylanase (Li et al., 2015). The endo-xylanase produced by *A. japonicus* grown on wheat bran was purified from the cell-free culture supernatant. Crude extract (300 mL) containing the extracellular enzyme was applied to a CM-cellulose chromatography column pre-equilibrated with 50 mM sodium acetate buffer pH 4.5. The fractions containing high amounts of purified endo-xylanase were eluted with a linear gradient of 0–1.0 M NaCl, resulting in a 38.9-fold purification with 83.3% recovery. A summary of the purification procedures is presented in Table 1. There are few examples in the literature where fungal xylanases from mesophilic strains were purified using a single-step purification strategy (Sandrim et al., 2005; Yegin, 2017). Generally, different purified enzymes are commonly used in the fine chemistry and pharmaceutical industries to obtain highly pure products with an increased value. A pure enzyme preparation can prevent other undesirable reactions that could be promoted by contaminant enzymes and metabolites from crude extracts, decreasing the costs for the final product separation. To obtain a purified catalyst, additional techniques can be expensive and make the process unfeasible. Thus, a simple method to obtain purified xylanase is desirable and can reduce the

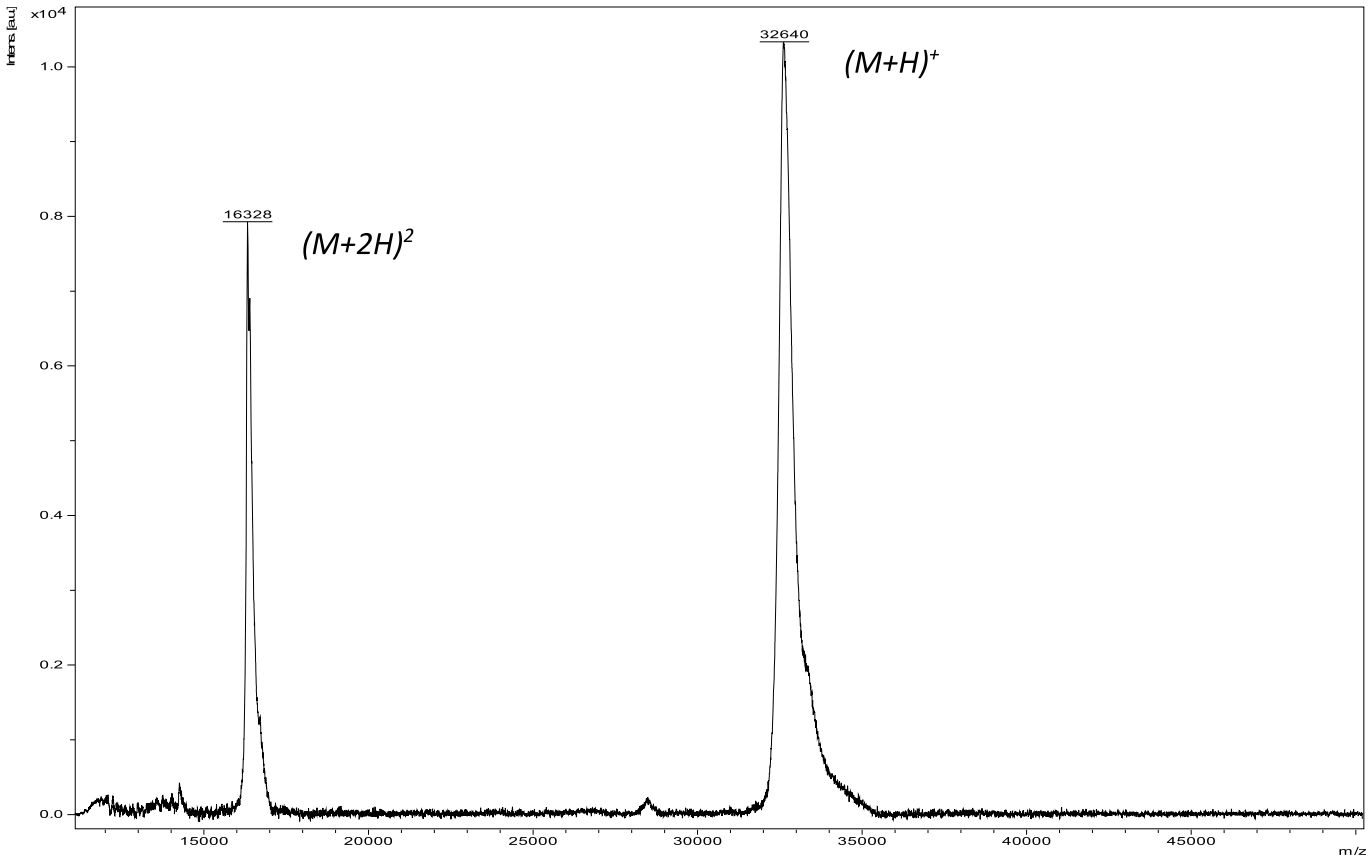

Fig. 2. MALDI-TOF mass spectrometry of the purified endo-xylanase of *A. japonicus*. Ionization was obtained with superDHB matrix, 50–55% laser intensity and spectrum acquisition in positive linear mode and external calibration. The graph shows mass/charge ratio (m/z) and ion intensity (arbitrary units), depicting a mono $(M + H)^+$ and double $(M+2H)^{2+}$ charged main component of 32,639 Da.

overall costs of biocatalysis reaction processes. The xylanase from *Aspergillus niger* BCC 14405, using ion exchange and hydrophobic chromatography, showed a 5-fold purification and 20.7% recovery (Krisana et al., 2005). Another *A. niger* xylanase purification using ion exchange and gel permeation chromatography showed a 36.97-fold purification with 38.9% recovery (Pal and Khanum, 2011). The apparent molecular mass of the purified endo-xylanase was 32 kDa, as estimated by SDS-PAGE (Fig. 1). This result was confirmed by MALDI-TOF mass spectrometry analysis, with a main component