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Novel Xylanase from a Holstein Cattle Rumen Metagenomic Library and Its Application in Xylooligosaccharide and Ferulic Acid Production from Wheat Straw

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Supporting Information

ABSTRACT: A novel gene fragment containing a xylanase was identified from a Holstein cattle rumen metagenomic library. The novel xylanase (Xyln-SH1) belonged to the glycoside hydrolase family 10 (GH10) and exhibited a maximum of 44% identity to the glycoside hydrolase from *Clostridium thermocellum* ATCC 27405. Xyln-SH1 was heterologously expressed, purified, and characterized. A high level of activity was obtained under the optimum conditions of pH 6.5 and 40 °C. A substrate utilization study indicated that Xyln-SH1 was cellulase-free and strictly specific to xylan from softwood. The synergistic effects of Xyln-SH1 and feruloyl esterase (FAE-SH1) were observed for the release of xylooligosaccharides (XOS) and ferulic acid (FA) from wheat straw. In addition, a high dose of Xyln-SH1 alone was observed to improve the release of FA from wheat straw. These features suggest that this enzyme has substantial potential to improve biomass degradation and industrial applications.

KEYWORDS: *xylanase, metagenomic library, xylooligosaccharides, ferulic acid, wheat straw*

INTRODUCTION

Agricultural wastes are the most abundant and cheapest raw materials available for lignocellulose biotransformation. The utilization of these renewable biomasses as fuel, pulp, and feed has been performed for centuries but are not economical, efficient, or essential.[1] Ferulic acid (FA) and oligosaccharides (OSs) derived from lignocelluloses are high-value products. FA is the most abundant, ubiquitous hydroxycinnamic acid and is distributed widely throughout the plant kingdom. Moreover, this compound is also a known antioxidant with potential food and medical applications.[2,3] OSs, such as xylooligosaccharides (XOS), are valuable as prebiotics and natural food preservatives.[4] Various agricultural residues and byproducts, such as stalks, straw, husks, and shells, are the ideal sources of XOS and FA. Despite their universal presence in plant cell wall materials, the complex structure of the plant cell wall renders the recovery of FA, XOS, and their derivatives from biomass a challenging process.[5] In general, cellulose, hemicellulose, and lignin, which vary in their compositions and cross-linking, constitute a rigid structure.[6] Under natural conditions, the degradation of any of these biomass constituents requires the synergistic action of several types of enzymes,[7] and two families of enzymes have garnered increasing attention. Xylanases constitute a class of enzymes that are crucial to the degradation of xylan, the major source of XOS. The second key class of enzymes is the feruloyl esterases (EC 3.1.1.73), which hydrolyze the ester linkages of ferulic and diferulic acids esterified to the polysaccharose in plant cell walls. These properties make these two enzymes key for the degradation of biomass and represent promising industrial applications, particularly because of their well-known synergism. Tapin et al. verified the synergistic enzymatic application of feruloyl esterase and xylanase for papermaking, which could improve its cost-effectiveness.[8]

The enzyme-catalyzed depolymerization of polysaccharides is an essential step for the conversion of lignocellulosic biomass.[7] However, a limited source for these enzymes hinders the development of a viable lignocellulosic industry. The identification and utilization of novel xylanases and/or feruloyl esterases are important topics for research.[9] The herbivore rumen, with abundant enzyme activities, functions as a natural and efficient organ for the degradation of biomass. However, traditional microbiological methods of cultivation recover <1% of the total bacterial species, which limits the genetic information available regarding rumen enzymes.[10] Metagenomics, a culture-independent technology, has exhibited tremendous potential for the identification of novel enzymes and metabolic pathways for industrial applications.[11]

In the present study, a novel xylanase (Xyln-SH1) was identified from a Holstein cattle rumen metagenomic library. The gene was cloned, heterologously expressed, and characterized. Moreover, the production of XOS and FA from wheat straw by Xyln-SH1 with the coapplication of the feruloyl esterase FAE-SH1 was also evaluated.

MATERIALS AND METHODS

Genomic Library Screening and Positive Transformant Sequencing. The fosmid metagenomic library of Holstein cattle rumen from China was constructed in the previous study.[12] The

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transformants were inoculated onto Luria−Bertani (LB) agar plates that contained 1% (w/v) birch wood xylan (Sigma, St. Louis, MO, USA). The formation of a zone of clearance around the inoculation point indicated xylanase production. One positive clone with the highest activity was chosen for further study. The insert DNA was shotgun sequenced by the Beijing Genomic Institute.

