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Characterization of a GH Family 43 β-Xylosidase Having a Novel Carbohydrate-binding Module from *Paenibacillus xylaniclasticus* Strain TW1

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Abstract: *Paenibacillus xylaniclasticus* **strain TW1, a gram-positive facultative anaerobic bacterium, was isolated as a xylanolytic microorganism from the wastes of a pineapple processing factory. A gene encoding one of its xylanolytic enzymes, a β-xylosidase, was cloned and sequenced. Sequence analysis revealed that this β-xylosidase, named *Px*Xyl43A, was composed of a glycoside hydrolase (GH) family 43 subfamily 12 catalytic module and an unknown function module (UM). The full-length *Px*Xyl43A (*Px*Xyl43A) was heterologously expressed in *Escherichia coli* and purified. Recombinant *Px*Xyl43A exhibited hydrolysis activity against both *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) and *p*-nitrophenyl-α-L-arabinofuranoside at specific activities of 250 and 310 mU/mg, respectively. The optimal reaction pH and temperature for *p*NPX hydrolysis were 7.1 and 54 °C, respectively. At pH 7.0 and 54 °C, the K_m and k_{cat} for *p*NPX were 1.2 mM and 2.8 ± 0.15 s^{-1}, respectively. It was also discovered that the recombinant unknown function module of *Px*Xyl43A (*Px*Xyl43A-UM) could bind to insoluble xylans like birchwood xylan and oat spelt xylan, whereas it did not bind to cellulosic substrates such as ball-milled cellulose, carboxymethyl cellulose or lichenan. The *Px*Xyl43A-UM's binding constant value K_a for oat spelt xylan was 2.0 × 10^{-5} M^{-1}. These results suggest that *Px*Xyl43A possesses a novel carbohydrate-binding module, named as CBM91, specific for xylan-containing polysaccharides.**

Key words: *Paenibacillus xylaniclasticus*, **β-xylosidase, glycoside hydrolase family 43, Carbohydrate-binding module**

INTRODUCTION

Lignocellulose is one of the most abundant polysaccharide biomass as a carbon source for sustainable energy production. For biofuels production, the rigid lignocellulose, which consists of cellulose, hemicellulose and lignin, is required to be degraded into fermentable monosaccharides. Lignocellulose degradation has been accomplished by variety of microbial enzymes, such as, cellulases, hemicellulases, β-glucosidases and β-xylosidases. Among them,

β-xylosidase is essential for complete digestion of hemicellulose, since it cleaves the β-glycoside bonds of xylan unit.[1] It is known that some of the β-xylosidases, which belong to glycoside hydrolase (GH) family 43, possess carbohydrate-binding module (CBM), that is non-catalytic sugar binding modules to facilitate the enzyme catalytic activity. So far, xylan-binding CBMs appended to GH family 43 β-xylosidases were identified and classified into CBM families 6, 13, and 35.[2)3)4)]

Paenibacillus xylaniclasticus strain TW1, a gram-positive facultative anaerobic bacterium, was isolated as a xylanolytic bacterium from sludge in an anaerobic digester fed with pineapple wastes in Thailand.[5] The partial genome sequencing of the TW1 strain was performed to show that this strain would possess many xylanolytic enzymes. The TW1 strain has been shown to produce many xylanolytic enzymes and extracellular multienzyme complexes when incubated with 0.5 % of birch wood xylan (BWX) and secretes cellulose-binding proteins when incubated with 0.5 % of corn hull.[6] Thus, the TW1 strain is a promising bacterium for degradation and saccharification of lignocellulosic biomass. The

TW1 strain showed especially high xylanolytic activity, since it could grow on oat spelt xylan (OSX) as the sole carbon source.[6] However, the various xylanolytic enzymes of the TW1 strain have yet to be identified and characterized. In this study, we focused on one of the putative β-xylosidase genes from the TW1 strain, named *Px*Xyl43A, because this gene contains an unknown function module at the C-terminus instead of a typical CBM. We investigated the xylanolytic activities of *Px*Xyl43A as well as the polysaccharide-binding activity of the unknown function module using purified recombinant proteins. Here, we report a novel CBM derived from *P. xylaniclasticus*.