of 32.6 kDa (Fig. 2). The molecular mass of the purified endo-xylanase of *A. japonicus* was similar to the xylanases from *Aspergillus caespitosus* (Sandrim et al., 2005), *Aspergillus versicolor* (Carmona et al., 2005), *Aspergillus awamori* (Teixeira et al., 2010) and *A. niger* DFR-5 (Pal and Khanum, 2011). Kulkarni et al. (1999) reported that microbial xylanases are single subunit proteins within a range of 8–145 kDa.

To verify the identity of the purified endo-xylanase, a peptide mass fingerprint was obtained after acrylamide band excision and in-gel trypsin digestion of the electrophoresed protein (Fig. 3). A survey in the protein sequence data banks using the MASCOT algorithm revealed significant identity with two sequences, both *Aspergillus aculeatus* endo-xylanases belonging to family 10 of glycoside hydrolases (GH). The UniProtKB/Swiss-Prot sequence deposited with access O59859.1 (score = 121; $p < 0.001$) had 71% coverage, while for the GenBank access ALI87002.1 (score = 99; $p < 0.02$) the coverage was 60%, suggesting that the similarity level of our enzyme with the *A. aculeatus* sequences is very high. Further analysis by top-down fragmentation rendered an amino-terminal sequence of 21 residues, which presented 84% similarity with *A. japonicus* var. *aculeatus* xylanases (not shown), strengthening the relatedness of our enzyme with the xylanase family.

The sequenced DNA of cluster region ITS1-5.8S-ITS4 was analysed using Geneious Software (Geneious Prime® 2019.0.4) (Kearse et al., 2012). These results were compared with GenBank database sequences (Benson et al., 2005) to identify the fungus homology and identity (http://www.ncbi.nlm.nih.gov/BLAST/). The genomic analysis showed 100% identity with *A. japonicus* var. *aculeatus* in BLAST under accession number MH567078. Filamentous fungi can exhibit a multiplicity of xylanases, in some cases three or more endo-xylanases have been separated from a single culture, which may differ by post-translational modifications and processing, probably due to an adaptive response of these microorganisms to optimize their biodegradation mechanisms (Rizzatti et al., 2004; Paës et al., 2012). However, genes involved in xylanase production can reflect phylogenetic relationships of closely related taxa (Degefu et al., 2004).

