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Screening, identification, and characterization of a GH43 family β-xylosidase/α-arabinofuranosidase from a compost microbial metagenome

Tomohiko Matsuzawa[1] · **Satoshi Kaneko**[2,3] · **Katsuro Yaoi**[1]

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Abstract A putative glycoside hydrolase family 43 β-xylosidase/α-arabinofuranosidase (CoXyl43) that promotes plant biomass saccharification was isolated via functional screening of a compost microbial metagenomic library and characterized. CoXyl43 promoted the saccharification of plant biomasses, including xylans (xylan and arabinoxylan), rice straw, and *Erianthus*, by degrading xylooligosaccharide residues to monosaccharide residues. The recombinant CoXyl43 protein exhibited both β-xylosidase and α-arabinofuranosidase activities for chromogenic substrates, with optimal activity at pH 7.5 and 55 °C. Both of these activities were inactivated by ethanol, dimethylsulfoxide, and zinc and copper ions but were activated by manganese ions. Only the β-xylosidase activity of recombinant CoXyl43 was enhanced in the presence of calcium ions. These results indicate that CoXyl43 exhibits unique enzymatic properties useful for biomass saccharification.

Keywords β-xylosidase · α-arabinofuranosidase · Biomass · Metagenome · Glycoside hydrolase family 43

✉ Katsuro Yaoi
k-yaoi@aist.go.jp

[1] Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan

[2] Food Biotechnology Division, National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

[3] Department of Subtropical Biochemistry and Biotechnology, Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan

Introduction

The replacement of fossil fuels on a global scale has the potential to solve multiple environmental problems in addition to fossil fuel depletion. Lignocellulosic biomass materials, such as various agricultural residues (e.g., rice straw, corn stover, and timber from forest thinning), can be converted into renewable biofuels and biochemicals. Lignocellulosic biomass is composed of cellulose, hemicelluloses, and lignin (Lynd et al. 2002) and is available in large quantities. Cellulose consists of linear chains of β-1,4-linked glucose and is the most abundant component of lignocellulosic biomass. Xylans are hemicelluloses and are the most common hetero-polysaccharides in lignocellulosic biomass (Saha 2003). Xylans are composed of homopolymeric backbone chains of β-1,4-linked xylopyranose units and may contain arabinose, glucuronic acid, and other small sugars (Table 1) (Kormelink and Voragen 1993; Saha 2003). In natural environments, lignocellulosic biomass is degraded by enzymes such as glycoside hydrolase, which is produced by environmental microorganisms. For example, cellulose is hydrolyzed by cellobiohydrolase (Teeri et al. 1983, 1987) and endo-β-1,4-glucanase (Penttilä et al. 1986; Okada et al. 1998; Saloheimo et al. 1988). Xylans are hydrolyzed by xylanase (Tenkanen et al. 1992; Xu et al. 1998) and xylosidase (Shallom et al. 2005). These glycoside hydrolases are vital for the saccharification and degradation of plant biomass.

The filamentous fungus, *Trichoderma reesei* (also known as *Hypocrea jecorina*), produces cellulases and hemicellulases in large quantities and efficiently degrades cellulosic biomass materials. Many mutants that produce large amounts of cellulase have been generated from wild-type *T. reesei* (Peterson and Nevalainen 2012).

Table 1 Monosaccharide compositions of alkaline-pretreated rice straw, alkaline-pretreated *Erianthus*, birch wood xylan, and wheat arabinoxylan

		Alkaline-pretreated rice straw	Alkaline-pretreated *Erianthus*	Birch wood xylan	Wheat arabinoxylan
Monosaccharide composition (%)	Glucose	66.1	70.9	1.4	0.3
	Xylose	26.7	24.5	89.3	65.8
	Arabinose	5.2	3.5	1.0	33.5
	Mannose	0.2	0.1	n.d.	0.1
	Galactose	1.4	0.8	n.d.	0.2
	Rhamnose	0.3	0.1	n.d.	n.d.
	others	0.1	0.1	8.3	n.d.
References		This study	This study	Kormelink and Voragen (1993)	Gruppen et al. (1992)

n.d. not detected

T. reesei PC-3-7, isolated from the QM9414 strain, is a cellulose hyperproducing mutant (Nogawa et al. 2001). Although *T. reesei* produces large amounts of cellulases and hemicellulases, these enzymes are too costly for use in commercial cellulosic biomass saccharification. Therefore, the discovery of new cellulases is important to facilitate biomass saccharification.

Although environmental microorganisms can be used to produce various glycoside hydrolases for the commercial degradation of lignocellulosic biomass, more than 99 % of environmental microorganisms are difficult to culture and have not been fully characterized (Torsvik and Øvreås 2002; Kimura et al. 2010). This indicates that the vast majority of microbial resources have not been accessed for biotechnology (Handelsman et al. 1998). This limitation can be overcome to a large extent by metagenomics, which is the culture-independent genomic analysis of microorganisms. Metagenomics has been used to screen for novel microbial enzymes in forest soil (Lee et al. 2008), activated sludge (Suenaga et al. 2007), marine environments (Okamura et al. 2010), and mammalian rumen (Beloqui et al. 2006; Ferrer et al. 2012).

In this study, a metagenomic approach was used to identify an enzyme that acts synergistically with *T. reesei* cellulase enzymes to improve the efficiency of lignocellulose saccharification. Genes encoding glycoside hydrolases were isolated from a compost metagenomic library using a chromogenic (*p*-nitrophenyl (*p*NP)) substrate mixture (*p*NP-β-D-lactopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-mannopyranoside, and *p*NP-β-D-galactopyranoside). Among the isolated hydrolases was a putative glycoside hydrolase family 43 (GH43) protein (named CoXyl43) that enhanced the saccharification of lignocellulosic biomass and xylans using *T. reesei* cellulase enzymes. CoXyl43 had bifunctional β-xylosidase/α-arabinofuranosidase activity and was able to hydrolyze xylooligosaccharide intermediates produced by *T. reesei* cellulases and hemicellulases into xylose.

Materials and methods

Materials

Xylan from birch wood, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-L-arabinopyranoside, *p*NP-β-D-cellobioside, *p*NP-α-L-fucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-L-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-α-L-rhamnopyranoside, and *p*NP-α-D-xylopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). *p*NP-α-D-galactopyranoside, *p*NP-α-D-glucopyranoside, and *p*NP-β-D-xylopyranoside were purchased from Nacalai Tesque (Kyoto, Japan). *p*NP-α-D-mannopyranoside was purchased from Wako Pure Chemical Industries (Osaka, Japan). *p*NP-β-D-xylopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-β-D-cellobioside, *p*NP-β-D-fucopyranoside, *p*NP-β-L-fucopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-galactopyranoside, and *p*NP-β-D-glucopyranoside were dissolved in water at a final concentration of 20 mM. *p*NP-α-L-arabinopyranoside, *p*NP-β-L-arabinopyranoside, *p*NP-α-L-fucopyranoside, *p*NP-β-D-mannopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-α-L-rhamnopyranoside, *p*NP-α-D-xylopyranoside, and *p*NP-α-D-glucopyranoside were dissolved in 25 % dimethyl sulfoxide (DMSO) at a final concentration of 20 mM. Arabinoxylan from wheat flour (low viscosity) was obtained from Megazyme (Wicklow, Ireland). Arabinofuranosyl-xylooligosaccharides, *O*-α-L-arabinofuranosyl-(1→3)-*O*-β-xylopyranosyl-(1→4)-D-xylopyranose (Ara*f*-X2) and *O*-β-D-xylopyranosyl-(1→4)-[*O*-α-L-arabinofuranosyl-(1→3)]-*O*-β-xylopyranosyl-(1→4)-D-xylopyranose (Ara*f*-X3) were prepared as reported previously (Fujimoto et al. 2004).

