Comparative Analysis of Enzymatic Hydrolysis of *Miscanthus* Xylan using *Aspergillus niger*, *Hypocrea orientalis*, and *Trichoderma reesei* Xylan-degrading Enzymes

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Xylan-degrading enzymes from *Aspergillus niger* and *Hypocrea orientalis* were characterized by enzyme activity assays and protein profiling with SDS-PAGE and LC-MS/MS. The hydrolysis of *Miscanthus* xylan by xylan-degrading enzymes from *A. niger*, *H. orientalis*, and *Trichoderma reesei* were comparatively studied by HPLC analysis. It was found that the glycoside hydrolase families 10 xylanase was the main xylanase secreted by *H. orientalis* and *A. niger* when using corn cob and wheat bran as inducers. Compared to the enzymes from *T. reesei*, the enzymes from *A. niger* showed better efficiency in the hydrolysis of *Miscanthus* xylan into monosaccharides. Nevertheless, the enzymes from *H. orientalis* were more preferable for the hydrolysis of *Miscanthus* xylan into xylo-oligosaccharides (XOS), especially xylobiose and xylotriose. *Miscanthus* xylan degradation was significantly influenced by the activities of β-xylosidase and α-L-arabinofuranosidase. Xylan-degrading enzymes with high ratios of β-xylosidase and α-L-arabinofuranosidase are necessary for the efficient conversion of *Miscanthus* xylan into monosaccharides. However, xylan-degrading enzymes with low β-xylosidase activity and high α-L-arabinofuranosidase activity were required for producing XOS.

Keywords: Aspergillus niger; Hypocrea orientalis; Trichoderma reesei; Xylan-degrading enzymes; Hydrolysis; Xylo-oligosaccharides

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INTRODUCTION

The efficient enzymatic conversion of cellulose and hemicellulose polymers into monosaccharides and xylo-oligosaccharides (XOS) is a topic of current interest. Hemicelluloses play an important role in biomass enzymatic digestion (Xu *et al*. 2012; Zhang *et al*. 2012; Li *et al*. 2013a). Xylan, the major hemicellulosic polysaccharide present in plant cell walls, has a backbone of β-1,4-linked xylose residues and side chains of different substituents (Vázquez *et al*. 2002). Enzymatic hydrolysis of xylan involves the synergistic action of several main chain- and side group-cleaving enzymes, including endo-β-1,4-xylanases (EC 3.2.1.8), β-xylosidases (EC 3.2.1.37), α-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases (EC 3.2.1.139), acetyl xylan esterases (EC 3.1.1.72), and feruloyl esterases (EC 3.1.1.73) (de Vries and Visser 2001). Endo-β-1,4-xylanases as the main xylan-degrading enzymes randomly cleave the internal β-1,4-glycosyl bonds in the xylan main chain producing xylooligomers (Zhang *et al*. 2011), and β-xylosidases convert xylooligomers to xylose monomers (Knob *et al* 2009). Species of *Trichoderma*

and *Aspergillus* are used as producers of enzymes that deconstruct lignocellulosic biomass by various companies (Banerjee *et al*. 2010). *Trichoderma reesei* has a highly efficient set of enzymes involved in cellulose degradation (Selig *et al*. 2008), while *Aspergillus niger* mostly produces enzymes that degrade hemicelluloses (Stricker *et al.* 2008).

Xylan-degrading enzymes with negligible amounts of exo-xylanase or β-xylosidase activity are required for the production of high-value added XOS because exo-xylanase and β-xylosidase produce xylose as the main product and inhibit the production of XOS (Vázquez *et al*. 2002). However, high-activity xylanase and β-xylosidase should be supplemented in saccharification processes for total hydrolysis of lignocellulosic biomass into monosaccharides because xylan clearly inhibited the hydrolysis of cellulose by cellulase and xylooligomers are stronger inhibitors of cellulase activity than are glucose and cellobiose (Selig *et al*. 2008; Qing *et al*. 2010; Zhang *et al*. 2012). Currently, most biotechnological processes are based on the use of crude enzymes. Strains with specificity toward xylan-degrading enzyme activity are of great application potential for the bioconversion of lignocellulosic biomass into monosaccharides or high-value added XOS.

