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Heterologous expression and functional characterization of a GH10 endoxylanase from *Aspergillus fumigatus* var. *niveus* with potential biotechnological application

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ABSTRACT

Xylanases decrease the xylan content in pretreated biomass releasing it from hemicellulose, thus improving the accessibility of cellulose for cellulases. In this work, an endo-β-1,4-xylanase from *Aspergillus fumigatus* var. *niveus* (AFUMN-GH10) was successfully expressed. The structural analysis and biochemical characterization showed this AFUMN-GH10 does not contain a carbohydrate-binding module. The enzyme retained its activity in a pH range from 4.5 to 7.0, with an optimal temperature at 60 °C. AFUMN-GH10 showed the highest activity in beechwood xylan. The mode of action of AFUMN-GH10 was investigated by hydrolysis of APTS-labeled xylohexaose, which resulted in xylotriose and xylobiose as the main products. AFUMN-GH10 released 27% of residual xylan from hydrothermally-pretreated corn stover and 14% of residual xylan from hydrothermally-pretreated sugarcane bagasse. The results showed that environmentally friendly pretreatment followed by enzymatic hydrolysis with AFUMN-GH10 in low concentration is a suitable method to remove part of residual and recalcitrant hemicellulose from biomass.

1. Introduction

The interest in producing fuels, energy and chemicals from renewable resources such as lignocellulosic biomass has increased in many industrial sectors. Moreover, concerns regarding sustainability and the effect of greenhouse gases generated from the combustion of fossil fuels have increased interest in implementation of environmentally friendly biorefineries, a concept analogous to petroleum refineries. Utilization of lignocellulosic biomass, such as sugarcane bagasse and corn stover, in biorefineries is a promising alternative to replace fossil fuels. However, depolymerization of lignocellulosic biomass still is one of the bottlenecks in commercialization of biorefineries due to the complex composition of the material [1,2].

Xylan is a constituent of plant cell walls and the second most abundant polysaccharide in nature. This polymer has branched sections, which are decorated by side groups, and is naturally degraded by different types of enzymes working in coordination. Xylanases (endo-β-1,4-xylanases – EC 3.2.1.8) are the main proteins that hydrolyze internal bonds in the xylan backbone [3,4]. According to the Carbohydrate Active Enzymes database (CAZy), xylanases are found in Glycoside Hydrolase (GH) families 3, 5, 8, 9, 10, 11, 12, 16, 26, 30, 43, 44, 51, 62, 98 and

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141 with the majority of xylanases in groups GH10 and GH11 in terms of sequences and characterized enzymes [5,6]. Other GH families also have bi-functional enzymes containing two catalytic domains that can present residual or secondary xylanase activity [4,7].

GH10 xylanases have higher molecular masses compared with GH11 xylanases. In both families the catalytic domain can be associated to carbohydrate binding modules (CBMs) linked on the N- or C-terminus [8]. These enzymes have been used as bleaching agents in the Kraft process, in combination with cellulases in starch-based ethanol and as additives in animal feed and bakery industries to improve final product quality [9]. Many microorganisms, including filamentous fungi from the genus *Aspergillus*, have in their genomes a diversity of genes encoding xylanases varying in structure, catalytic mechanism, biochemical properties, substrate specificity and released products. In addition, there are a large number of *Aspergillus* species which show different growth characteristics, such as the capacity to grow in elevated temperatures, making them candidates for the prospection of thermostable enzymes [10,11]. From the many species in this genus *A. fumigatus* var. *niveus* (previously reported as *A. niveus*) has had its genome sequenced and has been explored as a source for genes encoding plant cell wall degrading enzymes with thermostable characteristics. Although it's potential *A. fumigatus* var. *niveus* is a pathogenic fungus, which makes necessary the expression of proteins in heterologous host [12–15].

Biorefineries in Europe, USA and Brazil have investigated environmentally friendly biomass pretreatments in their production plants. However, the residual xylan in the pretreated material significantly restricts the accessibility of cellulose by cellulases, thus making the use of xylanases desirable [16]. Conversion of xylan to xylose and xylooligosaccharides (XOS) by xylanases instead of during pretreatment reduces formation of sugar degradation products and other fermentation inhibitors. Among xylanases described in the CAZy database, enzymes from family GH10 have been considered the most promising for such conversion [17]. In this study, we report the cloning and heterologous expression of an *A. fumigatus* var. *niveus* GH10 xylanase (AFUMN-GH10). The recombinant enzyme was purified and biochemically characterized and its mode of action on residual recalcitrant hemicellulose from sugarcane bagasse and corn stover pretreated by liquid hot water was investigated.

2. Materials and methods

2.1. Multiple sequence analysis

The protein sequence of endo-β-1,4-xylanase (AFUMN-GH10) of *A. fumigatus* var. *niveus* (accession number KEY83365) was used as a model to search for putative xylanases from family GH10 using the Basic Local Alignment Search Tool at the National Center for Biotechnology Information (Bethesda, MD, USA) server [18]. Xylanases from family GH11 were obtained from the CAZy database (www.cazy.org). Protein sequences were compared with available GH10 and GH11 xylanases using blastp from the dbCAN database (http://csbl.bmb.uga.edu/dbCAN/). Domains were verified by sequence search in the Pfam (https://pfam.xfam.org) and InterPro databases (https://www.ebi.ac.uk/interpro/). Validated sequences were submitted to multiple alignment analysis using MUSCLE software with default parameters and the alignment obtained was used to construct a phylogenetic tree by the neighbor-joining method using the MEGA 7 program [19]. The tree was visualized by iTOL and the evolutionary distances calculated by the Jones-Taylor-Thornton model [20].

2.2. Microbial strains, plasmids and culture conditions

The *A. fumigatus* var. *niveus* strain (AFUMN) was kindly provided by Prof. Rolf A. Prade from Oklahoma State University (Stillwater, OK, USA). *A. nidulans* strain A773 (*pyrG89; wA3; pyroA4*) was purchased from the Fungal Genetic Stock Center (FGSC, Manhattan, KS, USA). *A. nidulans* A773 was cultivated in minimal medium composed of 50 ml/L of salt solution (6 g/L $NaNO_3$, 1.5 g/L KH_2PO_4, 0.5 g/L KCl and 0.5 g/L $MgSO_4$) and 1 ml/L of trace elements (10 g/L EDTA, 4.4 g/L $ZnSO_4.H_2O$, 1.0 g/L $MnCl_2.4H_2O$, 0.32 g/L $CoCl_2.6H_2O$, 0.315 g/L $CuSO_4.5H_2O$, 0.22 g/L $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.47 g/L $CaCl_2.2H_2O$ and 1 g/L $FeSO_4.H_2O$), 1% glucose, pH 6.5 and supplemented with pyridoxine (1 mg/L), uracil/uridine (2.5 mg/L each), as previously described [21]. AFUMN was cultivated in potato dextrose agar medium (PDA – Sigma-Aldrich, St. Louis, MO, USA) at 45 °C for 2 days. The pEXPYR vector was used to clone and express the AFUMN-GH10 gene in *A. nidulans* A773 [21]. High efficiency NEB® Turbo competent *Escherichia coli* (New England Biolabs, Ipswich, MA, USA) was used to propagate the plasmid carrying the fragment purified from Polymerase Chain Reaction (PCR) corresponding to the AFUMN-GH10 gene. Oligonucleotides used for gene amplification in PCR were purchased from Exxtend Biotechnology Ltd. (Campinas, SP, Brazil). All chemicals were molecular biology grade and obtained from Sigma-Aldrich, except maltose used to induce protein expression, which was purchased from WE Consultoria (Porto Alegre, RS, Brazil).