3.2. Biochemical characterization of the endo-xylanase

The pH effect on the purified endo-xylanase activity is shown in Fig. 4a. The optimal endo-xylanase activity was at a range of pH from 5.0 to 6.0. The majority of purified fungal xylanases that have been reported are optimally active at the acidic pH range from 4.5 to 6.5 (Polizeli et al., 2005; Hmida-Sayari et al., 2012; Silva et al., 2015b; Sharma, 2017), and few neutral or alkaliphilic xylanases are reported in mesophilic fungus species of the *Aspergillus* genus (Polizeli et al., 2005). The pH stability was determined by verifying the remaining activity after incubating the purified enzyme for 24 h at room temperature. In Fig. 4b, it can be seen that pH stability for purified endo-xylanase at all pH studied was over 70% for 24 h. A xylanase isolated from *Aspergillus nidulans* has been found to be stable at pH 3.5–10.0 after 4 h of incubation (Reis et al., 2003; Juturu and Wu, 2012). Acidic xylanases can be very useful in the clarification of fruit juices and wines. Microbial xylanases from

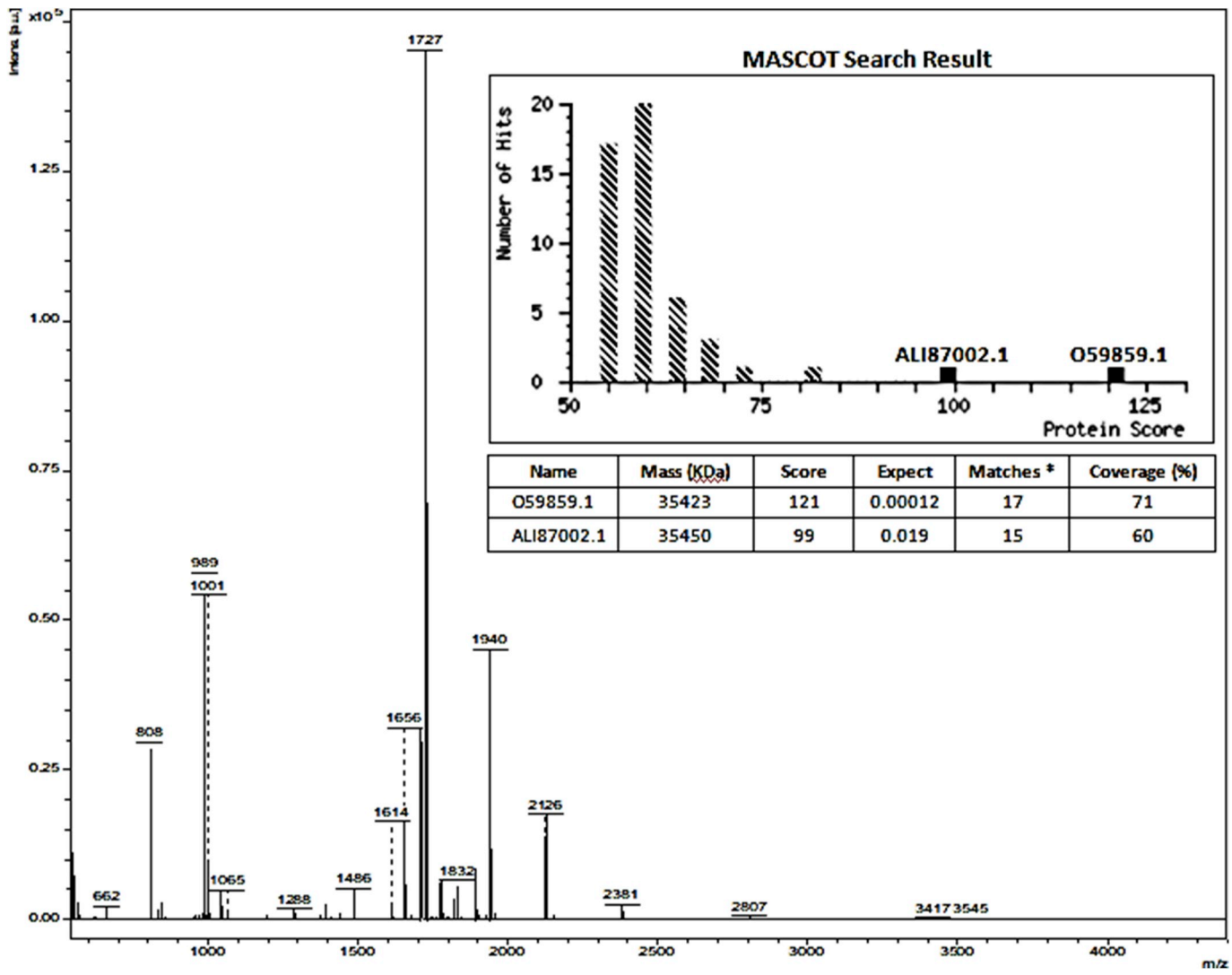

Fig. 3. Peptide mass fingerprint and MASCOT analysis of *A. japonicus* purified enzyme. Peptide mass fingerprint was obtained by MALDI-TOF MS after in-gel digestion with trypsin. Ion mass and intensity list was submitted to MASCOT analysis against SwissProt and NCBIprot databases, which significantly matched to two *A. aculeatus* xylanase sequences: UniProtKB/Swiss-Prot access **O59859.1**, with score = 121 ($p < 0.001$) and coverage = 71%; and GenBank access **ALI87002.1**, with score = 99 ($p < 0.02$) and coverage = 60%. * number of matched peptides out of 42 from *A. japonicus* xylanase peptide mass list.

mesophilic and thermophilic fungi and bacteria species are usually stable over a broad pH range (3.0–10.0) (Polizeli et al., 2005; Sharma, 2017; Walia et al., 2017). Moreover, the xylanases that are stable over a wide pH range could be very suitable for applications in the feed industry (Knob and Carmona, 2010; Juturu and Wu, 2012).