Miscanthus with high biomass yield and low nitrogen and water requirement is considered as one of leading feedstocks for biofuel and value-added chemicals production (Xu *et al*. 2012; Li *et al*. 2013a). The aim of this work was to investigate the application potential of xylan-degrading enzymes from *A. niger* BE-2 and *Hypocrea orientalis* EU7-22 for the bioconversion of *Miscanthus* into monosaccharides and XOS. In this paper, xylan-degrading enzymes obtained from *A. niger* BE-2 and *H. orientalis* EU7-22 using corn cob and wheat bran as substrates were characterized by enzyme activity assays and SDS-PAGE coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). In addition, the hydrolysis of *Miscanthus* xylan by xylan-degrading enzymes from *A. niger* BE-2, *H. orientalis* EU7-22, and *T. reesei* were comparatively studied by HPLC analysis.

EXPERIMENTAL

Materials

Xylohexaose (X_6), xylopentaose (X_5), xylotetraose (X_4), xylotriose (X_3), and xylobiose (X_2) were obtained from Megazyme (Bray, Ireland). Glucose (Glu), xylose (Xyl), arabinose (Ara), and HPLC-grade acetonitrile were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 1-phenyl-3-methyl-5-pyrazolone (PMP) was obtained from Acros Organics (Geel, Belgium). Beechwood xylan, *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX), and *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA) were purchased from Sigma-Aldrich (USA).

Commercial xylan-degrading enzyme preparation (xylanase activity, 696,345 IU/g; β-xylosidase activity, 469 IU/g; α-arabinofuranosidase activity, 281 IU/g) produced by *T. reesei* was purchased from Zaozhuang Jienuo Enzyme Co., Ltd (Shandong, China). *Miscanthus* xylan substrate (79.28% xylose, 13.08% arabinose, and 7.64% glucose) was isolated from *Miscanthus floridulus* as described previously (Li *et al*. 2013b).

Microorganism Xylan-degrading Enzyme Production

The xylan-degrading enzyme-producing strains *A. niger* BE-2 (GenBank accession No. JQ867187) and *H. orientalis* EU7-22 (GenBank accession No. KC751873) were preserved in the laboratory. Enzyme production was carried out in 250-mL flasks with 50 mL of medium. Each flask included 5 g/L tryptone, 0.4 g/L $CaCl_2$, 0.4 g/L $FeSO_4$, 0.8 g/L $MgSO_4$, 2.5 g/L KH_2PO_4, 1.5% (w/v) corncob, and 1.5% (w/v) wheat bran. The pre-cultured *H. orientalis* EU7-22 and *A. niger* BE-2 mycelium (10%, v/v) were inoculated into each flask and then incubated at 37 ^oC in a rotary shaker (180 rpm) for 72 h. The enzymes were obtained by centrifugation at 5000 *g* for 10 min to remove the mycelium and solid medium. Then, the enzymes were aliquoted into tubes and stored at -80 ^oC.

Enzyme Assays

Xylanase activity was assayed using 1% beechwood xylan as the substrate in sodium citrate buffer (50 mM, pH 5.0) at 50 ^oC for 10 min (Bailey *et al*. 1992). The amount of released sugar was determined by the dinitrosalicylic acid method described by Miller *et al*. (1960). One unit of xylanase activity was defined as the amount of enzyme that liberated 1 μmol of reducing saccharides per min from the substrate. β-xylosidase and α-arabinofuranosidase enzyme activities were measured using a final concentration of 5 mM *p*NPX and 2.5 mM *p*NPA as the substrates in citrate buffer (50 mM, pH 5), respectively. After incubation at 50 ^oC for 30 min, the reaction was terminated by addition of 1 mL of 0.5 M Na_2CO_3, and the absorbance was measured at 405 nm. One unit of β-xylosidase or α-arabinofuranosidase enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenyl per min from the synthetic substrate. Thermostability assays were determined by measuring residual xylanase and β-xylosidase activities after pre-incubation of enzymes at 50 ^oC for 0.5 h, 1 h, 3 h, 6 h, 12 h, and 24 h. The activity of the enzyme without pre-incubation was defined as 100%.

