Heterologous Expression of a New Acetyl Xylan Esterase from *Aspergillus niger* BE-2 and its Synergistic Action with Xylan-Degrading Enzymes in the Hydrolysis of Bamboo Biomass

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Efficient utilization of plant biomass by enzymatic hydrolysis is currently studied worldwide but still faces enormous challenges because of the inability to break down lignocellulosic materials with high sugar yields and low enzyme dosage. Therefore, the synergistic action between various enzymes plays an important role in reaching this goal. The synergistic cooperation between a novel acetyl xylan esterase (heterologous expressed at high levels in this study) and four other xylan-degrading enzymes (reported previously) were performed in this study. The acetyl xylan esterase (AnAxe) gene was cloned from *Aspergillus niger* BE-2 and expressed in *Pichia pastoris* GS115. The deduced amino acid (aa) sequence consisted of 304-aa and included a 23-aa signal peptide and 281-aa mature protein. The AnAxe was extracellularly expressed with a molecular weight of *ca*. 31 kDa. The purified AnAxe exhibited maximal specific activity of 480.2 IU/mg at pH 7.0 and 40 °C and was still thermostable below 50 °C. The metal ions used in this study and EDTA showed a slight effect on the AnAxe. A significant synergistic effect was determined between AnAxe and the other four xylan-degrading enzymes, including endo-β-1,4-xylanases, β-xylosidases, α-L-arabinofurano-sidases, and α-glucuronidases, on the degradation of bamboo biomass. The highest degree of synergism was obtained between AnAxe and endo-β-1,4-xylanases/β-xylosidases.

Keywords: Aspergillus niger; Acetyl xylan esterase; Pichia pastoris; Characterization; Synergistic cooperation

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INTRODUCTION

As the world continues to exhaust non-renewable energy sources such as fossil fuels, the need to take advantage of the clean renewable energy resources becomes increasingly urgent. Plant biomass, as a new type of renewable energy, has been proposed as one of the most promising alternatives because of its inexhaustible, ubiquitous, and green environmental protection advantages. There are many ways to utilize plant biomass, such as the production of xylitol (Horitsu *et al.* 1992; Inoue *et al.* 2016), ethanol (Taherzadeh and Karimi 2007), organic acids (Ohara *et al.* 2007), and other chemicals, but the bioconversion of plant biomass to sugars is the first key step. However, the ability to break down lignocellulosic materials into sugars with high yields and low enzyme dosage continues to be a challenge.

The main constituents in biomass include cellulose, hemicellulose, and lignin. Hemicellulose accounts for a great proportion in plant biomass and is the least utilized because of the complexity of its composition and structure (Beukes and Pletschke 2010). The major obstacle to efficient and economically feasible bioconversion of hemicellulose is its recalcitrance to enzymes.

Xylan, a major component of hemicellulose, consists of a β-1,4-linked D-xylose backbone with heterogeneous substituents such as L-arabinose, O-acetyl, D-galactose, feruloyl, and glucuronic acid residues (Coughlan and Hazlewood 1993; Bocchini *et al.* 2002). Thus, the complete degradation of hemicellulose requires the cooperative action of backbone-degrading enzymes, including endoxylanases and β-xylosidases, in addition to accessory enzymes such as acetyl xylan esterases, α-L-arabinofuranosidases, feruloyl esterases, and α-glucuronidases.

Acetylxylan, the most abundant hemicellulose in nature, appears to be almost all polysaccharide cell wall (Pawar *et al.* 2013). Acetyl xylan esterases (EC 3.1.1.6, AXEs) play a critical role in hemicellulose hydrolysis by removing acetyl ester groups from position 2 or 3 of D-xylopyranosyl residues in xylan chains (Poutanen *et al.* 1990; Christov and Prior 1993).

As early as 1986, studies (Biely *et al.* 1986) showed that the presence of AXEs could significantly accelerate the hydrolysis efficiency of hemicellulose. From then on, more and more reports (Puls *et al.* 1991; Raweesri *et al.* 2008) have suggested that deacetylation of xylan residues by AXEs not only can enhance the efficiency of lignocellulose bioconversion, but also expose their glycosidic linkages to enzymatic hydrolysis. Although the synergistic effect between AXEs and xylanases has been widely reported, few reports have focused on the synergistic cooperation of AXEs with other side-chain cleavage enzymes.

To date, multiple efforts have been devoted to the engineering of microorganisms. Various AXEs have been isolated and characterized from fungi and bacteria, but few have been applied on an industrial scale because of their low level of expression and weak catalytic activities. Heterologous expression of novel hemicellulases still remains a considerable concern. The *Pichia pastoris* expression system fits for heterologous expression because of its many advantages, such as effective protein folding, eukaryotic protein processing, accurate posttranslational modification, and high level of heterologous protein expression (Buckholz and Gleeson 1991; Zhang *et al.* 2000). Therefore, heterologous expression of the acetyl xylan esterase gene is of interest because it may provide one way to obtain a greater quantity of enzymes.