Biomass pretreatment

Pretreated plant biomass for enzymatic saccharification was prepared by the alkaline treatment of rice straw or *Erianthus*

as described previously with some modifications (Kawai et al. 2012). Alkaline-pretreated rice straw was treated with 0.5 % sodium hydroxide at 100 °C for 5 min. Alkaline-pretreated *Erianthus* was treated with 1 % sodium hydroxide at 120 °C for 5 min. The compositions of alkaline-pretreated rice straw and *Erianthus* were determined by acid hydrolysis and high-performance liquid chromatography (HPLC) according to the procedure published by NREL (http://www.nrel.gov/biomass/analytical_procedures.html). The monosaccharide compositions of alkaline-pretreated rice straw and *Erianthus* are listed in Table 1.

Construction of metagenomic library and screening of glycoside hydrolases with a positive effect on biomass saccharification

Metagenomic DNA extracted from composts was integrated into p18GFP plasmid vectors as described previously (Uchiyama et al. 2013).

Escherichia coli DH10B (Life Technologies Corporation, Carlsbad, CA, USA) harboring the metagenomic library were selected on Luria-Bertani (LB) agar plates containing ampicillin (LB + Amp), and 20 colonies were inoculated together into the same well of 96-well plates containing 900 μl LB + Amp medium with 10 μM isopropyl thiogalactoside (IPTG). After overnight cultivation at 37 °C, cells were collected by centrifugation (4000 rpm, 5 min) and resuspended in 220 μl Milli-Q water. Ten microliters of each cell suspension was mixed with 10 μl of a *p*NP substrate mixture (1 mM *p*NP-β-D-lactopyranoside, 1 mM *p*NP-β-D-xylopyranoside, 1 mM *p*NP-β-D-mannopyranoside, and 1 mM *p*NP-β-D-galactopyranoside) and incubated overnight at room temperature. After incubation, wells that had developed a yellow color, derived from *p*NP, were selected (*p*NP substrate screening). To isolate individual clones from the selected wells, a 20-clone mixture was diluted and cultured on LB + Amp plates. Clones that degraded the *p*NP substrate were selected (*p*NP substrate positive clones).

*p*NP substrate positive clones were then inoculated into 900 μl LB + Amp medium containing 10 μM IPTG. After overnight cultivation at 37 °C, cells were collected and resuspended in 145 μl Milli-Q water and 10 μl of 10× BugBuster (Novagen, Madison, WI, USA). The cell suspensions were incubated for 30 min at room temperature for protein extraction, and soluble fractions (4000 rpm, 20 min, 4 °C) of cell lysates were collected. Ten-microliter aliquots of the soluble fractions were mixed with 100 μl biomass premix (100 mM sodium acetate buffer (pH 5.0), 42.2 % (*w*/*v*) alkaline-treated rice straw, 0.02 % sodium azide), 70 μl 100 mM sodium acetate buffer (pH 5.0), and 20 μl 10 μg/ml cellulase CTec2 (Novozyme, Bagsværd, Denmark) and incubated overnight at 37 °C. To determine the polysaccharide degradation activity, reducing sugars were measured using the 3,5-dinitrosalicylic acid (DNS) reagent method (Miller 1959). Inserted DNA fragments encoding putative glycoside hydrolase, termed *coxyl43*, that had a positive effect on biomass saccharification were sequenced. The nucleotide sequence of *coxyl43* was deposited in DDBJ/EMBL/GenBank under accession number LC025936.

Cloning, expression, and purification of glycoside hydrolase

To express the mature region of CoXyl43 in *E. coli*, the *coxyl43* gene was synthesized in its codon-optimized form using DNA2.0 Inc. (Menlo Park, CA, USA) (DDBJ/EMBL/GenBank accession number LC027446) and cloned into a pET-28b vector (Novagen) digested with *Nde*I and *Xho*I. *E. coli* BL21(DE3) (NIPPON GENE, Tokyo, Japan) harboring the pET28b-putative glycoside hydrolase gene was cultured overnight in 100 ml Overnight Expression Instant LB medium (Novagen) containing 20 μg/ml kanamycin at 37 °C. After cultivation, the cells were harvested by centrifugation (5000 rpm, 3 min). The cell pellet was resuspended in BugBuster (Novagen) with Benzonase (Novagen) and incubated for 40 min at room temperature. Cell debris was removed by centrifugation (10,000 rpm, 20 min) at 4 °C. The supernatant was applied to a HisTrap HP Ni^{2+}-affinity column (GE Healthcare, Buckinghamshire, England) to purify the recombinant enzyme. The recombinant protein was eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4) and concentrated using an ultrafiltration membrane (Amicon Ultra 10K cutoff, Millipore, Darmstadt, Germany). The concentrated recombinant enzyme was then applied to a HiLoad 16/60 Superdex 200 prep-grade gel-filtration column (GE Healthcare) and eluted with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM disodium hydrogen phosphate, 1.8 mM potassium dihydrogen phosphate: pH 7.4). The enzyme fraction was concentrated using an ultrafiltration membrane (Amicon Ultra 10K cutoff, Millipore).

Biomass saccharification

Xylans (xylan from birch wood or arabinoxylan from wheat) were dissolved in Milli-Q water to a final concentration of 2 % and incubated at 98 °C for 5 min with agitation (1000 rpm) using a Thermomixer comfort (Eppendorf, Hamburg, Germany). After centrifugation (8000 rpm for 5 min), the supernatant was used as a source of xylans or arabinoxylans.

Culture supernatant of *T. reesei* PC-3-7 (ATCC 66589) (*T. reesei* PC-3-7 was cultured in the liquid medium containing Avicel) was used as a source of cellulases (Kawai et al. 2012; Kawamori et al. 1986). Saccharification of xylans by cellulases was performed in 200-μl tubes in 50 mM sodium phosphate buffer (pH 6.0) with or without CoXyl43. For xylan

saccharification, 20 μl xylan solution, 10 μl 50 μg/ml cellulases (or 10 μl sterile water), and 10 μl 50 μg/ml CoXyl43 in 200 mM sodium phosphate buffer (pH 6.0) (or 10 μl 200 mM sodium phosphate buffer) were mixed and incubated at 40 °C for 24 h. For arabinoxylan saccharification, 20 μl arabinoxylan solution, 10 μl 50 μg/ml cellulases (or 10 μl sterile water), and 10 μl 50 μg/ml CoXyl43 in 200 mM sodium phosphate buffer (pH 6.0) (or 10 μl 200 mM sodium phosphate buffer) were mixed and incubated at 40 °C for 24 h. After incubation, the produced sugars were measured by DNS and analyzed using a high-performance ion chromatography system (HPIC) as described previously (Nakazawa et al. 2012). The degree of saccharification was calculated relative to the complete saccharification of xylans by phenol-sulfuric acid reaction (Dubois et al. 1956). Briefly, 200 μl 5 % phenol and 200 μl diluted xylan or arabinoxylan solutions were mixed, and 1 ml concentrated sulfuric acid was then added. After incubation at room temperature for 20 min, the absorbance was measured at 490 nm. A standard curve was constructed using D-xylose.