Enzymatic Hydrolysis

Miscanthus xylan was hydrolyzed by crude enzymes from *H. orientalis* EU7-22, *A. niger* BE-2, and *T. reesei* as described previously (Li *et al*. 2013b). *Miscanthus* xylan (2 g) was suspended in 100 mL of citrate buffer (50 mM, pH 5.0) containing 0.02% azide, and enzymes were added at 100 to 200 IU/g (xylanase activity) dry matter. Hydrolysis was performed in shake-flasks at 50 ^oC for 0.5 h, 1 h, 2 h, 3 h, and 6 h. Hydrolysis samples were boiled for 10 min and then centrifuged at 8000 *g* for 5 min, filtered, and stored at 4 ^oC for further analysis.

HPLC Analysis

Monosaccharides and oligo-saccharides were analyzed by HPLC using a PMP pre-column derivatization method described previously (Li *et al*. 2013b). The analysis of PMP derivatives of saccharides was carried out on an Agilent 1200 HPLC system (U.S.) equipped with a diode array detector. The analytical column used was a CAPCELL PAK C_{18} MG column (3.0 mm i.d. × 250 mm, 5μm, Shiseido, Japan). Elution was carried out at a flow rate of 0.5 mL/min at 30 ^oC, with a sodium phosphate buffer (40 mM, pH 8.0)/-acetonitrile (79:21, v/v). The wavelength for UV detection was 245 nm.

SDS-PAGE and LC-MS/MS Analysis

SDS-PAGE on 15% polyacrylamide was performed using the method of Laemmli (1970). Protein bands were visualized by Coomassie Brilliant Blue R-250 staining. The most abundant protein bands from *H. orientalis* EU7-22 and *A. niger* BE-2 were excised from Coomassie-stained gels and subjected to in-gel trypsin digestion. Tryptic peptides were extracted from gels using 0.15% formic acid/67% acetonitrile. Dried peptides were dissolved in 0.1% formic acid/2% acetonitrile and separated on a fused silica capillary emitter (inner diameter, 75 μm; length, 15 cm; New Objective, Woburn, MA) packed in-house with 5 μm of C18 resin and analyzed on an AB SCIEX TripleTOF 5600 system. The resulting peak lists were used to query the Swiss-Prot database using the Mascot program. Only significant hits as defined by mascot probability analysis were considered.

RESULTS AND DISCUSSION

Analysis of Xylan-degrading Enzymes from *A. niger* BE-2 and *H. orientalis* EU7-22

The xylan-degrading enzymes obtained from *A. niger* BE-2 and *H. orientalis* EU7-22 using corncob and wheat bran as substrates were evaluated. The enzyme activity of xylanase, β-xylosidase, and α-L-arabinofuranosidase from *A. niger* BE-2 and *H. orientalis* EU7-22 after 72 h of cultivation are shown in Table 1.

Table 1. Enzyme Activity of Xylan-degrading Enzymes from *A. niger* BE-2 and *H. orientalis* EU7-22

Enzyme activity (IU/mL)	*A. niger* BE-2	*H. orientalis* EU7-22	Increased or decreased level[@]
Xylanase	91.5±1.38	109.5±2.29	-18%
β-Xylosidase	2.14±0.18	0.32±0.01	148%
α-L-Arabinofuranosidase	0.42±0.01	0.19±0.01	75%
Xylanase/β-xylosidase (EX/BX)	42.8	342.3	156%

All evaluations of samples were carried out in triplicate. The uncertainties represent the standard errors of three experiments. The differences in xylan-degrading enzymes between *A. niger* and *H. orientalis* were analyzed by the percentage of the increased or decreased level at pair: subtraction of two samples divided by means of two values at pair.

H. orientalis EU7-22 expressed higher xylanase activity, but much lower β-xylosidase and α-L-arabinofuranosidase activities, than the *A. niger* BE-2 strain. The xylanases from *H. orientalis* EU7-22 and *A. niger* BE-2 exhibited better thermo-tolerance than the xylanases from *T. reesei* at 50 ^{o}C and 60 ^{o}C (Fig. 1a, c). β-xylosidase plays a key role in the complete hydrolysis of XOS into xylose (Knob *et al* 2009). As shown in Fig. 1b and 1d, the β-xylosidase from *A. niger* BE-2 retained more than 90% enzyme activity after 24 h of incubation at 50 ^{o}C and 60 ^{o}C, which is more thermo-tolerance than β-xylosidase from either *H. orientalis* EU7-22 or *T. reesei*. XOS are stronger inhibitors of cellulase activity (Qing *et al* 2010). These results show that the thermo-tolerant β-

xylosidase from *A. niger* BE-2 has promise for use in lignocellulosic biomass hydrolysis processes.