The optimal *A. japonicus* endo-xylanase activity occurred from 50 to 60 °C, decreasing only 7.4% and 1.5% (respectively), when compared to the maximum endo-xylanase activity at 55 °C. The enzyme also retained its activity of approximately 50% at 65 °C and 25% from 70 °C to 80 °C (Fig. 5a). The optimal temperature for purified *A. japonicus* endo-xylanase activity was similar to other xylanases of *Aspergillus* species and other xylanase-producing microorganisms (mesophiles and thermophiles), which ranged from 25 to 80 °C (Polizeli et al., 2005; Chakdar et al., 2016; Sharma, 2017). The effect of temperature on purified endo-xylanase stability was determined by measuring the relative activity after intervals from 10 to 240 min of incubation the enzyme in McIlvaine buffer pH 5.0 (in the absence of substrate) at 40, 45, 50 and 55 °C (Fig. 5b). The enzyme was stable at 40 and 45 °C and retained 50% of its activity after 240 min ($t_{1/2}$) of incubation at 50 and 55 °C. Other mesophilic and thermophilic fungi, including various *Aspergillus* species, exhibit a broad xylanase thermostability range from 35 to 100 °C (Ahmed et al., 2009; Michelin et al., 2010; Peng et al., 2012).

It is known that xylanases may have their hydrolytic activity modulated by metal ions, organic solvents and detergents. Most xylanases are found to be inhibited by Mn^{2+} (Saha, 2002). However, *A. japonicus* endo-xylanase was stimulated by Mn^{2+} at 1 and 5 mM, corresponding to 34.5% and 22.7%, respectively (Table 2). Silva et al. (2015b) reported similar results for xylanase (xyl I) from *T. inhamatum* using 10 mM divalent ions (Mn^{2+}, Mg^{2+}, Co^{2+} and Ca^{2+}). However, strong inhibition of xylanolytic relative activity was observed for *A. ficuum* A-98 (Lu et al., 2008), *A. niger* US368 (Hmida-Sayari et al., 2012) and *A. terreus* (Vitcosque et al., 2016) in the presence of these ions. Juturu and Wu (2012) reported that the presence of the Ca^{2+} binding site plays a role in preventing the thermal inactivation, unfolding and proteolysis of hemicellulases. Other metal ions that were tested had minor influences on the endo-xylanolytic activity of *A. japonicus*. On the other hand, purified enzymes from the mesophilic fungi *A. ficuum*, *A. terreus* and *T. inhamatum* presented a range of effects, from small to substantial inhibition of xylanase activity (Lu et al., 2008; Hmida-Sayari et al., 2012; Silva et al., 2015b; Vitcosque et al., 2016).