MATERIALS AND METHODS

Strain, plasmid and media. *P. xylaniclasticus* strain TW1 was isolated previously from the wastes of a pineapple processing factory in Thailand.[5] The *Escherichia coli* strains αINV (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) and ME9806 (iVEC3) (National BioResource Project (NBRP), Mishima, Japan) were used as cloning hosts while *E. coli* JM109 and BL21(DE3)(TOYOBO CO., LTD., Japan) were used as protein expression hosts.[7] The pCR2.1 plasmid (Invitrogen) was used for cloning, and the pQE30 plasmid (QIAGEN Benelux B.V., Venlo, Netherland) and pET16b plasmid (Novagen Inc., Madison, WI, USA) were used for expression of recombinant His-tagged proteins. Transformed *E. coli* was cultivated in LB liguid medium supplemented with ampicillin (50 µg/mL). The recombinant proteins of the full length of *Px*Xyl43A (*Px*Xyl43A) and the unknown function module at the C-terminus of *Px*Xyl43A (*Px*Xyl43A-UM) were expressed using the plasmids pET16b-*Px*Xyl43A and pQE30-*Px*Xyl43A-UM, respectively.

Cloning of the full length of **Px*Xyl43A* *and the unknown function module at the C-terminal.*** A DNA fragment (1,585 bp) including the region encoding *Px*Xyl43A (1,560 bp) was obtained from genomic DNA of *P. xylaniclasticus* strain TW1 by PCR using primers #1 and #2 (Table 1). A DNA fragment of *Px*Xyl43A-UM (684 bp) was amplified from genomic DNA of *P. xylaniclasticus* strain TW1 using primers #7 and #8. The linear pET16b for iVEC cloning was amplified using primers #5 and #6. The DNA fragment of *Px*Xyl43A-UM was inserted into pCR2.1. After digestion with *Hind*III and *Bam*HI, the DNA fragment was ligated into the corresponding site of pQE30. The DNA fragment including the region encoding *Px*Xyl43A was amplified again using primers #3 and #4 and inserted into pET16b by *in vivo* cloning using *E. coli* strain ME9806 (iVEC3).[7] Each of the

inserted DNA fragments was sequenced to confirm the absence of mutations.

Expression and purification of recombinant proteins of **Px*Xyl43A.*** Transformed *E. coli* cells having pET16b-*Px*Xyl43A or pQE30-*Px*Xyl43A-UM were grown overnight at 37 ˚C in LB liguid medium supplemented with ampicillin (50 µg/mL). When the optical density of the cells at 600 nm reached 0.4, the lac operon trigger isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. After several hours of incubation, cells were collected by centrifugation at 12,000 rpm and disrupted with sonication in 50 mM sodium phosphate buffer (pH 7.0). The recombinant proteins were purified from the cell-free extracts with the Profinia system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's instructions. The purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined using a Protein Assay kit (Bio-Rad Laboratories) with bovine serum albumin (BSA) as the standard.

Substrate specificity assays of **Px*Xyl43A.*** The xylosidase activity of *Px*Xyl43A was measured by incubating the protein in the presence of 0.25 mM *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) in 50 mM sodium phosphate buffer (pH 7.0) at 54 ˚C for 10 min unless otherwise stated. The amount of *p*-nitrophenol released from *p*NPX was measured by the absorbance at 420 nm. All enzyme assays were repeated at least three times to confirm reproducibility. *p*NP-β-D-glucopyranoside (*p*NPG), *p*NP-α-L-arabinofranoside (*p*NPAf), and *p*NP-β-D-galactopyranoside (*p*NPGal) were used to determine the substrate specificity of *Px*Xyl43A. One unit (U) of enzyme activity for *p*NP-glycoside was defined as the enzyme amount required to liberate 1 µmol of *p*-nitrophenol per min. OSX was used as insoluble xylan at 1.5 % (w/v). After enzymatic reactions, reducing sugars from OSX were quantified using the 3,5-dinitrosalicylic acid method.[8]

Determination of optimal pH and temperature and thermo-stability of **Px*Xyl43A.*** To determine the optimal pH for β-xylosidase activity, the recombinant *Px*Xyl43A solution was incubated for 5 min at different pHs (pH 3.0 to pH 10) using Britton-Robinson's buffer at 54 ˚C in the presence of 0.25 mM *p*NPX as a substrate. For optimal temperature determination, *Px*Xyl43A was incubated at different temperatures (15 to 75 ˚C) for 5 min in 50 mM potassium phosphate buffer (pH 7.0) in the presence of 0.25 mM *p*NPX as a substrate. For thermostability assay, *Px*Xyl43A were incubated at different temperatures (40 to 57 ˚C) for 2h in 50 mM potassium phosphate buffer (pH 7.0), and then the residual β-xylosidase activity were determined. All enzyme assays

Table 1. List of primers.