Enzymatic Hydrolysis of *Miscanthus* Xylan

The time course of enzymatic hydrolysis of *Miscanthus* xylan by different xylan-degrading enzymes at 50 °C was studied. When equal amounts of xylanase activity units were used, the enzymes from *A. niger* BE-2 degraded crude xylan into primarily xylose, arabinose, xylobiose, xylotriose, and xylohexaose, with trace amounts of xylotetraose and glucose, within 0.5 h (Fig. 2a). The enzymes from *H. orientalis* EU7-22 and *T. reesei* produced primarily xylobiose, xylotriose, and xylotetraose, with small amounts of xylohexaose, xylopentaose, xylose, arabinose, and glucose, within 0.5 h (Fig. 2b, 2c).

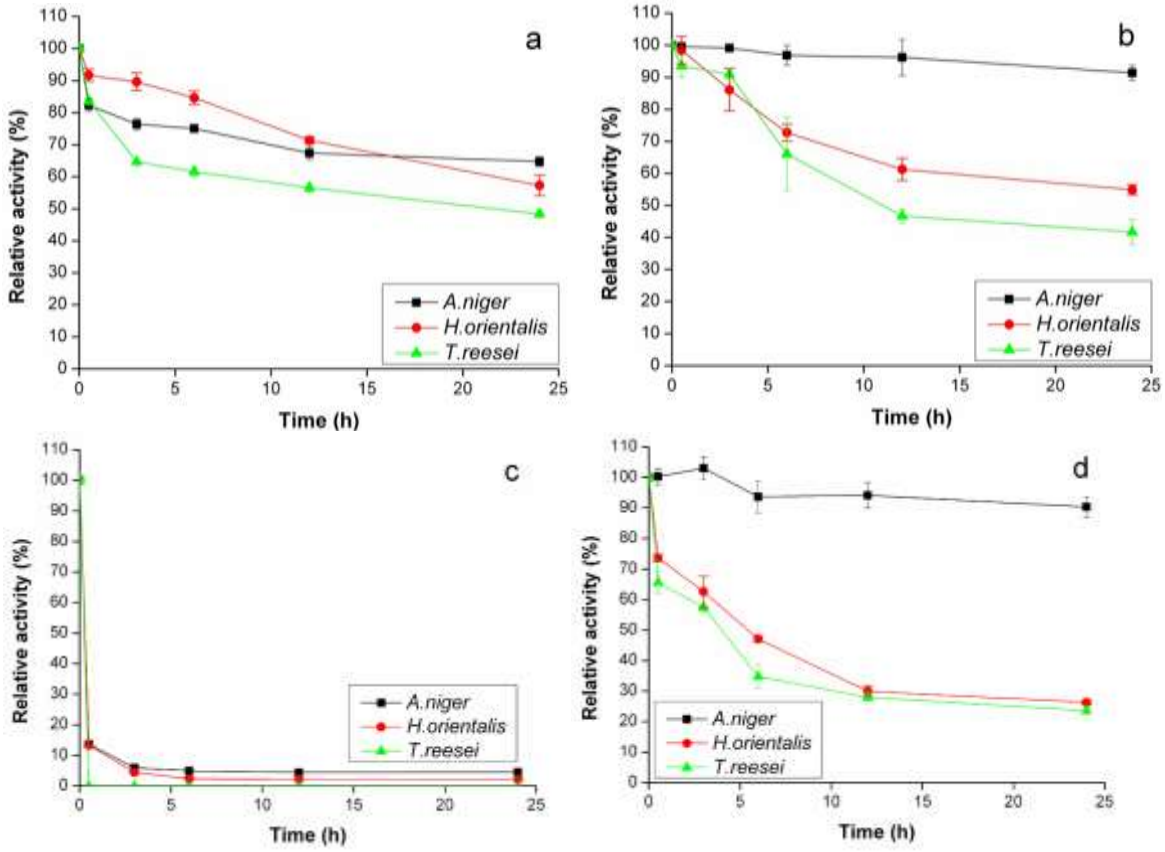

Fig. 1. The thermostability of xylan-degrading enzymes of *H. orientalis* EU7-22 and *A. niger* BE-2 : (a) thermostability of xylanase activity at 50 °C, (b) thermostability of β-xylosidase activity 