To further explore the hydrolytic ability of a complex substrate to obtain reducing sugars efficiently, bamboo was used as a substrate because it is ubiquitous and abundant in East Asia and South East Asia and has shown great potential as a bioenergy feedstock because of its fast growth rate and sustainable availability (Scurlock *et al.* 2000).

In this study, the gene encoding acetyl xylan esterase from *Aspergillus niger* (*A. niger*) BE-2 was cloned and expressed in *P. pastoris* GS115. The synergistic action of AnAxe with endo-β-1,4-xylanases was first studied, and on that basis, interesting enzyme cocktails of AnAxe in combination with four other xylan-degrading enzymes were prepared.

EXPERIMENTAL

Strains and Culture Media

A. niger BE-2 (GenBank accession No. JQ867187) was used as the source of the acetyl xylan esterase gene. *Pichia pastoris* (*P. pastoris*) GS115 (Invitrogen, Carlsbad, CA, USA) was preserved in YPD medium (1% yeast extract, 2% peptone, and 20% glucose), which was used for the heterologous expression of *Anaxe* cDNA. Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5α (Invitrogen, USA). Recombinant enzymes including AnXyn10C (glycosyl hydrolases family 10 endo-β-1,4-xylanase from *A. niger*), AnXln3D (glycosyl hydrolases family 3 β-xylosidase from *A. niger*), AnAxh62A (glycosyl hydrolases family 62 α-L-arabinofuranosidases from *A. niger*; Li *et al.* 2015), and AnGus67 (glycosyl hydrolases family 67 α-glucuronidase from *A. niger*; unpublished) were expressed in *P. pastoris* and preserved in the laboratory.

Cloning of *AnAxe* Gene

Based on the gene encoding acetyl xylan esterase in *A. niger* CBS513.88 from the Genbank database, we designed specific primers for amplifying *Anaxe* encoding the mature protein. The DNA fragment encoding the mature AnAxe protein was amplified with a pair of primers (forward primer, 5'-GAATCCTAGGCATGTCGCCAAGCGCAGT-3'; reverse primer, 5'-ATAAGAATGCGGCCGCTCAAGCAAACCCAAACCACTC-3'). The restriction endonuclease sites *Bln* I (*Avr* II) and *Not* I were underlined in primer sequences that were used for linking the target gene and plasmid. The gene *Anaxe* was inserted into the expression vector pPIC9K (Invitrogen) to generate the pPIC9K-*Anaxe* plasmid. Three positive clones were sequenced, and the correct genes were uploaded to the National Center for Biotechnology Information (NCBI) by sequence alignment. After sequencing, the results were identified using the Blast Server at the NCBI website (http://www.ncbi.nlm.nih.gov/). The phylogenetic tree was constructed using Mega 5.0 software (Molecular Evolutionary Genetics Analysis, Version 5.0).

Expression of *AnAxe* in *P. pastoris*

The plasmid pPIC9K-*Anaxe* was linearized by *Sal* I and transformed into *P. pastoris* GS115 by electroporation. Transformants were selected on minimal dextrose plates (0.004% biotin, 1.5% agar, 2% dextrose, and 1.34% YNB). The phenotypes of transformants were identified by colony polymerase chain reaction (PCR) using 5'AOX1 (5'-GACTGGTTCCAATTGACAAGC-3') and 3'AOX1 (5'-GCAAATGGCATTCT-GACATCC-3') primers. The selected transformants were cultured on buffered minimal glycerol medium (BMGY) (225 rpm, 28 °C) until the culture reached an optical density at 600 nm (OD_{600}) of 2.0 to 6.0. The cells were resuspended and cultured in 100 mL of buffered minimal methanol medium (1% yeast extract, 2% peptone, 100 mmol/L potassium phosphate, pH 6.0, 1.34% YNB, 0.004% biotin, and 1% methanol) to induce the expression of protein. The fermentation broth was centrifuged (1500 × *g*, 10 min), and the supernatant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and enzymatic activity analysis.

Enzyme Purification

All purification steps were performed at 4 °C. The culture supernatant was concentrated (4000 × *g*) using a Millipore (Billerica, MA, USA) Amicon Ultra centrifugation tubes with a molecular weight cut-off of 10 kDa. After that, the culture supernatant was loaded on a gel-filtration column (d × h = 1.5 cm × 60 cm) of Sephadex G-50 (Pharmacia, Uppsala, Sweden). The bound proteins were eluted with phosphate buffer (50 mmol/L, pH 6.8) at a flow rate of 0.6 mL/min. The purified enzyme was evaluated by SDS-PAGE.

