J. Microbiol. Biotechnol. (2014), 24(7), 943–953
http://dx.doi.org/10.4014/jmb.1405.05032

jmb

Novel Alkali-Tolerant GH10 Endo-β-1,4-Xylanase with Broad Substrate Specificity from *Microbacterium trichothecenolyticum* HY-17, a Gut Bacterium of the Mole Cricket *Gryllotalpa orientalis*

Do Young Kim[1†], Dong-Ha Shin[2,3†], Sora Jung[1], Hyangmi Kim[4], Jong Suk Lee[5], Han-Young Cho[1], Kyung Sook Bae[4], Chang-Keun Sung[3], Young Ha Rhee[6], Kwang-Hee Son[1*], and Ho-Yong Park[1*]

[1]*Industrial Bio-materials Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon 305-806, Republic of Korea*
[2]*Insect Biotech Co. Ltd., Daejeon 305-811, Republic of Korea*
[3]*Department of Food Science and Technology, Chungnam National University, Daejeon 305-764, Republic of Korea*
[4]*Microbial Resource Center, KRIBB, Daejeon 305-806, Republic of Korea*
[5]*Gyeonggi Bio-Center, Gyeonggi Institute of Science & Technology Promotion, Suwon 443-270, Republic of Korea*
[6]*Department of Microbiology and Molecular Biology, Chungnam National University, Daejeon 305-764, Republic of Korea*

Received: May 15, 2014
Revised: May 23, 2014
Accepted: May 24, 2014

First published online
May 27, 2014

*Corresponding authors
K.-H.S.
Phone: +82-42-8604650;
Fax: +82-42-8604659;
E-mail: sonkh@kribb.re.kr
H.-Y.P.
Phone: +82 42 8604650;
Fax: +82 42 8604659;
E-mail: hypark@kribb.re.kr

†These authors contributed equally to this work.

pISSN 1017-7825, eISSN 1738-8872

The XylH gene (1,167-bp) encoding a novel hemicellulase (41,584 Da) was identified from the genome of *Microbacterium trichothecenolyticum* HY-17, a gastrointestinal bacterium of *Gryllotalpa orientalis*. The enzyme consisted of a single catalytic domain, which is 74% identical to that of an endo-β-1,4-xylanase (GH10) from *Isoptericola variabilis* 225. Unlike other endo-β-1,4-xylanases from invertebrate-symbiotic bacteria, rXylH was an alkali-tolerant multifunctional enzyme possessing endo-β-1,4-xylanase activity together with β-1,3/β-1,4-glucanase activity, which exhibited its highest xylanolytic activity at pH 9.0 and 60°C, and was relatively stable within a broad pH range of 5.0–10.0. The susceptibilities of different xylose-based polysaccharides to the XylH were assessed to be as follows: oat spelts xylan > beechwood xylan > birchwood xylan > wheat arabinoxylan. rXylH was also able to readily cleave *p*-nitrophenyl (*p*NP) cellobioside and *p*NP-xylopyranoside, but did not hydrolyze other *p*NP-sugar derivatives, xylobiose, or hexose-based materials. Enzymatic hydrolysis of birchwood xylan resulted in the product composition of xylobiose (71.2%) and xylotriose (28.8%) as end products.

Keywords: Endo-β-1,4-xylanase, GH10 enzyme, *Microbacterium trichothecenolyticum* HY-17, mole cricket, gut bacterium

Introduction

Recalcitrant plant biomass consists primarily of cellulose, hemicelluloses (including β-1,4-xylan and β-1,4-mannan), and lignin, all of which may be biologically degraded. Of such components, cellulosic and xylosidic polysaccharides have attracted a great amount of attention in industry, as renewable resources for the production of fermentable sugars, which can be further converted to bioethanol *via* microbial transformations [5, 36]. Compared with celluloses, xylans present in lignocellulosic biomass are pentose-based polysaccharides, in which D-xylose molecules are linked by β-1,4-xylosidic bonds [30]. In addition, these polysaccharides are frequently present in a specific form, with substituents such as acetic acid or L-arabinofuranose, together with 4-*O*-methyl-D-glucuronic acid [14].

In general, β-1,4-xylans are completely hydrolyzed to D-xylose molecules by the cooperative action of various

hemicellulases [14]. Endo-β-1,4-xylanases are glycoside hydrolase (GH) enzymes, which randomly cleave internal β-1,4-xylosidic linkages, and are well known to play a key role in the deconstruction of β-1,4-xylosidic materials. These microbial enzymes are currently classified into six GH families (GHs 5, 8, 10, 11, 30, and 43) based on their primary structure (http://www.cazy.org/Glycoside-Hydrolases.html) [24], and the endo-β-1,4-xylanases belonging to GH families 10 and 11 have been the most extensively examined thus far [9]. In addition to the aforementioned GH enzymes, a retaining endo-β-1,4-xylanase from *Cellulosimicrobium* sp. strain HY-13, which is homologous to inverting GH6 cellobiohydrolases, has also recently been reported as a new type of endo-β-1,4-xylanase [22].

Similar to other fibrolytic microorganisms [4, 25], xylanolytic microorganisms are widely distributed in diverse ecosystems, and play a considerable role in the biological recycling of plant matter throughout the biosphere [4, 9]. In particular, these exosymbiotic bacteria in the guts of herbivorous invertebrates, including insects and higher animals, are very important ecologically, because they contribute significantly to the intestinal digestion of plant biomass taken up as food by the hosts [11, 28]. Until now, some xylanolytic gut bacteria have been isolated from different insects or their larvae, such as the longicorn beetle (*Moechotypa diphysis* [16] and *Batocera horsfieldi* [38]), sun beetle (*Pachnoda marginata* [6]), mole cricket (*Gryllotalpa orientalis* [29]), cotton bollworm (*Helicoverpa armigera* [1]), and wood-feeding higher termites [10], and the unique biological functions and molecular structures of their corresponding GH enzymes have been well characterized. The results of these previous studies have often shown that the endo-β-1,4-xylanases from the isolated symbionts are GH enzymes displaying peculiar enzymatic features in their molecular structure, biocatalytic properties, and substrate hydrolysis pattern. Therefore, more intensive study on diverse insect gut bacteria is expected to facilitate the discovery and application of novel xylanolytic enzymes.

In this study, we describe the molecular and biochemical characteristics of a novel alkali-tolerant GH10 endo-β-1,4-xylanase (XylH), identified from a gut bacterium (*Microbacterium trichothecenolyticum* HY-17) of the mole cricket *G. orientalis*, which assimilates lignocellulosic materials such as plant roots and seeds. The binding capacity of the non-modular enzyme without any additional substrate binding domains to various hydrophobic polymers is also demonstrated and emphasized. To the best of our knowledge, this is the first report of identifying an endo-β-1,4-xylanase from *Microbacterium* species and its study at a molecular level.

Materials and Methods

Materials

D-Xylooligosaccharides of xylobiose to xylohexaose, wheat arabinoxylan, xyloglucan, ivory nut mannan, and azo-xylan were purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland) and chitosan and pectin were provided by USB Co. (Cleveland, OH, USA). Hydrophobic poly(3-hydroxybutyrate) granules were prepared from *Wautersia eutropha* KHB-8862 cells grown on glucose. All other substances, including *para*-nitrophenyl (*p*NP) sugar derivatives, D-xylose, soluble starch, lignin, chitin, curdlan from *Alcaligenes faecalis*, carboxymethylcellulose, and β-1,4-xylans from birchwood, beechwood, and oat spelts, were obtained from Sigma-Aldrich (St. Louis, Mo, USA).

