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Heterologous Expression and Characterization of an Acidic GH11 Family Xylanase from *Hypocrea orientalis*

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Abstract A gene encoding glycoside hydrolase family 11 xylanase (HoXyn11B) from *Hypocrea orientalis* EU7–22 was expressed in *Pichia pastoris* with a high activity (413 IU/ml). HoXyn11B was partly N-glycosylated and appeared two protein bands (19–29 kDa) on SDS-PAGE. The recombinant enzyme exhibited optimal activity at pH 4.5 and 55 °C, and retained more than 90% of the original activity after incubation at 50 °C for 60 min. The determined apparent K_m and V_{max} values using beechwood xylan were 10.43 mg/ml and 3246.75 IU/mg, respectively. The modes of action of recombinant HoXyn11B on xylo-oligosaccharides (XOSs) and beechwood xylan were investigated by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), which indicated that the modes of action of HoXyn11B are different from HoXyn11A since it is able to release a significant amount of xylose from various substrates. This study provides an opportunity to better understand the hydrolysis mechanisms of xylan by xylanases from *Trichoderma.*

Keywords Heterologous expression · *Pichia pastoris* · Xylan hydrolysis · Xylanases · *Hypocrea orientalis* · Xylo-oligosaccharides

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Introduction

Hemicellulose is the second most abundant polysaccharides in plant cell wall. The efficient enzymatic hydrolysis of hemicellulose into fermentable monosaccharides or value-added xylo-oligosaccharides (XOSs) is of considerable interest [1, 2]. Hemicellulose is mainly composed of heteroxylans, which have a backbone of β-1,4-linked xylose residues and side chains of different substituent units such as L-arabinofuranose or 4-*O*-methyl-D-glucuronic acid [3]. Xylanases play a crucial role in the breakdown of heteroxylans, which randomly cleave xylan main chain and generate variously unsubstituted or branched XOS [4].

Xylanases in the various glycoside hydrolase (GH) family differ in their physicochemical properties, three-dimensional structure, and substrate specificity [5]. According to the sequence-based GH classification, most xylanases belong to GH10 and GH11 family, while minority xylanases are classified in GH5 and GH8 family. The substrate specificities of GH10 and GH11 family xylanases have been extensively studied. GH11 family xylanases have higher substrate specificity than GH10 family xylanases [4]. However, GH10 family xylanases show higher catalytic activity on highly substituted heteroxylans and produce smaller products than GH11 family xylanases [6]. GH5 family xylanases show distinct substrate specificity from that of GH10 or GH11 family xylanases. They show very low specific xylanases on polymeric xylan but are able to degrade several different xylans, mainly producing xylose [7]. GH8 family xylanases have low affinity for small XOS and are unable to engage in productive binding with highly substituted substrates [8].

Xylanases are routinely used in various biotechnological applications such as bread making, paper bio-bleaching, and xylan-rich lignocellulosic biomass biorefining. For these applications, not only are the biochemical properties but also their specific activity and substrate specificity are of high importance. A recent study showed that the classification of enzymes within a certain family does not predict their performance towards various substrates [9]. In order to looking for specific xylanase for various applications, therefore, more deep studies about xylanases from different family as well as the same family acting on various substrates are needed.

Hypocrea orientalis EU7–22 is in the genus of *Trichoderma*, which produces a large amount of cellulases and hemicellulases [10]. Compared to commercial xylan-degrading enzymes produced by *Trichoderma reesei*, the crude enzymes from *H. orientalis* EU7–22 exhibited better potential for the production of XOS [11]. The efficient expression of xylanase with high catalytic efficiency and negligible cellulases or β-xylosidases would reduce costs and make it possible to be widely used in various industries such as pulp bleaching or XOS production [12, 13]. Currently, heterologous expression is becoming one of the main strategies for the large-scale production of various industrial enzymes [6]. Compared to filamentous fungus expression system, *Pichia pastoris* lacks background contaminating activities and possess high levels of heterologous protein expression [14]. Besides, it has additional advantages of easy molecular and genetic manipulations [15]. In this study, the heterologous expression of HoXyn11B from *H. orientalis* EU7–22 in *P. pastoris* under the control of the methanol inducible alcohol oxidase 1 (*AOX1*) promoter was reported for the first time. The biochemical properties of HoXyn11B were characterized. In addition, the modes of action of HoXyn11B towards XOS and beechwood xylan were also investigated.