50 °C, (c) thermostability of xylanase activity at 60 °C, (d) thermostability of β-xylosidase at 60 °C; All tests were done in triplicate. The error bars represent the standard errors of three experiments.

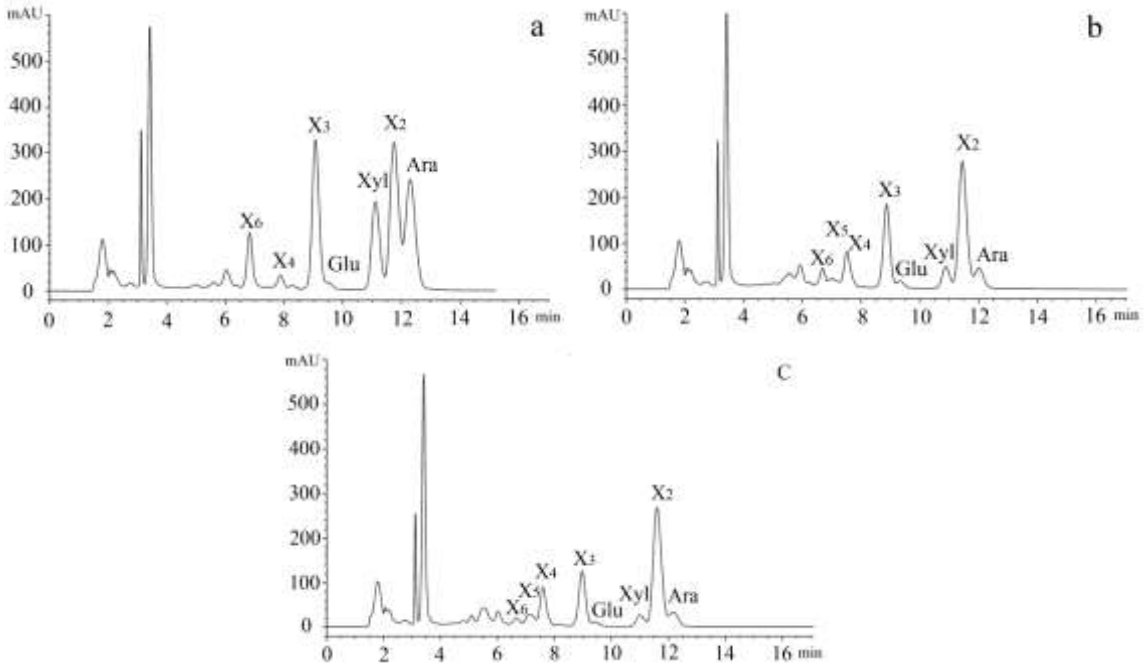

Fig. 2. Analysis of enzymatic hydrolysates of *Miscanthus* xylan by HPLC (100 IU/g, 0.5 h). (a) *A. niger* BE-2, (b) *H. orientalis* EU7-22, (c) *T. reesei.* Xylohexaose (X_6), xylopentaose (X_5), xylotetraose (X_4), xylotriose (X_3), xylobiose (X_2), glucose (Glu), xylose (Xyl), and arabinose (Ara).

The presence of arabinose as a side group on xylan limits the access of xylanase to the main chain of xylan, and the cleavage of arabinose from xylan provides more binding sites for xylanase (Sakamoto *et al.* 2011). Most arabinose from *Miscanthus* xylan was released within 0.5 h by the enzymes from *A. niger* BE-2 (Fig. 3a, b).