1	AACAGTATAGCAGTTAAATACGAAA
2	TTAGGACAATCCTCATTAAGCTCCG
3	CGGCCATATCGAAGGTCGTCATATGAACAGTATAGCAGTTAAATA
4	GCCCCAAGGGGGTTATGCTAGTTAGGACAATCCTCATTAAGCTCCG
5	CATATGACGACCTTCGATATGGC
6	TAACTAGCATAACCCCTTGGGG
7	GGATCCATGAATGTCGAAATTCTCGCAGGA
8	AAGCTTTCAAGACAGCGGTGTATAGTCCAG

were repeated at least three times to confirm reproducibility.

***Kinetic analysis of β-xylosidase activity of Px*Xyl43A* for pNPX.** To determine the reaction kinetics of *Px*Xyl43A for *p*NPX, the reaction was performed at 54 ˚C in 50 mM potassium phosphate buffer (pH 7.0) for 1.5 min in the presence of different concentrations of *p*NPX (1.8 to 9.0 mM).

Assessment of the inhibitory effect of xylose on β-xylosidase activity of Px*Xyl43A*. The inhibitory effect of excess xylose against the β-xylosidase activity of *Px*Xyl43A was assessed using 0.25 mM *p*NPX as a substrate under the presence of different concentrations of xylose (10 to 200 mM) at 54 ˚C for 5 min.

Analysis of hydrolysis product of xylooligosaccharides. Xylooligosaccharides with different molecular weights, xylobiose (X2) (Wako), xylotriose (X3) (Wako), xylotetraose (X4) (Megazyme), xylopentaose (X5) (Megazyme) and xylohexaose (X6) (Megazyme) (10 μg of each), were incubated with 0.1 U of purified *Px*Xyl43A at 54 ˚C for 1 h in 10 μL of 50 mM potassium phosphate buffer (pH 7.0). The reaction products were analyzed by thin-layer chromatography (TLC) on a DC-Fertigplatten SIL G-25 plate (Macherey-Nadgel) developed with a 1-butanol/acetic acid/water (1:2:1, v/v/v) solvent mixture. The products were visualized by soaking the plate in methanol-sulfuric acid solution (5 % sulfuric acid in methanol) and baking at 130 ˚C for several minutes.

Macroarray assay for Px*Xyl43A-UM* binding to polysaccharides. A macroarray assay of the recombinant protein of the unknown function module (*Px*Xyl43A-UM) was performed using polysaccharides, including BWX, OSX, ball-milled cellulose (BMC), carboxymethyl cellulose (CMC) and lichenan as the substrates, according to previously reported procedures.[9)10)] Aliquots (1 μL of 0.2−10 μg/μL) of each polysaccharide were applied to an untreated nitrocellulose sheet (MS-Millipore membrane filters) (Millipore Co., Burlington, MA, USA). The nitrocellulose sheet was left at room temperature for 30 min to dry. After blocking with 5 % (w/v) skim milk in 5 × phosphate buffer (PBS) for 1 h, the sheet was incubated with *Px*Xyl43A-UM (12.5 μg/mL) in 5 × PBS for 1 h. After extensively washing with 5 × PBS, the nitrocellulose sheet was incubated at room temperature with a 1,000-fold dilution of anti-His horseradish peroxidase (HRP)-conjugated antibody (Cat#1014981) (QIAGEN) in 5 × PBS for 1 h. The sheet was washed with distilled water and incubated with freshly prepared HRP substrate (0.05 % 3,3′-diaminobenzidine tetrahydrochloride and 0.005 % H_2O_2) to detect *Px*Xyl43A-UM bound to each polysaccharide. The reaction was stopped by washing the nitrocellulose sheet with distilled water.