A. japonicus endo-xylanase was activated by 0.01 and 0.1 M β-mercaptoethanol (40.52% and 29.85%, respectively), Triton X-100

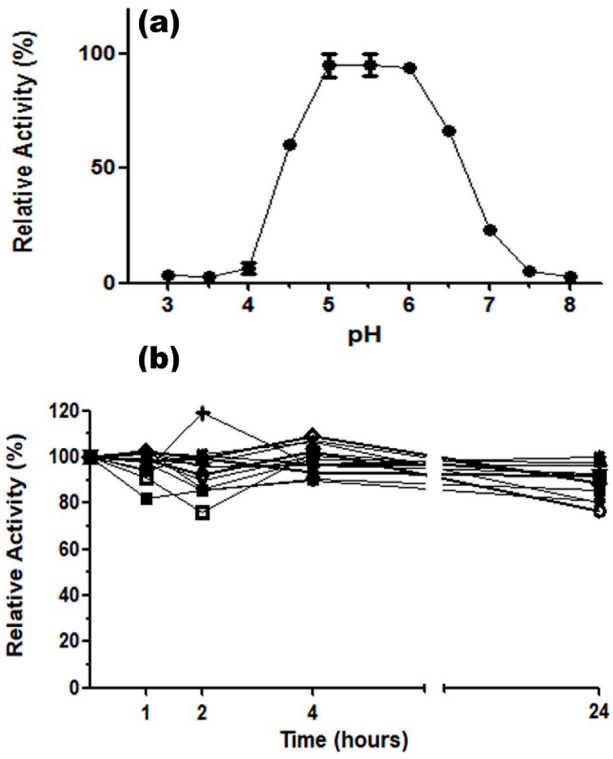

Fig. 4. Influence of pH on the activity and stability of *A. japonicus* endo-xylanase. Assay conditions: (a) 1% (w/v) birchwood xylan in pH 3.0–8.0 McIlvaine buffer, 50 °C; (b) the enzymatic preparations were incubated in pH 3.0 (■); 3.5 (□); 4.0 (○); 4.5 (●); 5.0 (+); 5.5 (◆); 6.0 (◊); 6.5 (Δ); 7.0 (ˣ); 7.5 (▾) and 8.0 (▴), without substrate. All experiments were performed in triplicate.

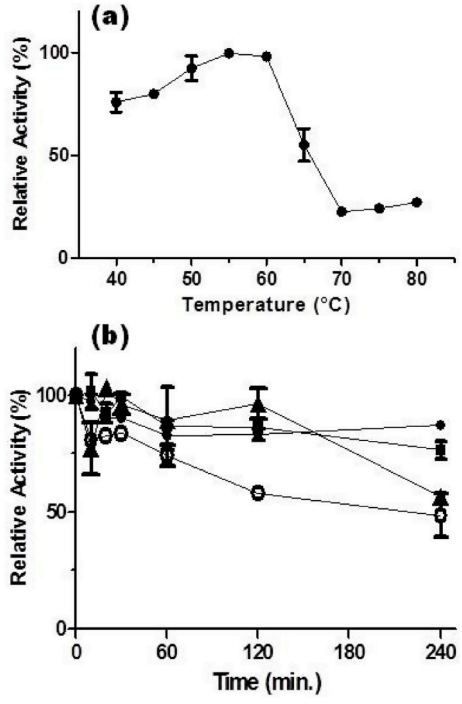

Fig. 5. Influence of temperature on the activity and stability of *A. japonicus* endo-xylanase. Assay conditions: (a) 1% (w/v) birchwood xylan in McIlvaine buffer pH 5.0 at temperatures ranging from 40 to 80 °C; (b) enzymatic preparation aliquots were incubated at 40 °C (●); 45 °C (■); 50 °C (▴) and 55 °C (○). All experiments were performed in triplicate.

Table 2
Effect of metal ions (1 and 5 mM) and Ethylenediaminetetraacetic acid (EDTA) on the activity of *A. japonicus* endo-xylanase.

Ions	Relative activity (%)	
	1 mM	5 mM
Control	100,0 ± 0,01	100,0 ± 0,06
NH_4Cl	106,0 ± 4,92	90,8 ± 1,15
$CuSO_4$	92,3 ± 2,09	43,1 ± 4.23
$MgSO_4$	104,2 ± 0,11	77,3 ± 7,19
$FeSO_4$	99,6 ± 3,45	81,8 ± 7,03
KCl	99,8 ± 5,12	83,0 ± 1,93
$CaCl_2$	103,0 ± 3,86	83,3 ± 1,73
$CoCl_2$	105,6 ± 0,52	70,0 ± 7,78
EDTA	108,0 ± 0,19	66,1 ± 3,88
$ZnCl_2$	116,3 ± 1,36	86,8 ± 1,08
$MnCl_2$	134,5 ± 0,41	122,7 ± 3,28
$BaCl_2$	109,0 ± 1,46	90,2 ± 2,70

Control: without addition of ions.