However, the yield of arabinose kept increasing in hydrolysates of *H. orientalis* EU7-22 (Fig. 3c, 3d) and *T. reesei* (Fig. 3e, 3f). This shows that the enzymes from *A. niger* BE-2 exhibit high catalytic activity for arabinosyl side-chains of *Miscanthus* xylan. Despite *A. niger* BE-2 having the highest amount of β-xylosidase, the yields of xylose and XOS produced by the enzymes from *A. niger* BE-2 were both higher than the enzymes from *H. orientalis* EU7-22 and *T. reesei* at 0.5 h (Table S1). This indicated that the cleavage of arabinose side chains at the initial hydrolysis phase enhanced the conversion of *Miscanthus* xylan into XOS. When the hydrolysis time was extended, the enzymes from *A. niger* BE-2 with a lower ratio of xylanase to β-xylosidase (EX/BX) efficiently degraded xylan into xylose (Fig. 3a, b). The enzymes from *H. orientalis* EU7-22 and *T. reesei* with higher EX/BX ratios also degraded xylan into XOS (Fig. 3c, d, e, f). The yields of xylose produced by the enzymes (200 IU/g) from *H. orientalis* EU7-22 and *T. reesei* (Fig. 3d, f) were much lower than that produced by the enzymes (100 IU/g) from *A. niger* BE-2 (Fig. 3a). These results revealed that *Miscanthus* xylan degradation was significantly influenced by the activity of β-xylosidase.