Isolation and Identification of a Xylan-Degrading Bacterium

Aerobic, heterotrophic bacteria in the digestive tract of mole crickets were isolated by incubating them on either R2A agar medium (Scharlau Chemie S.A.) or plate count agar (Difco) at 25ºC for 48–72 h. For this, mole crickets were initially washed with 70% ethanol, after which they were rewashed twice with sterilized water. The digestive tracts were then separated from the bodies of the mole crickets by dissection. The intestinal contents were carefully recovered, diluted in phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, and 0.024% KH_2PO_4), and spread onto plate count agar and R2A agar plates to isolate the aerobic gut bacteria. Of the morphologically different heterotrophic bacteria isolated from the primary screening, xylanolytic bacteria were rescreened for their halo-forming ability using M9 minimal salts agar medium (Difco) containing yeast extract (5 g/l) together with azo-xylan (2 g/l). A xylan-degrading bacterium, strain HY-17, formed a translucent halo around the developed colonies on the medium and was preferentially chosen for further work. A partial 16S rDNA sequence from strain HY-17 was amplified using universal polymerase chain reaction (PCR) primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-AAGTCGTAACAAGGTAACC-3′) for phylogenetic identification. The sequence analysis of the resulting PCR products was conducted using an ABI prism BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 and an ABI 3730x1 DNA Analyzer (Applied Biosystems) at Genotech Co. (Seoul, Republic of Korea). To determine its phylogenetic affiliation, the 16S rDNA sequence from the isolate was compared with 16S rDNA sequences available in GenBank using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) and EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/). The 16S rDNA sequence of strain HY-17 was then aligned with sequences of strains belonging to the genus *Microbacterium* using the CLUSTAL_X program. Strain HY-17 was deposited in the Korean Collection for Type Cultures under code no. *M. trichothecenolyticum* HY-17 KCTC 12338BP.

Cloning and Overexpression of the Xylanase Gene

A partial fragment of the XylH gene from the genomic DNA was amplified using degenerate oligonucleotides designed on the basis of conserved regions (WDVVNE and VTEADV) in the GH10 endo-β-1,4-xylanases. The upstream primer (MF-10) was 5′-TGGGACGTCSTCAACGAG-3′ and the downstream primer (MR-10) was 5′-GACGTCSGCCTCSGTGAC-3′, which yielded a 375 bp DNA fragment. The complete XylH gene was cloned into a pGEM-T easy vector (Promega) by repeated DNA walking and nested PCR methods, using a DNA Walking SpeedUp premix kit (Seegene). For the recombinant production of XylH, PCR amplification of the gene encoding mature XylH with *Nde*I and *Hin*dIII sites in the N-terminus and C-terminus, respectively, was conducted using the pGEM-T easy/*xylH* vector as a template, as described elsewhere [20]. The PCR primers used were as follows: the upstream primer (FMF) was 5′-CATATGGCGCCTCCCGGA TTCAGC-3′ and the downstream primer (RMF) was 5′-AAGCTT TCAGCCGCGTCGGGGC-3′. The amplified gene product was then separated on a 1.0% agarose gel, and was purified using a NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). The obtained PCR products were cloned into a pGEM-T easy vector (Promega), after which the recombinant vectors were excised with *Nde*I and *Hin*dIII. The resulting *xylH* fragments with the corresponding sticky ends were isolated and ligated into a pET-28a(+) vector (Novagen) with the same ends, followed by transformation of the resulting pET-28a(+)/*xylH* into *Escherichia coli* BL21. Overproduction of the recombinant mature protein (rXylH) with N-terminal His-tag was then performed by culturing the recombinant cells harboring pET-28a(+)/*xylH* using a 2 L baffle-flask that contained 500 ml of Luria-Bertani broth (Difco), in a rotary shaker (180 rpm) for 7 h at 30°C. The overexpression of the mature XylH gene was induced by adding 1 mM IPTG after the absorbance of the culture at 600 nm reached approximately 0.4. After cultivation, the recombinant cells were recovered by centrifugation (8,650 ×*g*) and then stored at −20°C.

Purification and Analysis of Recombinant Enzymes

To purify active recombinant enzymes, the frozen cells were suspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole) (pH 7.4), after which they were disrupted by sonication. The soluble fraction containing the rXylH proteins was collected by centrifugation (12,000 ×*g*) for 20 min at 4°C, filtered, and then directly subjected to affinity chromatography using a HisTrap HP (GE Healthcare, Sweden) (5 ml) column attached to a fast-protein liquid chromatography system (Amersham Pharmacia Biotech, Sweden). Affinity column chromatography was accomplished according to the protocols provided by the manufacturer, after which the rXylH proteins were eluted from the column using a linear gradient of 0.02−0.5 M imidazole at a flow rate of 5.0 ml/min. The fractions displaying high endo-β-1,4-xylanase activity were pooled and desalted with a HiPrep 26/10 desalting column (GE Healthcare, Sweden), using 50 mM sodium phosphate buffer (pH 6.5) as the mobile phase. The resulting enzyme solution was re-applied onto a HiPrep 16/10 DEAE FF (GE Healthcare, Sweden) column that had been pre-equilibrated with 50 mM sodium phosphate buffer (pH 6.5). Enzyme elution was conducted using a gradient of 0 to 0.5 M NaCl at a flow rate of 5.0 ml/min, and the fractions displaying endo-β-1,4-xylanase activity were pooled and desalted for further analysis. To determine the relative molecular mass, the purified rXylH was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% gel. The separated protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The protein contents were quantified using Bio-Rad Protein Assay Dye Reagent Concentrate, and bovine serum albumin was used as a standard.

Enzyme Assays

Endo-β-1,4-xylanase activity was routinely determined by quantifying the amount of reducing sugars released from beechwood xylan using 3,5-dinitrosalicylic acid reagent. D-Xylose was used as a standard. The enzyme reaction was accomplished at 55°C for 15 min using the standard assay mixture (0.5 ml), which was made of 1.0% beechwood xylan with suitably diluted enzyme solution (0.05 ml) in 50 mM glycine-NaOH buffer (pH 9.0). One unit (U) of rXylH activity toward xylan polysaccharides was defined as the amount of enzyme required to produce 1 μmol of reducing sugar per minute under standard assay conditions. Chromogenic saccharides substituted with *p*NP (each 5 mM) were employed to assess the other biocatalytic activities of rXylH. The transferase activity of rXylH toward *p*NP-sugar derivatives was evaluated under the same reaction conditions used in the standard assay of endo-β-1,4-xylanase activity. In this case, the enzyme reactions were stopped by adding 0.5 ml of 2 M sodium carbonate into the reaction mixtures. One unit (U) of transferase activity for *p*NP-sugar derivatives was defined as the amount of enzyme required to release 1 μmol of *p*NP per minute under standard assay conditions.

Effects of pH, Temperature, and Chemicals on Endo-β-1,4-Xylanase Activity

The effect of pH on the endo-β-1,4-xylanase activity of rXylH was assessed by subjecting samples to pH values ranging from 3.5 to 11.0 at 55°C for 15 min, employing the following buffer systems (50 mM): sodium citrate (pH 3.5 to 5.5), sodium phosphate (pH 5.5 to 7.5), Tris-HCl (pH 7.5 to 9.0), and glycine-NaOH (pH 9.0 to 11.0). The pH stability of rXylH was assayed by measuring the residual activity of the enzyme after incubation at the aforementioned pH values for 1 h at 4°C. The hydrolytic reaction was performed at 55°C for 15 min after the addition of 1.0% beechwood xylan to the reaction mixture. The effect of temperature on the endo-β-1,4-xylanase activity was determined by reacting the purified rXylH with beechwood xylan at temperatures ranging from 30°C to 70°C in 50 mM glycine-NaOH buffer (pH 9.0). The thermal stability of rXylH was determined by measuring the residual activity of the enzyme after incubation at different

Table 1. Effects of metal ions (1 mM) and chemical reagents (5 mM) on rXylH activity.