Table 3
Effect of various substances on endo-xylanase activity from *A. japonicus*.

Phenolic compounds (1mg/mL)	Detergent (10 %)	Organic solvent (10 %)	Reducing agents	Residual activity (%)
Control	Control	Control	Control	100 ± 0.14
Vanillin				78.25 ± 2.09
Ferulic acid				117.26 ± 2.66
Cinnamic acid				70.45 ± 0.35
4-hydroxybenzoic acid				84.40 ± 0.43
Coumaric acid				86.29 ± 0.57
Tannic acid				8.75 ± 0.98
			2-ME (0.01M)	140.52 ± 0.35
			2-ME (0.1M)	129.85 ± 0.99
	Triton X-100			201.61 ± 0.05
	Tween- 20			191.94 ± 0.03
	Tween- 80			138.87 ± 0.13
	SDS			0 ± 0.05
		Acetonitrile		72.10 ± 0.06
		Butanol		53.75 ± 0.04
		Acetone		78.28 ± 0.06
		Isopropanol		68.91 ± 0.05
		Methanol		54.87 ± 0.05
		DMSO		68.16 ± 0.12
		Ethanol		59.36 ± 0.03

ME = 2-mercaptoethanol (0.1M).

(101.61%), Tween-20 (91.94%) and Tween-80 (38.97%). Considering that both Tween and Triton are nonionic surfactants, these would promote proteins disaggregation improving enzymatic hydrolysis by exposure of their catalytic sites (Monclaro et al., 2016) (Table 3). On the other hand, the enzyme was inactivated by SDS, indicating the importance of hydrophobic interactions for the maintenance of its three-dimensional structure since SDS is an anionic detergent and a strong denaturant of proteins (Manning and Colón, 2004). These results

Table 4
Kinetic parameters of xylanases produced by filamentous fungi.

Strain	Vmax (μmol/min/mg)	Km (mg/mL)	Kcat (s^{-1})	Kcat/Km (mL s^{-1} mg^{-1})	References
A. japonicus	467.4	2.59	253.95	98.05	(Present study)
A. ficuum	11.1	3.74	–	–	Fengxia et al. (2008)
A. awamori	10000.0	1.0	–	–	Subramaniyan and Prema (2002)
A. awamori	333.0	0.33	–	–	Subramaniyan and Prema (2002)
A. awamori	455.0	0.09	–	–	Subramaniyan and Prema (2002)
A. nidulans	1091.0	0.97	–	–	Subramaniyan and Prema (2002)
A. terreus	17.4	1.20	1.6	1.3	Vitcosque et al. (2016)
A. niger	811.0	1.03	472.9	459.2	Hmida-Sayari et al. (2012)
C. graminicola	481.3	3.7	136.4	36.9	Carli et al. (2016)
M. flava (MFX I)	3333.0	6.6	700.2	106.1	Sharma et al. (2010)
M. flava (MFX II)	1923.0	5.0	357.5	71.5	Sharma et al. (2010)
P. thermophila	1424.7	1.6	612.6	382.9	Li et al. (2006)
T. lanuginosus CBS 288.54	2402.3	4.0	1049	262.3	Li et al. (2005)