Biomass (alkaline-treated rice straw and *Erianthus*) saccharification by cellulases (*T. reesei* PC-3-7 strain) and CoXyl43 was performed in 20-ml plastic bottles. The total reaction volume was 2 ml. The reaction mixture contained 0.54 g alkaline-treated rice straw (water content: 81.4 %; final concentration 5 % (*w*/*v*)) or 0.56 g alkaline-treated *Erianthus* (water content: 82.1 %, final concentration 5 % (*w*/*v*)), 500 μl of 400 mM sodium phosphate buffer (pH 6.0), 10 μl 0.5 mg/ml CoXyl43 (or 10 μl sterile water), 117.6 μl 1.7 mg/ml cellulases (culture supernatant of *T. reesei* PC-3-7), 20 μl 2 % sodium azide, and sterile water. The reaction was performed at 40 °C with shaking at 150 rpm for 72 h. To inactivate the enzymes, 200 μl supernatant were incubated at 100 °C for 5 min. The produced sugars were measured by DNS and analyzed using an HPIC system as described previously (Nakazawa et al. 2012). A standard curve for DNS was constructed using D-glucose.

Substrate specificity

The total reaction volume was 20 μl and contained 0.15 μg of the recombinant enzyme, 50 mM sodium phosphate buffer (pH 7.5), and 5 mM *p*NP-substrate (*p*NP-β-D-xylopyranoside, *p*NP-α-D-xylopyranoside, *p*NP-α-L-arabinofuranoside, *p*NP-α-L-arabinopyranoside, *p*NP-β-L-arabinopyranoside, *p*-NP-β-D-cellobioside, *p*NP-α-L-fucopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-L-fucopyranoside, *p*NP-α-D-galactopyranoside, *p*NP-β-D-galactopyranoside, *p*NP-α-D-glucopyranoside, *p*NP-β-D-glucopyranoside, *p*NP-α-D-mannopyranoside, *p*NP-β-D-mannopyranoside, or *p*NP-α-L-rhamnopyranoside). The reaction mixture was incubated at 50 °C for 5 min. To stop the reaction, 50 μl of a 1.0-M sodium bicarbonate solution were added. The concentration of released *p*NP was determined by measuring the solution absorbance at 405 nm (Infinite M200 PRO, Tecan (Zurich, Switzerland)).

The substrate specificity of CoXyl43 for xylan and arabinoxylan was analyzed using xylan and arabinoxylan solutions (described above) in a final reaction volume of 20 μl. The reaction mixture contained 0.2 μg of the recombinant enzyme, 10 μl of the xylan or arabinoxylan solution, and 50 mM sodium phosphate buffer (pH 7.5). The reaction mixture was incubated at 50 °C for 10 min. The produced sugars were measured by DNS.

Arabinofuranosyl-xylooligosaccharide hydrolysis

Each reaction (100 μl final volume) contained 25 μg of the recombinant enzyme, 0.3 % Ara*f*-X2 or Ara*f*-X3, and 50 mM sodium phosphate buffer (pH 7.5). Reaction mixtures were incubated at 50 °C for 24 h. The produced sugars were analyzed by HPIC as described previously (Nakazawa et al. 2012).

Effects of pH and temperature on hydrolysis activity

The optimum pH for recombinant glycoside hydrolase activity with 3 mM *p*NP-β-D-xylopyranoside was evaluated at 40 °C for 5 min in McIlvaine's buffer ranging from pH 3.0 to pH 9.0 (McIlvaine 1921). The optimum temperature for recombinant glycoside hydrolase activity with 2.86 mM *p*NP-β-D-xylopyranoside in McIlvaine buffer (pH 7.5) was evaluated from 20 to 80 °C for 5 min.

Kinetic analyses

The kinetic parameters of CoXyl43 for chromogenic substrates were determined using 0.025 μg recombinant CoXyl43 at *p*NP-β-D-xylopyranoside or *p*NP-α-L-arabinofuranoside concentrations from 0.125 to 8 mM in 50 mM sodium phosphate buffer (pH 7.5) at 50 °C for 5 min. The total reaction volume was 20 μl. To stop the reaction, 50 μl of a 1.0-M sodium bicarbonate solution were added. The kinetic parameters of CoXyl43 for xylobiose were determined using 0.5 μg recombinant CoXyl43 at xylobiose concentrations from 0.125 to 50 mM in 50 mM sodium phosphate buffer (pH 7.5) at 50 °C for 5 min. The reaction was stopped by heating the reaction mixture to 98 °C for 10 min. The xylose concentration was determined using a D-Xylose assay kit obtained from Megazyme (Wicklow, Ireland). Kinetic constants (K_m and k_{cat}) were calculated using a nonlinear regression

of the Michaelis-Menten equation using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA).

Effects of organic solvents, metal ions, and chelating agent

The effects of additives including organic solvents (ethanol and DMSO), metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}), and ethylenediaminetetraacetic acid (EDTA) were evaluated by measuring enzyme activity in the presence of these additives with 5 mM *p*NP-β-D-xylopyranoside or *p*NP-α-L-arabinofuranoside and 50 mM sodium phosphate buffer (pH 7.5) at 50 °C for 5 min following the measurement of *p*NP release.

Results

Screening of clones with glycoside hydrolase activity from the metagenomic library

About 40 clones exhibiting glycoside hydrolase activity in a mixture of *p*NP substrates (mixture of *p*NP-β-D-lactopyranoside, *p*NP-β-D-xylopyranoside, *p*NP-β-D-mannopyranoside, and *p*NP-β-D-galactopyranoside) were screened from the metagenomic library (approximately 30,000 colonies). Among these *p*NP substrate positive clones, one showing a positive effect on biomass saccharification