Binding assay of Px*Xyl43A-UM* for polysaccharides. OSX, BWX, BMC, and lichenan were used as substrates, after washed three times with 50 mM potassium phosphate buffer (pH 7.0) to remove soluble saccharides. The protein solutions (0.4 mg/mL) of *Px*Xyl43A-UM or BSA as a control were incubated with 5 mg of each substrate for 10 min at room temperature in 50 mM potassium phosphate buffer (pH 7.0). After centrifugation (15,000 rpm, 5 min, 4 ˚C), the supernatant was collected as the unbound proteins, and the precipitate rinsed with 50 mM potassium phosphate buffer (pH 7.0) as the bound proteins. The unbound and bound proteins were denatured with SDS sample buffer containing 2 % SDS and 1 % β-mercaptoethanol and then analyzed by SDS-PAGE.

Determination of the binding affinity parameters of Px*Xyl43A-UM* for OSX. Different concentrations (1.6 μM−16 μM) of *Px*Xyl43A-UM were added to 20 mL of 3 % OSX and incubated for 5 min on ice with gentle mixing. The solutions were centrifuged (15,000 rpm, 2 min, 25 ˚C), and the free protein concentration in the supernatant was measured by Lowry's method.[11)] The bound protein concentration was determined from the difference between the total protein concentration and the protein concentration in the supernatant. Absorbance parameters were estimated using the following equation based on the Langmuir adsorption isotherm: $1/[PC] = (1/K_a[PC]_{max})(1/[PC]) + (1/[PC]_{max})$, where [PC], where [PC], K_a (M^{-1}) and $[PC]_{max}$ are the concentration of the protein complex with carbohydrate, the equilibrium absorption constant and the maximum amount of protein bound to polysaccharide, respectively.[12)]

RESULTS AND DISCUSSION

DNA sequence analysis of Px*Xyl43A*.

Sequence analysis showed that the open reading frame of the *Px*Xyl43A gene consists of 1,560 nucleotides encoding a protein of 520 amino acids with a predicted molecular mass of 57 kDa (Fig. 1A). The DNA sequence coding *Px*Xyl43A was registered with DDBJ with the accession number LC648486. A BLAST homology analysis indicated that the first half of the DNA sequence of *Px*Xyl43A* (1−881 bp) was homologous to that of the GH family 43 enzymes, with 72 % similarity to GH43_XYL1 from *Paenibacillus lautus* and 69 % similarity to the enzyme from *Paenibacillus* sp. JDR-2. Phylogenetic analysis with eleven GH43 enzymes, whose three-dimensional structures are registered in the PDB, indicated that *Px*Xyl43A belongs to GH43 subfamily 12 (Fig. 1B). However, there was no homologous sequence match to the latter half of the sequence of the *Px*Xyl43A* gene (882−1,560 bp). The signal peptide analysis using SignalP 5.0 predicted that *Px*Xyl43A has no signal peptide, suggesting either that this protein is an intracellular enzyme or that this bacterium has another expression system independent of signal peptides. Thus, *Px*Xyl43A is expected to be an intercellular enzyme belonging to GH43 subfamily 12, defined as being composed of a GH43 β-xylosidase catalytic module at the N-terminus and an unknown-function module, named UM, at the C-terminus (Fig. 1A). According to the database, some of the known GH43 enzymes have a catalytic module at the N-terminus and an additional unknown-function module, similar to *Px*Xyl43A-UM, at the C-terminus.[13)14)15)16)17)] However, the amino acid sequences of these unknown-function modules did not show similarity to that of the function-characterized modules, classified as CBM. Therefore, we decided to characterize the catalytic activity of the full-length *Px*Xyl43A as well as the carbohydrate-binding activity of the unknown-function module (UM) derived from *Px*Xyl43A.

Cloning, Expression and purification of Px*Xyl43A*.