Enzymatic Assay

The enzymatic activity was measured using *p*-nitrophenyl acetate (Sigma, USA) as the substrate according to the modified protocol of Biely (1985). The substrate was dissolved in dimethyl sulphoxide (DMSO) with a final concentration of 50 mmol/L, and then the reaction was carried out in potassium phosphate buffer (100 mM, 50 °C, pH 6.5). The release of *p*-nitrophenol was determined through the absorption at 405 nm. One unit of enzymatic activity was defined as the amount of enzyme release, 1 μmol of *p*-nitrophenol per minute. The measurements were performed in triplicate.

To study the thermal stability, the samples were pre-incubated in a water bath at 45, 50, 55, 60, and 65 °C. The residual activity was determined every 10 min. The optimum temperature and pH were measured between the ranges of 10 and 80 °C and 4.0 and 9.0, respectively. The effects of metal ions and chemicals were assessed by pre-incubating the enzyme with the test substrates (25 °C, 30 min) and then assaying the residual enzymatic activity under standard conditions. Nine different metal ions (Fe^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, K^{+}, Mg^{2+}, Al^{3+}, Cu^{2+}, and Ba^{2+}) and ethylenediaminetetraacetic acid (EDTA) were added to the reaction system with a final concentration of 5 mmol/L to determine their effects on enzymatic activity.

The Michaelis–Menten kinetic parameters K_m and V_{max} of AnAxe were determined from Lineweaver–Burk plots with different substrate (*p*-nitrophenyl acetate) concentrations ranging from 10 mmol/L to 200 mmol/L.

Enzyme Synergistic Hydrolysis

The bamboo used in this study was obtained locally from Xiamen (Fujian, China). The composition of the ball mill-treated bamboo powder (BBP) was cellulose 41.24%, hemicellulose 23.48%, lignin 27.85%, and ash 2.68%.

The reaction mixtures contained 20 mg of BBP in 2 mL of phosphate buffer (50 mM, pH 5.0) mixed well with AnXyn10C and/or AnAxe and incubated at 50 °C for 24 h on a thermo mixer incubator at 300 rpm. The reaction was terminated by boiling for 10 min to terminate the reaction, and the mixture was then centrifuged at 12,000 × *g* for 10 min to obtain the supernatant for analysis. Instead of enzymes, the buffer was used as the control. The same reaction system was conducted with AnAxe (20 IU/g substrate), AnXln3D (20 IU/g substrate), AnAxh62A (20 IU/g substrate), AnGus67 (20 IU/g substrate), and AnXyn10C (300 IU/g substrate) acting respectively or simultaneously. The complete enzyme list used in the hydrolysis experiments is given in Table. 1. To ensure that there was sufficient volume of side-chain cleavage enzymes, we added excessive doses of enzymes. Reducing sugars in the supernatant were measured as xylose equivalents referring to the modified protocol of Miller (1959). The degree of synergism

(DS) was calculated as reducing sugars released from enzyme combinations to the sum of single enzymes acting alone. All evaluations of samples were performed in duplicate.

Table 1. Recombinant Enzymes from *A. niger* Expressed in *P. pastoris* and Used in this Study

Enzyme	Family	Abbreviation	Specific Activity
acetyl xylan esterase	CE1	AnAxe	480.2 IU/mg
endo-β-1,4-xylanase	GH10	AnXyn10C	785 IU/mg
β-xylosidase	GH3	AnXln3D	58.7 IU/mg
α-L-arabinofuranosidase	GH62	AnAxh62A	177 IU/mg
α-glucuronidase	GH67	AnGus67	n.d.

n.d. not determined

RESULTS AND DISCUSSION

Cloning and Sequencing of *AnAxe*

The cDNA (912 bp) and genomic DNA (1063 bp) fragments (Fig. 1) encoding acetyl xylan esterase from *A. niger* were obtained using the above-mentioned primers. The *Anaxe* gene consisted of three exons and two introns with lengths of 71 bp and 80 bp, respectively. The genomic DNA sequence has been submitted to the GenBank database with an accession number of KT945039. Based on the topology of the phylogenetic tree, AnAxe shows a close relationship with *Aspergillus ficuum* ATCC 16882 (AF331757.1), which is attributed to the CE1 family (Fig. 2).