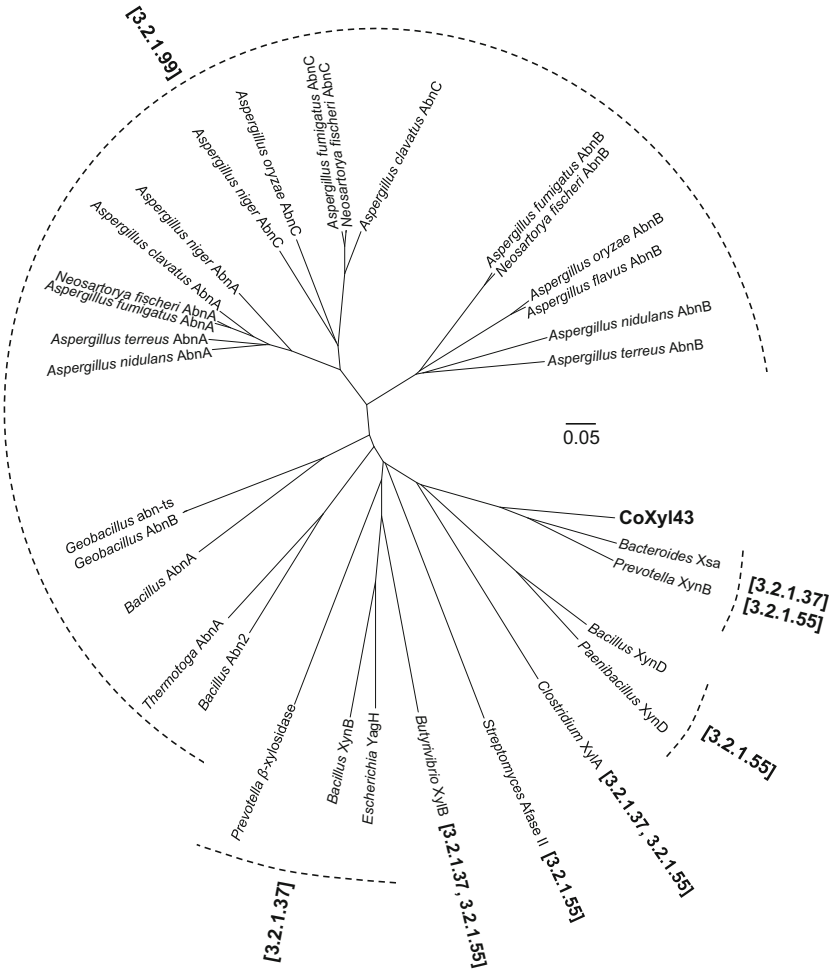

Fig. 1 Phylogenetic tree of GH43 enzymes. The amino acid sequences of GH43 enzymes sharing a high sequence similarity with CoXyl43 were obtained from UniProtKB (www.uniprot.org). Sequence alignment was performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/), and the phylogenetic tree was constructed using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). UniProtKB accession numbers are as follows: *Bacteroides ovatus* Xsa, P49943; *Prevotella ruminicola* XynB, P48791; *Bacillus subtilis* XynD, Q45071; *Paenibacillus polymyxa* XynD, P45796; *Clostridium stercorarium* XylA, P48790; *Streptomyces chartreusis* Afase, P82594; *Butyrivibrio fibrisolvens* XylB, P45982; *Escherichia coli* YagH, P77713; *B. subtilis* XynB, P94489; *Prevotella ruminicola* β-xylosidase, Q9WXE8; *B. subtilis* Abn2, P42293; *Thermotoga petrophila* AbnA, A5IKD4; *B. subtilis* AbnA, P94522; *Geobacillus stearothermophilus* AbnB, B3EYM8; *Geobacillus thermodenitrificans* abn-ts, Q93HT9; *Aspergillus nidulans* AbnA, Q5BA96; *Aspergillus terreus* AbnA, Q0CS14; *Aspergillus fumigatus* AbnA, Q4WYX7; *Neosartorya fischeri* AbnA, A1D5W1; *Aspergillus clavatus* AbnA, A1CLG4; *Aspergillus niger* AbnA, A2QT85; *A. niger* AbnC, A5AAG2; *Aspergillus oryzae* AbnC, Q2U1X8; *N. fischeri* AbnC, A1DKY5; *A. fumigatus* AbnC, Q4W930; *A. clavatus* AbnC, A1CN18; *A. fumigatus* AbnB, B0XTS5; *N. fischeri* AbnB, A1DHW8; *A. oryzae* AbnB, Q2UI74; *Aspergillus flavus* AbnB, B8N803; *A. nidulans* AbnB, Q5AZC8; *A. terreus* AbnB, Q0CY27. EC numbers: 3.2.1.37, β-xylosidase; 3.2.1.55, α-L-arabinofuranosidase; 3.2.1.99, endo-1,5-α-L-arabinanase

was isolated. Plasmid DNA was extracted from this clone, and the inserted DNA was sequenced. A gene encoding a putative glycoside hydrolase, named CoXyl43, was isolated from the sequencing data. CoXyl43 contains an open reading frame of 1107 bp encoding a putative protein of 369 amino acids. The N-terminus of the mature protein may begin at the 47th residue from the first Met, because the first 46 amino acids from the N-terminus are predicted to be a signal sequence. N-terminal proteolytic cleavage resulted in a protein of 323 amino acid residues with a predicted molecular mass of 36,186 Da. CoXyl43 was predicted to belong to the glycoside hydrolase family 43 (GH43), which contains β-xylosidase (EC 3.2.1.37) (Shallom et al. 2005; Brüx et al. 2006), α-arabinofuranosidase (EC 3.2.1.55) (Flipphi et al. 1993b), and endo-α-arabinase (EC 3.2.1.99) (Flipphi et al. 1993a). Comparisons against the BLAST database (NCBI BLAST: http://blast.ncbi.nlm.nih.gov/Blast.cgi, nonredundant UniProtKB/SwissProt sequences) revealed that CoXyl43 is similar to *Bacteroides ovatus* Xsa (identity: 63 %, similarity: 75 %) (Whitehead 1995) and *Prevotella ruminicola* XynB (identity: 57 %, similarity: 69 %) (Gasparic et al. 1995), both of which belong to the

