Journal of Bioscience and Bioengineering
VOL. 116 No. 2, 152–159, 2013

www.elsevier.com/locate/jbiosc

Characterization of a recombinant bifunctional xylosidase/arabinofuranosidase from *Phanerochaete chrysosporium*

Nguyen Duc Huy,[1,2] Palvannan Thayumanavan,[1,3] Tae-Ho Kwon,[4] and Seung-Moon Park[1,*]

Division of Biotechnology, College of Environmental and Bioresource Sciences, Chonbuk National University, Iksan, Jeonbuk 570-752, Republic of Korea,[1] Institute of Resources, Environment and Biotechnology, Hue University, Vietnam,[2] Laboratory of Bioprocess and Engineering, Department of Biochemistry, Periyar University, Salem, Tamil Nadu 636 011, India,[3] and Natural Bio-Materials Co., Jeonju, Jeonbuk 561-360, Republic of Korea[4]

Received 13 November 2012; accepted 3 February 2013
Available online 7 March 2013

A bifunctional xylosidase/arabinofuranosidase gene (*PcXyl*) was cloned from the cDNA library of *Phanerochaete chrysosporium* and further expressed in *Pichia pastoris*. Enzymatic assay indicated that *P. pastoris* produced rPcXyl at a level of 26,141 U l^{-1}. The xylosidase and arabinofuranosidase activities of rPcXyl were maximized, respectively, at pHs of 5.0 and 5.5 and temperatures of 45°C and 50°C. SDS-PAGE revealed a single band of purified rPcXyl of 83 kDa. Cu^{2+} and Zn^{2+} completely inhibited the enzyme activity of rPcXyl. The enzyme activity of rPcXyl was increased 151%, 126% and 123%, respectively, in the presence of glucose, xylose and arabinose at concentrations of 5 mM. rPcXyl hydrolyzed xylobiose to xylose and xylotriose to xylose and xylobiose, indicating rPcXyl acts as an *exo*-type enzyme. Additionally, rPcXyl enhanced xylose release from xylan substrates in synergy with rPcXynC.

[**Key words:** *Phanerochaete chrysosporium*; *Pichia pastoris*; Xylosidase; Arabinofuranosidase; Xylan; Xylose]

Hemicellulose comprises a complex polysaccharide structure mainly composed of pentose (xylose, arabinose) and hexose (glucose, mannose) sugars, the most abundant of which is xylose. For complete hydrolysis of hemicellulose, the synergetic actions of numerous enzymes are required. These enzymes include endo-xylanases (E.C 3.2.1.8), β-xylosidases (E.C 3.2.1.37), endo-arabinan ases (E.C.3.2.1.99), α-L-arabinofuranosidases (E.C 3.2.1.55), α-glu-curonidases (E.C 3.2.1.139), endo-mannanases (E.C 3.2.1.78), β-man osidases (3.2.1.25), α-galactosidases (E.C 3.2.1.22), β-glucosidases (E.C 3.2.1.21) and esterases (E.C.3.1.1.-). Among these, xylanases and β-xylosidases are the key enzymes responsible for the hydrolysis of xylan, the major component of hemicelluloses. Xylanases hydrolyze the internal β-1,4 linkage of the main chain of xylan, yielding short xylo-oligomers, which β-xylosidases cleave to release single units of xylose (1,2).