Gene Annotation and Analysis of Sequence Features. The open reading frame (ORF) was analyzed using the online software ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf). Sequence analysis was performed with the Basic Local Alignment Search Tool (BLAST) and several other programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MEGA 4.0 software (http://www.megasoftware.net/) was employed to generate the phylogenetic tree, and the most similar protein sequences to the characterized xylanase were retrieved from the CAZY database (http://www.cazy.org/). Multiple alignments of the xylanase catalytic domains were performed using the ClustalX program. Estimations of the molecular weight (MW), p*I*, and protein sequence translation were performed using the ExPASy proteomics server (http://expasy.org/tools/). The signal peptide was analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). The predicted proteins were classified according to the Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The tertiary structure was predicted using the online I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/), which was built using multiple-threading alignments by LOMETS and iterative TASSER assembly simulations.[13]

Recombinant Expression and Protein Purification. The full-length ORF of the putative xylanase gene was PCR amplified with the following primers: MXylF 5′-ATGAACAGCGCACTGCAATCCCC-3′ and MXylR 5′-GGATCGGGCGGCTTCCAGGA-3′. The PCR products were electrophoresed in a 1.0% agarose gel. The appropriate bands were purified and cloned into the pEASY-E2 expression vector (Transgene, Beijing, China) to fuse a hexahistidine to the C-terminus of the recombinant protein. The ligation was completed with DNA topoisomerase. The presence of an insert in the plasmid was confirmed with white-blue selection, colony PCR, and sequencing. The *Escherichia coli* BL21 (DE3) strain was used as the host for protein expression.

The recombined *E. coli* was inoculated on 100 mL of LB liquid medium in a 1 L triangular flask and cultured at 37 °C in a 200 rpm shaker. At the exponential growth stage, the expression of the enzyme was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma) for 4 h at 28 °C. The crude enzyme was harvested as previously described.[14] The recombinant enzyme was then purified with the Ni Sepharose 6 Fast Flow kit (GE Healthcare, USA) according to the manufacturer's instructions. The enzyme was eluted with a linear imidazole gradient of 30−100 mM. The protein solutions were dialyzed overnight in phosphate buffer solution (PBS, 0.01 M, pH 7.0). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the purity and the molecular weight of the enzyme samples.

Enzyme Activity and Protein Concentration Assay. The xylanase activity was determined by measuring the release of sugar from birch wood xylan using the 3,5-dinitrosalicylic acid (DNS) method with D-xylose to generate the standard curve. One unit (U) of xylanase activity was defined as the amount of enzyme that released 1 μmol of xylosyl reducing sugar units per minute.[15] FAE-SH1 was isolated from the same fosmid library and heterologously expressed as previously described.[12] The feruloyl esterase activity was determined as previously described.[5] The protein concentration was determined using the Bradford method with albumin from bovine serum (BSA) as the standard. All of the reactions were performed in triplicate.

Biochemical Characterization of the Purified Xyln-SH1. The optimum pH for Xyln-SH1 activity was determined at 37 °C in different buffers with pH values ranging from 3.0 to 9.0, and the optimum temperature was determined between 15 and 85 °C at the optimum pH. The thermostability of the purified Xyln-SH1 was assayed under the optimum conditions after the enzyme was incubated, without substrate, in a range of temperatures from 30 to 60 °C. To determine the pH stability, the purified enzyme was incubated at 4 °C in different buffers over a pH range of 3.0−9.0 for 1 h without substrate, and the residual enzyme activities were measured under the optimum conditions. The buffers used in the study were 0.1 M citric acid−Na_2HPO_4 (pH 3.0−7.5) and 0.1 M Tris-HCl (pH 7.0−9.0).

Various metal salts and several chemical reagents at a concentration of 20 mM were added to the assay system to study the effect of metal ions and chemical reagents on the activity of the purified enzyme.

Substrate Specificity and Hydrolysis Product Analysis. Birch wood xylan, oat xylan, beech wood xylan, locust bean gum, sodium carboxymethylcellulose, microcrystalline cellulose (Sigma), and methyl ferulate were utilized to determine the substrate specificity of the purified Xyln-SH1. Each substrate was added to the hydrolysis buffer to a final concentration of 1.0% (w/v). The activities were determined as described above.

Thin layer chromatography (TLC) was employed for the qualitative determination of the enzyme hydrolysis products. The hydrolysis reaction mixture consisted of birch wood xylan in 10 mM citric acid−Na_2HPO_4 buffer (pH 6.5) mixed with 100 U of enzyme and was incubated at 40 °C. Aliquots (100 μL) were withdrawn at each 10 min interval during hydrolysis. A total of 2 μL of the hydrolysis reaction mixture was spotted on an aluminum sheet coated with Silicagel 60 (Merck, Darmstadt, Germany) in a covered chamber. The solvent system used for the resolution was 2:1:1 butanol/ acetic acid/double-distilled water.[16] The results were visualized by heating at 110 °C for 2 min after a methanol/sulfuric acid mixture (90:10, v/v) treatment.

Degradation of Wheat Straw by the Combination of Xyln-SH1 and FAE-SH1. Wheat straw was sampled immediately after the wheat harvest in Beijing (longitude, 116.2° E; latitude, 40.22° N). Any decayed and malnourished wheat straws were discarded; the ears and roots of the straw were chopped. After being dried overnight at 50 °C, the straw was ground in analytical mill (IKA laboratory technology, Germany) to fine flour by passage through a 250 μm sieve. The total XOS content of 5 g wheat straw was obtained after hydrolysis with 0.2 M H_2SO_4 in a 100 °C water bath for 1 h[17] and neutralization to pH 7.0 with NaOH. The XOS content was determined using an HPLC equipped with a refractive index detector and an Aminex HPX-87H column.[18] The total FA content was prepared using the alkaline lysis method[19] and determined using HPLC.

Enzymatic hydrolysis of the wheat straw was performed in a 100 mL Erlenmeyer flask. Xyln-SH1 and FAE-SH1 were added to 5 g of the sample in 50 mL of citric acid−Na_2HPO_4 buffer (pH 6.5) and stirred in a 40 °C water bath for 1 h. The release of the XOS and FA was determined as described above after centrifugation (8600*g*, 15 min).