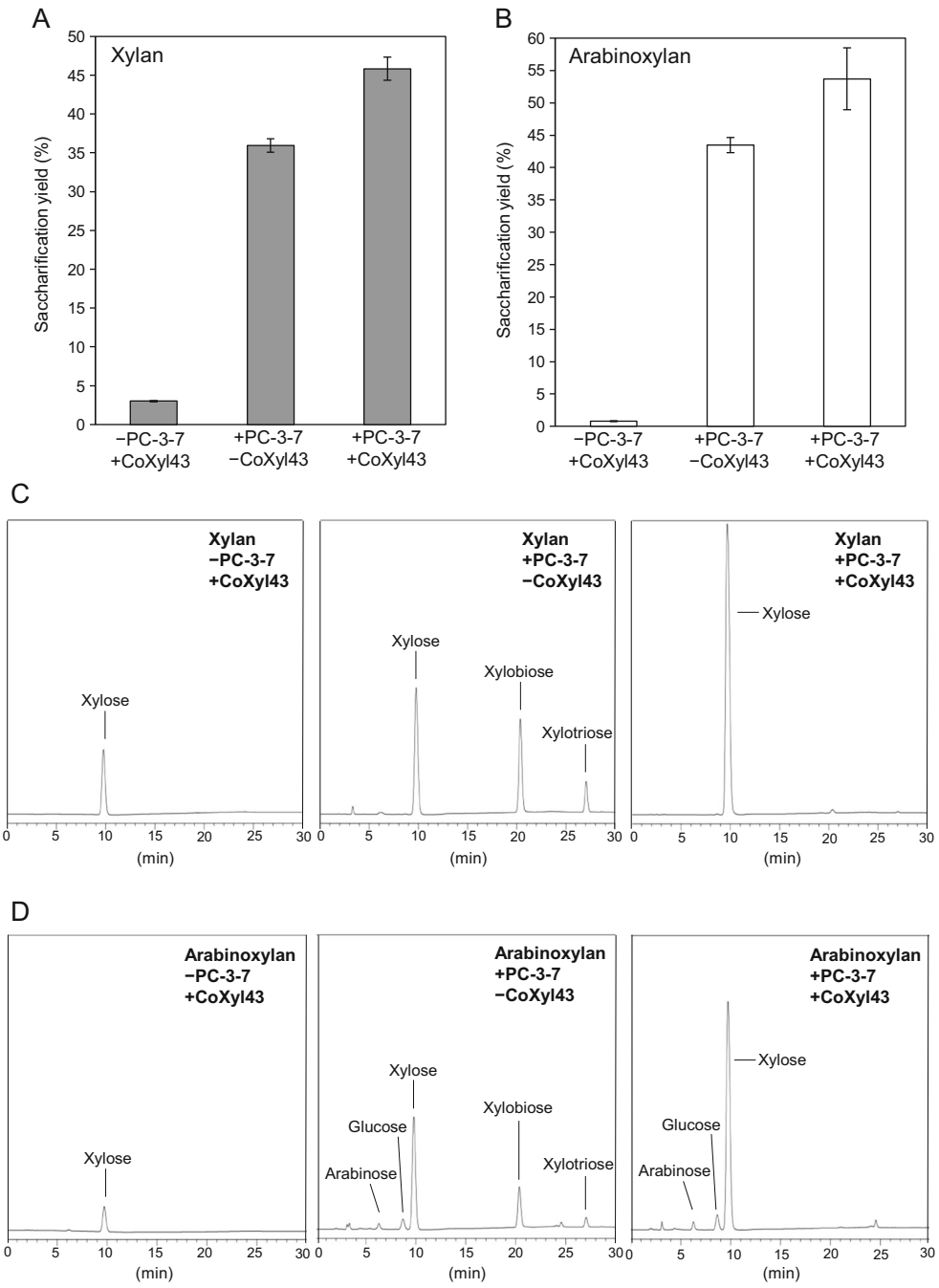

Fig. 2 Synergism between *T. reesei* cellulases and CoXyl43 in xylan saccharification. *T. reesei* PC-3-7 cellulases and CoXyl43, either in combination (+PC3-7 + CoXyl43) or alone (+PC3-7 - CoXyl43, −PC3-7 +CoXyl43), were incubated with birch wood xylan (**a** and **c**) or wheat flour arabinoxylan (**b** and **d**) at 40 °C for 24 h. Saccharification yields (**a** and **b**) and HPIC chromatograms of the hydrolysates (**c** and **d**) are shown

GH43 class of carbohydrate-active enzymes in the CAZy database (Fig. 1). Both *B. ovatus* Xsa and *P. ruminicola* XynB are bifunctional β-xylosidases/α-arabinofuranosidases (EC 3.2.1.37, EC 3.2.1.55). CoXyl43 had a relatively low degree of similarity with the characterized GH43 arabinanases (EC 3.2.1.99) (Fig. 1).

The recombinant CoXyl43 enzyme was expressed in *E. coli* for characterization. His-tagged recombinant CoXyl43 was purified using Ni^{2+}-affinity chromatography and gel filtration.

Synergism of cellulases and CoXyl43 on biomass saccharification

CoXyl43 and PC-3-7 cellulases (alone or combined) were incubated with birch wood xylan (Fig. 2a, c) or wheat flour arabinoxylan (Fig. 2b, d) to evaluate any synergistic effects on saccharification. Some reducing sugar (xylose) was released from both xylan and arabinoxylan in the presence of CoXyl43 alone, suggesting that CoXyl43 has a slight exo-xylanase activity

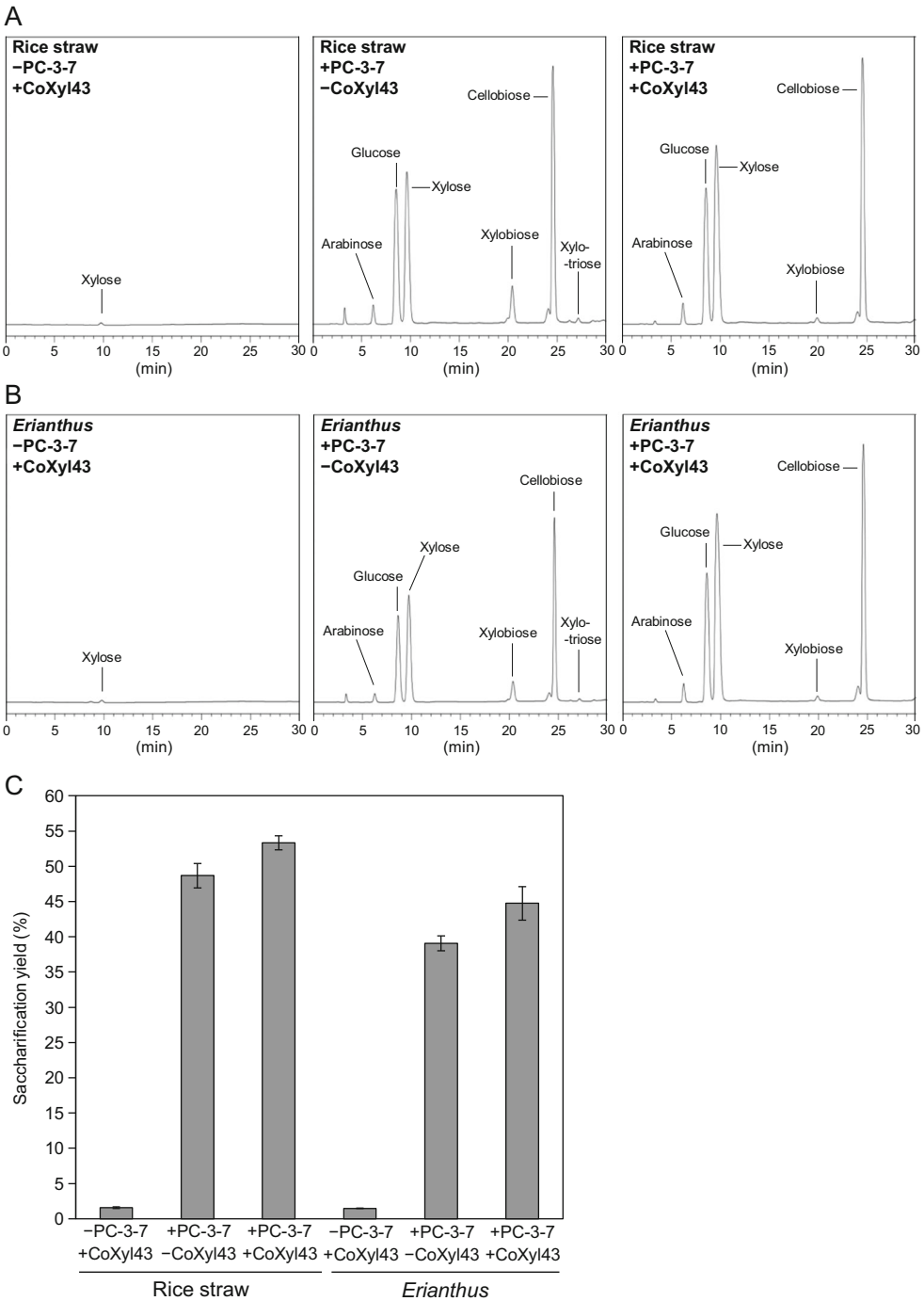

Fig. 3 Synergism between *T. reesei* cellulases and CoXyl43 in NaOH-pretreated biomass saccharification. PC-3-7 cellulases and CoXyl43, either in combination (+PC3-7 +CoXyl43) or alone (+PC3-7 -CoXyl43, −PC3-7 +CoXyl43) were incubated with NaOH-pretreated rice straw (**a** and **c**) and *Erianthus* (**b** and **c**) at 40 °C for 72 h. HPIC chromatograms (**a** and **b**) and saccharification yields (**c**) are shown