β-Xylosidases are classified into eight families of glycoside hydrolases based on their amino acid sequence similarities and biochemical properties. Enzymes from families 3, 30, 39, 52, 54, 116, and 120 catalyze the hydrolysis of xylo-oligomers via a retaining mechanism, while enzymes from family 43, however, perform hydrolysis by inverting the anomeric configuration of xylo-oligomers (2,3). β-Xylosidases are essential enzymes of xylanolytic systems in microorganisms, along with xylanases, prevent end-product inhibition of hydrolysis enzymes by xylo-oligomers. Interestingly, numerous β-xylosidases isolated from microbial systems have been reported to exhibit bifunctional β-xylosidase/α-L-arabinofuranosidase activity, including those from a compost starter mixture metagenome (4), *Paenibacillus woosongensis* (5), *Thermoanaerobacter ethanolicus* (6), and *Penicillium purpurogenum* (7). The bifunctional activities of β-xylosidases are due to their spatial similarities with D-xylopyranose and L-arabinofuranose, particularly their glycosidic bonds and hydroxyl groups (1). Xylosidases and arabinofuranosidases are important enzymes in biomass saccharification. These enzymes not only increase the release of reducing sugars, but also prevent the inhibition of other hydrolysis enzymes in synergistic action with xylanase or cellulase (8–10). Thus, these enzymes are of great potential in biotechnology and industrial applications due to their synergetic activities.

Xylose is an abundant sugar in hemicelluloses. Moreover, xylose is found in agricultural and agro-industrial materials, such as barley straw, corn stalks, corn stover, rice straw, and wheat straw, comprising 15%–35% of each plant's dry weight (11). Xylose can be utilized to produce bioethanol or valuable compounds used in food, pharmaceutical and industry (12,13). To produce xylose from biomass sources, numerous methods have been developed including chemical and enzymatic methods (11). Enzymatic treatments are preferred due to their environment friendliness, recyclability, and effectiveness.

In this study, we cloned a putative β-xylosidase gene from the cDNA library of *Phanerochaete chrysosporium*, which was then expressed in *Pichia pastoris*. The biochemical characterization of the

* Corresponding author. Tel.: +82 63 850 0837; fax: +82 63 850 0834.
E-mail address: smpark@chonbuk.ac.kr (S.-M. Park).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2013.02.004

resultant enzyme was investigated and found to exhibit bifunctional β-xylosidase/α-L-arabinofuranosidase activity.

MATERIALS AND METHODS

Strains, plasmids and media The white rot fungus *P. chrysosporium* BKM-F-1767 was obtained from the Korean Collection for Type Culture and maintained in medium as described by Tien and Kirk (14). Gene cloning was carried out using a pGEM-T Easy vector (Promega, Madison, WI, USA). The recombinant vector was transferred into *Escherichia coli* Top10, which was subsequently screened on LB agar plate containing 50 μg ml^{-1} ampicillin, supplemented with IPTG and X-Gal. *P. pastoris* GS115 (*his4*) and vectors pPICZ and pPICZα were purchased from Invitrogen (Carlsbad, CA, USA) and utilized as the expression host and vectors, respectively. The *PcXyl* gene was constructed with pPICZC or pPICZαC. The resulting construct was then introduced into *E. coli* Top10 and selected using low-salt LB medium containing 25 μg ml^{-1} of Zeocin. Recombinant β-xylosidase (rPcXyl) expression was studied by inoculating recombinant *P. pastoris* in YP medium (1% yeast extract, and 2% peptone) containing 1% methanol.

cDNA library Total mRNA was isolated from *P. chrysosporium* mycelia using the Oligotex mRNA Mini kit (Qiagen, Valencia, CA, USA). The cDNA library was synthesized using the SMARTer PCR cDNA Synthesis kit following the manufacturer's instructions (Clontech, Mountain View, CA, USA).