N-Terminal Sequence Analysis and the Nucleotide Sequence Accession Number. The purified Xyln-SH1 was blotted onto a PVDF membrane according to the standard protocol. The N-terminal protein sequences of the xylanase were determined using Edman degradation with an automatic PPSQ-21A protein sequencer (Shimadzu Corp., Japan). The nucleotide sequence of the insert DNA containing Xyln-SH1 was deposited in the GenBank database under the accession no. JN571491.

RESULTS AND DISCUSSION

Screening and Analysis of the High Molecular Weight Insert DNA. A metagenomic fosmid library was constructed to isolate novel enzymes from the environmental DNA (eDNA) that was recovered from a Holstein cattle rumen. One clone exhibited a high hydrolysis activity toward xylan and was sequenced for further study. The ORF and BlastP analyses of the insert DNA indicated that a hypothetical protein displayed the highest identity (74%) to the acyl-coenzyme A synthetases/AMP-(fatty) acid ligases from *Ruminococcus* sp. The other 66 putative proteins (encoded by >300 bp of nucleotide) exhibited identities <70% to known protein sequences (Supporting Information). Moreover, a maximum of seven hits, which represents 10% of all the putative proteins, exhibited a consistent identity to *Clostridium* sp. sequences. Such a result

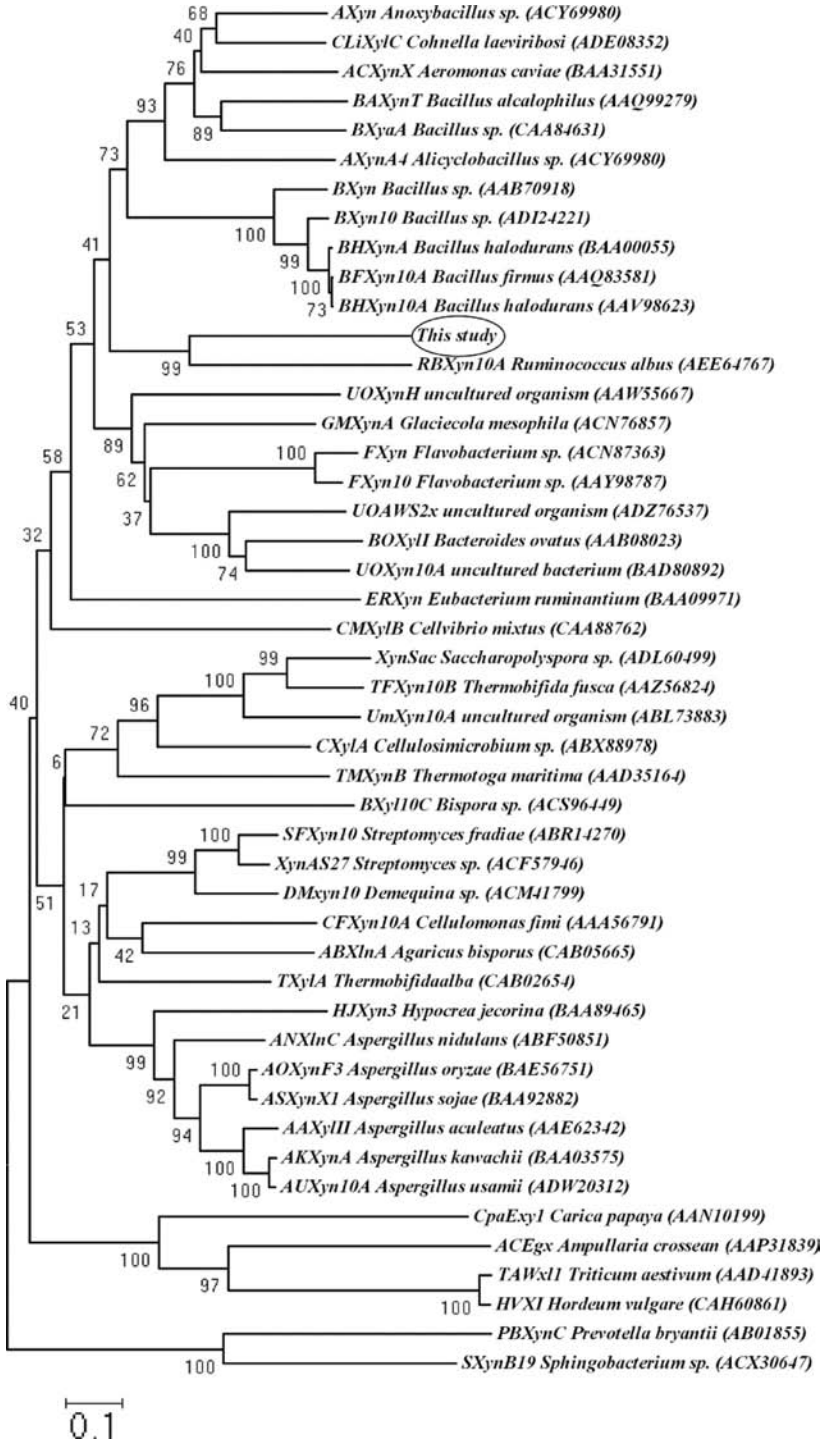

Figure 1. Phylogenetic tree of Xyln-SH1 (this study, outlined with an oval) with GH10 family proteins using the neighbor-joining (NJ) method. The bootstrap values (n = 500 replicates) are reported as percentages. The scale bar represents the number of changes per amino acid position. GenBank accession numbers are indicated in parentheses.