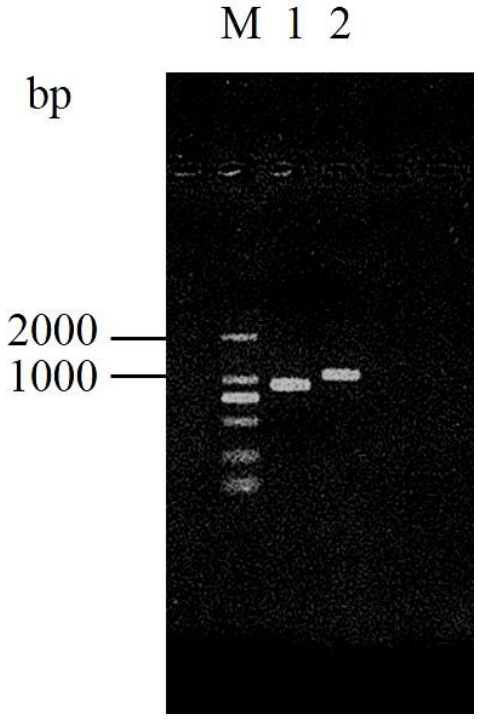

Fig. 1. Cloning of the full-length gene encoding the AnAxe from *A. niger* BE2. M: marker; 1: cDNA (912 bp); 2: genomic DNA (1063 bp)

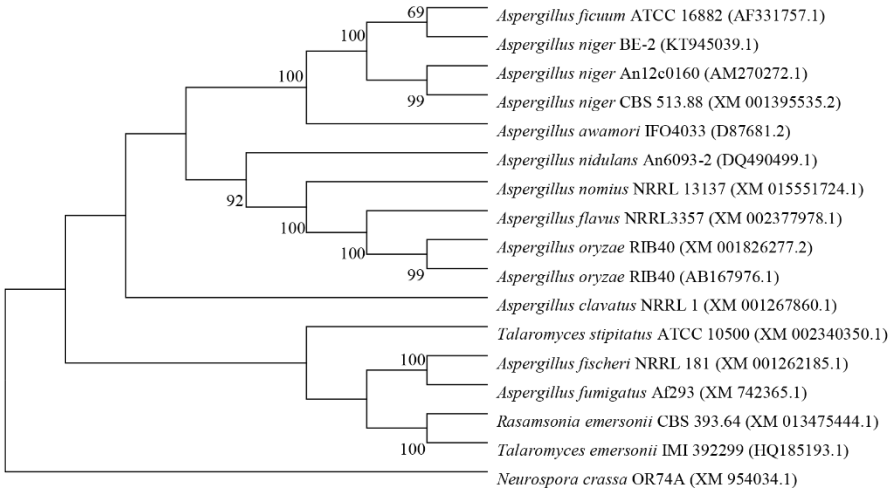

Fig. 2. Phylogenetic tree showing the evolutionary relativity and the homological degrees among the gene of *Anaxe*. The estimated genetic distance between sequences is proportional to the lengths of the horizontal lines connecting one sequence to another. Genbank accession numbers are shown in brackets.

Expression of the Transformants in *P. pastoris*

The plasmid pPIC9K-*Anaxe* was transformed into *P. pastoris* by electro-transformation. The selected transformants were cultured in BMMY medium inducted by 10 mL/L methanol. After 144 h of culture, the supernatant was harvested and preserved in a frozen state at -80 °C for activity and SDS-PAGE analyses. The AnAxe showed one single protein band as analyzed by SDS-PAGE with an apparent molecular weight of approximately 31 kDa both before and after purification (Fig. 3).

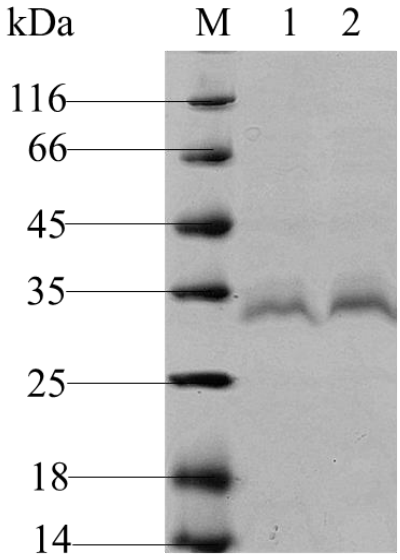

Fig. 3. SDS-PAGE of the AnAxe expressed in *P. pastoris*. M: protein marker; 1: unpurified AnAxe; 2: purified AnAxe

The specific activity of AnAxe was 480.2 IU/mg, which was a higher activity and demonstrated higher expression in *P. pastoris* as compared with previous reports (Koseki *et al.* 2006). The K_m and V_{max} values of AnAxe at standard assay conditions were graphically determined to be 7.24 mmol/L and 102 μmol/min, respectively. This is a lower K_m value than that (11 mM) from *Talaromyces emersonii* (Waters *et al.* 2012) which indicates that AnFaeA has a stronger affinity to the substrates. However, compared to other work (Shao and Wiegel 1995) which used 4-methylumbelliferyl acetate as substrate, the K_m (0.45 mM and 0.52 mM) of two acetyl xylan esterases from *Thermoanaerobacterium* sp. Strain JW/SL-YS485 were much lower than that from the enzyme in this study.