Materials and Methods

Materials

Beechwood xylan (BWX), oat spelt xylan (OSX), *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA), *p*-nitrophenyl-β-D-cellobiose (*p*NPC), and *p*-nitrophenyl-β-D-glucose (*p*NPG) were obtained from Sigma-Aldrich (Milwaukee, USA). Wheat arabinoxylan (WAX), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), xylopentaose (X_5), and xylohexaose (X_6) were purchased from Megazyme (Bray, Ireland). Caboxy methyl cellulose (CMC), xylose (Xyl), and arabinose (Ara) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Acros Organics (Geel, Belgium).

Strains, Vectors, and Culture Media

H. orientalis EU7–22 (GenBank accession no. KC751873) was used for total RNA extraction. The shuttle vector pPIC9K was used to obtain secreted expression of HoXyn11B. Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α cultivated at 37 °C in Luria-Bertani (LB) medium or LB agar. *P. pastoris* GS115 was cultivated in following media that were prepared according to the manual of Multi-copy *P. pastoris* Expression Kit: minimal dextrose (MD), yeast extract peptone dextrose (YPD), buffered glycerol complex (BMGY), and buffered methanol complex (BMMY).

Construction and Transformation of the Plasmid

The gene fragment encoding HoXyn11B without the signal peptide sequence was isolated from *H. orientalis* EU7–22 cDNA by RT-PCR using gene-specific primers (upstream 5′-ATAGAATTCATGCCCACGGGCCTTGAG-3′, downstream 5′-TTAGCGGCCGCCTAGTTGCTGACACTCTGTG-3′) supplied with the *Eco*RI and *Not*I restriction sites. The primers of HoXyn11B were based on the sequence of *H. orientalis* EU7–22 *XynB* (GenBank accession no. JQ238611). The amplified fragment was gel-purified and digested with *Eco*RI and *Not*I and then inserted into the *Eco*RI/*Not*I site of pPIC9K vector and transformed into *E. coli* DH5a. The constructed vector pPIC9K-*HoXyn11B* was confirmed by DNA sequencing before transformation. Then, the vector pPIC9K-*HoXyn11B* was linearized with *Bg*lII, and transformed into *P. pastoris* GS115 by electroporation methods. The transformants were first screened on an MD plate, and then, the phenotype of transformants was confirmed by colony PCR using 5′*AOX1* and 3′*AOX1* primers.

Expression and Purification of Recombinant Protein

Large-scale expression was carried out as previously described [6]. After 168 h of growth, cultures were centrifuged at 12000 rpm for 10 min and the supernatant was stored at −80 °C before SDS-PAGE and biochemical property analysis.

Enzyme Assay

Xylanase activity was assayed using 1% BWX as the substrate in sodium citrate buffer (50 mM, pH 4.5) at 55 °C for 10 min [16]. One unit of xylanase activity was defined as the

amount of enzyme that forms reducing groups corresponding to 1 μmol of xylose in 1 min under the above conditions.

SDS-PAGE Analysis

Proteins analyses were carried out at via SDS-PAGE on 13% polyacrylamide by the method of Laemmli [17]. Proteins in the gel were visualized by Coomassie Brilliant Blue R-250 staining. The protein concentration was determined by the Bradford assay [18].

Deglycosylation Assay

HoXyn11B was N-deglycosylated using Endoglycosidase H (Endo H; New England Biolabs) at 37 °C for 1 h in 50 mM sodium citrate (pH 5.5). Molecular weights (MWs) were predicted using ProtParam tool (http://web.expasy.org/protparam/). N-glycosylation sites were predicted using NetNglyc1.0 (http://www.cbs.dtu.dk/services/NetNglyc/).