suggests that the DNA fragment may originate from an uncultured organism. The low similarities of the hypothetic proteins with their closest homologues from public databases were also found in other complex metagenomic libraries.[20] This may result from the limited knowledge about the genomes and encoded genes of uncultured microbes.[21]

Sequence Analysis of Xyln-SH1. The sequenced DNA fragment contained the ORF of a putative glycoside hydrolase family protein. The Megablast analysis indicated that the nucleic acid sequence displayed no significant similarity to the nucleotide collection (nr/nt) database. The deduced amino acid sequences exhibited the highest identity (44%) to the glycoside hydrolase family protein from *Clostridium thermocellum* ATCC 27405 (GenBank accession no. YP001038252) and to the endo-β-1,4-xylanases from *Acetivibrio cellulolyticus* CD2 (GenBank accession no. ZP07329203). This protein was named Xyln-SH1, and it is 1056 bp in length and codes for 351 amino acids. The phylogenetic relationship of Xyln-SH1 to

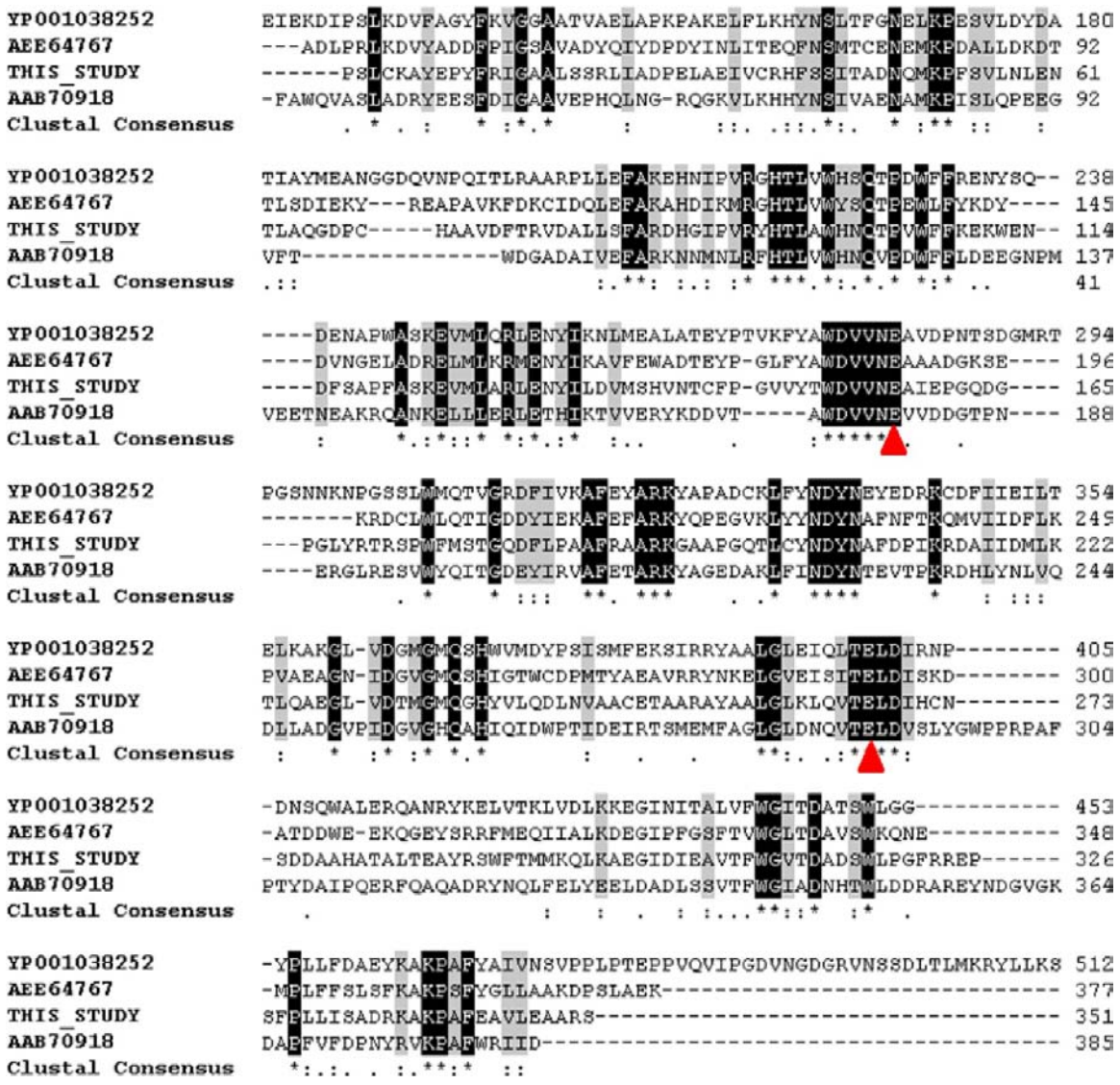

Figure 2. Multialignment analysis of the Xyln-SH1 protein sequence: rectangle, conserved domain; dots, predicted catalytic residues; AAB70918, alkaline thermostable endoxylanase from *Bacillus* sp. NG-27; AEE64767, Xyn10A from *Ruminococcus albus* 8; this study, Xyln-SH1 protein sequence (JN571491); YP001038252, glycoside hydrolase family protein from *Clostridium thermocellum* ATCC 27405.