Effects of Temperature and pH on the Stability and Activity of AnAxe

The effect of temperature on AnAxe was investigated. AnAxe exhibited maximum activity at 40 °C and retained only 50% maximum activity at 60 °C (Fig. 4a). The optimum pH of AnAxe hydrolytic activity was 7.0 (Fig. 4b), similar to the recombinant enzyme from *Chrysosporium lucknowense* AXE2 (Pouvreau *et al.* 2011). AnAxe was thermostable below 50 °C but decreased dramatically after 60 min of incubation at 60 °C (Fig. 4c). The stability of developed enzyme in this research is similar to a report (dos Santos *et al*. 2013) in which the enzymes were produced by *A. niger*.

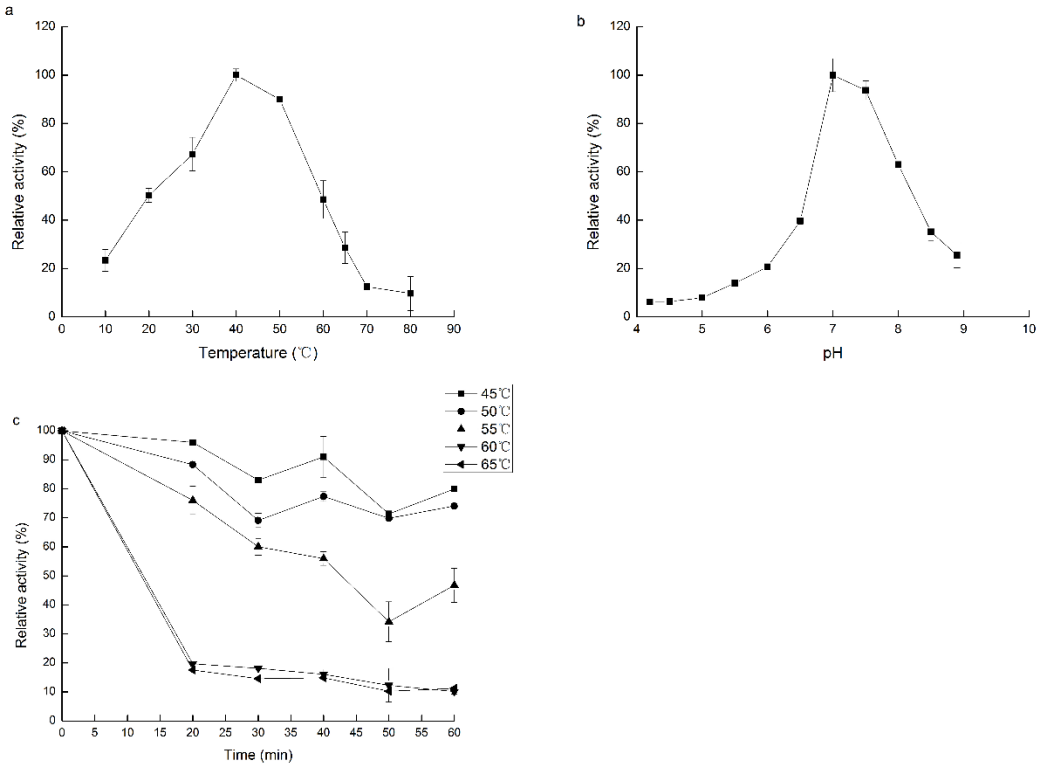

Fig. 4. Effect of temperature and pH on activity of the enzyme: (a) effect of temperature on AnAxe activity; (b) effect of pH on AnAxe activity; (c) thermo-stability of AnAxe

Effects of Various Metal Ions and EDTA on the Activity of AnAxe

The residual activities were all above 70%, which suggests that metal ions used in this study and EDTA had a slight effect on the stability (Table 2). As for previous reports,

the enzyme activity of acetyl xylan esterases from *Caldanaerobacter subterraneus* (Moriyoshi *et al.* 2013) is not influenced by metal ions, while that from *Streptomyces lividans* is completely inhibited (Taylor *et al.* 2006). Therefore, there are corresponding effects of metal ions on various sources of acetyl xylan esterases.