Gene manipulation The nucleotide sequence of the putative cDNA of *PcXyl* was obtained from the RP78 genome database (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html). The full-length cDNA of *PcXyl* was amplified using the forward PcXyl-F 5′-GAATTCATGCACCGTATTGCGAGGGC-3′ and reverse PcXyl-R 5′-GGGCCCATAAACGTTCTCTACAGGTT-3′ primers. Additionally, *PcXyl* cDNA without secretion signal was generated with the forward PcXylα-F and reverse PcXylα-R primers of 5′-GAATTCGGTCGTGTCGCTCACTCGAA-3′ and 5′-CCGCGGAATAAACGTTCTCTACAGGT-3′, respectively, resulting in an amplicon deemed *PcXylα*. The PCR conditions of 94°C for 1 min, 94°C for 30 s, 62°C for 30 s, 72°C for 2 min (for 30 cycles), and 72°C for 10 min were employed for PCR amplification using *Pfu* DNA polymerase (Solgent, Daejeon, South Korea). The expected PCR products were excised and purified from a 0.8% agarose gel, after which an A-tail was added by *Taq* DNA polymerase (Takara, Shiga, Japan) at 72°C for 30 min, followed by ligation with pGEM-T Easy Vector. *E. coli* transformants were screened by colony PCR with PcXyl-F and PcXyl-R or PcXylα-F and PcXylα-R primers. Three recombinant plasmids were randomly isolated and sequenced. The *PcXyl*-pGEM-T gene was excised using *Eco*RI and *Apa*I, whereas PcXylα-pGEM-T was incubated with *Eco*RI and *Sac*II. The *PcXyl* and *PcXylα* genes were separated and further purified on a 0.8% agarose gel. The purified *PcXyl* and *PcXylα* genes were then inserted into the pPICZA and pPICZαA vectors, respectively, resulting in pPICZA-*PcXyl* and pPICZαA-*PcXylα*. A sequence analysis was performed on pPICZA-*PcXyl* or pPICZαA-*PcXylα* using AOX1 primer according to the manufacturer's instructions.

The pPICZA-*PcXyl* and pPICZαA-*PcXylα* plasmids were linearized using *Pme*I, after which one of the two was then transformed into *P. pastoris* GS115 using an electroporation method, as recommended by the manufacturer (Bio-Rad, Hercules, CA, USA). Transformed cells were selected on YPD agar plates containing 100 μg ml^{-1} of zeocin and 1 M sorbitol at 30°C, until colonies were observed (2–3 days). The integration of *PcXyl* or *PcXylα* into the *P. pastoris* genome was confirmed by PCR using AOX1 primers.

Enzyme induction The qualification expression of twenty positive *P. pastoris* transformants were examined by dot blot analysis using His-tag antibody as described by Vasu et al. (15). The highest secreted *P. pastoris* transformants were grown in 5 ml YPD medium at 30°C and 200 rpm for 24 h, and then transferred to 50 ml of fresh YP medium containing 1% glycerol in a shaking incubator at 30°C and 180 rpm overnight. Enzyme induction was carried out in 100 ml of YP medium at an initial cell optical density of 1.0, supplemented with 1% methanol for every 24 h for 7 days. One milliliter of cultivation fluid was collected every 24 h and centrifuged for 5 min at 15,000 rpm, after which enzyme activity was measured.

Enzyme activity determination Xylosidase activity was determined by measuring the release of *p*-nitrophenol (pNP) from *p*-nitrophenyl-β-D-xylopyranoside (pNPβX) (Sigma, St. Louis, MO, USA) as described by Kim and Yoon (5). Briefly, 90 μl of supernatant was mixed with 100 μl of 100 mM sodium acetate, pH 5.0, at 40°C. The reaction was initiated by adding 10 μl of 50 mM pNPX in ethanol and incubating for 10 min at 40°C. The reaction was terminated by adding 1 ml of 1 M sodium carbonate. The amount of pNP released was determined at 410 nm wavelength using a TCC-240A UV spectrophotometer (Shimadzu, Kyoto, Japan). A standard curve was generated using pNP as a substrate, and the absorbance was converted into moles of pNP released. One unit of xylosidase activity was defined as the release of 1 nmol per minute of pNP under experimental conditions.