The amplified DNA encoding the full-length protein (*Px*Xyl43A) and the unknown-function module (*Px*Xyl43A-UM) were 1,585 and 684 bp, respectively (Fig. 1A). The molecular sizes of the recombinant proteins of *Px*Xyl43A
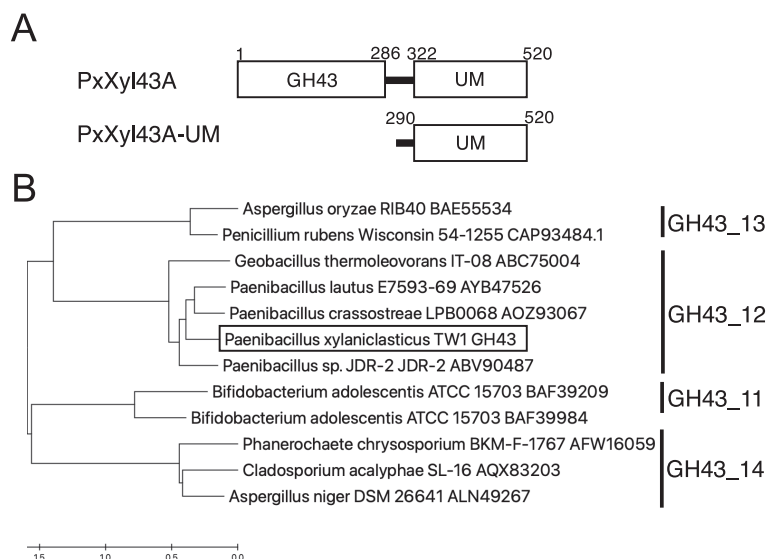
Fig. 1. The domain structure of *Px*Xyl43A and the phylogenetic analysis with GH43 enzymes.

A: Schematic diagram of *Px*Xyl43A and its recombinants, *Px*GH43 and *Px*GH43-UM. The numbers represent the number of amino acids. *Px*GH43 family proteins consist of a GH43 catalytic domain (GH43) and an unknown function module (UM), which are connecting by a linker peptide between base pairs 287 and 321. B: Phylogenetic tree of GH43 subfamily 11 to 14. Each alignment was obtained using NCBI. The phylogenetic tree was built using the neighbor-joining method in Mega X. The following enzymes were used in this analysis: GH family 43 protein from *Cladosporium acalyphae* SL-16 (AQX83203), xylosidase/arabinofuranosidase from *Aspergillus niger* DSM 26641 (ALN49267), β-xylosidase/α-L-arabinofuranosidase from *Phanerochaete chrysosporium* BKM-F-1767 (AFW16059), two β-1,4-xylosidases (BAF39209 and BAF39984) from *Bifidobacterium adolescentis* ATCC 15703, unnamed protein product from *Aspergillus oryzae* RIB40 (BAE55534), Pc16g08140 from *Penicillium rubens* Wisconsin 54-1255 (CAP93484), β-xylosidase from *Geobacillus thermoleovorans* IT-08 (ABC75004), XynB from *Paenibacillus* sp. JDR-2 (ABV90487), GH family 43 protein from *Paenibacillus lautus* E7593-69 (AYB47526), GH family 43 protein from *Paenibacillus crassostreae* LPB0068 (AOZ93067).

and *Px*Xyl43A-UM were estimated at 57 and 25 kDa, respectively by SDS-PAGE, corresponding to the predicted molecular sizes (data not shown). The concentration of purified recombinant proteins of *Px*Xyl43A and *Px*Xyl43A-UM were 11 and 1.1 mg/mL, respectively.

Assay for β-xylosidase activity of Px*Xyl43A*.

The β-xylosidase activity of the recombinant *Px*Xyl43A toward *p*NPX as a soluble substrate was measured. As a result, *Px*Xyl43A showed hydrolytic activity for *p*NPX, with a specific activity of 250 ± 37 mU/mg. The kinetic parameters K_m and k_{cat} of the β-xylosidase activity of *Px*Xyl43A for *p*NPX were 1.2 mM and 2.8 ± 0.15 s^{-1}, respectively. As the k_{cat} values for *p*NPX of other GH43 β-xylosidases were reported to be 1.3−57 s^{-1}, *Px*Xyl43A has a relatively slow turnover rate for *p*NPX compared to other GH43 β-xylosidases.[18)19)20)21)22)] The K_m data also suggest that *Px*Xyl43A has relatively high affinity for *p*NPX, as the K_m values of other GH43 β-xylosidases for *p*NPX were 0.38−17 mM.[18)19)20)23)] The β-xylosidase activity of *Px*Xyl43A toward insoluble xylan OSX was also tested because the TW1 strain possesses high xylanolytic activity toward OSX.[6)] It has been reported that some GH43 enzymes have β-xylosidase activity toward insoluble xylan.[24)25)26)] However, it was found that *Px*Xyl43A did not show β-xylosidase activity toward OSX (data not shown). These results suggest that the TW1 strain possesses other β-xylosidases responsible for the degradation of insoluble xylan.