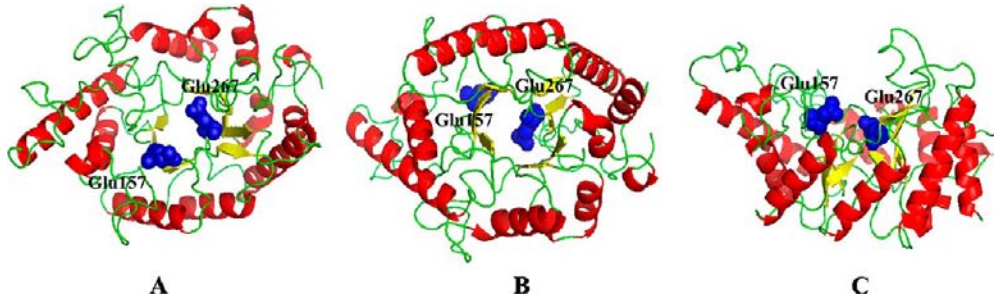

Figure 3. Predicted 3D structure of Xyln-SH1: (A) top view; (B) bottom view; (C) lateral view; red, α-helix; green, β-sheet; green, random coil; blue spheres, putative catalytic residues.

characterized xylanases is illustrated in Figure 1. Xyln-SH1 exhibited the closest genetic relationship to the prokaryotic xylanase from *Bacillus* sp. and RBxyn10A from *Ruminococcus albus* (AEE6476), which belongs to glycoside hydrolase family 10 (GH10) and originated from the rumen.[22]

Several conserved domains and residues from the deduced Xyln-SH1 primary sequence were identified using multialignment with the most similar characterized protein sequences, as demonstrated in Figure 2 (shaded in gray and black). Two residues (E157 and E267, marked with a triangle) were observed in the conserved regions (WDVVNE and TELD) and may function as sites of catalysis.[23] The typical conserved regions were also found in many other GH10 xylanases, such as Umxyn10A from a rice straw-degrading enrichment culture,[24]

xyn10 from *Flavobacterium* sp.,[25] and xylanase 10B from *Thermotoga maritima* MSB8.[26] The low similarities of these enzymes to Xyln-SH1 may provide more information on the causal relationship between their experimental functions and protein primary sequences.

Recent advances in computer algorithms for the prediction of protein structure and function make it possible to derive important information from primary sequences.[13] X-ray studies indicated that the tertiary structure of GH10 family proteins were generally formed by a "salad bowl" $(\beta/\alpha)_8$ barrel fold.[23] In this study, the I-TASSER platform for automated protein structure and function prediction was applied to the Xyln-SH1 tertiary structure (Figure 3). The results indicated that Xyln-SH1 tertiary structure was formed from eight parallel β-sheets surrounded by eight α-helices. The eight α-helixes formed the outer brim of the bowl. One face of the molecule possessed a large radius, which may shield the structure from the solvent.[27] The conserved domains and putative catalytic sites (E157 and E267, highlighted with spheres in Figure 3) were located in the center of this "salad bowl" structure. The above features indicated that Xyln-SH1 was a typical member of glycoside hydrolase family 10 (GH10).

Cloning, Expression, and Purification of Xyln-SH1. The PCR-amplified gene fragment that coded for the xylanase was ligated to the pEASY-E2 vector and transformed into *E. coli* BL21 (DE3). The transformants were screened on plates containing birch wood xylan for the formation of a clear and transparent zone surrounding the colonies. The xylanase activity of one particular clone chosen for further study was detected after induction, and a distinct protein band was observed in the cytoplasm using SDS-PAGE analysis (Figure 4, lane B, highlighted with an arrow). The calculated distinct protein molecular weight was approximately 41.5 kDa, which was in accordance with the predicted data (39.5 kDa) linking with the hexahistidine tags and amino acid residues on the vector. The predicted p*I* of Xyln-SH1 was 5.2 according to the ExPASy proteomics server analysis. These properties of Xyln-SH1 were in agreement with the typical GH10 endoxylanases, which have a high molecular weight (≥30 kDa) and a low p*I*.[28]

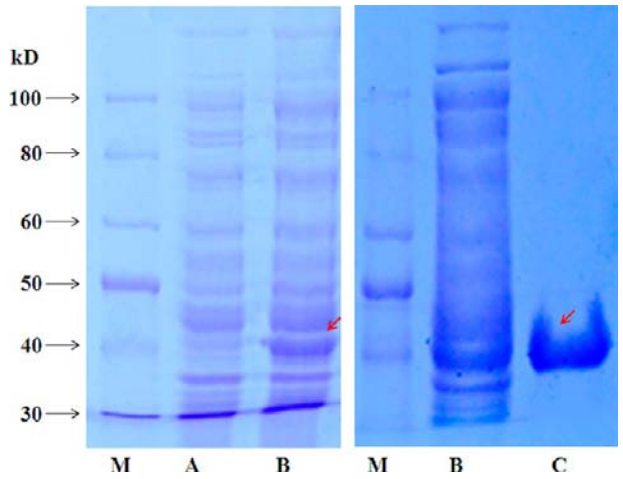

Figure 4. SDS-PAGE analysis of Xyln-SH1. Lanes: M, marker from 30 to 100 kDa; A, uninduced cytoplasmic total protein; B, induced cytoplasmic total protein; C, purified protein.