Enzyme purification and deglycosylation Cell-free supernatant was collected by centrifugation at 2000 rpm for 5 min, filtered through 0.45 μm filters, and then mixed with 10× binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole; pH 7.4). Enzyme was purified using a Ni^{2+} His-tag column (Histrap-GE Healthcare, Picataway, NJ, USA) with an ÄKTA fast protein liquid chromatography purification system. Enzyme was eluted with an elution buffer (20 mM sodium phosphate, 0.5 M NaCl and 500 mM imidazole; pH 7.4) and collected into 15 ml conical tubes. Purified enzyme (rPcXyl) was dialyzed with a cellulose dialysis tubing membrane (Sigma, St. Louis, MO, USA) against distilled water at 4°C overnight. Protein concentration was estimated by the Bradford method using a Thermo Scientific Protein Assay kit (Rockford, IL, USA), taking serum albumin as the standard.

The glycosylation of rPcXyl was analyzed using Endoglycosidase H. Approximately 5 μg of purified rPcXyl were incubated with 500 unit of enzyme for 30 min, 37°C. The molecular mass of purified rPcXyl and deglycosylated rPcXyl were determined by SDS-PAGE.

Effect of pH, temperature, metal ions and sugars on enzyme activity The effect of pH on rPcXyl activity was determined by assaying for enzyme activity at 40°C at different pH values of 3.0–6.0 using 50 mM sodium acetate and 7.0–8.0 using 50 mM sodium phosphate buffer. The reaction mixture was incubated at the specified temperature for 30 min, after which 10 μl of purified enzyme was added for 10 min. The amount of pNP released was then measured as described above. The optimal temperature for rPcXyl activity was examined at temperatures of 30–60°C at the optimal pH.

The effect of metal ions (Mn^{2+}, K^{+}, Ca^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, and Ni^{2+}) on rPcXyl activity was investigated to further characterize the enzyme. rPcXyl was incubated with 10 mM each of metal ion at the optimal pH and temperature. The retained activity of rPcXyl was compared with the enzyme activity thereof in a non-metal reaction.

Additionally, the effect of sugars (glucose, xylose and arabinose (Sigma, St. Louis, MO, USA)) on rPcXyl activity was also studied. To do so, rPcXyl was incubated for 1 h at optimal pH and temperature with 5 mM, 10 mM, 50 mM, or 100 mM of each sugar. The retained activity of rPcXyl was measured and compared to that in the absence of sugar.

Substrate specificity The substrate specificities of rPcXyl were investigated using sugar beet arabinan, debranched arabinan, wheat arabinoxylan (Megazyme, Bray, Co. Wicklow, Ireland), birchwood xylan, beechwood xylan, as well as *p*-nitro phenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-α-D-xylopyranoside (pNPαX), *p*-nitrophenyl-α-L-arabinofuranoside (pNPA), and *p*-nitrophenyl-α-D-xylopyranoside (pNPαX) (Sigma, St. Louis, MO, USA). pNPG, pNPαX, and pNPA hydrolysis were carried out in the same manner performed in the pNPβX assay. The sugars released by hydrolysis reactions of sugar beet arabinan, debranched arabinan, wheat arabinoxylan, birchwood xylan, and beechwood xylan were measured by the 3,5-dinitrosalicylic acid (DNSA) method (16). One unit of enzymatic activity was defined as the amount of enzyme that produced 1 μmol reducing sugar per minute.

The competitive inhibition of α-L-arabinofuranosidase substrate on xylosidase activity of rPcXyl was performed by adding pNPA into 100 μl of 50 mM sodium acetate pH 5.0 reaction containing 5 mM of pNPβX. The reaction was carried out for 10 min at 45°C, after which the amount of pNP released was determined as described above.

Hydrolysis of xylo-oligomers and xylan substrates in synergistic action with xylanase The mode of action of rPcXyl was examined using xylo-oligomers (xylobiose, xylotriose, and xylopentaose), while the synergistic action of rPcXyl and rPcXynC (17) were evaluated using birchwood and beechwood xylans. Hydrolytic products of xylo-oligomers and xylan substrates were analyzed by thin layer chromatography (TLC) using chloroform/acetic acid/H_2O (6:7:1) as a mobile phase. Reaction products were visualized by spraying TLC plates with sulfuric acid/ethanol (5:95, v/v) containing 1 mg/ml of orcinol, followed by baking at 110°C for 5 min. Xylose, xylobiose, xylotriose, and xylopentaose were used as standards.