with xylan and arabinoxylan (left panels of Fig. 2c, d). With *T. reesei* cellulases (PC-3-7), saccharification yields of xylan and arabinoxylan were approximately 36 and 43 %, respectively, and xylooligosaccharide (such as xylobiose and xylotriose) accumulation was detected (middle panels of Fig. 2c, d). When CoXyl43 was added to a xylan saccharification solution containing PC-3-7 cellulases, the accumulation of xylooligosaccharides was negligible (right panels of Fig. 2c, d), resulting in a significant increase in saccharification yields (Fig. 2a, b).

Next, the synergistic effects of CoXyl43 with PC-3-7 cellulases in NaOH-pretreated rice straw and *Erianthus* saccharification were investigated (Fig. 3). As with xylan, some xylose was released from NaOH-pretreated rice straw and *Erianthus* in the presence of CoXyl43 alone (Fig. 3a, b). Accumulation of xylooligosaccharides and some cellooligosaccharides, including cellobiose, was also observed during the saccharification of NaOH-pretreated rice straw and *Erianthus* using PC-3-7 cellulases (Fig. 3a, b). The addition of CoXyl43 dramatically reduced the amounts of accumulated xylooligosaccharides (Fig. 3a, b), and the relative saccharification yield increased by approximately 5 % (Fig. 3c). These results indicated that CoXyl43 could almost completely hydrolyze the xylooligosaccharides derived from plant biomasses into xylose.

Characterization of recombinant CoXyl43

The hydrolytic activity of recombinant CoXyl43 toward various chromogenic substrates was measured as a means of characterizing substrate specificity (Table 2). CoXyl43 exhibited hydrolytic activity with *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinofuranoside but not with other *p*NP substrates (such as *p*NP-β-D-mannopyranoside and *p*NP-β-D-galactopyranoside) (Table 2). The specific activities of recombinant CoXyl43 toward birch wood xylan and wheat arabinoxylan were 22.0 U/mg protein and 4.2 U/mg protein, respectively. One unit was defined as the amount of enzyme that released 1 μmol of xylose equivalents as reducing sugars per minute. CoXyl43 showed substantial activity between pH 5.5 and 9.0 and between 20 and 60 °C (Fig. 4a, b). The CoXyl43 activity with *p*NP-β-D-xylopyranoside was maximal at pH 7.5 and 55 °C.

Table 2 Substrate specificity of recombinant CoXyl43

Substrate	Relative activity (%)
*p*NP-β-D-xylopyranoside	100.0±2.5
*p*NP-α-D-xylopyranoside	<5
*p*NP-α-L-arabinofuranoside	119.9±3.6
*p*NP-α-L-arabinopyranoside	<5
*p*NP-β-L-arabinopyranoside	<5
*p*NP-β-D-cellobioside	<5
*p*NP-α-L-fucopyranoside	<5
*p*NP-β-D-fucopyranoside	<5
*p*NP-β-L-fucopyranoside	<5
*p*NP-α-D-galactopyranoside	<5
*p*NP-β-D-galactopyranoside	<5
*p*NP-α-D-glucopyranoside	<5
*p*NP-β-D-glucopyranoside	<5
*p*NP-α-D-mannopyranoside	<5
*p*NP-β-D-mannopyranoside	<5
*p*NP-α-L-rhamnopyranoside	<5

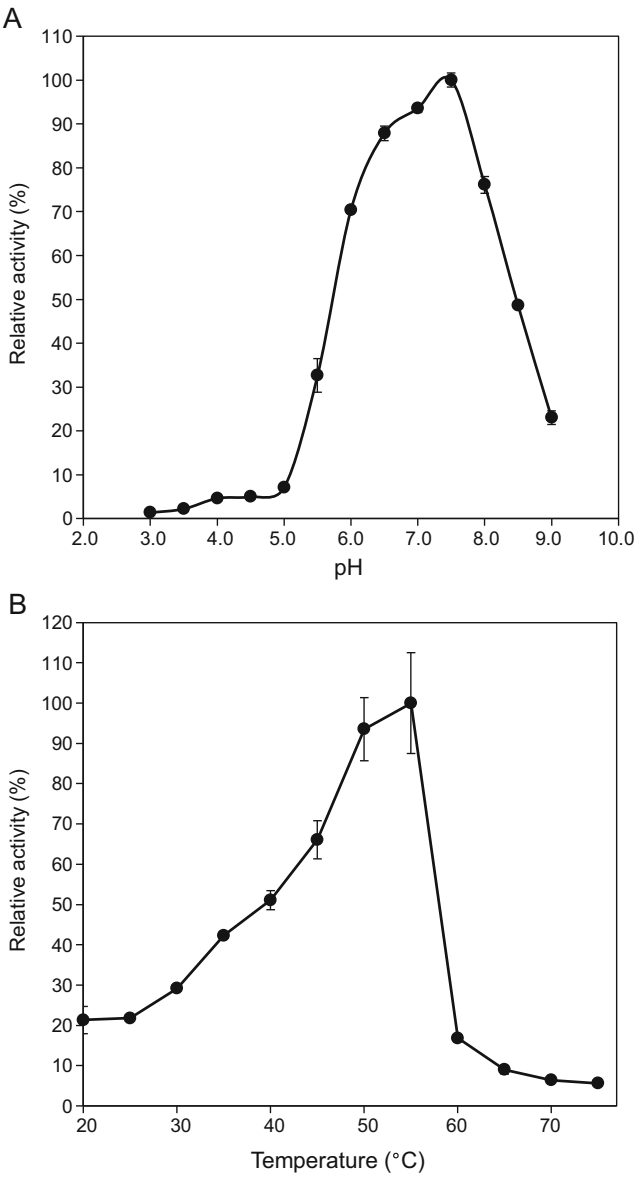

Fig. 4 The activity of recombinant CoXyl43 is shown at (**a**) various pH values and (**b**) temperatures. *Error bars* represent standard deviations (SD)