Compound	Relative activity (%)[a]
None	100.0
$HgCl_2$	0.0
$CaCl_2$	90.6 ± 1.6
$NiSO_4$	100.1 ± 2.3
$CuCl_2$	99.8 ± 1.4
$ZnSO_4$	105.0 ± 1.9
$MgSO_4$	99.2 ± 1.2
$MnCl_2$	131.1 ± 1.4
$SnCl_2$	97.0 ± 4.8
$BaCl_2$	100.6 ± 0.7
$CoCl_2$	122.7 ± 1.4
$FeSO_4$	105.6 ± 0.4
N-Bromosuccinimide	39.6 ± 0.3
Iodoacetamide	96.5 ± 3.2
Sodium azide	104.3 ± 2.8
N-Ethylmaleimide	102.5 ± 3.1
EDTA	96.6 ± 1.9
Tween 80 (0.5%)	121.3 ± 5.7
Triton X-100 (0.5%)	123.2 ± 3.5

[a]Relative activity was obtained from three repeated experiments.

temperatures (37°C, 45°C, 50°C, 55°C, and 60°C) for 15, 30, and 60 min in 50 mM glycine-NaOH buffer (pH 9.0). In this case, the hydrolytic reaction was initiated by adding 1.0% beechwood xylan to the reaction mixture. The stimulatory or inhibitory effects against the endo-β-1,4-xylanase activity of divalent cations (1 mM) and chemical reagents [5 mM or 0.5% (wt/v)] were assessed after incubation of rXylH at 4°C for 10 min in 50 mM sodium phosphate buffer (pH 6.5) containing the compound of interest (Table 1).

Analysis of the Hydrolysis Products

Enzymatic hydrolysis of xylooligosaccharides (each 1 mg) and birchwood xylan (10 mg) was performed using purified rXylH (0.5 U) in 0.1 ml of 50 mM glycine-NaOH buffer (pH 9.0) for 12 h at 40°C. The degradation reaction was then finished by heating the reaction mixture at 100°C for 5 min. The hydrolysis products were analyzed by liquid chromatography/tandem mass spectrometry (LC-MS/MS). High-performance liquid chromatography (HPLC) analysis was conducted using a Finnigan Surveyor Modular HPLC system (Thermo Electron Co.) equipped with an Asahipak NH2P-50 2D column (5 μm, 2.0 × 150 mm; Shodex), as described in our previous paper [22].

Binding Assay

Diverse insoluble polymeric materials such as xylan from oat spelts, mannan from ivory nut, Avicel PH-101, lignin, poly(3-hydroxybutyrate) granules, chitin from crab shells, chitosan from crab shells, and curdlan from *Alcaligenes faecalis* were used as candidate substrates to assess the binding capacity of rXylH. For the binding assay, the polymeric materials were initially washed four times with distilled water, after which they were rewashed with 50 mM glycine-NaOH buffer (pH 9.0). The binding of rXylH to insoluble materials was conducted by incubating the appropriately diluted enzyme preparation (approximately 5.0 U/ml) with an equal volume of substrate material in 1.5 ml Eppendorf tubes on ice for 2 h. During the incubation period, the reaction mixtures were vigorously stirred every 5 min. The binding capacity was assayed by determining the residual endo-β-1,4-xylanase activity in the supernatant, which was recovered from the reaction mixtures by centrifugation (11,400 ×*g*). In addition, the binding of rXylH to the insoluble substrates was quantitatively confirmed by assaying the concentration of proteins in the respective supernatants recovered from the reaction mixtures.

Nucleotide Sequence Accession Number

The 16S rDNA sequence of the isolate has been deposited in GenBank under the accession number KC405581. The nucleotide sequence of the XylH gene has been deposited in GenBank under the accession number KF233593.

Results and Discussion

Isolation and Identification of Xylanolytic Gut Bacteria

Insects, including termite and scarab larvae, have been reported to contain a variety of aerobic and facultative anaerobic bacteria in their hind guts [3, 17]. Thus, in this study, we performed the isolation of aerobic, heterotrophic bacteria from the gut contents of *G. orientalis*. As a result, a total of 32 aerobic and culturable bacterial species, which displayed distinct morphological characteristics, were selectively isolated from the gut contents of the mole cricket. The results of activity-based screening using azo-xylan clearly showed that of the 32 heterotrophic symbionts isolated from the digestive tract of *G. orientalis*, 13 bacterial species (approximately 40.6% of all isolates) were capable of degrading β-1,4-xylan. The phylogenetic analysis of these xylanolytic gut symbionts indicated that they were systematically classified into the gram-positive phyla of Actinobacteria and Firmicutes together with the gram-negative phylum of Proteobacteria (Fig. 1). In particular, Actinobacteria was the most abundant phylum in the gut of *G. orientalis*, and made up of diverse β-1,4-xylan-degrading bacteria belonging to the genera *Cellulosimicrobium*, *Gryllotalpicola*, *Leifsonia*, *Luteimicrobium*, *Microbacterium*, and *Streptomyces*. Conversely, only *Paenibacillus cineris* (RU-07) was isolated as a member of the Firmicutes. These

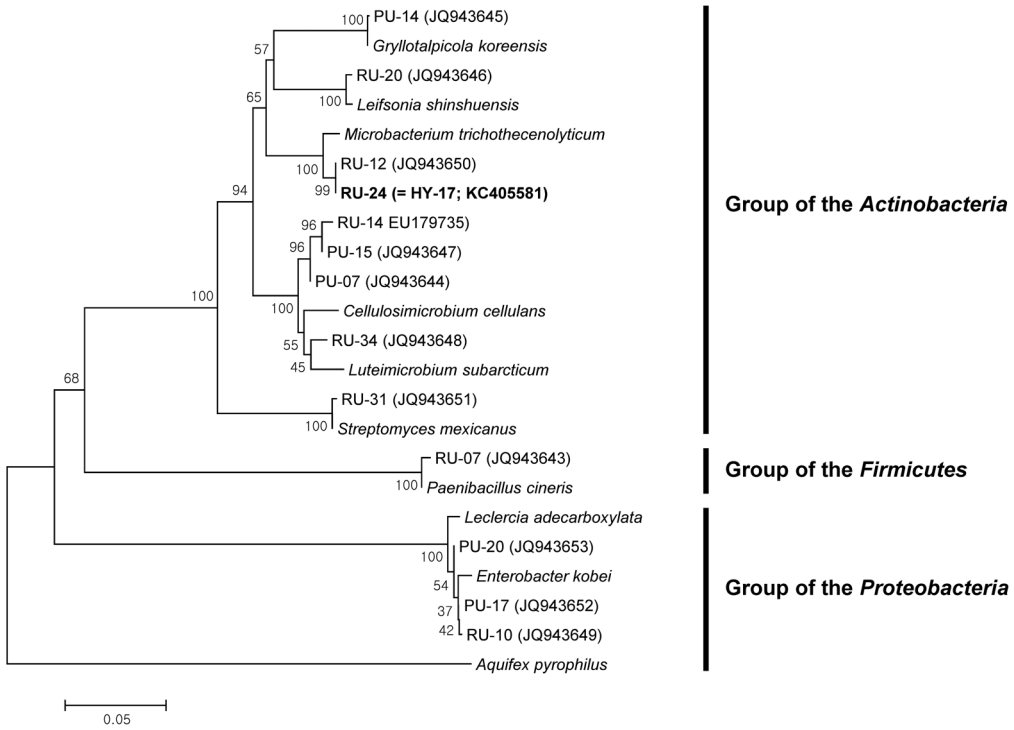

Fig. 1. Phylogenetic positions of xylanolytic bacteria isolated from the gut of *G. orientalis* among neighboring species.
The phylogenetic tree was constructed by the method of neighbor joining, based on pairwise comparisons of 16S rDNA sequences. Numbers at nodes indicate levels of bootstrap support (%) based on 1,000 replicated datasets. Bar, 0.05 substitutions per nucleotide position.

results reflect that a variety of gram-positive bacteria belonging to the phylum Actinobacteria may play a considerable role in the digestion of β-1,4-xylan in the gut of the mole cricket.