Substrate specificity of Px*Xyl43A*.

To assess the substrate specificity of *Px*Xyl43A for sugars other than xylose, the hydrolytic activities toward *p*NPAf, *p*NPG, and *p*NPGal were also measured. Some of the GH43

enzymes from the rumen metagenome and from *Bacillus* sp. HJ14 exhibit activity for both *p*NPX and *p*NPAf while some GH43 enzymes possess hydrolytic activity toward only one of the two.[21)22)24)27)] As the exception, the GH43 enzyme from *Geobacillus stearothermophilus* T-6 hydrolyzes a wide variety of *p*NP substrates: namely, *p*NPX, *p*NPAf, *p*NPG, *p*NPGal, *p*NP-α-L-rhamnopyranoside, *p*NP-β-D-fucopyranoside, and *p*NP-β-D-mannopyranoside.[19)] We found that *Px*Xyl43A showed hydrolytic activity for *p*NPAf, but not for *p*NPG or *p*NPGal. The specific activity for *p*NPAf was determined as 310 ± 29 mU/mg, a value comparable to that of the activity for *p*NPX (250 ± 37 mU/mg). Thus, *Px*Xyl43A is a GH43 enzyme that acts on both *p*NPX and *p*NPAf. However, *Px*Xyl43A did not show hydrolytic activity for *p*NPGal and *p*NPG, similar to most other reported GH43 enzymes.
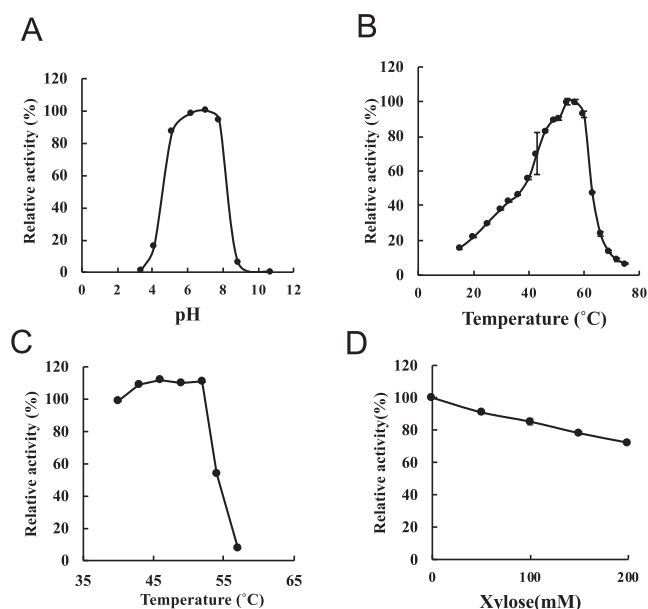
Optimal pH and temperature and thermostability of Px*Xyl43A*.

The optimal reaction condition of *Px*Xyl43A was determined using *p*NPX as a substrate. The data showed that the optimal pH of *Px*Xyl43A was 7.0 and that over 80 % of its maximum activity was retained at pH 5.0 to 8.0 (Fig. 2A). The optimal pHs of other GH43 β-xylosidases have been reported to be pH 5.5 to pH 7.5, so the optimal pH of *Px*Xyl43A falls within the usual range.[19)20)21)22)28)29)] The optimal temperature of *Px*Xyl43A was 54 ˚C, and over 50 % of its maximum activity was retained at temperatures from 40 to 60 ˚C (Fig. 2B). Similarly, the optimal reaction temperatures reported for *p*NPX of GH43 β-xylosidases from other mesophilic microbes are 50 to 65 ˚C.[19)20)29)30)] As to the thermostability, the activity of *Px*Xyl43A was stably maintained at 40 to 52 ˚C, but decreased drastically at temperatures higher than 52 ˚C (Fig. 2C). Considering that the optimal growth temperature of

P. xylaniclasticus TW1 is around 40 ˚C, it seems reasonable that *Px*Xyl43A exhibits its enzymatic activity at this temperature.[5] Thus, the data indicated that *Px*Xyl43A possesses thermal resistance up to 52 ˚C.