Table 2. Effect of EDTA and Metal Ions (all at 5 mM concentration) on the Activity of AnAxe

Additives (5 mM)	Relative activity
Control	100.00 ± 0.00
EDTA	81.02 ± 0.32
Fe^{2+}	81.35 ± 0.41
Co^{2+}	80.10 ± 0.50
Mn^{2+}	81.55 ± 0.27
Ca^{2+}	76.36 ± 0.15
Zn^{2+}	79.10 ± 0.83
K^{+}	83.77 ± 0.70
Mg^{2+}	69.62 ± 0.23
Al^{3+}	79.77 ± 0.10
Cu^{2+}	80.98 ± 0.22
Ba^{2+}	82.09 ± 0.01

Synergistic Action between AnAxe and Other Xylan-Degrading Enzymes

The application of AnAxe in the hydrolysis of BBP in combination with xylanase (AnXyn10C) was investigated (Fig. 5). The amount of reducing sugars from BBP hydrolyzed by AnXyn10C alone with activity ranging from 0 to 1000 IU/g substrate is shown in Fig. 5a. Up to 0.72 mg/mL were released at 300 IU/g substrate, after which no obvious increase was detected. It has been reported that there is a synergistic effect between xylanases and acetyl xylan esterases in the hydrolysis of acetylation xylan (Puls *et al.* 1991; Selig *et al.* 2008; Zhang *et al.* 2011). Biely's work concluded that acetylation hindered the action of xylanase (Biely 1985). Therefore, in this work, AnAxe was added with activity ranging from 0 to 5 IU/g substrate on the basis of 300 IU/g substrate AnXyn10C in 2 mL of BBP suspension (10 mg/mL). The amount of reducing sugars was increased from 11.2% to 19.6% when the addition of AnAxe was up to 5 IU/g substrate, but hardly increased with increasing amounts of AnAxe (Fig. 5b). Similar results were presented in a study (Huy *et al.* 2013) that reported 19.4%, 11.2%, and 6.3% increases in reducing sugars by the cooperation of xylanase and acetyl xylan esterase in hydrolyzing birch wood xylan, beech wood xylan, and wheat arabinoxylan, respectively. In a previous study (Christov and Prior 1993), xylanases could hydrolyze xylan to substituted xylooligomers which thus made it possible for acetyl xylan esterase to attack and cleave *O*-acetyl groups, which will facilitate the release of xylooligomers.

Although the synergistic effect of hemicellulases has been widely investigated, only a few reports have studied the cooperation between acetyl xylan esterase and other side chain cleavage enzymes. Therefore, we report for the first time that AnAxe cooperated with three side-chain xylan-degrading enzymes (AnXln3D, AnAxh62A, and AnGus67) on the hydrolysis of BBP (Fig. 6). There was a certain amount of reducing sugar released from BBP by the cooperation of AnXln3D/AnXyn10C, AnAxh62A/AnXyn10C, and AnGus67/AnXyn10C. When AnAxe was added, there was a prominent enhancement of reducing sugar generated from BBP.