When grown on the azo-xylan (2 g/l)-containing medium, strain HY-17 (designated as strain RU-24 in Fig. 1) formed a translucent clear zone around the developed colonies, indicating that this bacterium produces xylanolytic enzymes capable of efficiently decomposing the polysaccharide. However, it could not degrade azo-carboxymethylcellulose and azo-mannan. Based on the nucleotide sequence of the 16S rRNA gene of strain HY-17, the gram-positive xylanolytic gut isolate of a mole cricket, *G. orientalis*, was exclusively identified as *M. trichothecenolyticum*, which was closely related to *M. trichothecenolyticum* IFO 15077^T, with a sequence similarity of 98.68%. To the best of our knowledge, no endo-β-1,4-xylanase from *Microbacterium* species has been isolated and characterized to date. Strain HY-17 in this study was preferentially selected for further work.

Molecular Characterization of the β-1,4-Xylanase Gene

The XylH gene, which expresses an extracellular endo-β-1,4-xylanase from *M. trichothecenolyticum* HY-17, was cloned by genome mining methods. It was predicted to contain a 1,167 bp open reading frame that encodes an acidic protein of 388 amino acids (Fig. 2) with a deduced molecular mass of 41,584 Da and a calculated pI of 4.84. The result of signal peptide prediction using the SignalP 4.1 server indicated that premature XylH may be post-translationally processed between Ala30 and Ala31, which is expected to yield a mature XylH of 358 amino acids, with a deduced molecular mass of 38,632 Da and a calculated pI of 4.67. A protein BLAST survey revealed that XylH was a non-modular endo-β-1,4-xylanase composed of a single catalytic GH10 domain (Leu43 to Leu376) without any additional functional domains (Fig. 2). The primary structure of XylH was determined to be most similar to that of *Isoptericola variabilis* 225 GH10 endo-β-1,4-xylanase (NCBI reference sequence: YP_004541186), which had not been biochemically characterized but was identified through a genome survey. In addition, multiple sequence alignment showed that the catalytic GH10 domain in XylH from *M. trichothecenolyticum* HY-17 had 74% similarity to that of *I. variabilis* 225 GH10 endo-β-1,4-xylanase. Moreover, the functional domain of

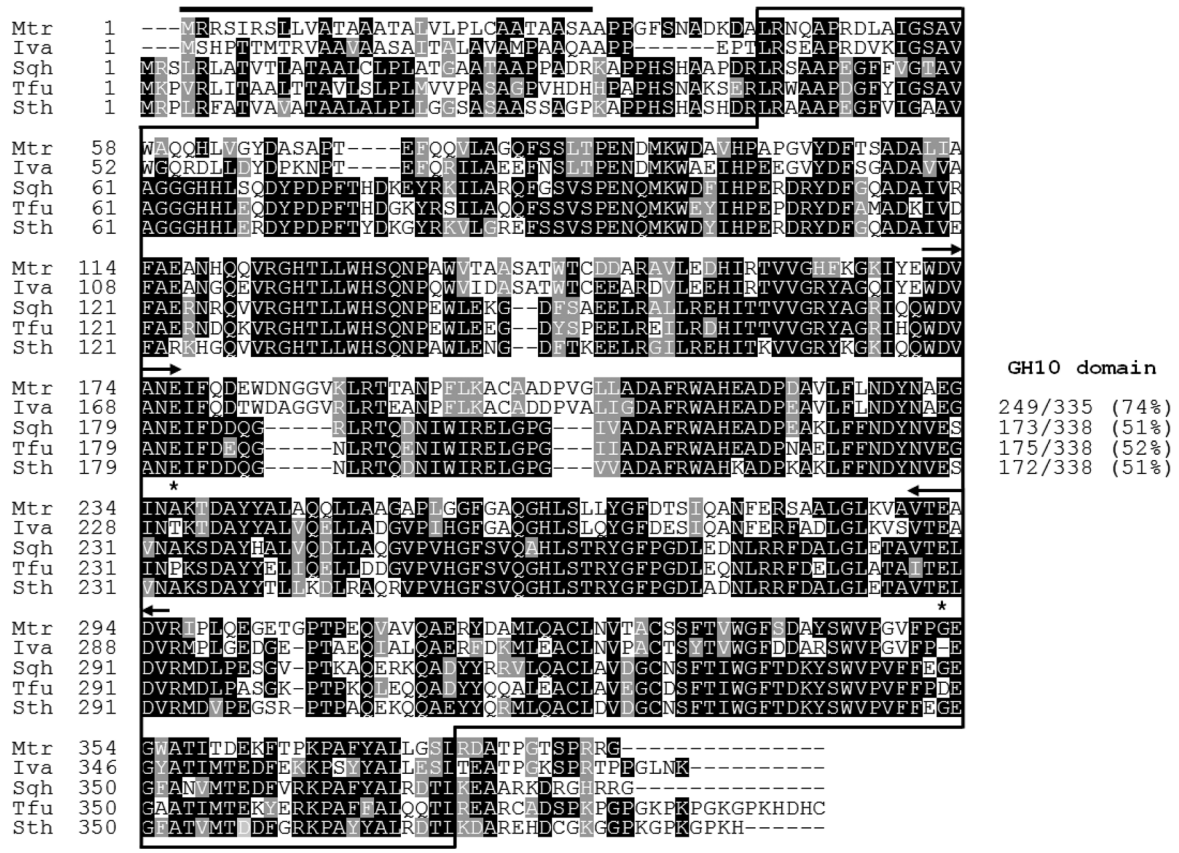

Fig. 2. Primary sequence alignment of *M. trichothecenolyticum* HY-17 GH10 endo-β-1,4-xylanase and its structural homologs.
Sequences (NCBI or GenBank accession numbers): Mtr, *M. trichothecenolyticum* HY-17 endo-β-1,4-xylanase (KF233593); Iva, *Isoptericola variabilis* 225 endo-β-1,4-xylanase (YP_004541186); Sgh, *Streptomyces ghanaensis* endo-β-1,4-xylanase (WP_004984539); Tfu, *Thermobifida fusca* YX endo-β-1,4-xylanase (YP_290847); Sth, *Streptomyces thermocarboxydus* HY-15 endo-β-1,4-xylanase (ACJ64840). The identical and similar amino acids are shown by black and gray boxes, respectively. The predicted signal peptide is indicated by a black bar. The internal peptide sequences used in the design of degenerate oligonucleotides for PCR are marked by arrows. Highly conserved amino acid residues that play a critical role in the biocatalytic reaction are indicated by asterisks. The GH10 domain is outlined by a solid-line box.