Stability of β-xylosidase activity of Px*Xyl43A* in the presence of high xylose.

It has been reported that most GH43 enzymes show lower β-xylosidase activity in the presence of high concentrations of xylose. [23)31)] For example, a GH43 subfamily 12 enzyme from *Lactobacillus brevis*, XynB2, lost 66 % of its β-xylosidase activity in the presence of 100 mM xylose.[23)] By contrast, *Px*Xyl43A retained 85 % of its β-xylosidase activity in the presence of 100 mM of xylose and 74 % of its activity at 200 mM xylose (Fig. 2D). These data indicate that *Px*Xyl43A possesses high tolerance for product inhibition by D-xylose. This characteristic of *Px*Xyl43A would be advantageous for lignocellulosic biomass saccharification in the presence of xylose at high concentrations.

Analysis of hydrolysis products.

To study the mode of action of *Px*Xyl43A on different-length xylooligosaccharides, reactions using X2, X3, X4, X5, and X6 as substrates were analyzed by TLC (Fig. 3). When using X2 as a substrate, xylose was released as a product of *Px*Xyl43A after 2 h reaction and increased every hour. When X3 was used as a substrate, xylose was released slowly and detected after 4 h reaction. On the other hand, when X4, X5, and X6 were used as substrates, no reaction products were detected. These results suggest that *Px*Xyl43A is an exo-acting enzyme which releases xylose from small xylooligosaccharides up to DP 3.

Binding specificity of Px*Xyl43A-UM* for insoluble polysaccharides.

To investigate the binding specificity and affinity of *Px*Xyl43A-UM for insoluble polysaccharides, a binding test and macroarray assay were performed (Figs. 4A and 4B). For the binding test, BWX, OSX, BMC or lichenan were

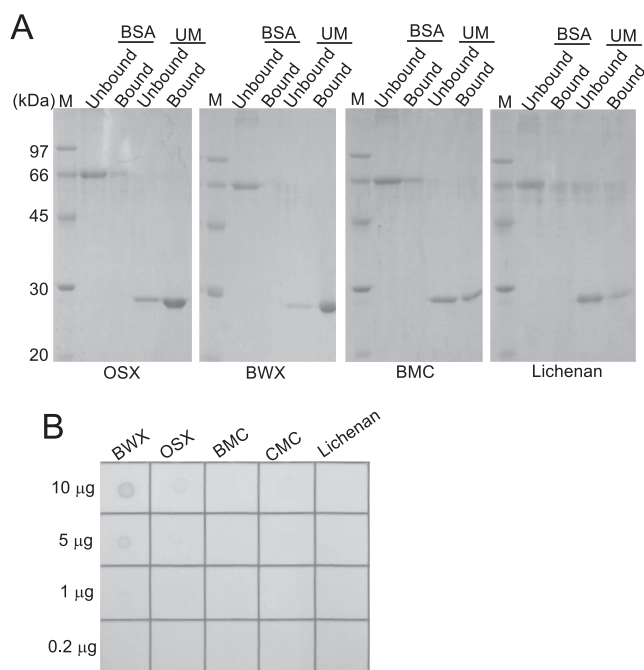
Fig. 2. Characterization of β-xylosidase activity of PxGH43.
The optimal pH (A), optimal temperature (B), thermostability (C) and effect of xylose concentration (D) were determined for the β-xylosidase activity of *Px*GH43 in the presence of 0.25 mM *p*NPX as a substrate. Values are the means of triplicate experiments ± the standard deviations.