are similar to those described for *A. niger* US368 (Hmida-Sayari et al., 2012), *T. inhamatum* (Silva et al., 2015b) and *A. terreus* (Vitcosque et al., 2016). A strong inhibition by SDS was also observed for *Penicillium sclerotiorum* xylanase (Knob and Carmona, 2010). The *A. japonicus* endo-xylanase was also inhibited by organic solvents and phenolic compounds, except ferulic acid, which is a plant cell wall-bound component and a precursor in lignocellulosic biomass; it has been suggested that it plays key roles in cell defence and cell wall development (Wallace and Fry, 1999). Studies have demonstrated that the inhibitory effects of phenolic compounds are due to conformational changes that induce protein steric inactivation (Boukari et al., 2011). A similar result was found by Monclaro et al. (2016) in which *Aspergillus tamarii* xylanase activity increased with the addition of ferulic acid. According to Monclaro et al. (2019), the inhibitory effect of phenolic compounds on the cellulases and hemicellulases activities has been described. But interestingly, in the present study, the endo-xylanase activity from *A. japonicus* was stimulated by ferulic acid. A comparative study about the hydrolysis of different types of xylans in the presence of phenolic compounds showed that the xylanase activity from *A. tamarii* (AtXyl1) was also increased in the presence of vanillin and tannic acid using birchwood xylan. Moreover, the hydrolysis of beechwood xylan by AtXyl1 was stimulated by these phenolic acids above and also by *p*-coumaric, cinnamic, ferulic and 4-hydroxybenzoic acids (Monclaro et al., 2019). Although the mechanism of modulation of enzyme activity by phenolic acids still remains to be clarified, Monclaro et al. (2019), using molecular docking of three-dimensional structure of GH11 xylanase from *A. niger* model, in the presence or absence of xylooligosaccharides, launched a better understand about the interactions between phenolic acids and the GH11 xylanases. In this simulation, the Tyr164, Ser 94 and Tyr89 residues in catalytic domain can be involved in these interactions with phenolic acids. Other studies also corroborate the role of these amino acids in phenolic acids interactions with xylan hydrolysis by GH11 xylanases (Krengel and Djikstra, 1996; Vandermarliere et al., 2008; Tison et al., 2009; Paës et al., 2012).

The *A. japonicus* purified endo-xylanase was also assayed for hydrolytic activity against a variety of substrates. As previously described (Silva et al., 2015a; Vitcosque et al., 2016) the *A. japonicus* purified endo-xylanase showed a high specificity for birchwood xylan and did not act towards avicel, starch or carboxymethylcellulose (not shown), indicating the specific breakdown of β-anomeric glycosidic linkages of xylose residues, as demonstrated by Carli et al. (2016). The *A. japonicus* endo-xylanolytic activity showed a single stimulation curve in the presence of different birchwood xylan concentrations (ranging from 0.02 to 0.5%, w/v). The endo-xylanase maximal velocity (Vmax) was 467.4 ± 30.38 μmol/min/mg, with a km value of 2.59 ± 0.17 mg/mL, a kcat value of 253.95 ± 16.51 s^{-1} and a kcat/km value of 98.05 ± 4.41 mL s^{-1} mg^{-1}. Kinetic studies of other fungi producers of xylanases are presented in Table 4.

Fig. 6. Thin-layer chromatography (TLC) of hydrolysis products using 1% (w/v) birchwood xylan substrate upon reaction with endo-xylanase from *A. japonicus*. Lane 1: xylose 5 mg/mL; Lanes 2–4: hydrolysis products using xylanase after 0, 30 and 60 min of incubation, respectively.

The resulting products from the hydrolysis of birchwood xylan by the purified endo-xylanase were analysed by TLC (Fig. 6). The hydrolysis products obtained from *A. japonicus* purified xylanase consisted of xylooligosaccharides of various lengths and xylose. It is known that endo-xylanases randomly cleave β-1,4-glycosidic linkages inside the xylan main chain and release xylooligosaccharides of different lengths, while β-xylosidases release xylose from xylobiose and xylooligosaccharides (Zimbardi et al., 2013). Since xylooligosaccharides were released by xylan hydrolysis, the *A. japonicus* purified enzyme was classified as an endo-xylanase, corroborating the similarity of the endo-xylanases found in the MASCOT analysis. The production of xylooligosaccharides as product has also been observed by TLC using endo-xylanases from *Aspergillus flavus* (Chen et al., 2019), *Neosartorya tatenoi* (Seemakram et al., 2016), *Thermoascus aurantiacus* (Chanwicha et al., 2015), *A. terreus* (Sorgatto et al., 2012) and *Aspergillus carneus* (Fang et al., 2008) from birchwood xylan; and *Trichoderma inhamatum* (Silva et al., 2015b) from oat spelt xylan. *Aspergillus oryzae* LC1 produced xylooligosaccharides (xylobiose, xylotriose and xylotetraose) by the enzymatic hydrolysis of sugar cane bagasse, wheat straw and wheat bran from lignocellulosic biomass (Bhardwaj et al., 2019).

3.3. Fruit juice clarification and hydrolysis of fruit peel wastes by endo-xylanase from A. japonicus

Carbohydrate polymers such as pectin, starch and hemicellulosic components present in fruit juices make them cloudy and viscous. These cloudy juices are less attractive to consumers and also make them

Table 5
Fruit juice clarification and hydrolysis of fruit peel wastes by endo-xylanase of *A. japonicus*.