Effect of Temperature and pH on HoXyn11B

The effect of pH on the enzymes activity of HoXyn11B was determined at pH values from 3 to 8 using McIlvaine's buffer at 50 °C. The effect of temperature on the enzyme activity of HoXyn11B was determined by standard methods at different temperatures (30~80 °C). The thermostability of HoXyn11B was determined by measuring the residual enzyme activity after pre-incubation of enzymes at different temperature (50, 55, 60, and 65 °C) for different time (10, 20, 30, 40, 50, and 60 min). The activity of the enzyme without pre-incubation was defined as 100%. All evaluations of samples were carried out in triplicate.

Substrate Specificity and Analysis of Kinetic Parameters

The substrate specificity of recombinant protein was evaluated using BWX (1%), WAX (1%), OSX (1%), CMC (1%), *p*NPX (10 mM), *p*NPA (10 mM), *p*NPC (10 mM), and *p*NPG (10 mM). For the kinetic experiment, the purified protein was incubated with six concentrations of BWX (2–20 mg/ml) in sodium citrate buffer (50 mM, pH 4.5) at 55 °C for 10 min. K_m and V_{max} were determined using Lineweaver–Burk plots.

Enzymatic Hydrolysis

To investigate the modes of action of HoXyn11B on XOS and xylan, X_2–X_6 (1 mg/ml) were incubated with HoXyn11B (300 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h individually. BWX (20 mg/ml) were incubated with HoXyn11B (300 IU/g substrate), AnXln3D (12.5 IU/g substrate), and AnAxh62A (10 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h individually or combination sequentially. Hydrolysis was terminated by boiling the samples in a water bath for 10 min. Thereafter, the samples were incubated with another recombinant enzyme for 24 h under the same conditions or directly analyzed by TLC, HPLC, and MALDI-TOF-MS.

Hydrolysis Product Analysis

The hydrolysis products of X_2~X_6 and BWX released by HoXyn11B were detected by thin-layer chromatography (TLC) (GF 254, Qingdao, China) with a solvent system consisting of chloroform, acetic acid, and water (30:35:5, *v*/*v*/*v*). The plate was then sprayed with the reagent aniline-diphenylamine-phosphoric acid and heated for 10 min at 85 °C in an oven to visualize the products.

The hydrolysis products of BWX were analyzed by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a PMP pre-column derivatization method described previously [19, 20]. The analysis of PMP derivatives of saccharides was carried out on a Waters HPLC system (USA) equipped with a CAPCELL PAK C_{18} MG column (3.0 mm i.d. × 250 mm, 5 μm, Shiseido, Japan) and a diode array detector. Elution was carried out at a flow rate of 0.4 ml/min at 30 °C, with a sodium phosphate buffer (40 mM, pH 8.0)—acetonitrile (79:21, *v*/*v*). The wavelength for UV detection was 245 nm. MALDI-TOF-MS was performed on a MicroFlex (Bruker, USA) equipped with a nitrogen laser at 337 nm.

Results and Discussion

Gene Cloning and Expression of HoXyn11B

The gene encoding HoXyn11B was cloned from *H. orientalis* into the pPIC9K plasmid and expressed in *P. pastoris* as a recombinant protein using the native *Saccharomyces cerevisiae* α-factor secretion signal sequence under the control of the *AOX1* promoter. After 168-h induction by adding 1% (*v*/*v*) methanol at 24-h intervals, HoXyn11B exhibited high level of xylanase activity (413 U/ml) with a high protein concentration (0.22 mg/ml). As shown in Table 1, the xylanase activity of HoXyn11B was higher than those reported in previous studies [6, 14, 21, 22]. The predicted MW of HoXyn11B was 19 kDa; however, SDS-PAGE analysis indicated that HoXyn11B displays two protein bands with an apparent MW of 19 and 29 kDa (Fig. 1). Both glycosylated and non-glycosylated enzymes were present in HoXyn11A and HoXyn11B [6]. Bioinformatics analysis showed that there is one potential N-glycosylation site in the HoXyn11B sequence. After treatment with Endo H, the 29-kDa band in HoXyn11B was changed to 26-kDa band, whereas a single protein band (21 kDa) was found in HoXyn11A, which contained three potential N-glycosylation sites and displayed two protein bands (21–25 kDa) before treatment with Endo H [6]. These results indicated that HoXyn11B was partly N-glycosylated and some other post-translational modifications such as O-glycosylation and assembly of disulfide bonds may be existed.