2.3. Cloning, heterologous expression and purification of AFUMN-GH10

For genomic DNA (gDNA) extraction, spores of AFUMN cultivated in a Petri dish were harvested and inoculated in a 1 L Erlenmeyer flask containing 250 ml of liquid minimal medium followed by incubation under agitation of 200 rpm at 45 °C for 2 days [10,22]. The resulting mycelia were filtered in a Buchner funnel with a filter paper, washed with 300 ml of sterile water and frozen in liquid nitrogen before being ground in a mortar for gDNA extraction using the kit Wizard® Genomic DNA Purification (Promega, Madison, WI, USA).

The coding sequence of AFUMN-GH10 was analyzed using the SignalP 4.1 Server (Center for Biological Sequence Analyses, Lyngby, Denmark), and the native signal peptide was maintained in the designed oligonucleotides [23]. The gene was amplified from AFUMN gDNA by PCR using Phusion® High-Fidelity DNA Polymerase (New England Biolabs) with the oligonucleotide primers AFGH10 F (5′ – **CATTACACCTCAGCAATG**GTCGTCCTCAG-CAAGCTCGTCAGC – 3′), and AFGH10R (5′ – **GTCCCGTGCCGGTTA**T-CAGAGAGCAGCAATGATGGCATTATACG – 3′) designed with specific site regions (in bold) that allowed the amplified fragment to assemble to the vector by the Gibson Assembly Method [24]. The pEXPYR vector was first digested with *Xba*I enzyme (New England Biolabs) and amplified with the oligonucleotide primers pEXPYR-F (5′ – **CCGGCACGGGAC**TTCTAGTGATTTAATAGCTCCATGTCAACA – 3′) and pEXPYR-R (5′ – **CATTGCTGAGGTGTA**ATGATGCTGGGGGTG – 3′) in a PCR to add regions (in bold) to assemble the gene [22,25].

The assembly was performed by mixing 250 ng of the gene with 25 ng of vector in a final volume of 5 μl. To this reaction was added 5 μl of 2X Gibson Assembly master mix (New England Biolabs), followed by incubation at 50 °C in a thermocycler for 1 h [24]. The reaction was used to transform high efficiency Turbo competent *E. coli* cells (New England Biolabs) by heat shock method. A positive clone for AFUMN-GH10 was confirmed by colony PCR and used to propagate the plasmid of interest, which was then extracted by Wizard® Plus SV Minipreps DNA Purification System kit (Promega) and transformed into *A. nidulans* A773 as previously described [26]. The *A. nidulans* recombinant strains were further selected by

their ability to grow in the absence of uracil and uridine. Eight strains were analyzed for the production of the recombinant AFUMN-GH10 protein and 10^7 to 10^8 spores/ml of selected transformant were inoculated in 500 ml of liquid minimal medium supplemented with 2% maltose and pyridoxine (1 mg/L), distributed in a metal tray (35 × 15 × 8 cm). The culture was incubated for 2 days at 37 °C without shaking. Heterologous protein production was quantified by Bradford method and monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [27,28].

The mycelial mat was separated from the medium containing the protein AFUMN-GH10 by filtration through a qualitative membrane (Miracloth, MilliporeSigma, Burlington, MA, USA). The medium was concentrated by ultra-filtration (10,000 Da cut-off membrane in an Amicon® Stirred-cell, MilliporeSigma) followed by buffer exchange with 50 mM citrate-phosphate, pH 5.5. The enzyme was purified by chromatography using 5 ml of SP-Sepharose Fast Flow resin (GE Healthcare Life Sciences, Marlbourough, MA, USA) packed in an Econo-Pac® Chromatography Column (Bio-Rad, Hercules, CA, USA) equilibrated with the same buffer. Elutions were performed with 20 ml of 50 mM citrate-phosphate buffer, pH 5.5 with NaCl added in different concentrations ranging from 0.05 to 1 M. The resulting fractions were analyzed by SDS-PAGE and the elutions containing the target protein were mixed, concentrated in a Vivaspin™ centrifugal concentrator (10,000 Da cut-off, GE Healthcare Life Sciences) and dialyzed with 50 mM citrate-phosphate buffer, pH 5.5. The concentrated elution was injected in a ÄKTA Pure 25 M System (GE Healthcare Life Sciences) connected with a Superdex 75 10/300 G L column equilibrated with the same buffer at a flow rate of 0.5 ml/min. The fractions (1.5 ml) were collected and monitored for xylanase activity using beechwood xylan (Xbe) (1% w/v) as substrate and reducing sugars were analyzed by the dinitrosalicylic acid (DNS) method [29]. Fractions showing xylanase activity were verified by SDS-PAGE, mixed and used for further analysis.

2.4. Liquid chromatography-tandem mass spectrometry (LC–MS/MS)

The band corresponding to the purified AFUMN-GH10 in Coomassie stained SDS-PAGE was excised, reduced, alkylated and submitted to *in situ* trypsin gel digestion (Promega). An aliquot (1 μl) of tryptic peptides was desalted in a C18 column (75 μm x100 mmol/L). The fragments were analyzed by RP-nanoUPLC (nanoAcquity, Waters, Milford, MA, USA) coupled with a Q-TOF Ultimamass Spectrometer (Waters) with a nano-electrospray source at a flow rate of 0.6 μl/min. The gradient was 2–90% (v/v) acetonitrile in 0.1% (v/v) formic acid over 45 min. The equipment was set up on the top three modes. Spectra were obtained though the software MassLynx v.4.1 (Waters) and the raw data files were converted to a peak list format (mgf) by the software Mascot Distiller v.2.3.2.0, 2009 (Matrix Science Ltd., London, UK). The MS/MS profiles were searched against predicted protein sequences of *A. fumigatus* var. *niveus* using engine Mascot v.2.3 (Matrix Science Ltd) [30].