XylH was 52% (175/338), 51% (173/338), and 51% (172/338) identical to the GH10 domain of *Thermobifida fusca* YX endo-β-1,4-xylanase (NCBI reference sequence: YP_290847), *Streptomyces ghanaensis* endo-β-1,4-xylanase (NCBI reference sequence: WP_004984539), and *Streptomyces thermocarboxydus* HY-15 endo-β-1,4-xylanase (GenBank Accession No. ACJ64840), respectively. In XylH, the putative catalytic residues of Glu176 (acid/base catalyst) and Glu292 (catalytic nucleophile), which perform the double-displacement of retaining GH enzymes [27], were found in strictly conserved regions of the active site (Fig. 2). A double-displacement mechanism mediates transfer of sugar molecules between oligosaccharides.

Enzymatic Characterization of rXylH

In this study, rXylH was purified to electrophoretic homogeneity by affinity and anion-exchange column chromatographies (data not shown). The result of SDS-PAGE analysis revealed that the relative molecular mass of purified recombinant enzymes was approximately 41.0 kDa, which corresponded to the deduced molecular mass (40.9 kDa) of intact His-tagged XylH. The highest biocatalytic activity of rXylH toward beechwood xylan was observed when the enzyme was reacted with the substrate in 50 mM glycine-NaOH buffer (pH 9.0) at 60°C (Figs. 3A and 3C). Additionally, rXylH exhibited over 80% of its highest biocatalytic activity within a broad pH range of 5.5–10.0 (Fig. 3A). The enzyme was relatively stable within a broad pH range of 5.0–10.0 because it retained over 75% of its original biocatalytic activity at the pH values, even after pre-incubation for 1 h at 4°C (Fig. 3B). However, it was drastically inactivated when exposed to pH values less

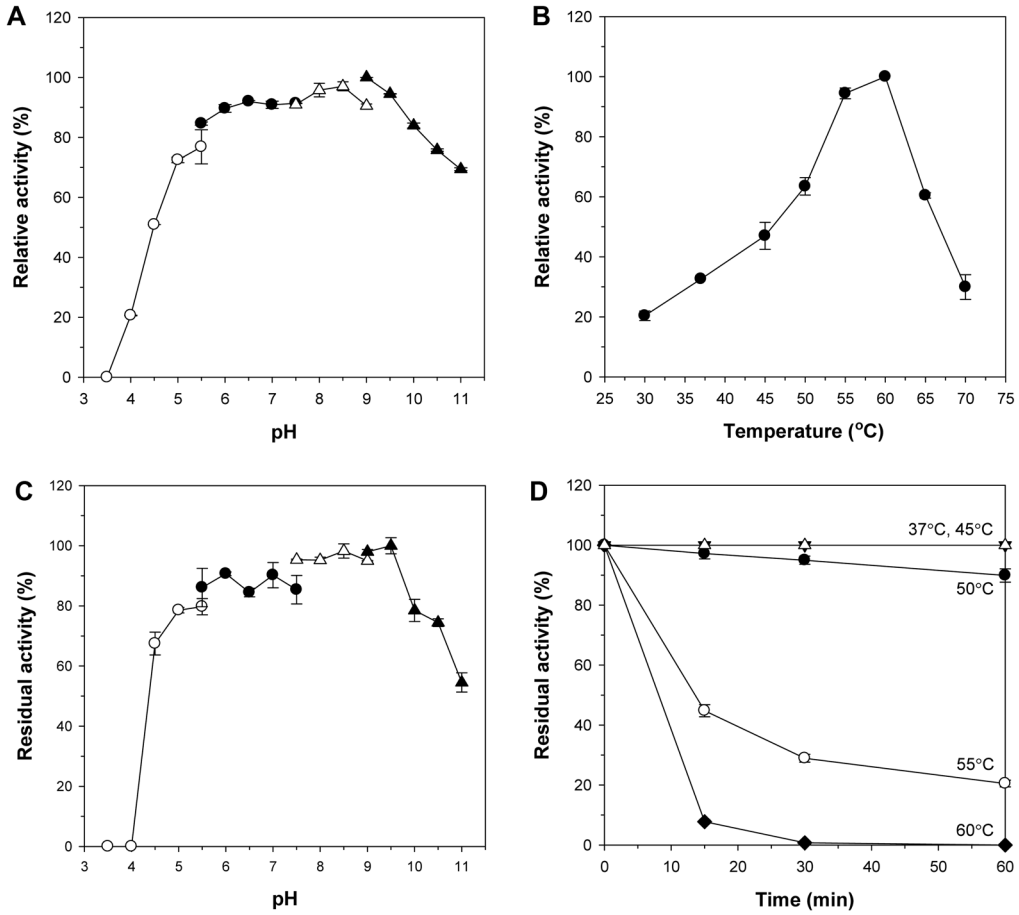

Fig. 3. Effects of pH and temperature on the endo-β-1,4-xylanase activity and stability of rXylH.
(**A**) Effect of pH on rXylH activity; (**B**) effect of temperature on rXylH activity; (**C**) the pH stability of rXylH; and (**D**) the thermal stability of rXylH. The optimal pH of the enzyme was determined using the following buffers (50 mM): sodium citrate (○), sodium phosphate (●), Tris-HCl (△), and glycine-NaOH (▲).

than 4.5. rXylH seemed to be particularly stable at temperatures below 45°C, whereas its xylan-degrading activity was significantly decreased when exposed to temperatures above 55°C (Fig. 3D). The half-life of rXylH at the suboptimal temperature of 55°C was determined to be approximately 12 min. These thermal properties indicated that the enzyme is a thermolabile biocatalyst, similar to functional homologs from other invertebrate gut microorganisms [19, 21, 29, 38].

It has been reported that the majority of endo-β-1,4-xylanases are completely or considerably inactivated in the presence of *N*-bromosuccinimide and Hg^{2+} ions, which modify Trp and Cys residues [18]. One of these outstanding inhibitory effects of endo-β-1,4-xylanases by the chemical reagents are considered to be because some Trp residues in the active sites of the enzymes play a crucial role in enzyme-substrate interaction, as elucidated by site-directed mutagenesis [20, 31] and mapping of GH enzyme substrate subsites [39]. In this study, rXylH was also completely inhibited by 1 mM Hg^{2+}, while the inhibition of the enzyme exerted by 5 mM *N*-bromosuccinimide was assessed to be approximately 60% of its original biocatalytic activity (Table 1). Based on the above descriptions, the three residues in premature XylH (Trp129, Trp337, and Trp345) were predicted to play a key role in substrate binding of the enzyme and catalysis. On the other hand, it has been demonstrated that the inhibition of endo-β-1,4-xylanases from *Bacillus halodurans* S7 [26] and *Bacillus* sp. NG-27 [15] by Hg^{2+} is insignificant, which suggests that catalytically critical Cys residues may be absent in their active sites. It is interesting to note that the stimulatory or inhibitory effects of sulfhydryl reagents (each 5 mM), EDTA (5 mM), and