Table 1 Comparison of properties of various recombinant GH11 family xylanases

Organism	Name	Enzyme activity	MW (kDa)	pH_{opt}	T_{opt} (°C)	Reference
Hypocrea orientalis	HoXyn11B	413 IU/ml	19~29	4.5	55	This study
Hypocrea orientalis	HoXyn11A	284 IU/ml	21~25	6	60	[6]
Cellulomonas uda	CuXyn11A	161.5 IU/ml	45	6.5	50	[22]
Trichoderma reesei	TrXyn2	261 IU/ml	21	6	60	[14]
Aspergillus niger	Anxyn11A	240 IU/ml	25	5	60	[21]

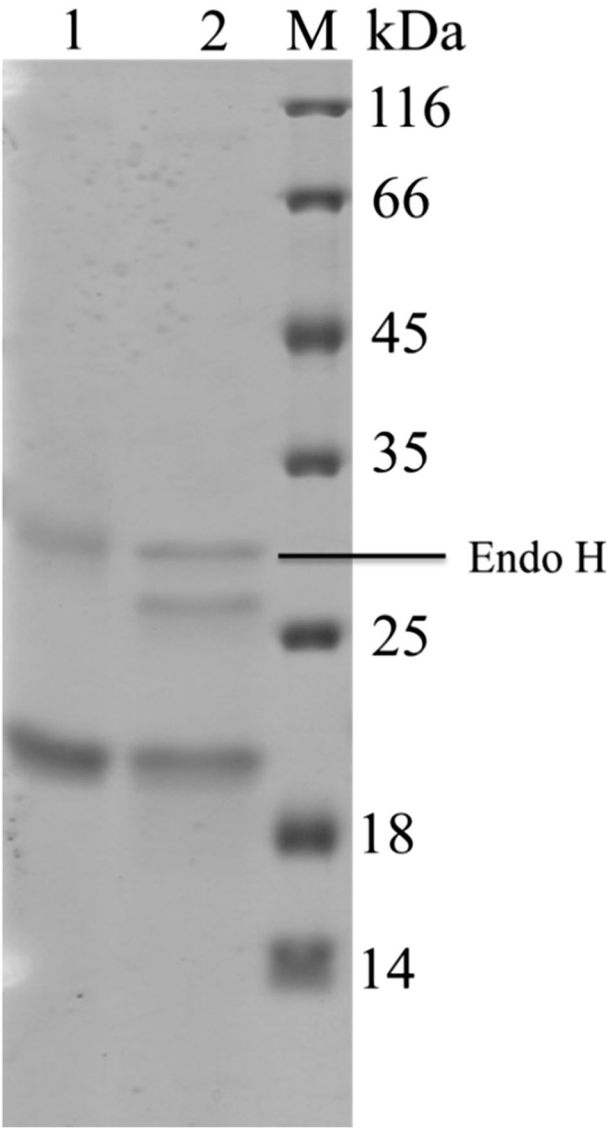

Fig. 1 SDS-PAGE analysis of HoXyn11B in *P. pastoris*. *1* without treatment with Endo H, *2* treatment with Endo H, 3 M protein marker

Biochemical Characterization of Recombinant HoXyn11B

The effect of pH on the activity of HoXyn11B was studied. HoXyn11B exhibited maximal activity at pH 4.5 (Fig. 2a), whereas HoXyn11A exhibited maximal activity at pH 6.0 [6]. The effect of temperature on HoXyn11B was investigated (Fig. 2b). HoXyn11B exhibited optimum activity at 55 °C, whereas HoXyn11A exhibited optimum activity at 60 °C [6]. The thermal stability of HoXyn11B was determined at 50, 55, 60, and 65 °C. HoXyn11B was highly stable at 50 °C and retained more than 80% activity after 60-min incubation at 55 °C (Fig. 2c). At 60 °C, HoXyn11A entirely lost its activity