2.5. Size exclusion chromatography - multi angle light scattering (SEC-MALS)

The SEC-MALS experiment was performed in a system composed with a Waters 600 High Performance Liquid Chromatograph (HPLC) (using a WTC-030S5 column, Wyatt Technology, Santa Barbara, CA, USA) coupled to a DAWN® HELEOS® light scattering device and an Optilab® T-rEX refractometer (Wyatt Technology). The size exclusion column was equilibrated with 25 mM Tris-HCl and 300 mM NaCl solution at pH 8.0. A sample volume of 100 μl (2 mg/ml) was applied in the SEC-MALS system at a flow rate of 0.5 ml/min. Experimental data and molecular mass were reported by software ASTRA version 7 (Wyatt Technology).

2.6. Circular dichroism and thermal denaturation

The far-UV circular dichroism (CD) spectra of AFUMN-GH10 (0.2 mg/ml) was obtained using a JASCO J-815 CD spectropolarimeter in 20 mM phosphate buffer, pH 7.35, at 25 °C by applying a 0.1 cm path-length cuvette and wavelengths ranging from 190 to 270 nm. A total of six spectra were collected for each measurement, averaged and the final spectra were obtained subtracting a buffer blank. The spectral analysis of temperature increase was performed by monitoring the ellipticity at 222 nm from 25 to 80 °C at 2 °C intervals with a setting time of 60 s. The transition curve was calculated through the unfolded fraction of AFUMN-GH10, which is calculated by finding the difference between the ellipticity of folded AFUMN-GH10 (25 °C) and the ellipticity in any temperature and dividing it by the difference between the ellipticities of folded and unfolded AFUMN-GH10 over the temperature range mentioned above. The CDSSTR method through the DichroWeb server (University of London, UK) was used to access the secondary structure of AFUMN-GH10 and the data deconvolution set with reference was defined as 4 [31–33].

2.7. Biochemical characterization of AFUMN-GH10

The enzymatic activity of recombinant endoxylanase AFUMN-GH10 was measured by colorimetric method against 7 substrates. Reactions were performed with 50 μl of substrate (1% w/v, solubilized in water), 45 μl of 50 mM citrate-phosphate buffer (pH 5.0) and 5 μl of purified enzyme (final concentration of 0.8 μg). The reactions were incubated at 50 °C for 10 min in a T100 thermocycler (Bio-Rad). Reducing sugars were quantified according to the DNS method, stopping the reaction by adding 100 μl of DNS reagent and boiling for 5 min [29]. Absorbance was measured in an Infinite 200 PRO spectrophotometer (Tecan, Maennedorf, Switzerland) at 540 nm. One enzymatic activity unit (U), was defined as the amount of enzyme that produced 1 μmol of reducing sugars per minute using xylose as standard. The tested substrates, Xbe, birchwood xylan (Xbi), rye arabinoxylan, wheat arabinoxylan and xyloglucan were purchased from Megazyme (Bray, Ireland); and 4-nitrophenyl β-D-xylopyranoside (pNPX) and carboxymethylcellulose (CMC) from Sigma-Aldrich.

To determine the optimal pH, enzymatic reactions were carried out at pH values ranging from 2 to 10 in a 50 mM citrate-phosphate-glycine buffer system for 5 min. For optimal temperature, reactions were incubated in optimal pH with temperatures ranging from 30 to 85 °C for 5 min. The reactions consisted of 0.8 μg of purified AFUMN-GH10 enzyme in 50 μl of Xbe (1% w/v) as substrate and buffer to a final reaction mixture of 100 μl [34]. The pH stability was assessed by incubating AFUMN-GH10 in a 50 mM citrate-phosphate-glycine buffer system at pH 4.0, 5.0, 6.0 and 7.0 from 2 to 72 h at 30 °C, followed by measuring the enzyme activity at optimal temperature. Thermal stability was evaluated by incubating the enzyme at 50, 55, 60 and 65 °C for up to 360 min in its optimal pH followed by incubation with Xbe (1% w/v) at optimal conditions. The effect of metal ions on AFUMN-GH10 activity were determined in the presence of $ZnSO_4$, $MnSO_4$, $FeSO_4$, NaCl, $MgSO_4$, KCl, $CuSO_4$, $CoCl_2$, $CaCl_2$ (10 mM) in 50 mM sodium acetate buffer (pH 5.0) with Xbe as substrate. The reactions were incubated at 60 °C for 5 min using a sample without ion addition as control. Further evaluation of different concentrations of $MnSO_4$ and $CaCl_2$ (1 to 30 mM) were performed as previously described. For kinetic parameters, reactions were performed as previously described and determined according to the Michaelis-Menten model with Xbe and Xbi as substrates (concentrations from 0.5 to 15 mg/ml)

incubated for 3 min at 60 °C. The Graph Pad Prism 7.03 (GraphPad, San Diego, CA, USA) was used to calculate K_m, V_{max}, K_{cat} and K_{cat}/K_m by non-linear regression.

2.8. Enzymatic hydrolysis of pretreated sugarcane bagasse and corn stover

Sugarcane bagasse (BP) and corn stover (CSP) were pretreated by liquid hot water (LHW) at 5% solid content at 180 °C for 10 and 20 min using a heating rate of 1.0 °C/min in a 5 L M/K Systems digester (M/K Systems Inc, Elk Groove Village, IL, USA). The pretreated materials were exhaustively washed with distilled water to remove residual sugars, dried at room temperature and analyzed for composition. For hydrolysis, approximately 22.5 mg of BP or CSP (pretreated for 10 or 20 min) were hydrated for 18 h in 1 ml of 50 mM sodium acetate buffer, pH 5.0, then 0.25 nmol (8.3 μg) of purified AFUMN-GH10 was added to make a final volume of 1.5 ml. Reactions were incubated in an orbital shaker at 55 °C, 140 rpm and samples were taken at defined periods from 30 min to 24 h of incubation and released sugars were monitored by DNS method [29]. After incubation the tubes were centrifuged at 10,000 rpm for 15 min at 4 °C and an aliquot of the hydrolyzate was used to determine the amount of solubilized xylose [35]. The concentration of xylose was measured by HPLC equipped with a refractive index detector at 35 °C using an Aminex HPX-87H column (Bio-Rad) at 45 °C, eluted with sulfuric acid at 0.005 mol/L using an external standard. Xylan conversion yield was calculated as the ratio of xylose released and the initial amount of xylose in pretreated biomass. To evaluate how the pretreatment time (10 and 20 min) could affect the residual hemicellulose hydrolysis, an analysis of variance (ANOVA) was performed with pretreatment time as the independent variable and xylan conversion yield as the dependent variable. Significant differences were determined by Tukey's test with 95% confidence level for both types of biomass using STATGRAPHICS Centurion XVI.I software. All experiments were performed in triplicate.