The recombinant enzyme was purified to apparent homogeneity by Ni^+ affinity chromatography and dialyzed (Figure 4, lane C). The identity of the purified recombinant enzyme was further confirmed by N-terminal protein sequence analysis (data not shown).

Biochemical Characterization of Xyln-SH1. The biochemical characterization of the xylanase was performed using birch wood xylan as the substrate. The majority of the xylanases studied are observed to be optimally active at, or near, mesophilic temperatures (approximately 40−60 °C) and at neutral (particularly for the bacterial xylanases) or slightly acidic (particularly for the fungal xylanases) pH values.[23] The purified, recombinant Xyln-SH1 exhibited optimal activity at approximately 40 °C and pH 6.5 (Figure 5A,B). The results were in agreement with the majority of xylanases and close to the typical cattle rumen temperature and pH values, which are 39.4 °C and pH 5.5−7.0, respectively.[29] Xyln-SH1 was stable below 40 °C and retained almost 100% activity after 1 h of incubation (Figure 5D). The enzyme activity decreased rapidly at temperatures >50 °C and lost almost 90% of its initial activity after a 20 min incubation at 60 °C (Figure 5D). Little activity loss was detected for the samples that were incubated in different buffers ranging from pH 3.5 to 9.0 for 1 h (Figure 5C). Similarly high pH stability was also observed in xylanases XylC from *Fusarium graminearum* GH10,[30] and this extreme pH stability is a valuable property for industrial applications.

The effects of different reagents on Xyln-SH1 activity were determined after incubation with the enzyme in the reaction solution. As illustrated in Table 1, organic solvents, such as dimethyl sulfoxide (DMSO), Tween 20, and *N*,*N*-dimethylformamide (DMF), exhibited no effects on the enzyme activity, whereas isopentanol, *n*-butanol, SDS, and ethanol inhibited the enzyme activity. Heavy metal ions including Mn^{2+}, Cu^{2+}, Ni^+, Zn^{2+}, and Co^{2+} significantly inhibited enzyme activity, which suggests that Xyln-SH1 is a thiol-sensitive enzyme because these heavy metal ions bind the free mercapto groups (−SH) in cysteine residues.[15]

Substrate Specificity of Xyln-SH1. The hydrolysis reactions were performed with a variety of substrates. The purified Xyln-SH1 exhibited high specificity toward birch wood xylan and oat spelt xylan, the activities of which were 13009.53 ± 81.54 and 896.10 ± 15.52 U/mg, respectively. Most characterized GH10 xylanases are capable of utilizing various sources of xylan and exhibited similar hydrolysis profiles on birch wood xylan and beech wood xylan.[14] However, Xyln-SH1 was a special case and showed no activity on beech wood xylan. The mechanism of this phenomenon needs further studies.

Some endo-β-1,4-xylanases exhibit cellulase activity, which results in cellulose loss, pulp quality degradation, and increased effluent treatment costs.[23] Xyln-SH1 demonstrated no activity toward sodium carboxymethylcellulose or microcrystalline cellulose. Because of these characteristics, Xyln-SH1 can be exploited for the development of environmentally friendly technologies in the paper and pulp industries.[31] In addition, no hydrolysis reaction was detected using locust bean gum or methyl ferulate as substrate. From an industrial perspective, high reaction specificity will achieve a targeted hydrolysis reaction, which will render the posthydrolysis process easier, less expensive, and more efficient.

Hydrolytic Product Analysis of Xyln-SH1. Birch wood xylan was utilized as the substrate for Xyln-SH1 under the optimum conditions, and the enzymatic hydrolysis products were analyzed using TLC. As indicated in Figure 6, there was an evident increase in XOS content during the first 30 min, and the Xyln-SH1 released at least five types of XOS. Xylotriose