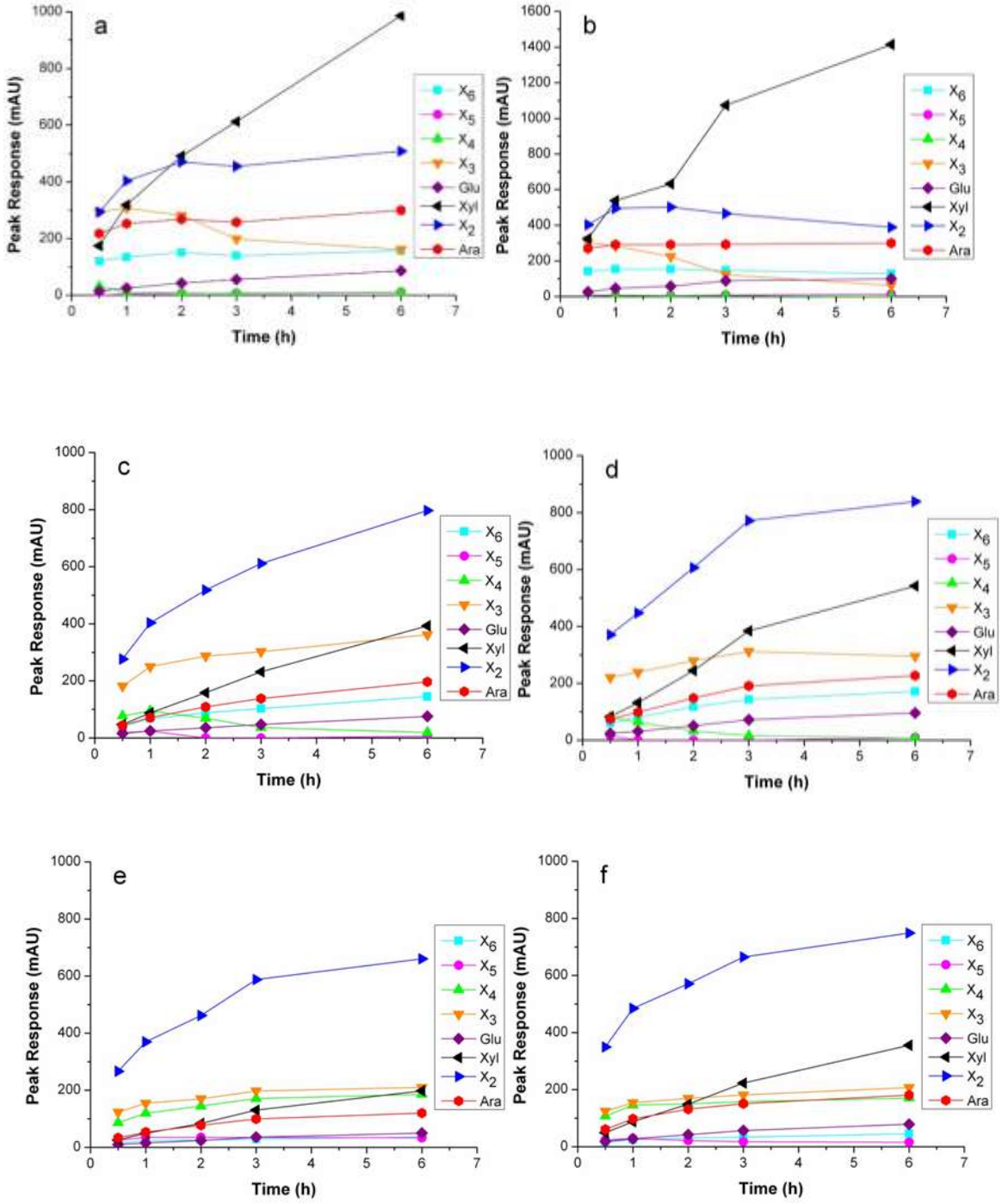

Fig. 3. Time course of enzymatic hydrolysis of xylan by xylan-degrading enzymes from *A. niger* BE-2 (a 100 IU/g and b 200 IU/g), *H. orientalis* EU7-22 (c 100 IU/g and d 200 IU/g), and *T. reesei* (e 100 IU/g and f 200 IU/g) when different amounts of xylanase activity units were added. The saccharide yield was quantitated by the peak response (mAU).

SDS-PAGE and LC-MS/MS Analysis of Xylan-degrading Enzymes

The yield of xylotetraose decreased and the yield of xylohexaose increased when the enzymes from *H. orientalis* EU7-22 were used, while the yield of xylotetraose increased and little xylohexaose was found when the enzymes from *T. reesei* were used (Fig. 3c, d, e, f). In addition, compared to the enzymes from *T. reesei*, more xylobiose and xylotriose were produced by the enzymes from *H. orientalis* EU7-22, even though a smaller dosage of enzymes was used (Fig. 3c, f). The results indicated that the action patterns of xylan-degrading enzymes from *H. orientalis* EU7-22 and *T. reesei* on *Miscanthus* xylan are different. The two major inducible endo-1,4-beta-xylanases expressed by *T. reesei* are xyn1 and xyn2, accounting for more than 90% of the xylan-degrading activity of this fungus (Torronen *et al.* 1992; Torronen *et al*. 1994). *H. orientalis* is in the genus of *Trichoderma*, which shows high homology with *T. reesei*. The deduced amino acid sequences of xyn1 (GenBank accession No. AFD50198.1) and xyn2 (GenBank accession No. AFD50199.1) from *H. orientalis* showed 95% and 96% homology to that of *T. reesei* xyn2 (GenBank accession No. P36217.1) and xyn1 (GenBank accession No. P36218.1), respectively.

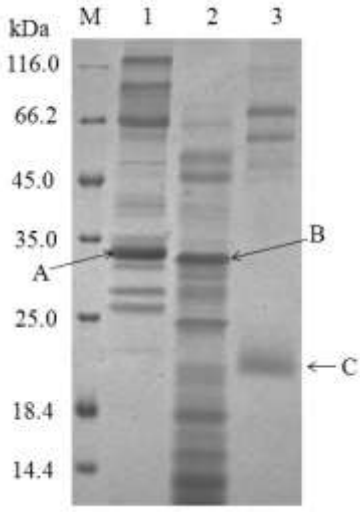

Fig. 4. SDS-PAGE analysis of xylan-degrading enzymes. Lane M, protein molecular weight marker; Lane 1, proteins secreted by *A. niger* BE-2; Lane 2, proteins secreted by *H. orientalis* EU7-22; Lane 3, commercial xylan-degrading enzymes produced by *T. reesei.*