metal ions (each 1 mM), such as Ca^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Sn^{2+}, Ba^{2+}, and Fe^{2+}, on the enzyme activity were only marginal. These results were highly comparable to the effects of the same compounds on the biocatalytic activities of other endo-β-1,4-xylanases. Recently, Shi *et al.* [32] reported that the biocatalytic activity of *Thermotoga thermarum* GH10 endo-β-1,4-xylanase (Xyl10A) could be greatly enhanced by more than 4-fold of its original activity in the presence of 1 mM Ca^{2+}, but its enzyme activity was almost completely inhibited by 1 mM Cu^{2+}. In addition, the hydrolytic activity of *Cellulosimicrobium* sp. strain HY-13 GH10 endo-β-1,4-xylanase (XylK1) toward birchwood xylan was stimulated to 1.4-fold of its original activity in the presence of 1 mM Fe^{2+}, whereas the enzyme was highly sensitive to 5 mM EDTA [19]. Moreover, the xylan-degrading activity of *Streptomyces thermocarboxydus* HY-15 GH10 endo-β-1,4-xylanase (XylG) was significantly suppressed by Cu^{2+} (1 mM), EDTA (5 mM), and sulfhydryl reagents (each 5 mM) such as iodoacetamide and *N*-ethylmaleimide [21]. In contrast to the above results, rXylH displayed increased hydrolytic activity (>1.2-fold of its original activity) toward beechwood xylan when incubated in the presence of Mn^{2+} (1 mM), Co^{2+} (1 mM), and non-ionic surfactants (each 0.5%) such as Tween 80 and Triton X-100 (Table 1). The stimulation of endo-β-1,4-xylanases by the compounds was also observed in GH10 enzymes from *T. thermarum* [32], *S. thermocarboxydus* HY-15 [21], and *Flavobacterium johnsoniae* [7]. However, Mn^{2+} ions have been reported to strongly inhibit an endo-β-1,4-xylanase from *B. halodurans* S7 [26].

Table 2. Hydrolysis activity of rXylH for different substrates.

Substrate	Specific activity (U/mg)[a]
Birchwood xylan	75.1 ± 2.0
Beechwood xylan	83.4 ± 0.2
Oat spelts xylan	97.2 ± 0.5
Wheat arabinoxylan	52.5 ± 2.7
Xyloglucan	ND[b]
Soluble starch	ND
Pectin	ND
Locust bean gum	ND
β-1,3/β-1,4-D-Glucan from barley	2.3 ± 0.5
Carboxymethylcellulose	ND
*p*NP-cellobioside	260.9 ± 0.6
*p*NP-glucopyranoside	ND
*p*NP-xylopyranoside	46.7 ± 0.5
*p*NP-mannopyranoside	ND
*p*NP-galactopyranoside	ND

[a]Specific activity was obtained from three repeated experiments.
[b]Not detected.

Substrate Specificity and Hydrolysis Products

rXylH was able to preferentially decompose various types of β-1,4-xylan polysaccharides (Table 2) in addition to xylooligosaccharides of xylotriose (X_3) to xylohexaose (X_6) (Table 3). However, it was found that carbohydrate polymers such as xyloglucan, soluble starch, pectin, locust bean gum, and carboxymethylcellulose were practically insensitive to the enzyme (Table 2). The susceptibility of xylosidic polysaccharides to rXylH was evaluated to be in the order of xylan from oat spelts > xylan from beechwood > xylan from birchwood > arabinoxylan from wheat. This substrate specificity of rXylH toward the xylosidic polysaccharides was similar to that of *Cellulosimicrobium* sp. strain HY-13 GH10 endo-β-1,4-xylanase [19] for the same substrates. On the other hand, it has been demonstrated that the GH10 endo-β-1,4-xylanases from *Streptomyces thermonitrificans* NTU-88 [8] and *Cohnella laeviribosi* HY-21 [20] efficiently degrade the carbohydrates in the order of xylan from oat spelts > xylan from birchwood > xylan from beechwood. Additionally, *B. halodurans* S7 GH10 endo-β-1,4-xylanase displayed high biocatalytic activity toward the carbohydrates in the order of xylan from birchwood > xylan from beechwood > xylan from oat spelts [26]. In this study, the specific activity of rXylH toward xylan from oat spelts was 97.2 U/mg, and this activity was approximately 1.85-fold higher than the hydrolytic activity (52.5 U/mg) of the enzyme toward arabinoxylan from wheat (Table 2). It is noteworthy that rXylH could hydrolyze β-1,3/β-1,4-D-glucan from barley, although its biocatalytic activity (2.3 U/mg) for the unrelated substrate was insignificant. These substrate specificities of rXylH strongly suggested that it was a particular GH10

Table 3. LC analysis of the hydrolysis products of xylosidic materials by rXylH.

Substrate	Composition (%) of products formed by hydrolysis reaction				
	X_2	X_3	X_4	X_5	X_6
X_2	100.0				
X_3	45.8	38.9	12.3	3.1	
X_4	34.1	38.3	22.7	5.0	
X_5	25.9	35.9	23.4	12.1	2.8
X_6	21.0	36.7	27.1	10.9	4.2
Birchwood xylan	71.2	28.8			

multifunctional enzyme having endo-β-1,4-xylanase activity together with β-1,3/β-1,4-glucanase activity, distinguishing it from other GH10 endo-β-1,4-xylanases. It is presumed that the ability of rXylH to degrade β-1,3/β-1,4-D-glucan from barley might be attributed to the active site cleft of the enzyme, which is large enough to bind to the substrate, as shown in *Paenibacillus* sp. strain E18 bifunctional GH10 enzyme (XynBE18) with endo-β-1,4-xylanase and β-1,3/β-1,4-glucanase activities [33]. Of the family 10 endo-β-1,4-xylanases, XynBE18 has only been reported to exhibit specific activities of 61.6 and 25.9 U/mg, respectively, toward both xylan from birchwood and β-1,3/β-1,4-D-glucan from barley.

In this study, compared with X_2, birchwood xylan and a series of xylooligosaccharides of xylotriose (X_3) to xylohexaose (X_6) were found to be readily degraded by rXylH (Table 3). Specifically, the enzyme degraded birchwood xylan to X_2 (71.2%) and X_3 (28.8%) without forming noticeable amounts of X_1 or xylooligomers [≥xylotetraose (X_4)]. The absence of X_1 as an end product in the reaction mixture strongly suggests that rXylH is a strict endo-β-1,4-xylanase, which generally hydrolyzes xylosidic substrates in an endo-fashion. In contrast, β-1,4-xylanases from *Streptomyces* sp. S9 [23] and *Clostridium acetobutylicum* ATCC 824 [2] predominantly produce X_1 from birchwood xylan as the hydrolysis product. It is also important to note that compared with birchwood xylan, biocatalytic degradation of X_3, X_4, and xylopentaose (X_5) resulted in the production of a mixture that contained longer xylooligosaccharides. For example, rXylH produced a mixture of xylooligosaccharides composed of X_2 (45.8%), X_3 (38.9%), X_4 (12.3%), and X_5 (3.1%) from X_3 used as the substrate of biocatalytic reaction. Moreover, enzymatic hydrolysis of X_4 yielded a mixture consisting of xylooligosaccharides of X_2 (34.1%), X_3 (38.3%), X_4 (22.7%), and X_5 (5.0%). It is now considered that these results might be due to the transxylosylation activity of rXylH, as previously shown in some retaining GH10 endo-β-1,4-xylanases [13, 20, 27] that can transfer sugars to another acceptor molecule. Similar phenomena related to the formation of longer xylooligosaccharides than substrates used in the biocatalytic reaction were also observed when the indicated xylooligosaccharides were hydrolyzed by GH10 endo-β-1,4-xylanases from some invertebrate-symbiotic bacteria [19, 21]. However, many endo-β-1,4-xylanases have been known to preferentially hydrolyze the respective xylooligomers to shorter molecules [12, 35, 37].