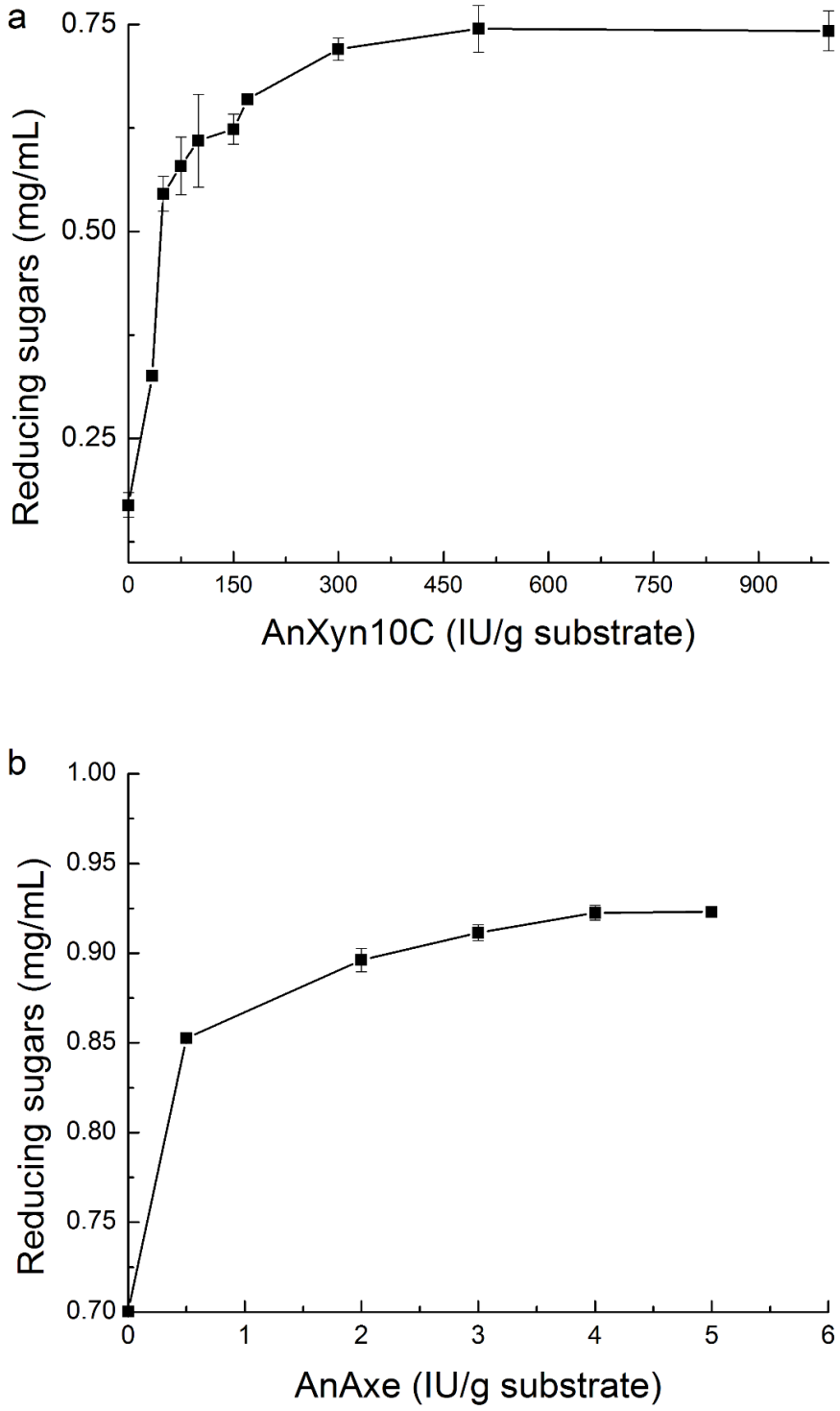

Fig. 5. Reducing sugar yields in enzyme hydrolysis of BBP: (a) the amount of reducing sugars released from BBP by AnXyn10C alone with activity ranging from 0 to 1000 IU/g substrate at 50 °C for 24 h; (b) the amount of reducing sugars released from BBP by AnXyn10C (300 IU/g substrate) and AnAxe with activity ranging from 0 to 5 IU/g substrate at 50 °C for 24 h