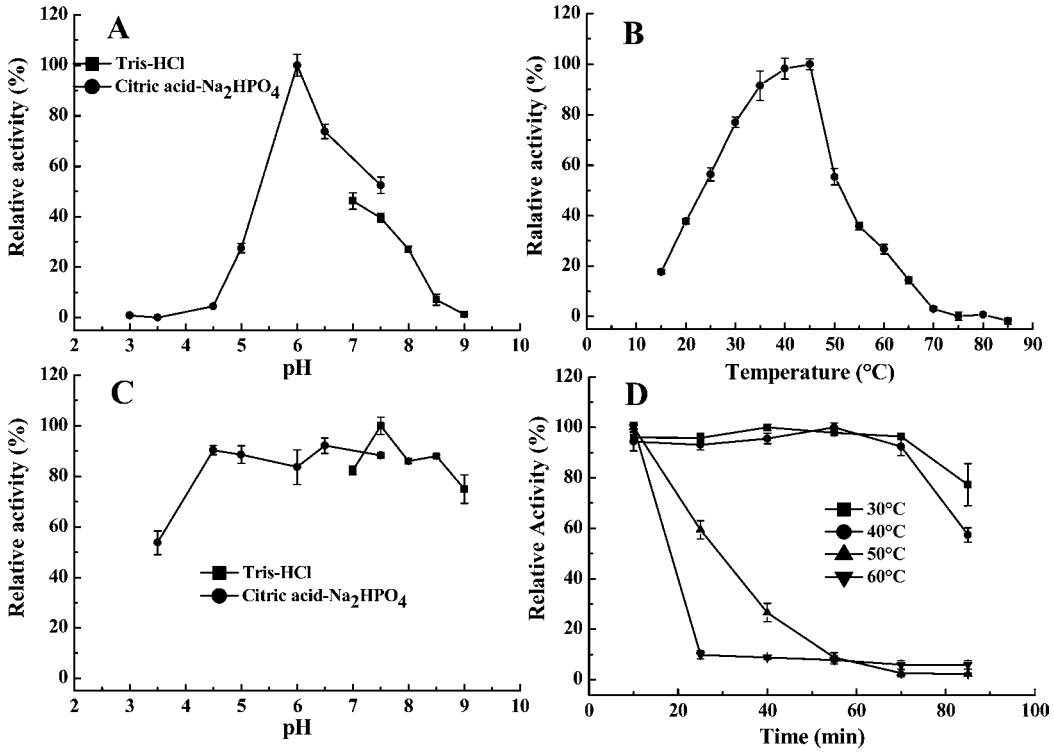

Figure 5. Effect of pH (A) and temperature (B) on the xylanase activity, pH stability (C), and thermostability (D) of Xyln-SH1. Values are given as the mean of three experiments ± standard deviation.

Table 1. Effects of Various Regents on Xyln-SH1 Activities[a]

chemical	relative activity (%)	chemical	relative activity (%)
distilled water	100 ± 2.48	Mg^{2+}	73.61 ± 3.66
Triton	94.44 ± 2.69	Mn^{2+}	32.22 ± 2.20
DMSO	112.5 ± 7.35	Cu^{2+}	6.94 ± 0.30
ethanol	68.06 ± 4.23	Fe^{2+}	80.83 ± 5.22
Tween 20	107.78 ± 6.01	Ni^{+}	26.94 ± 2.60
glutaraldehyde	82.22 ± 2.22	Zn^{2+}	8.06 ± 6.03
isopentanol	8.89 ± 0.80	Ca^{2+}	93.61 ± 3.60
SDS	10.56 ± 1.51	K^{+}	95.56 ± 5.10
n-butanol	23.61 ± 2.22	Co^{2+}	19.17 ± 5.01
acetone	103.06 ± 3.60	Ba^{2+}	91.67 ± 7.23
DMF	116.11 ± 5.23	Na^{+}	99.78 ± 4.50

[a]The final concentrations of the test regents were 20 mM. Each value in the panel represents the mean of three experiments ± standard deviation.

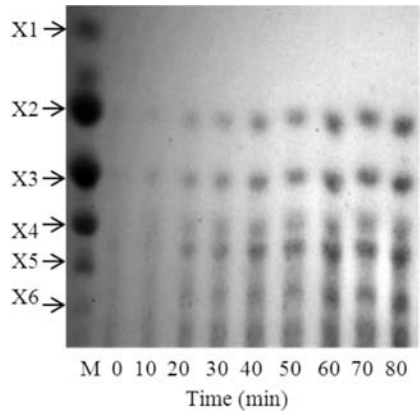

Figure 6. Hydrolysis product analysis of birch wood xylan after Xyln-SH1 treatment for different times. Lanes: M, standard XOS (X1−X6: xylose, xylobiose, xylotriose, xylotetraose, xylopentaose and xylohexaose); 0, distilled water sample; 10−80, samples at different treatment times.

(X2) release was observed after 10 min, which was prior to the release of other XOS. Xylohexaose (X6) was observed after 30 min of hydrolysis. Moreover, no xylose was detected throughout the entire hydrolysis process.

Many characterized endo-*β*-1,4-xylanases can hydrolyze xylan into XOS with a degree of polymerization (DP) no greater than 5,[32] and X2 or a mixture of X2 with xylotetraose (X4) are the main hydrolysis products of GH10 xylanases.[33,34] Additionally, most GH10 xylanases can produce xylose during hydrolysis reaction, such as xynAS9 from *Streptomyces* sp. S9[35] and TaXyn10A from *Thermoascus aurantiacus*,[33] whereas few reported are incapable of releasing xylose.[32] Xyln-SH1 was capable of releasing XOS with a DP from 2 to 6 with X2, xylotriose (X3) and xylopentaose (X5) being the main products, but no xyloses were generated. The results suggested Xyln-SH1 was a strict endo-*β*-1,4-xylanase and potential in XOS production.