Similar to some GH10 enzymes, rXylH displayed relatively high hydrolytic activities toward *p*NP-sugar derivatives substituted with D-xylose or D-cellobiose, even though the enzyme did not cleave *p*NP-sugar derivatives substituted with D-glucose, D-mannose, or D-galactose (Table 2). In this case, the biocatalytic activity of rXylH toward *p*NP-cellobioside was assessed to be approximately 260.9 U/mg, which was higher than those of *Cellulosimicrobium* sp. strain HY-13 GH10 endo-β-1,4-xylanase [19] and *C. laeviribosi* HY-21 GH10 endo-β-1,4-xylanase [20] for the same chromogenic substrate (171.7 and 140.5 U/mg, respectively). However, this cleavage activity of rXylH for *p*NP-cellobioside was evaluated to be relatively low when compared with that (474.0 U/mg) of *S. thermocarboxydus* HY-15 GH10 endo-β-1,4-xylanase [21]. The results shown in Table 2 also indicated that the cleavage activity (46.7 U/mg) of rXylH toward *p*NP-xylopyranoside was approximately 20.3-fold and 6.2-fold higher than those of *S. thermocarboxydus* HY-15 GH10 endo-β-1,4-xylanase [21] and *Cellulosimicrobium* sp. strain HY-13 GH10 endo-β-1,4-xylanase [19], respectively. Considering that rXylH was not able to hydrolyze xylobiose (X_2) to two D-xylose (X_1) molecules (Table 3), it is believed that the cleavage activities of the enzyme toward *p*NP-cellobioside and *p*NP-xylopyranoside were originated from its relatively high transferase activity.

Binding Affinity of rXylH to Insoluble Polymers

It has been shown that endo-β-1,4-xylanases can bind to insoluble xylosidic substrates by their catalytic domain, regardless of the presence or absence of additional functional binding modules in the N-terminus and/or C-terminus regions [7, 19]. In addition, a variety of non-modular endo-β-1,4-xylanases with a single catalytic domain belonging to GH10 [2, 29, 38] and GH11 [16,34] families, which exhibited hydrolytic activity toward xylan polysaccharides, have been identified and biochemically characterized to date. However, the binding capacity of non-modular GH10 endo-β-1,4-xylanases to diverse insoluble polymers has not been well documented. Thus, through the use of diverse hydrophobic polymers with different microstructures, we extensively investigated the substrate binding ability of rXylH as a model endo-β-1,4-xylanase consisting of only a single catalytic GH10 domain. In this study, rXylH was tightly bound to some insoluble materials, such as Avicel, ivory nut mannan, and chitin, and also displayed relatively high binding affinity (>75%) to oat spelts xylan, chitosan, and poly(3-hydroxybutyrate) granules (Fig. 4). Conversely, the enzyme did not attach to curdlan from *A. faecalis*. These results clearly suggest that the catalytic GH10 domain in rXylH plays a considerable role in substrate binding as well as catalysis, and displays broad substrate binding affinity for a variety of structurally nonrelated hydrophobic materials,

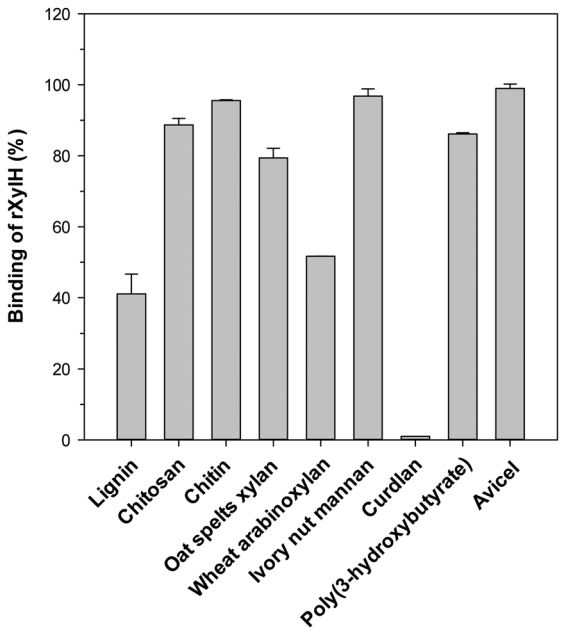

Fig. 4. Binding of rXylH to insoluble polymers.

together with xylan polysaccharides.

In conclusion, the unique characteristics of XylH shown in its primary amino acid sequence, broad pH adaptability, hydrolytic ability toward various carbohydrates and *p*NP-sugar derivatives, and substrate binding affinity for hydrophobic materials indicate that the enzyme is a novel biocatalyst that is considerably distinct from other functional GH10 homologs. XylH has broad substrate specificity, has been shown to be relatively active and stable in a wide pH range of 5.0–10.0, and is expected to be useful for industrial purposes, such as pulp bleaching and additives for animal feeds.

Acknowledgments

This work was supported by grants from the KRIBB Research Initiative Program (KGM2131413; KCS3071413) of the Ministry of Science, ICT & Future Planning, and a grant from the Ministry of Agriculture, Food and Rural Affairs (AGM0111213), Republic of Korea.

References

1. Adlakha N, Rajagopal R, Kumar S, Reddy VS, Yazdani SS. 2011. Synthesis and characterization of chimeric proteins based on cellulase and xylanase from an insect gut bacterium. *Appl. Environ. Microbiol.* **77:** 4859-4866.
2. Ali MK, Rudolph FB, Bennett GN. 2005. Characterization of thermostable Xyn10A enzyme from mesophilic *Clostridium acetobutylicum* ATCC 824. *J. Ind. Microbiol. Biotechnol.* **32:** 12-18.
3. Brune A, Friedrich M. 2000. Microecology of the termite gut: structure and function on a microscale. *Curr. Opin. Microbiol.* **3:** 263-269.
4. Calderón-Cortés N, Quesada M, Watanabe H, Cano-Camacho H, Oyama K. 2012. Endogenous plant cell wall digestion: a key mechanism in insect evolution. *Annu. Rev. Evol. Syst.* **43:** 45-71.
5. Cardona CA, Quintero JA, Paz IC. 2010. Production of bioethanol from sugarcane bagasse: status and perspectives. *Bioresour. Technol.* **101:** 4754-4766.
6. Cazemier AE, Verdoes JC, van Ooyen AJJ, Op den Camp HJM. 1999. Molecular and biochemical characterization of two xylanase-encoding genes from *Cellulomonas pachnodae*. *Appl. Environ. Microbiol.* **65:** 4099-4107.
7. Chen S, Kaufman MG, Miazgowicz KL, Bagdasarian M. 2013. Molecular characterization of a cold-active recombinant xylanase from *Flavobacterium johnsoniae* and its applicability in xylan hydrolysis. *Bioresour. Technol.* **128:** 145-155.
8. Cheng H-L, Tsai C-Y, Chen H-J, Yang S-S, Chen Y-C. 2009. The identification, purification, and characterization of STXF10 expressed in *Streptomyces thermonitrificans* NTU-88. *Appl. Microbiol. Biotechnol.* **82:** 681-689.
9. Collins T, Gerday C, Feller G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **29:** 3-23.
10. Dheeran P, Nandhagopal N, Kumar S, Jaiswal YK, Adhikari DK. 2012. A novel thermostable xylanase of *Paenibacillus macerans* IIPSP3 isolated from the termite gut. *J. Ind. Microbiol. Biotechnol*. **39:** 851-860.
11. Flint HJ, Bayer EA, Rincon MT, Lamed R, White BA. 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. *Nature Rev. Microbiol.* **6:** 121-131.
12. Fontes CMGA, Gilbert HJ, Hazlewood GP, Clarke JH, Prates JAM, Mckie VA, *et al*. 2000. A novel *Cellvibrio mixtus* family 10 xylanase that is both intracellular and expressed under non-inducing conditions. *Microbiology* **146:** 1959-1967.
13. Gallardo O, Diaz P, Pastor FIJ. 2003. Characterization of a *Paenibacillus* cell-associated xylanase with high activity on aryl-xylosides: a new subclass of family 10 xylanases. *Appl. Microbiol. Biotechnol*. **61:** 226-233.
14. Gírio FM, Fonseca C, Carvalheiro F, Duarte S, Marques S, Bogel-Lukasik R. 2010. Hemicelluloses for fuel ethanol: a review. *Bioresour. Technol.* **101:** 4775-4800.
15. Gupta N, Vohra RM, Hoondal GS. 1992. A thermostable extracellular xylanase from alkalophilic *Bacillus* sp. NG-27. *Biotechnol. Lett.* **14:** 1045-1046.
16. Heo S, Kwak J, Oh H-W, Park D-S, Bae KS, Shin D-H, Park H-Y. 2006. Characterization of an extracellular xylanase in