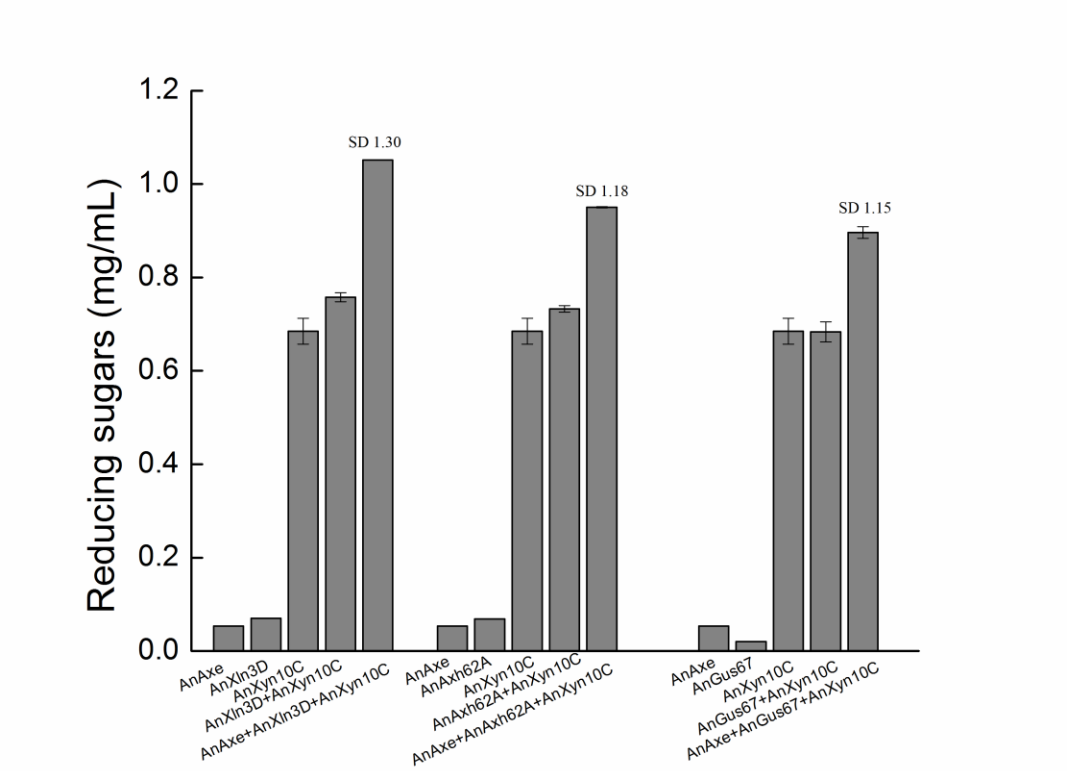

Fig. 6. Synergistic hydrolysis of BBP with recombinant hemicellulases. The BBP was incubated with AnXyn10C (300 IU/g substrate), AnAxe (20 IU/g substrate), AnXln3D (20 IU/g substrate), AnGus67 (20 IU/g substrate), and AnAxh62A (20 IU/g substrate) in sodium citrate buffer (50 mM, pH 5.0) containing 0.02% azide at 50 °C for 24 h individually or simultaneously. DS: degree of synergism, as (ab)/(a)+(b).

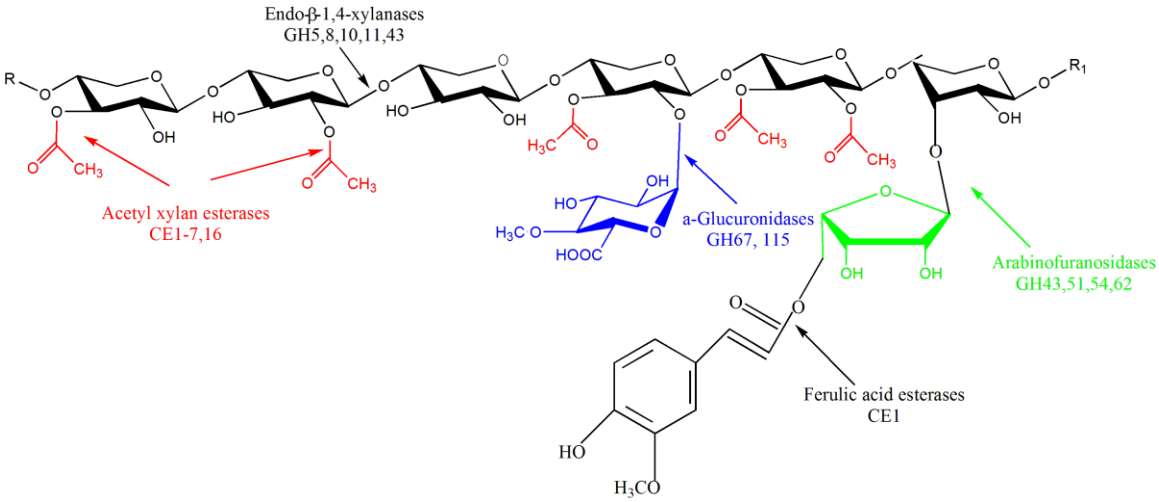

Fig. 7. Various xylan-degrading enzyme active sites in hemicellulose

The degree of synergism between AnAxe and other xylan-degrading enzymes (AnXln3D/AnXyn10C, AnAxh62A/AnXyn10C, and AnGus67/AnXyn10C) were 1.30, 1.18, and 1.15, respectively. Several impressive reports have shown that more cleavage sites can be exposed with a branch side chain being cleaved off (Liab *et al.* 2000; Li *et al.* 2016). The cleavage of the side groups may contribute to the relaxation of the cell wall structures and reduce the steric hindrance to provide more binding sites for

hemicellulases. As noted previously, in a process of enzyme hydrolysis of agricultural residues, the maximal reducing sugars was attained as all three side chain enzymes mixed with xylanases (Raweesri *et al.* 2008).

Figure 7 shows the various enzyme active sites, giving a visual guide to the potential synergistic effect between hemicellulases. The synergistic cooperation among various xylan-degrading enzymes rendered useful information to facilitate the development of highly efficient enzyme cocktails on hydrolysis of lignocellulosic biomass. Furthermore, the present work proved that saccharification of bamboo biomass without any chemical pretreatment can be performed.

CONCLUSIONS

1. A novel acetyl xylan esterase from *A. niger* BE-2 was cloned and expressed in *P. pastoris* at high levels. The expressed AnAxe displayed high specific activity, thermostability, and strong resistance to metal ions and EDTA, which confirmed that the enzyme could be used under relatively broad conditions and applied in various industries.
2. Bamboo was used as the substrate in this study and was pretreated with mechanical force, demonstrating a potential environmentally friendly method for pretreatment of raw materials.
3. The prominent synergistic effect between AnAxe and four other xylan-degrading enzymes may provide one method to obtain high sugar yields with low enzyme dosage, which can be acknowledged as a potential building block to unlock a key market of renewable resources.

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