Fig. 4. Binding assay of *Px*GH43-UM to insoluble polysaccharides.
A, The binding of *Px*GH43-UM to insoluble polysaccharides, BMS, OSX, BWX and lichenan, were analyzed by SDS-PAGE using BSA as a control. M, molecular weight marker. B, Macroarray assay of *Px*GH43-UM binding to each insoluble polysaccharide (0.2−10 μg): BWX, OSX, BMC, CMC and lichenan.
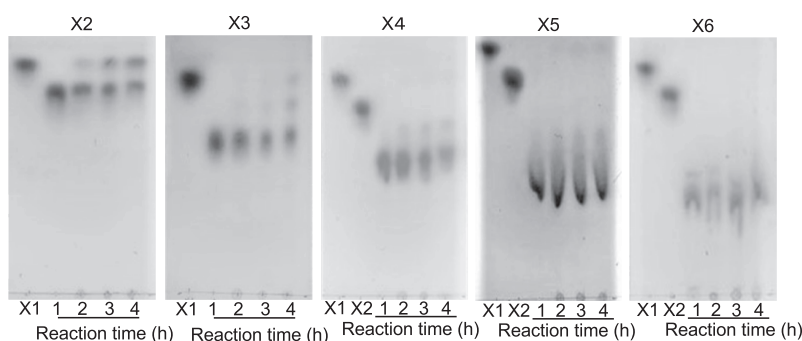
Fig. 3. Thin-layer chromatography of hydrolysis products of *Px*GH43 released from xyloorigosaccharides.
Reactions were carried out for 1−4 h using 1 mg/mL of each oligosaccharide substrate: X2, X3, X4, X5 or X6. Xylose (X1) and X2 were applied as standards.

used as insoluble polysaccharides (Fig. 4A). In cases of OSX and BWX as insoluble xylan, almost all the *Px*Xyl43A-UM protein was detected in the bound fraction, whereas BSA was detected mostly in the unbound fraction (Fig. 4A). These results suggest that *Px*Xyl43A-UM possesses binding activity to insoluble xylan. On the other hand, *Px*Xyl43A-UM did not bind to cellulosic substrates, such as BMC and lichenan (Fig. 4A). The macroarray assay confirmed that *Px*Xyl43A-UM bound to BWX and OSX but not to BMC, CMC or lichenan (Fig. 4B). Among these polysaccharides, *Px*Xyl43A-UM showed the highest affinity for BWX. These results suggest that *Px*Xyl43A-UM specifically binds to insoluble xylan.

Binding affinity of Px*Xyl43A-UM* for OSX.

Different concentrations of *Px*Xyl43A-UM were incubated with OSX, and the bound protein concentration was determined from the difference between the total protein concentration and the protein concentration in the supernatant. The binding constant value (K_a) of *Px*Xyl43A-UM for OSX was determined. As a result, the K_a value was 2.0×10^5 M^{-1} and $[PC]_{max}$ was 10 μmol/g for 0.5 g/L OSX. This K_a value is comparable to that of the already reported xylan-specific CBMs, belonging to CBM families 6, 13, and 35.[2)3)4)] The amino acid sequence of *Px*Xyl43A-UM showed no remarkable similarity to those of these already characterized CBMs. There is a report that GH43 β-xylosidase from *Thermobifida fusca* also has a C-terminus additional module, which is 30 % identical to *Px*Xyl43A-UM. However, in this case, the C-terminus module seemed not to be required for xylan-binding activity of the enzyme; indeed, no xylan-specific binding was shown by this module.[14)] Our present study shows that *Px*Xyl43A-UM is a novel CBM that specifically binds to xylan, the first representative of CBM91. However, the physiological significance of *Px*Xyl43A-UM̓s binding ability for insoluble xylan is not clear, because *Px*Xyl43A itself showed no β-xylosidase activity toward insoluble xylan. In addition, *Px*Xyl43A seems to be an intracellular enzyme, since it does not possess a sequence for a typical signal peptide. There are some GH43 β-xylosidases, CoXyl43A from a compost metagenome and XacGH43_1 from *Xanthomonas citri*, that do not have C-terminal module.[32)33)] Hence, it is likely that the xylan-binding ability may not be involved in GH43 β-xylosidase activity. Nevertheless, the xylan-binding activity as well as the β-xylosidase activity of *Px*Xyl43A could be utilized as a tool for efficient saccharification of xylan-containing biomass by combination with other degradative enzymes for insoluble xylan.

CONFLICTS OF INTEREST

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