Based on the amino acid sequences in the catalytic domain, most fungal xylanases are clustered into glycoside hydrolase families 10 and 11 (GH10 and GH11). Members of GH10 have a molecular weight greater than 30 kDa and are highly specific for small xylo-oligosaccharides, while members of GH11 show a low molecular weight and high activity on long chains of xylo-oligosaccharides (Biely *et al.* 1997; Pollet *et al.* 2010). Both xyn1 (19 kDa) and xyn2 (21 kDa) from *T. reesei* belong to GH11. Xyn3 (32 kDa) in *T. reesei*, which belongs to GH10, was found expressed in a mutant strain (*T. reesei* PC-3-7), but silenced in the most studied strain of *T .reesei*, QM9414 (Xu *et al*. 2000). SDS-PAGE analysis showed that a major band from 19 to 21 kDa was found in commercial xylanase preparation, but a major band from 32 to 33 kDa was found in *H. orientalis* EU7-22 and *A. niger* BE-2 (Fig. 4). The results revealed that GH11 xylanase may be the dominant protein in the commercial xylan-degrading enzyme preparation; nevertheless, GH10 xylanase may be the major xylanase expressed in *H. orientalis* EU7-22, which resulted in different hydrolyzates from *T. reesei*.

Table 2. Mascot Protein Identification of Major Proteins Produced by *A. niger* BE-2, *H. orientalis* EU7-22, and *T. reesei*

Spot	Protein Name	Match organism	Accession No.	Score	Peptides matches (95%)	Coverage (%)
A	Endo-1,4-beta-xylanase C	*Aspergillus niger*	A2QFV7	5616	138	77.1
B	Glycoside hydrolase family 10	*Trichoderma reesei*	G0RA32	1047	24	28.2
C	Endo-1,4-beta-xylanase	*Trichoderma reesei*	G0RUP7	10211	217	32.7

The most abundant protein bands from *A. niger* BE-2. *H. orientalis* EU7-22, and *T. reesei* were excised from Coomassie-stained gels and analyzed by LC-MS/MS. As shown in Table 2, the mass spectrum identified the protein band A as GH10 endo-1,4-beta-xylanase C from *A. niger*, the protein band B as GH10 endo-1,4-beta-xylanase 3 from *T. reesei*, and the protein band C as GH11 endo-1,4-beta-xylanase from *T. reesei*. These results showed that corn cob and wheat bran are fine substrates for *H. orientalis* EU7-22 and *A. niger* BE-2 to produce GH10 endo-1,4-beta-xylanase. SDS-PAGE and LC-MS/MS analysis confirmed the presumption that a GH10 xylanase is expressed by *H. orientalis* EU7-22. Compared to GH11 xylanases, GH10 xylanases with broader substrate specificities show better synergistic effects to boost the hydrolytic potential of cellulases with pretreated lignocellulosic substrates (Zhang *et al*. 2011; Hu *et al*. 2013).

CONCLUSIONS

1. *Miscanthus* xylan degradation was significantly influenced by the activity of β-xylosidase and α-L-arabinofuranosidase. Xylan-degrading enzymes with a high ratio of β-xylosidase and α-L-arabinofuranosidase were necessary for the efficient conversion of *Miscanthus* xylan into monosaccharides. However, xylan-degrading enzymes with low β-xylosidase activity and high α-L-arabinofuranosidase activity were required for producing XOS from *Miscanthus*.
2. SDS-PAGE coupled with LC-MS/MS is a powerful tool for the identification of the most abundant protein from crude enzymes. The GH10 xylanase was the primary xylanase expressed by *H. orientalis* EU7-22 and *A. niger* BE-2 when corn cob and wheat bran were used as substrates.
3. Compared to commercial xylan-degrading enzymes produced by *T. reesei*, the enzymes from *A. niger* BE-2 showed higher catalytic efficiency for complete hydrolysis of xylan into monosaccharides, while the enzymes from *H. orientalis* EU7-22 exhibited better potential for the production of XOS, especially xylobiose and xylotriose.

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APPENDIX: SUPPLEMENTARY INFORMATION

Table S1. Yield of Xylose and XOS Produced by Enzymatic Hydrolysis of *Miscanthus* xylan (100 IU/g, 0.5 h)

	A. niger BE-2	*H. orientalis* EU7-22	*T. reesei*
X_6（mAU）	121	40	15
X_5（mAU）	0	19	24
X_4（mAU）	29	77	85
X_3（mAU）	325	181	122
X_2（mAU）	320	276	266
XOS（mAU）	795	593	512
Xyl（mAU）	191	47	25

The saccharide yield was quantitated by the peak response (mAU). The yields of XOS were the sum of the peak response of X_2~X_6.