Production of XOS and FA from Wheat Straw with Xyln-SH1 and FAE-SH1 Cotreatment. The Xyln-SH1 and FAE-SH1 enzymes were applied to wheat straw for the production of XOS and FA. FA is commonly found in the plant cell wall and attached to polysaccharides, and they have also been suggested to be a linker between polysaccharides and lignin.[36] Ferulate cross-linking may restrict the enzymatic degradation of structural polysaccharides and limit the utilization of graminaceous crops and crop residues.[37] Theoretically, the release of FA may render it more accessible for xylanase to hydrolyze xylan, and the release of feruloylated oligosaccharides can be enhanced.[38,39] Enzyme mixtures with

feruloyl esterase activity, which are produced by many microorganisms, are observed to release sugars.[5] However, the individual functions of a feruloyl esterase and its synergistic action with xylanase for the release of XOS from wheat straw need to be studied.

The total extractable XOS and total alkali-extractable FA of wheat straw were 12.9 ± 2.5 g/100 g and 159.6 ± 10.3 mg/100 g, respectively. As demonstrated in Figure 7, FAE-SH1 action

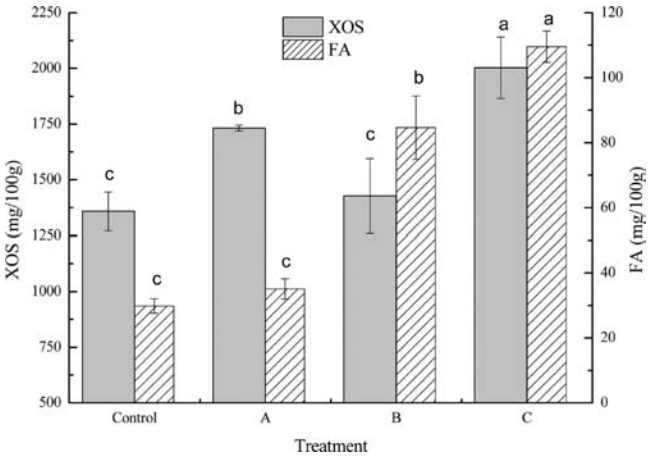

Figure 7. Release of XOS and FA from wheat straw using enzymatic treatment of Xyln-SH1 and FAE-SH1: control, distilled water treated; A, 1000 U Xyln-SH1 treatment; B, 1000 U FAE-SH1 treatment; C, 1000 U Xyln-SH1 + 1000U FAE-SH1 treatment. Each value in the panel represents the mean of three independent experiments; error bars represent standard deviation. The statistical analysis was performed using analysis of variance (ANOVA), followed by the least significant difference test (LSD). a, b, and c: statistically significant difference ($p < 0.05$).

has no significant ($p < 0.05$) effect on total XOS release. Significant XOS production was observed after a 1 h treatment with Xyln-SH1 and resulted in 1.73 ± 0.0125 g/100 g, which represents approximately 13.4% (w/w) of the total extractable XOS. Higher XOS production was obtained by the joint action of 1000 U of Xyln-SH1 and 1000 U of FAE-SH1, and this combination produced 2.01 ± 0.1378 mg/100 g. The higher yields of XOS may result from the destruction of the ferulated, rigid structure by FAE-SH1 and the increased accessibility of Xyln-SH1 to xylan.[40]

The yield of FA was 84.6 ± 9.75 mg/100 g from hydrolysis with 1000 U FAE-SH1 under the optimum hydrolytic conditions. In comparison, the synergistic action of 1000 U of FAE-SH1 and 1000 U of Xyln-SH1 released higher yields of FA (109.55 ± 4.86 mg/100 g), which represents 68.6% of the total FA in the wheat straw. *Neosartorya spinosa* NRRL185 has been reported to produce a full complement of enzymes for the complete recovery of ferulic acid from corn bran and corn fibers.[5] The suboptimal hydrolysis in this study may result from the materials utilized and can be remedied by further optimization and pretreatment technology.[41,42] As previously reported, the right choice of enzymes is important for FA release.[18,45] The type A feruloyl esterase from *Aspergillus niger* was more effective with a family 11 xylanase from *Trichoderma viride* in the release of FA from brewers' spent grain. The synergistic action of this feruloyl esterase with a family 10 xylanase from *Thermoascus aurantiacus* results in the release of diferulic acid.[43] In the present study, the efficient release of FA can also be achieved by the joint action of Xyln-SH1 (GH10) and FAE-SH1 (type C) on wheat straw.

The effects of xylanase alone on FA release from various biomass materials were investigated by many previous studies, and no significant effects were detected.[44] However, Huang et al. found that the *Thermobifida fusca* xylanase alone causes a slight increase in FA release.[45] In this study, a 2000 U dose of Xyln-SH1 enhanced the release of FA from wheat straw (57.94 ± 5.52 mg/100 g) significantly ($p < 0.05$), whereas no esterase activity or esterase-conserved domain was detected in this type of enzyme. The detailed mechanism for FA release may rely on the dose of xylanase and the structural characteristics of the biomass materials.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

BlastP analysis of the function of hypothetical proteins in JN571491. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BLAST, basic local alignment search tool; BSA, albumin from bovine serum; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DNS, 3,5-dinitrosalicylic acid; eDNA, environmental DNA; FA, ferulic acid; GH10, glycoside hydrolase family 10; IPTG, isopropyl-*β*-D-thiogalactopyranoside; LB, Luria−Bertani; MW, molecular weight; ORF, open reading frame; OS, oligosaccharide; PBS, phosphate buffer solution; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TLC, thin layer chromatography; XOS, xylooligosaccharides

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