2.8.1. Capillary zone electrophoresis

After the incubation of pretreated BP or CSP with AFUMN-GH10 in 50 mM sodium acetate buffer pH 5.0 at 55°C for 1, 7 and 24 h, the resulting products were derivatized with 8-aminopyreno-1,3,6-trisulfonic acid (APTS) by reductive amination and verified by capillary zone electrophoresis (CZE) [36]. To analyze the AFUMN-GH10 mode of action, hydrolysis of APTS-labeled xylohexaose (X6) was performed at 60°C for 5 and 30 min of incubation. The CZE of substrate-breakdown products were performed using a P/ACE MQD instrument (Beckman Coulter, Brea, CA, USA) equipped with a laser-induced fluorescence detector. A fused silica column (TSP 050375, Polymicro Technologies, Phoenix, AZ, USA) with an internal diameter of 50 μm and total length of 31 cm was used to separate oligosaccharides. The electrophoresis conditions were as follow: 30 kV/70–100 mA at 20°C using sodium phosphate buffer (40 mM, pH 2.5). Because of the small volumes of capillary electrophoresis combined with the small variations in buffer strength, retention times varied slightly when comparing separate electrophoresis runs [37].

3. Results and discussion

3.1. AFUMN-GH10 is a GH10 xylanase type III

Most xylanases are classified in the families GH10 and GH11 based on amino acid sequence similarities [5,38]. To further analyze AFUMN-GH10, a phylogenetic tree grouping 30 fungal xylanases from families GH10 and GH11 was constructed using the neighbor joining method [39]. GH10 xylanases were grouped into three subfamilies, defined according to sequences showing or lacking CBM1 as well as CBM1 localization. GH10 xylanases without CBM1 are classified as type III (GH10-III), while those containing CBMs at N- or C-terminus are type I and II (GH10-I and GH10-II), respectively [8]. The sequence analysis showed the AFUMN-GH10 has 324 amino acid residues, which contain a secretion signal peptide (1 to 19) and a catalytic domain of 292 amino acids (30 to 322). AFUMN-GH10 does not have a CBM1, thus it was grouped into subclass GH10-III along with xylanases of other ascomycetes such as *A. nidulans*, *Trichoderma reesei*, and the thermophiles *Chaetomium thermophilum*, *Thielavia terrestris* and *Thermothelomyces thermophila* (previously classified as *Myceliophthora thermophila*), accession numbers ABF50851, XP_006962419, XP_006695063, XP_003650974 and XP_003664565, respectively (Fig. 1) [40–42].

3.2. The recombinant AFUMN-GH10 is a monomer in solution

The linearized pEXPYR expression plasmid (6594 bp) and the full-length AFUMN-GH10 encoding gene (975 bp) were assembled *in vitro* and transformed in *E. coli* to propagate construction. Colony PCR was performed to verify positive colonies and showed an efficiency of 80% in *E. coli* transformation. *A. nidulans* A773 was transformed with this construction and the resulting recombinant strains were selected by the reversion of the auxotrophic mark that allows growth in the absence of uracil and uridine [21]. Eight recombinant strains were evaluated for the capacity to express and secrete AFUMN-GH10 in the culture supernatant. The expression was mediated by *glaAp* promoter induced by 2% of maltose, which allows the secretion of large amounts of recombinant proteins [21]. The recombinant strain showing the highest extracellular level of AFUMN-GH10 in SDS-PAGE had the band excised and analyzed by LC–MS/MS. The identified peptides covered 59% of the predicted protein sequence, confirming heterologous expression of AFUMN-GH10 in *A. nidulans* A773 (Fig. 2A).

The selected transformant produced 0.57 mg/ml of AFUMN-GH10 when cultivated in a metal tray without shaking for 2 days at 37°C. The protein sequence analysis by NetOGlyc 4.0 and NetNGlyc 1.0 servers predicted two sites for O-glycosylation at the positions 16 and 118 in the mature sequence and none for N-glycosylation. The purified protein showed a single and homogeneous band in SDS-PAGE with an estimated molecular weight of 33 kDa (Fig. 2B). The molecular weight was also confirmed by SEC-MALS showing an empirical molecular mass of 31.2 (± 0.1) kDa, which is 6.6% lower than ≅ 33.4 kDa predicted by ProtParam tool and visualized in SDS-PAGE, indicating that AFUMN-GH10 corresponds to a monomer in solution (Fig. 2B and Supplementary Fig. 1) [43].

3.3. Biochemical characterization of AFUMN-GH10

AFUMN-GH10 showed an optimal activity at pH 5.0 and retained more than 80% of its activity in pHs ranging from 4.5 to 7.0 (Fig. 3A). AFUMN-GH10 also exhibited stability at these pH values, retaining a residual activity of 80% after incubation at pH 4, 5, 6 and 7 for 72 h (Fig. 3B). These observations revealed that AFUMN-GH10 has characteristics similar to previously described xylanases from *Penicillium sp* [44,45], *T. terrestris* [41] and *Malbranchea pulchella* [46]. Stability over a wide range of pH allows for the application of the enzyme in several industrial processes [3].

AFUMN-GH10's optimal temperature was 60 °C and more than 60% of maximum activity was retained at temperatures ranging from 30 to 75 °C (Fig. 3A). GH10 family xylanases from other filamentous fungi also exhibited optimal temperatures ranging from 60 to 75 °C [3]. AFUMN-GH10 was stable at 50 and 55 °C, while activity decreased to 60% when incubated at 60 °C for 360 min and 19% after 15 min at 65 °C (Fig. 3B). A native *P. citrinum*