Paenibacillus sp. HY-8 isolated from an herbivorous longicorn beetle. *J. Microbiol. Biotechnol*. **16:** 1753-1759.
17. Huang S, Sheng P, Zhang H. 2012. Isolation and identification of cellulolytic bacteria from the gut of *Holotrichia parallela* larvae (Coleoptera: Scarabaeidae). *Int. J. Mol. Sci*. **13:** 2563-2577.
18. Keskar SS, Srinivasan MC, Deshpande VV. 1989. Chemical modification of a xylanase from a thermotolerant *Streptomyces*: evidence for essential tryptophan and cysteine residues at the active site. *Biochem. J.* **261:** 49-55.
19. Kim DY, Han MK, Park D-S, Lee JS, Oh H-W, Shin D-H, *et al*. 2009. Novel GH10 xylanase, with a fibronectin type 3 domain, from *Cellulosimicrobium* sp. strain HY-13, a bacterium in the gut of *Eisenia fetida*. *Appl. Environ. Microbiol*. **75:** 7275-7279.
20. Kim DY, Han MK, Oh H-W, Bae KS, Jeong T-S, Kim SU, *et al*. 2010. Novel intracellular GH10 xylanase from *Cohnella laeviribosi* HY-21: biocatalytic properties and alterations of substrate specificities by site-directed mutagenesis of Trp residues. *Bioresour. Technol*. **101:** 8814-8821.
21. Kim DY, Han MK, Oh H-W, Park D-S, Kim S-J, Lee S-G, *et al*. 2010. Catalytic properties of a GH10 endo-β-1,4-xylanase from *Streptomyces thermocarboxydus* HY-15 isolated from the gut of *Eisenia fetida*. *J. Mol. Catal. B Enzym*. **62:** 32-39.
22. Kim DY, Ham S-J, Kim HJ, Kim J, Lee M-H, Cho H-Y, *et al*. 2012. Novel modular endo-β-1,4-xylanase with transglycosylation activity from *Cellulosimicrobium* sp. strain HY-13 that is homologous to inverting GH family 6 enzymes. *Bioresour. Technol*. **107:** 25-32.
23. Li N, Meng K, Wang Y, Shi P, Luo H, Bai Y, *et al*. 2008. Cloning, expression, and characterization of a new xylanase with broad temperature adaptability from *Streptomyces* sp. S9. *Appl. Microbiol. Biotechnol*. **80:** 231-240.
24. Lombard V, Ramulu HG, Drula E, Coutinho PM, Henrissat B. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res.* DOI: 10.1093/nar/gkt1178.
25. Lynd LR, Weimer PJ, van Zyl WH, Pretorius IS. 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev*. **66:** 506-577.
26. Mamo G, Hatti-Kaul R, Mattiasson B. 2006. A thermostable alkaline active endo-β-1,4-xylanase from *Bacillus halodurans* S7: purification and characterization. *Enzyme Microb. Technol*. **39:** 1492-1498.
27. MacLeod AM, Lindhorst T, Withers SG, Warren RAJ. 1994. The acid/base catalyst in the exoglucanase/xylanase from *Cellulomonas fimi* is glutamic acid 127: evidence from detailed kinetic studies of mutants. *Biochemistry* **33:** 6371-6376.
28. Morrison M, Pope PB, Denman SE, McSweeney CS. 2009. Plant biomass degradation by gut microbiomes: more of the same or something new? *Curr. Opin. Biotechnol*. **20:** 358-363.
29. Oh H-W, Heo S-Y, Kim DY, Park D-S, Bae KS, Park H-Y. 2008. Biochemical characterization and sequence analysis of a xylanase produced by an exo-symbiotic bacterium of *Gryllotalpa orientalis*, *Cellulosimicrobium* sp. HY-12. *Antonie van Leeuwenhoek* **93:** 437-442.
30. Polizeli MLTM, Rizzatti ACS, Monti R, Terenzi HF, Jorge JA, Amorim DS. 2005. Xylanases from fungi: properties and industrial applications. *Appl. Microbiol. Biotechnol.* **67:** 577-591.
31. Roberge M, Shareck F, Morosoli R, Kluepfel D, Dupont C. 1999. Characterization of active-site aromatic residues in xylanase A from *Streptomyces lividans*. *Protein Eng*. **12:** 251-257.
32. Shi H, Zhang Y, Huang Y, Wang L, Wang Y, Ding H, Wang F. 2013. A novel highly thermostable xylanase stimulated by Ca^{2+} from *Thermotoga thermarum*: cloning, expression and characterization. *Biotechnol. Biofuels* **6:** 26.
33. Shi P, Tian J, Yuan T, Liu X, Huang H, Bai Y, *et al*. 2010. *Paenibacillus* sp. strain E18 bifunctional xylanase-glucanase with a single catalytic domain. *Appl. Environ. Microbiol.* **76:** 3620-3624.
34. Singh RK, Tiwari MK, Kim D, Kang YC, Ramachandran P, Lee J-K. 2013. Molecular cloning and characterization of a GH11 endoxylanase from *Chaetomium globosum*, and its use in enzymatic pretreatment of biomass. *Appl. Microbiol. Biotechnol.* **97:** 7205-7214.
35. Usui K, Suzuki T, Akisaka T, Kawai K. 2003. A cytoplasmic xylanase (XynX) of *Aeromonas caviae* ME-1 is released from the cytoplasm to the periplasm by osmotic downshock. *J. Biosci. Bioeng*. **95:** 488-495.
36. Wackett LP. 2008. Biomass to fuels *via* microbial transformations. *Curr. Opin. Chem. Biol*. **12:** 187-193.
37. Yan Q, Hao S, Jiang Z, Chen W. 2009. Properties of a xylanase from *Streptomyces matensis* being suitable for xylooligosaccharides production. *J. Mol. Catal. B Enzym.* **58:** 72-77.
38. Zhou J, Huang H, Meng K, Shi P, Wang Y, Luo H, *et al*. 2009. Molecular and biochemical characterization of a novel xylanase from the symbiotic *Sphingobacterium* sp. TN19. *Appl. Microbiol. Biotechnol.* **85:** 323-333.
39. Zolotnitsky G, Cogan U, Adir N, Solomon V, Shoham G, Shoham Y. 2004. Mapping glycoside hydrolase substrate subsites by isothermal titration calorimetry. *Proc. Natl. Acad. Sci. USA* **101:** 11275-11280.