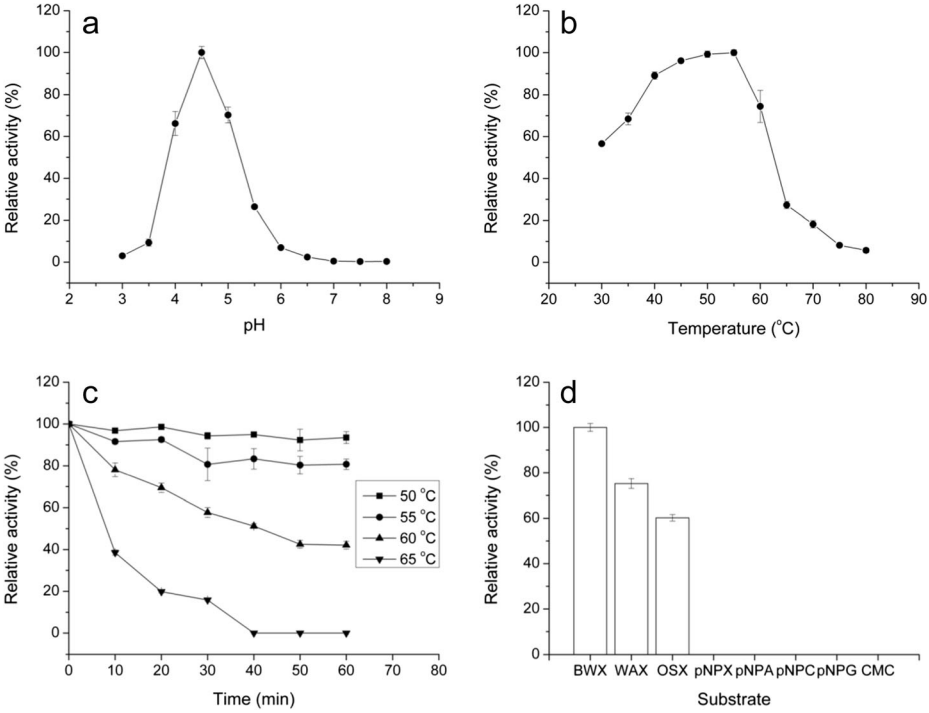

Fig. 2 Effect of temperature and pH on HoXyn11B in *P. pastoris*. **a** The effect of pH on HoXyn11B. **b** The effect of temperature on HoXyn11B. **c** The thermostability of HoXyn11B. **d** Substrate specificity activity (IU/mg) of HoXyn11B on various substrate. The values of relative activities are the means of three replicates

after 60-min incubation [6], whereas HoXyn11B retained more than 40% activity after 60-min incubation. The results indicated that HoXyn11B is more thermostable than HoXyn11A. The substrate specificity of HoXyn11B on various substrates was investigated. As shown in Fig. 2d, HoXyn11B exhibited hydrolytic activity on all xylan substrates but negligible activity on other substrates, indicating that HoXyn11B is specific for xylan degradation. The highest hydrolytic activity of HoXyn11B was observed with BXW followed by WAX and OSX. The hydrolytic activity of HoXyn11B towards BXW was evaluated by variation of the substrate concentration from 2 to 20 mg/ml at standard assay conditions. The K_m and V_{max} values of HoXyn11B were graphically determined to be 10.43 mg/ml and 3246.75 IU/mg, respectively. The K_m of HoXyn11B was higher than TrXyn2 (K_m of 2.1 mg/ml) but lower than AnXyn11A (K_m of 13.67 mg/ml) [14, 21].

Modes of Action of Recombinant HoXyn11B

To investigate the modes of action of HoXyn11B on XOS, the end hydrolysis products of XOS by HoXyn11B after incubation at 50 °C for 24 h were analyzed by TLC. As shown in Fig. 3, X_4~X_6 were hydrolyzed mainly to xylose, xylobiose, and xylotriose by HoXyn11B. Interestingly, X_3 was partly degraded into xylose and xylobiose by HoXyn11B, whereas xylobiose and xylotriose were the main hydrolysis products released from X_3 by HoXyn11A [6]. However, HoXyn11B was unable to hydrolyze X_2.