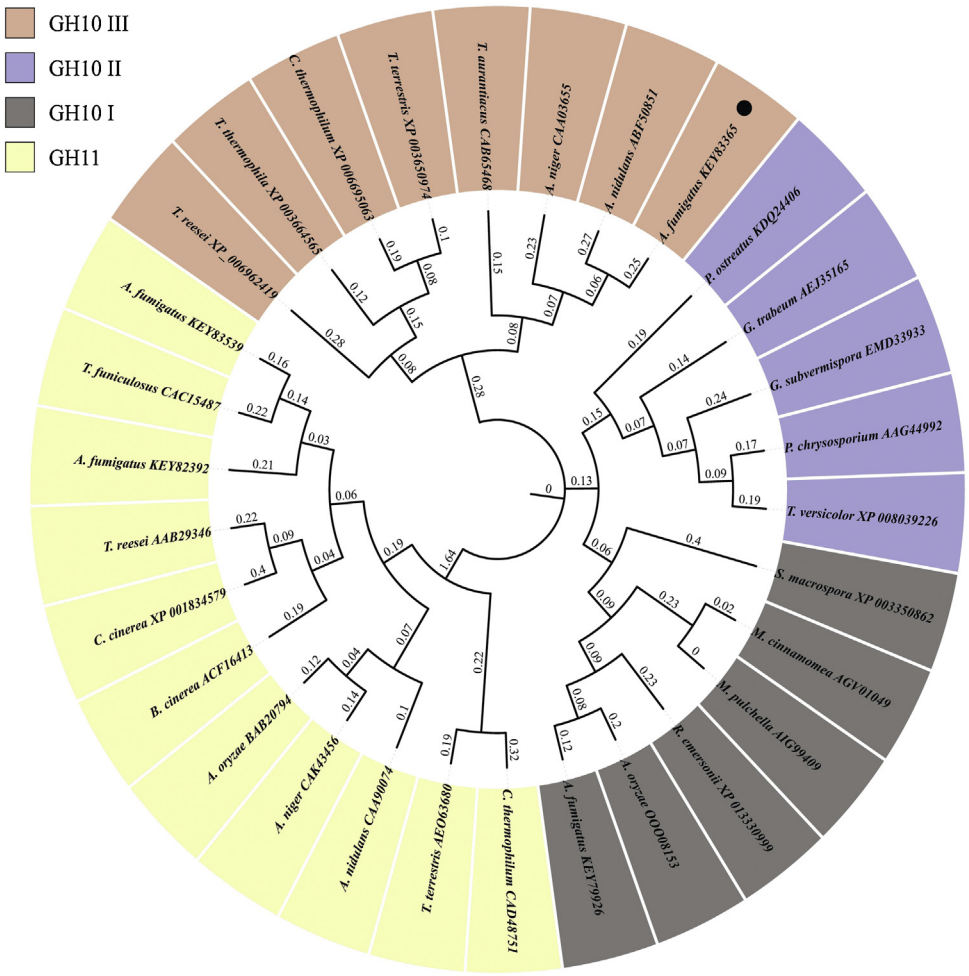

Fig. 1. Representative phylogeny of xylanases from families GH10 and GH11 from mesophilic and thermophilic fungi. AFUMN-GH10 (accession number KEY83365) characterized in this study is highlighted with a black dot. The protein sequences were taken at NCBI, CAZy and dbCAN databases.

GH10 xylanase demonstrated thermal stability in temperatures ranging from 55 to 70 °C maintaining 80% of its residual activity for 180 min [47]. The search for proteins with specific characteristics for industrial applications is desirable and has an emerging scientific demand since most preparations exhibit optimal activity in pHs and temperatures around 5.0 and 50 °C, respectively. Generally, the biochemical characteristics showed by enzymes are related to the microorganism used as source in production, which are not suitable for some industrial applications, being *T. reesei* the most explored microorganism.

The CD technique was applied to evaluate the secondary structure of AFUMN-GH10 (Fig. 4A) and DichroWeb server was used to analyze the resulting data (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [48]. The deconvolution analysis of AFUMN-GH10 showed a predominance of α-helix structures (38.0%), followed by β-strands (19.0%), turns (17.0%) and random coils

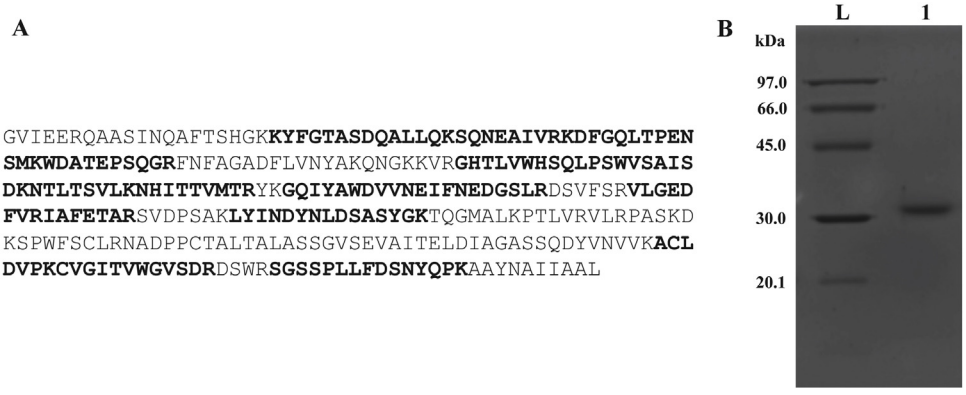

Fig. 2. Purification of AFUMN-GH10: (A) Identification of AFUMN-GH10 peptides by LC–MS/MS yielding 59% coverage (identified peptides are highlighted in bold). (B) SDS-PAGE 15% stained with Coomassie brilliant blue of AFUMN-GH10 after gel filtration chromatography; L – Ladder, 1 – purified AFUMN-GH10 showing a molecular weigh corresponding to approximate 33 kDa.

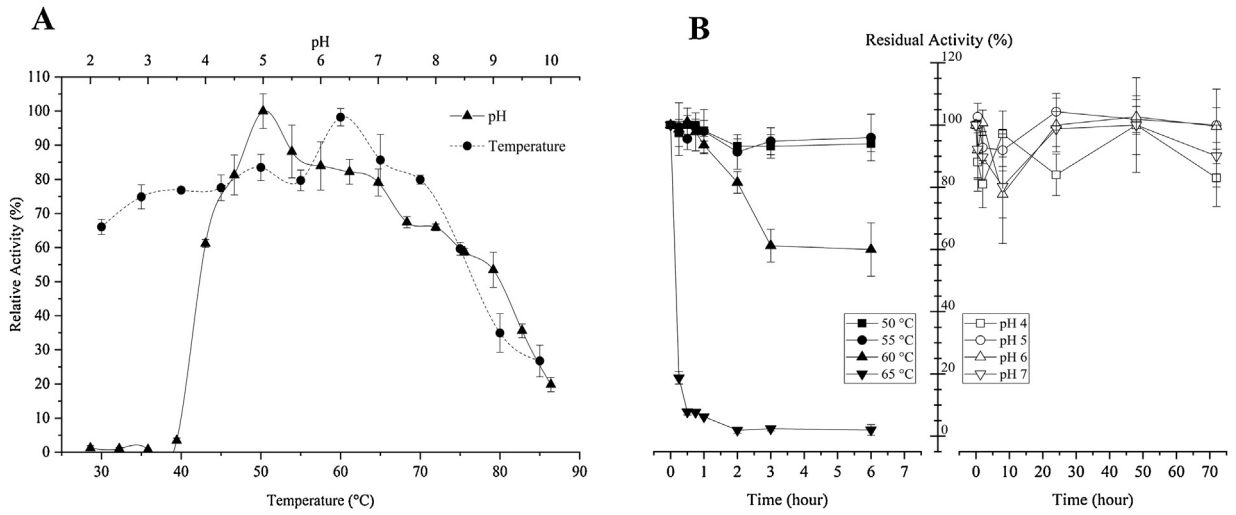

Fig. 3. Effect of pH and temperature in recombinant AFUMN-GH10 activity. (A) Optimal pH (▲) and temperature (●). For pH determination the activity was carried out at 50 °C in 50 mM citrate-phosphate-glycine buffer at different pH values. The optimal temperature was determined in the same buffer at pH 5.0 varying the temperature from 30 to 85 °C. (B) Stability in different temperatures and pHs. The thermal stability was evaluated incubating the enzyme at 50 °C (■), 55 °C (●), 60 °C (▲) and 65 °C (▼) in optimal pH and different periods followed by residual activity measurement. The pH stability was evaluated by incubating the enzyme at 30 °C for different periods in the previous buffer at pH 4.0 (□), 5.0 (○), 6.0 (Δ) and 7.0 (▽), followed by measurement in residual activity at 60 °C. All assays were performed in triplicate using xylan from beechwood (1% w/v) as substrate.