Fruits (*in nature*)	Reducing sugars yield in fruit peel waste hydrolysis by endo-xylanase (μmol)	Fruit juice clarification (% Clarification)	Fruit juice pH
Pineapple	2.89 ± 1.63	6.11 ± 0.10	3.5–4.0
Banana	3.36 ± 1.05	9.99 ± 1.34	4.0–4.5
Orange	1.85 ± 0.10	2.66 ± 0.09	4.0–4.5
Mango	2.69 ± 0.08	51.11 ± 0.08	4.0–4.5
Tangerine	1.91 ± 0.09	8.54 ± 0.08	4.0–4.5
Nectarine	1.00 ± 0.05	ND	4.0–4.5
Peach	0.66 ± 0.09	ND	4.0–4.5
Ponkan	2.16 ± 0.03	1.39 ± 0.08	4.0–4.5

ND = not detectable.

difficult to pasteurize and concentrate. Progressive degradation of the middle lamella between the cells can be done by hydrolytic enzymes, weakening the wall and resulting in the release of cell bound materials including water, thus making the juice recovery easier. When fruit juices are treated with xylanases, reducing carbohydrate units are released, allowing for better pulp processing and improvement of the yield of the substances contained in the fruit. Thus, the amount of reducing sugars released is the indicator for the breakdown of hemicellulose materials by the enzyme, being the calculation in transmittance percentage a measure of the clarity of the juice (Rosmine et al., 2017). The *A. japonicus* endo-xylanase activity in the clarification of fruit juices and in the hydrolysis of fruit peel wastes are illustrated in Table 5. Enzyme-treated juice gave a 51.11%, 9.99% and 8.54% increase in the clarity of mango, banana and tangerine juices, respectively. Bacterial xylanases used in juice clarification processes have been described in the literature with *Pediococcus acidilactici* GC25 (Adiguzel et al., 2019), *Bacillus licheniformis* (Bajaj and Manhas, 2012) and *Bacillus stearothermophilus* (Dhiman et al., 2011). However, there are few reports on fungal xylanases in fruit juice clarification processes, mainly using only purified xylanases. Usually, pectinases are used in these processes or along with xylanases, cellulases and amylases (Padma et al., 2017; Sharma, 2017; Dhiman et al., 2011). The xylanase produced by *Streptomyces* sp. showed increases in the clarity of orange, mousambi and pineapple juices by 20.87%, 23.64% and 27.89%, respectively (Rosmine et al., 2017). A study using hot water extraction with a combination of pectinases and cellulases in apple pomace resulted in a 37% increase in juice yield (Will et al., 2000). An approximately 25% increase in pineapple juice recovery was obtained when a mixture of two commercial enzymes, pectinase and hemicellulase, were used at 40 °C compared to the control in studies of Tochi et al. (2009). In the current study, endo-xylanase (0.42 mg/mL protein) was incubated for 15 min at 55 °C and showed activity toward all fruit peels. The liberation of reducing sugar towards theses fruit peel wastes was higher at banana peel (3.36 μmol) and pineapple peel (2.85 μmol), decreasing to mango (2.69 μmol) and ponkan (2.16 μmol) peels. Xylanase showed efficiency for releasing reducing sugars from fruit peel wastes, showing potential to be used in an alternative route for fruit waste use in the economical and sustainable production of biofuel. In addition, the hydrolysis of the fruit peel wastes by the endo-xylanase liberating reducing sugars from their hemicellulose components is important as it can be used to improve the nutritional properties of feed and reduce environmental pollution.

4. Conclusion

The present study features a promising approach for the production of *A. japonicus* endo-xylanase in liquid cultures with wheat bran, and the use of this enzyme in biotechnological processes. Furthermore, the purification was carried out through a single chromatography step, which reduces the cost and time for obtaining the pure enzyme. The peptide mass fingerprint MASCOT results, together with the amino-terminal sequence of our *A. japonicus*-purified enzyme, strongly suggest its identity as a new member of the xylanase family. Other pivotal properties such as its high efficiency in juice clarification and in the saccharification of peel wastes from the fruit juice industry indicate this enzyme as a promising and excellent candidate for applications in biotechnological processes.

Conflicts of interest

The authors declare that they have no conflict of interest.

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