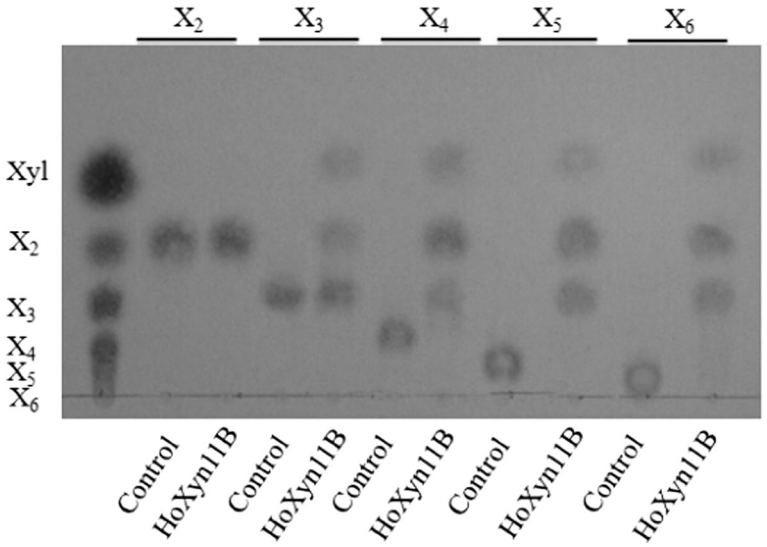

Fig. 3 Analysis of end hydrolysis products by TLC. X_2~X_6 (1 mg/ml) were incubated with HoXyn11B (300 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h

These results indicated that HoXyn11B shows better potential than HoXyn11A in the hydrolysis of xylan into fermentable sugars.

The modes of action of HoXyn11B on BWX were investigated by sequential enzymatic hydrolysis. As shown in Fig. 4, the end products were analyzed by TLC and HPLC. HoXyn11B released xylose, xylobiose, and 4-*O*-methylglucuronic acid substituted XOS (B2 > B3) as the main hydrolysis products from BWX. The addition of

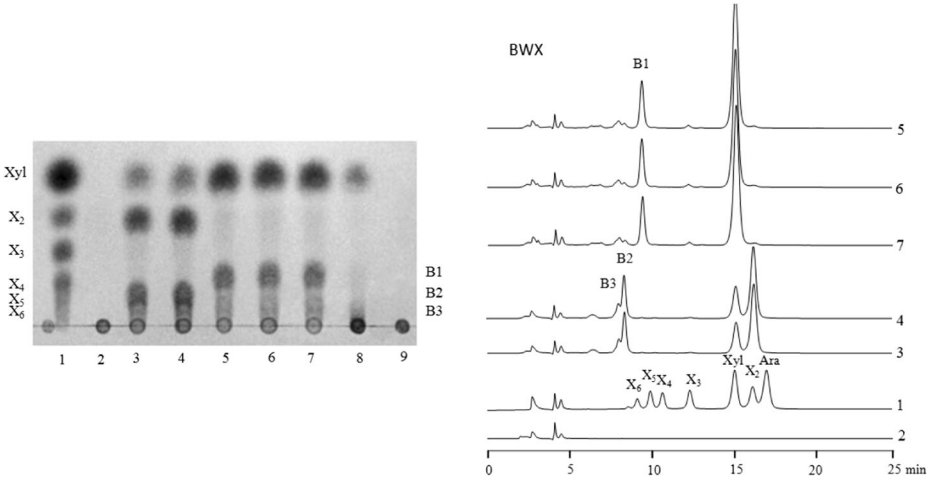

Fig. 4 TLC and HPLC analyses of produced end products by enzymatic hydrolysis of BWX. *1* X_2~X_6, *2* control, *3* HoXyn11B, *4* HoXyn11B + AnAxh62A, *5* HoXyn11B + AnAxh62A + AnXln3D, *6* HoXyn11B + AnXln3D, *7* HoXyn11B + AnXln3D + AnAxh62A, *8* AnXln3D, *9* AnAxh62A. BWX (20 mg/ml) were incubated with HoXyn11B (300 IU/g substrate), AnXln3D (12.5 IU/g substrate), and AnAxh62A (10 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h individually or combination sequentially