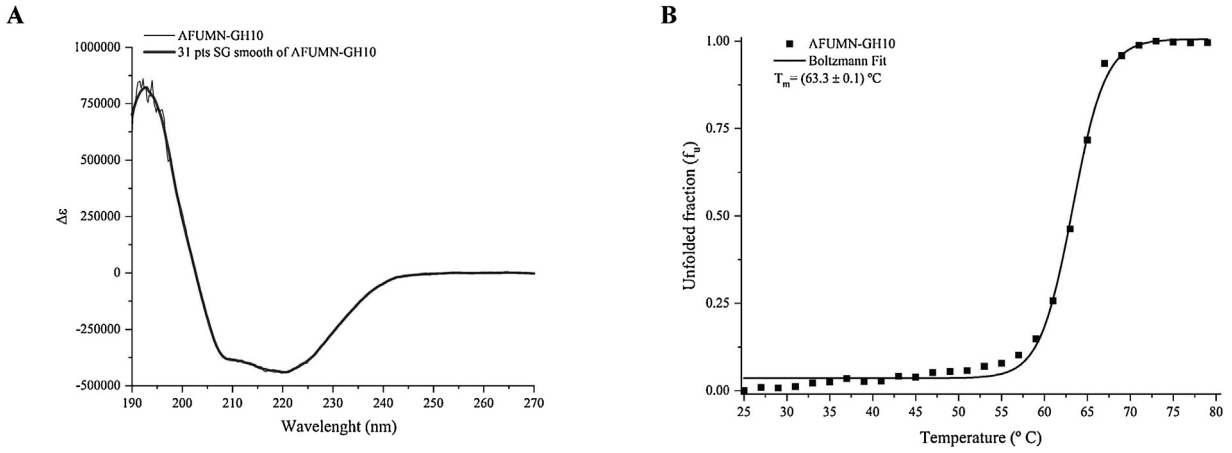

Fig. 4. (A) Far-UV CD spectra of purified AFUMN-GH10 as control protein (at 25 °C). (B) The curve shows experimental data of unfolded fraction of AFUMN-GH10 along temperature rise by monitoring the ellipticity at 222 nm. The sigmoidal fitting resulted in a Tm of 63.3 ± 0.1 °C for AFUMN-GH10.

(26.0%) (Supplementary Table 1). In addition, AFUMN-GH10 showed in CD analysis a melting temperature of 63.3 °C (Fig. 4B), corroborating with the optimal temperature previously determined in biochemical assay.

AFUMN-GH10 demonstrated the highest activity on Xbe, which was 14% higher than on Xbi (Table 1). Activity declined sharply with wheat arabinoxylan and rye arabinoxylan. The activity on Xbe is typical for GH10 xylanases, which show preference to hydrolyze linear xylans [49]. K_m and V_{max} were determined using Xbe and Xbi as substrates, and comparisons with other previously reported GH10 xylanases are shown in Table 2. AFUMN-GH10 showed K_m values similar to other GH10 xylanases when incubated with Xbi; however, V_{max} and K_{cat} values for AFUMN-GH10 were greater than reported for other GH10 xylanases incubated with Xbi. Kinetic characteristics exhibited by AFUMN-GH10 with Xbe as substrate were similar to other xylanases from the same family, except for a higher K_m when compared with reported xylanases from *Penicillium oxalicum* [50], *Caldicellulosiruptor bescii* [51] and a commercial xylanase from *Penicillium* sp. [52].

The catalytic efficiency (K_{cat}/K_m) is a convenient parameter to compare hydrolytic performance of enzymes [54]. AFUMN-GH10 showed a moderate catalytic efficiency value compared with a xylanase from *Streptomyces lividans* (XlnA), which showed the highest catalytic efficiency in this evaluation due to its affinity for Xbi [53]. AFUMN-GH10 showed a greater catalytic efficiency than

Table 1
Relative activity of purified AFUMN-GH10 on different substrates. CMC (carboxymethylcellulose), pNPX (4-nitrophenyl β-D-xylopyranoside), n.d. (non-detected).

Substrate	Relative activity (%)
Xylan from beechwood	100 ± 1.44
Xylan from birchwood	86 ± 0.61
Wheat arabinoxylan	45 ± 0.48
Rye arabinoxylan	3.2 ± 0.34
Xyloglucan	1.8 ± 0.32
CMC	0.1 ± 0.04
pNPX	n.d.

Table 2
Comparison in kinetic parameters of AFUMN-GH10 with GH10 xylanases from other microorganisms.

Entry name	Organism	Substrate	V_{max} μmol min^{-1} mg^{-1}	K_m mg ml^{-1}	K_{cat} s^{-1}	K_{cat}/K_m ml mg^{-1} s^{-1}	References
Xln A	*Streptomyces lividans*	Xbi	–	0.1	134	1327.0	[53]
XynB	*Caldicellulosiruptors sp*	Xbe	–	1.2	101	84.2	[51]
MpXyn 10A	*M. pulchella*	Xbi	82.0	4.6	748	162.6	[46]
Xyl10A	*P. oxalicum*	Xbi	144.5	3.3	82.3	24.8	[50]
Xyl 10A	*P. oxalicum*	Xbe	247.4	3.0	141	47.2	
Xyl 10B	*P. oxalicum*	Xbi	1140.6	1.3	787	626.4	
Xyl 10B	*P. oxalicum*	Xbe	914.4	1.0	631	648.1	
AFUMN-GH10	*A. fumigatus*	Xbi	2413.7	4.8	1341	279.4	This work
AFUMN-GH10	*A. fumigatus*	Xbe	11705.7	11.55	6483.7	561.3	This work

other GH10 xylanases on Xbe and similar values compared with xylanase Xyl10B from *P. oxalicum* (Table 2) [50].

The influence of metal ions on the activity of AFUMN-GH10 was also evaluated (Supplementary Table 2). The metal ions Mn^{2+}, Na^{+}, Mg^{2+}, K^{+} and Ca^{2+} enhanced the enzymatic activity of AFUMN-GH10, especially Mn^{2+} and Ca^{2+}, which increased activity by 86.8 and 64.1%, respectively. When AFUMN-GH10 was incubated in different concentrations of Mn^{2+} and Ca^{2+}, enzymatic activity increased in 65.2 and 64.1% with the addition of 5 and 10 mM of Ca^{2+} and 81.1 to 89.6% with the addition of 5 to 30 mM of Mn^{2+} (Fig. 5). Several studies report that the presence of ion Ca^{2+} in concentrations between 1 to 10 mM plays an important role on enzyme function and stability corroborating with the effect observed in AFUMN-GH10 and xylanases from *P. citrinum* [47], *Tramella fuciformis* [55], *A. niger* [56] and *Xanthomonas axonopodis pv. citri* [49]. However, most evaluations regarding the effect of Ca^{2+} are performed in low concentrations (around 5 mM). There is a lack of studies showing the effect on xylanase activity caused by higher concentrations of Ca^{2+} [57,58]. Studies that evaluated the effect of this ion in concentrations above 10 mM on Ca^{2+}-dependent carbohydrate active enzymes demonstrated a similar behavior showed by AFUMN-GH10 (Fig. 5). This result suggests the presence of Ca^{2+} in concentrations of 20 and 30 mM decreased the activity due the destabilization in enzyme structure affecting the substrate specificity [49,59]. In contrast, most previous studies did not report a beneficial effect from the addition of Mn^{2+}. The addition of Fe^{2+} and Cu^{2+} to the reaction media in a concentration of 10 mM strongly inhibited the AFUMN-GH10 activity, which was similar to the xylanases mentioned above.