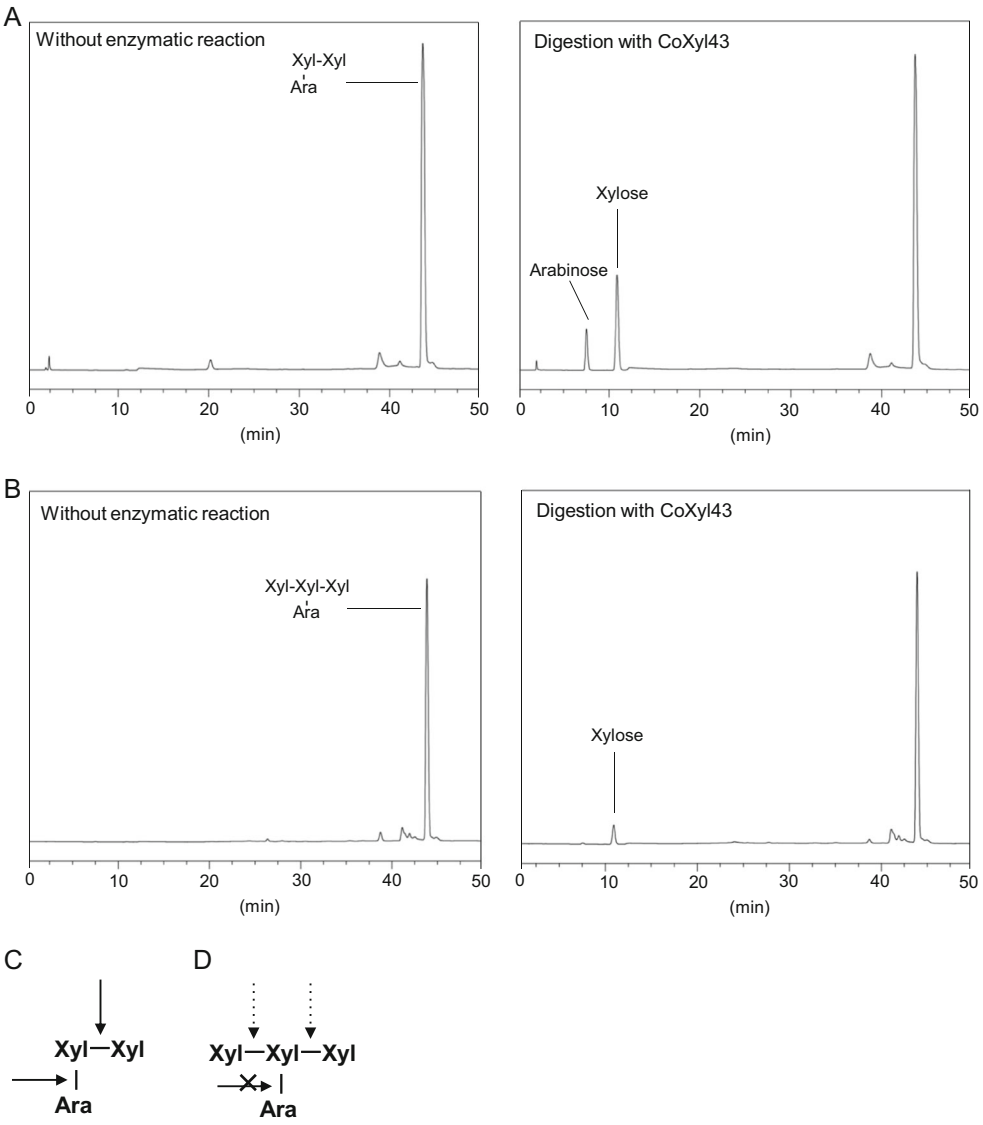

Fig. 5 Arabinofuranosyl-xylooligosaccharide hydrolysis by CoXyl43. (**a**) *O*-α-L-arabinofuranosyl-(1→3)-*O*-βxylopyranosyl-(1→4)-D-xylopyranose (Ara*f*-X2) and (**b**) *O*-β-D-xylopyranosyl-(1→4)-[*O*-α-L-arabinofuranosyl-(1→3)]-*O*-β-xylopyranosyl-(1→4)-D-xylopyranose (Ara*f*-X3) were hydrolyzed by CoXyl43. The predicted mechanisms of Ara*f*-X2 and Ara*f*-X3 hydrolysis using CoXyl43 are shown in **c** and **d**, respectively

We also investigated the hydrolytic activity of CoXyl43 against the arabinofuranosyl-xylooligosaccharides Ara*f*-X2 and Ara*f*-X3, which are degradation products of arabinoxylan (Fujimoto et al. 2004). Although CoXyl43 partially degraded Ara*f*-X2 into xylose and arabinose (Fig. 5a), little activity was observed against Ara*f*-X3 (Fig. 5b). These results indicate that CoXyl43 was able to degrade both xylooligosaccharides and the arabinose side chain of Ara*f*-X2 (Fig. 5c). However, the xylose residue at the nonreducing end of Ara*f*-X3 inhibited the release of an arabinofuranose residue, thereby inhibiting hydrolysis of the xylotriose backbone of Ara*f*-X3 (Fig. 5d).

Table 3 Steady-state kinetic constants of recombinant CoXyl43

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
*p*NP-β-D-xylopyranoside	1.43±0.07	4.60±0.08	3.21
*p*NP-α-L-arabinofuranoside	2.60±0.13	7.91±0.17	3.04
Xylobiose	2.02±0.06	17.82±0.14	8.83

Kinetic constants of CoXyl43

The kinetic parameters (Michaelis constant: K_m, turnover number: k_{cat}, and k_{cat}/K_m) of CoXyl43 were measured for *p*-NP-β-D-xylopyranoside, *p*NP-α-L-arabinofuranoside, and xylobiose (Table 3). The K_m of CoXyl43 for xylobiose was 2.02, and the k_{cat} for xylobiose was 17.82. The K_m of CoXyl43 for *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinofuranoside were 1.43 and 2.60, respectively, suggesting that *p*NP-β-D-xylopyranoside was the preferred substrate for CoXyl43. However, the k_{cat} of CoXyl43 for *p*NP-β-D-xylopyranoside was much lower than that for *p*NP-α-L-arabinofuranoside, and the catalytic efficiency constant k_{cat}/K_m for *p*NP-β-D-xylopyranoside was almost equivalent to that for *p*NP-α-L-arabinofuranoside.

Almost all of the GH43 bifunctional β-xylosidase/α-arabinofuranosidases have preferential activities. Some enzymes show much higher activity toward *p*NP-β-D-xylopyranoside than toward *p*NP-α-L-arabinofuranoside (Dougherty et al. 2012; Jordan et al. 2013; Shallom et al. 2005; Viborg et al. 2013; Whitehead and Cotta 2001; Kim and Yoon 2010; Zhou et al. 2012b) while the reverse relationship has been reported for other enzymes of the same family (Sakka et al. 1993; Shao and Wiegel 1992; Utt et al. 1991; Wagschal et al. 2009). Therefore, the almost equivalent catalytic efficiency constants for *p*NP-β-D-xylopyranoside and *p*NP-α-L-arabinofuranoside are unique to CoXyl43.