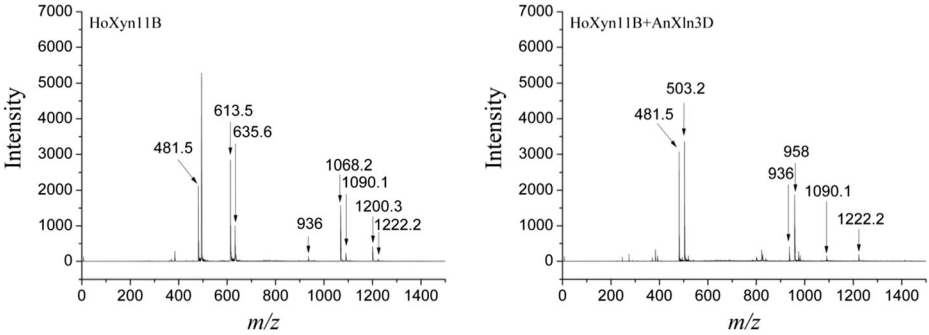

Fig. 5 MALDI-TOF-MS analysis of produced end products by enzymatic hydrolysis of BWX. BWX (20 mg/ml) were incubated with HoXyn11B (GH11, 300 IU/g substrate) and AnXln3D (GH3, 12.5 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h individually or combination sequentially

AnAxh62A in the preparation of HoXyn11B showed no effect in the hydrolysis products since L-arabinofuranose side groups were not present in BWX. The addition of AnXln3D in the preparation of HoXyn11B produced xylose and 4-*O*-methylglucuronic acid-substituted XOS (B1 > B2) as the end products. This indicated that 4-*O*-methylglucuronic acid-substituted XOS released by HoXyn11B can be further degraded by AnXln3D. The hydrolysis products were determined by MALDI-TOF-MS (Fig. 5), which indicated that B1, B2, and B3 are MeGlcAXyl3, MeGlcAXyl4, and MeGlcAXyl5, respectively (Table 2). It has previously been reported that HoXyn11A releases xylobiose, xylotriose, and 4-*O*-methylglucuronic acid substituted XOS (MeGlcAXyl4 = MeGlcAXyl5) as the main hydrolysis products with a small amount of xylose from BWX [6]. Compared to HoXyn11A, HoXyn11B released remarkable amounts of xylose but negligible amounts of xylotriose. HoXyn11B tends to produce shorter hydrolysis products than HoXyn11A. This indicated that HoXyn11A and HoXyn11B exhibit different hydrolysis properties on BWX, although they belong to the same GH family. GH10 family xylanases tend to attack glycosidic linkages next to a substituted xylose residue [7]. Interestingly, both of HoXyn11A and HoXyn11B that belong to GH11 family xylanases were unable to cleave glycosidic linkages next to a branch. Therefore, the results also suggested that the modes of action of GH11 family xylanases were significantly different with GH10 family xylanases.

Table 2 Summary of the PMP-derived saccharide peak identification in MALDI-TOF-MS spectra

Saccharides	M_r	*m/z*	
		$[M + H]^+$	$[M + Na]^+$
Xyl	480	481.5	503.2
X_2	612	613.5	635.6
X_3	744	745.7	767.8
X_4	877	877.9	899.9
X_5	1009	1010.2	1032.1
X_6	1141	1142.3	1164.2
$MeGlcAX_3$	935	936	958
$MeGlcAX_4$	1067	1068.2	1090.1
$MeGlcAX_5$	1099	1200.3	1222.2

Conclusions

In this work, an acidic GH11 family xylanase, HoXyn11B from *H. orientalis* EU7–22, was successfully expressed in *P. pastoris* for the first time. Recombinant HoXyn11B displayed high specific activity and good thermostability, indicating a potential for various industrial application. Modes of action analyzed in this study indicated that HoXyn11B is able to release a significant amount of xylose from various substrates, which shows different hydrolysis properties from HoXyn11A, although they belong to the same GH11 family. This study provided an opportunity to better understand the hydrolysis mechanisms of xylan by xylanases from *Trichoderma*.

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