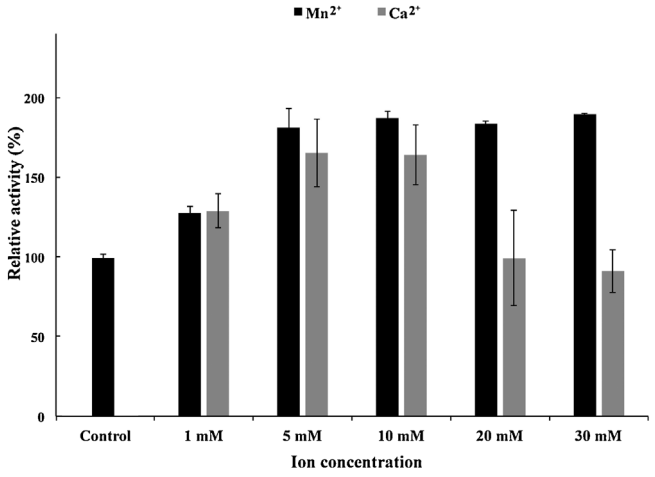

Fig. 5. Effect of Mn^{2+} and Ca^{2+} concentration on AFUMN-GH10 activity.

3.4. Pretreated sugarcane bagasse and corn stover hydrolysis

Physical and chemical biomass pretreatment methods such as hydrogen peroxide, alkali and steam explosion can be applied in biomass pretreatment to increase enzymatic accessibility of polysaccharides [60]. LHW is a promising, environmentally friendly pretreatment that depolymerizes mainly hemicellulose into oligosaccharides and monomers soluble in the liquid fraction while retaining cellulose and insoluble lignin in the solid portion. The residual hemicellulose in pretreated materials blocks the access of cellulases to cellulose due to its recalcitrance. However, addition of xylanases can overcome this problem since the enzyme can remove the residual and recalcitrant hemicellulose in the pretreated biomass, thus increasing the accessibility of cellulases [61–63]. Therefore, an efficient extraction of hemicelluloses is desirable to improve enzymatic digestibility of cellulose [64]. Here, the effect and performance of a xylanase at low concentration (0.37 mg/g of substrate) for hydrolysis of residual hemicellulose from BP and CSP pretreated with LHW at 180 °C for 10 and 20 min was evaluated. After pretreatment both BP and CSP showed a moderate content of residual xylan ranging from 13.1 to 16.0%, while residual lignin content showed a fluctuation between 25.1 to 30.1% (Table 3).

BP and CSP were hydrolyzed for 24 h in order to evaluate the efficiency of AFUMN-GH10 to release residual xylan. Xylan conversion by AFUMN-GH10 was higher in CSP than in BP independently of the pretreatment condition (Fig. 6). Pretreatment time for BP did not affect xylan conversion yield by AFUMN-GH10 with a yield of 14% for BP10 and 13% for BP20 ($p > 0.05$). Pretreatment time for CSP did affect xylan conversion yield with a yield of 27% for CSP10 and 18% for CSP20 ($p < 0.05$; Fig. 6). Longer LHW pretreatment times can increase the *in situ* production of hydronium ions (H_3O^{+}) from ionized water and dissociated acetic acid from acetyl groups in hemicellulose, which works as a catalyst attacking glycosidic bonds in xylan [65]. Consequently, hemicellulose available to enzymatic attack decreases (Table 3). Despite residual xylan content in the tested materials being similar,

Table 3
Chemical composition of evaluated feedstock in AFUMN-GH10 hydrolysis. BN – sugarcane bagasse *in natura*; BP10 – sugarcane bagasse pretreated for 10 min; BP20 – sugarcane bagasse pretreated for 20 min; CSN – corn stover *in natura*; CSP10 – corn stover pretreated for 10 min and CSP20 – corn stover pretreated for 20 min.

Biomass	Glucan*	Xylan*	Lignin*
BN	39.3 ± 0.6	21.4 ± 0.4	21.5 ± 0.3
BP10	47.0 ± 1.0	16.0 ± 0.4	23.6 ± 0.2
BP20	49.2 ± 0.6	13.1 ± 0.1	27.9 ± 0.2
CSN	30.6 ± 0.8	17.8 ± 0.1	18.1 ± 0.4
CSP10	37.4 ± 1.6	14.9 ± 0.4	25.1 ± 0.1
CSP20	48.5 ± 0.1	13.2 ± 0.1	30.1 ± 0.1

* Mass in percentage (%).

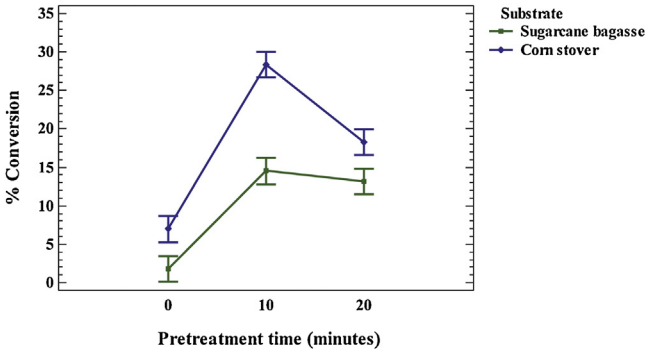

Fig. 6. Interaction graph showing the effect of AFUMN-GH10 in conversion of xylan from sugarcane bagasse and corn stover pretreated with liquid hot water for 10 and 20 min after 24 h of incubation.

enzymatic xylan conversions in CSP10 and CSP20 were higher than in BP10 and BP20. Lignin content was 16.7% lower in CSP10 than in CSP20, resulting in a higher xylan release from CSP10 (33.3%). This result can be related to non-productive adsorption of enzyme to lignin reducing enzyme efficiency (Table 3) [66–68].

The utilization of GH10 xylanase from *M. pulchella* (MpXyn10A) in hydrolysis of sugarcane bagasse pretreated with steam explosion achieved a xylan conversion of 41%. However, when the same material was submitted to a delignification step only 16% conversion was achieved, which indicated that solubilization of lignin also resulted in loss of hemicellulose. Furthermore, in this case, substrate availability was more decisive for enzymatic action than the amount of lignin in the material, which showed that the decrease in xylan content also affected its hydrolysis [46].