Effects of various organic solvents, metal ions, and chelating agent on CoXyl43 activity

The effects of various organic solvents, metal ions, and EDTA chelating agent on CoXyl43 were investigated (Table 4). Significant inactivation of CoXyl43 was observed in the presence of ethanol, DMSO, $ZnCl_2$, and $CuSO_4$. The CoXyl43 β-xylosidase activity was decreased by about 45 % with the addition of 100 mM EDTA, but no effect on α-arabinofuranosidase activity was observed with the addition of EDTA. The addition of $MnCl_2$ resulted in a slight activity enhancement of both β-xylosidase and α-arabinofuranosidase activities (156.6 and 162.5 %, respectively). Significant activation of the β-xylosidase activity of CoXyl43 was observed upon addition of Ca^{2+}, $CaCl_2$, or $CaSO_4$ (Table 4). The β-xylosidase activity was approximately 3-fold higher in the presence of 0.01 mM $CaCl_2$ or $CaSO_4$ and more than 5-fold higher in the presence of 1 mM $CaCl_2$ or $CaSO_4$. The β-xylosidase activity for 12.5 mM xylobiose at pH 7.5 (50 °C for 5 min) was enhanced by about 2.4-fold by addition of 1 mM $CaCl_2$. In contrast, the α-arabinofuranosidase activity of CoXyl43 was enhanced by only about 25 % in the presence of 1 mM $CaCl_2$ or $CaSO_4$.

Table 4 Effects of organic solvents, metal ions, and chelating agent on the β-D-xylosidase activities of recombinant CoXyl43

Reagent	Concentration	Relative activities (%) for	
		*p*NP-β-D-Xyl	*p*NP-α-L-Ara*f*
No additive		100.0±6.4	100.0±9.5
Ethanol	10 % (*v*/*v*)	25.2±1.2	17.1±0.3
	25 % (*v*/*v*)	7.9±0.8	6.5±1.1
DMSO	10 % (*v*/*v*)	57.8±2.0	31.5±2.4
	25 % (*v*/*v*)	8.6±1.9	4.9±0.7
$MgCl_2$	1 mM	92.0±3.9	118.3±4.7
	5 mM	93.9±5.7	123.2±13.8
$ZnSO_4$	1 mM	8.7±1.5	8.6±1.0
	5 mM	16.7±1.4	21.8±3.9
$CuSO_4$	1 mM	3.2±0.5	2.3±0.4
	5 mM	4.5±0.1	3.2±0.2
$FeSO_4$	1 mM	66.9±5.3	97.2±8.1
	5 mM	69.7±17.8	85.7±8.5
$MnCl_2$	1 mM	138.3±6.6	147.0±7.8
	5 mM	156.6±4.7	162.5±11.2
$CaCl_2$	0.01 mM	290.4±19.3	110.0±1.7
	0.1 mM	482.8±35.2	126.7±6.6
	1 mM	513.7±17.0	124.4±5.8
$CaSO_4$	0.01 mM	294.5±20.2	103.2±1.4
	0.1 mM	484.8±21.7	119.3±4.3
	1 mM	519.0±43.5	125.9±5.1
EDTA	10 mM	67.1±3.7	108.3±2.7
	100 mM	44.7±1.3	105.3±9.2

Discussion

Metagenomic analyses of environmental microorganisms are a highly effective means of screening for useful genes such as those suitable for biomass utilization and bioremediation. In the present study, we isolated a new β-xylosidase/α-arabinofuranosidase, CoXyl43, from a compost metagenome focusing on biomass saccharification. CoXyl43 displayed both β-D-xylosidase and α-L-arabinofuranosidase activities, similar to other members of the GH43 family of dual-functional enzymes and also showed xylooligosaccharide hydrolysis activity. CoXyl43 degraded xylooligosaccharides derived from plant biomasses, including both xylobiose and xylotriose, into xylose. Although CoXyl43 displayed high α-arabinofuranosidase activity toward *p*NP-α-L-arabinofuranoside, CoXyl43 removed few arabinofuranosyl units from the backbone chains of arabinoxylan and Ara*f*-X3 (Figs. 2d and 5b). These results suggest that the backbone chains of β-1,4-linked xylopyranose units interfere with α-arabinofuranosidase activity toward the arabinofuranosyl units of arabinoxylan and Ara*f*-X3.

In recent years, many genes encoding glycoside hydrolases have been obtained using metagenomic approaches. For example, Dougherty et al. (2012) isolated and characterized several endo-xylanases, α-fucosidase, and a bifunctional β-xylosidase/α-arabinofuranosidase from a compost metagenome. In addition, various cellulases and hemicellulases, including endoglucanase (Pang et al. 2009; Alvarez et al. 2013), endoxylanase (Gong et al. 2013), β-glucosidase/xylosidase (Zhou et al. 2012a; Bao et al. 2012), β-glucosidases (Uchiyama et al. 2013; McAndrew et al. 2013), β-galactosidase (Gupta et al. 2012), and β-xylosidase/α-arabinofuranosidase (Zhou et al. 2012b), were isolated from soil, compost, hot spring, or rumen using metagenomic methods. It was reported previously that a GH family 3 β-glucosidase/xylosidase and GH43 β-D-xylosidase/α-L-arabinofuranosidase from a yak rumen

metagenome showed synergisms with endo-xylanase in xylan hydrolysis (Bao et al. 2012; Zhou et al. 2012b). Synergism between CoXyl43 and *T. reesei* cellulases was observed not only in xylan (from birch wood) and arabinoxylan (from wheat flour) saccharification but also in NaOH-pretreated biomass (rice straw and *Erianthus*) saccharification, resulting in a significant increase in saccharification efficiency. These results indicate that CoXyl43 would be useful for the saccharification of various cellulosic/hemicellulosic biomasses and that metagenomic approaches to environmental microorganisms have the potential to identify genes that may be valuable for the utilization of plant biomass. The metagenomic isolation of genes encoding non-GH family proteins that improve the efficiency of biomass saccharification will be explored in future studies.

Various chemical reagents affected CoXyl43 activity. Organic solvents, such as ethanol and DMSO, and zinc ions, and copper ions all strongly inhibited both the β-xylosidase and α-arabinofuranosidase activities of CoXyl43. Zinc and copper ions may inhibit the catalytic reaction or hinder substrate binding at the active site. Manganese ions enhanced both the β-xylosidase and α-arabinofuranosidase activities. The inhibition by zinc and copper ions, and activation by manganese ions, has been observed with other GH43 bifunctional β-xylosidase/α-arabinofuranosidases (Gong et al. 2013; Lee et al. 2013; Yang et al. 2014). Interestingly, the β-xylosidase activity of CoXyl43 was dramatically enhanced by the addition of calcium ions, while the α-arabinofuranosidase activity was not. The presence of EDTA inhibited β-xylosidase activity but not α-arabinofuranosidase activity. These results indicate that manganese ions enhance both β-xylosidase and α-arabinofuranosidase activities while calcium ions enhance only β-xylosidase activity. Further studies, incorporating X-ray crystal structure analyses of CoXyl43 with and without calcium ions, will aim to elucidate the mechanism by which calcium ions activate β-xylosidase activity while having no effect on α-arabinofuranosidase activity. Previous studies have reported distinctly different metal ion sensitivities among the GH43 enzymes (Viborg et al. 2013). Our ongoing studies will clarify how metal ions assist the catalytic function of GH43 enzymes.

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Conflict of interest The authors declare no conflicts of interest.

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