The incubation of AFUMN-GH10 with BN and CSN released 1.8 and 7.4% of xylan content, respectively. Low conversion yields are probably related to amount of enzyme used with the chemical composition and structural organization of non-pretreated materials, which limit enzyme access to the polysaccharides. AFUMN-GH10 was able to release almost the same amount of reducing sugar from CSP10 after 2 h as from CSP20 and BP10 after 24 h and 3-fold more than from BP20 after 24 h (Supplementary Fig. 2).

3.5. Capillary zone electrophoresis analyzes

The mode of action of AFUMN-GH10 was investigated based on the hydrolysis of APTS-labeled xylohexaose (X6 - Fig. 7A). AFUMN-GH10 completely converted X6 to xylotetraose (X4 – in small amounts) followed by higher amounts of xylotriose (X3) and xylobiose (X2) after 5 min of incubation (Fig. 7B). After 30 min of incubation, the final products were X3 and X2, with an increase in X2 compared to after 5 min, suggesting the enzyme did not act in APTS labeled X3, but instead hydrolyzed X4 to X2 (Fig. 7C). Even after incubating the enzyme for periods longer than 30 min, the final products profile (X2 and X3) did not change (data not shown). This degradation pattern shows that the AFUMN-GH10 is an endo-acting enzyme preferentially cleaving internal glycosydic bonds. Similar results were observed for xylanases from *M. pulchella*, *C. bescii* and non-identified meta-transcriptome discovered enzymes, where xylanases were able to hydrolyze xylan from hemicellulose to XOS containing X4 to X6 with decreasing catalytic efficiency towards XOS containing less 4 units [46,69,70].

Products released from BP and CSP hydrolysis by AFUMN-GH10 were analyzed by CZE, showing production of small amounts of xylose, X2, X3 and X4 (Supplementary Fig. 3). Similar to incubation with X6, AFUMN-GH10 released X2 and X3 as the main products in most of tested conditions and substrates. However, we observed an increase in xylose production for extended periods of incubation with BP10 and CSP10 (7 and 24 h) (Supplementary Fig. 3A and C). It is likely that xylose accumulation was related to hydrolysis of xylans with larger degrees of polymerization between 1 and 7 h of incubation. Particularly, incubation of CSP10 with AFUMN-GH10 produced more X4 after 7 h and started to release X5 in small amounts after 24 h (Supplementary Fig. 3C). However, incubation of AFUMN-GH10 with BP20 and CSP20 did not show any significant changes after 1 h, which was probably due to the lower amounts of xylan in these materials. The detection of xylose during hydrolysis

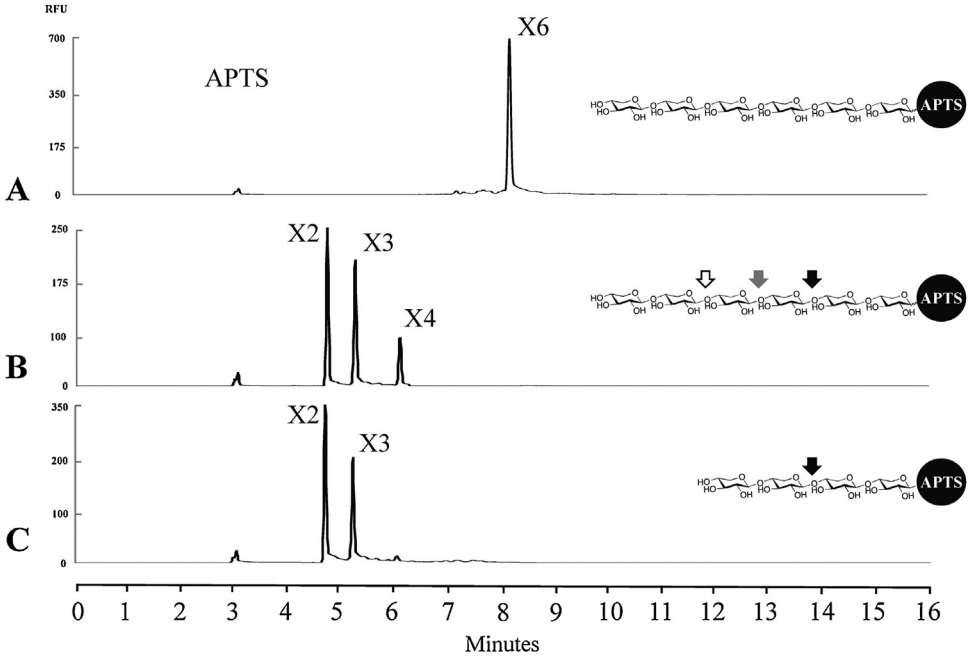

Fig. 7. CZE analysis of APTS-labeled xylohexaose (X6) after hydrolysis with AFUMN-GH10. (A) X6 without addition of enzyme (control). Products released from X6 after incubation with AFUMN-GH10 at 60 °C for 5 min (B) and 30 min (C). Arrows indicate the cleavage site and the enzyme affinity represented by colors: black (high), grey (moderate), and white (low). Relative fluorescence units (RFU) and retention time are shown in vertical and horizontal axis, respectively. Xylohexaose (X6), xylotetraose (X4), xylotriose (X3), xylobiose (X2) and 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS). The vertical axes were rescaled in the figures.

of BP20 and CSP20 was lower than with hydrolysis of BP10 and CSP10 and the formation of X5 was not detected (Supplementary Fig. 3B and D).

While only 14, 13, 27 and 18% of available xylans in BP10, BP20, CSP10 and CSP20 were released after hydrolysis with AFUMN-GH10, respectively; the main products detected in the hydrolyzates were XOS. Previous studies showed that removal of lignin is a crucial step to improve enzymatic activity, since it can affect the degree of polymerization of released XOS [71]. Likewise, the residual and recalcitrant hemicellulose mainly in the form of oligomers can strongly inhibit activity of cellulases [61]. The results showed the pretreatment of BP and CSP with LHW followed by enzymatic hydrolysis by AFUMN-GH10 is a suitable method to remove the residual hemicellulose from biomass mainly as oligomers.

4. Conclusions

This work showed successful cloning, heterologous expression in pEXPYR system and functional characterization of a GH10 endoxylanase (AFUMN-GH10). AFUMN-GH10 showed high specific activity on Xbe compared with previously studied endoxylanases. Moreover, AFUMN-GH10 was stable in different temperatures and pH range and was activated by Ca^{2+} and Mg^{2+}. The effect of AFUMN-GH10 at low concentration in pretreated BP and CSP demonstrated this enzyme was suitable to remove part of residual and recalcitrant hemicellulose as oligomers from these renewable materials.

Declaration of Competing Interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2019.e00382.

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