The synergistic action of rPcXyl on xylan (birchwood xylan, beenchwood xylan and arabinoxylan) was investigated using the rPcXynC. The reducing sugar released by hydrolysis reaction was measured as method described above.

Additionally, the transglycosylation activity of rPcXyl was determined using pNPβX and various alcohol receptors (ethanol, methanol, 2-propanol) as described by Shao et al. (3). The hydrolysis products were separated and visualized by TLC assay as described above.

RESULTS

Cloning and sequence analysis of *PcXyl* Previously, we reported the successful cloning and expression of a *P. chrysosporium* xylanase (17). In order to degrade hemicellulose substrates, we investigated other *P. chrysosporium* hemicellulases for use in enhancing the synergistic action of xylanase. Based on the *P. chrysosporium* RP78 genome database, we found a putative family 43 glycoside hydrolase that comprises a xylosidase conserved domain. The putative xylosidase gene is composed of 10 exons and 9 introns that encode for 598 amino acids with a predicted signal peptide of 26 residues. The predicted cDNA sequence of this putative xylosidase was used as a template to design primers for gene amplification from the total *P. chrysosporium* BKM-F-1767 cDNA library. The PCR amplicon was

perfectly matched with the expected size of the predicted *Xyl* gene of *P. chrysosporium* RP78, and hence, was named *PcXyl*. The nucleotide sequence indicated that *PcXyl* contains 1797 nucleotides, encoding for 598 amino acids. *PcXyl* shared 99% nucleotide and amino acid similarities to the predicted putative *P. chrysosporium* RP78 xylosidase. The full-length cDNA nucleotide sequence of *PcARA* in this study was deposited in GenBank (accession no. JX625152).

Homologous searching of a protein crystal structure database using the Phyre server (http://www.sbg.bio.ic.ac.uk/phyre2) revealed that *PcXyl* highly matched β-xylosidases from *Bacillus subtilis* (PDB 1YIF), *Clostridium acetobutylicum* (PDB 1YI7), *Geobacillus stearothermophilus* (PDB 2EXK), and *Bacillus halodurans* (PDB 1YRZ). Based on a model of *G. stearothermophilus* (18), three catalytic residues of *PcXyl* Asp48, Asp169, and Glu223 were identified as a general base, a modulator of pK_a, and a general acid, respectively. Ten amino acid residues, namely Ser63, Phe65, Trp109, Ala110, Phe191, Thr243, His284, Leu300, Arg313, and Phe564, are known to play an important role in substrate binding. These amino acids are highly conserved in *PcXyl*, *G. stearothermophilus* xylosidase, *Aspergilus niger* putative xylosidase (EIT77121), *Butyrivibrio fibrisolvens* xylosidase/arabinosidase (19), and *Selenomonas ruminantium* xylosidase/arabinosidase (20) (Fig. 1). We also compared *PcXyl* with previously published xylosidase/arabinofuranosidase sequences. The results indicated that *PcXyl* exhibited similarities to xylosidases/arabinofuranosidases from *B. fibrisolvens* (31%) (19), starter mixture metagenome (30%) (4), *S. ruminantium* (28%) (20), and *Fusarium graminearum* (33%) (21). While there were no observable similarities between *PcXyl* and xylosidases/arabinofuranosidases from rumen metagenome (22), *P. purpurogenum* (7), *P. woosongensis* (5), *Clostridium stercorarium* (23), and *T. ethanolicus* (6). No fungal carbohydrate binding domain was found; however, twelve potential *N*-glycosylation sites (Asn−Xaa−Ser/Thr) were identified in the *PcXyl* sequence.

Expression and purification of rPcXyl We expressed rPcXyl in *P. pastoris* using both *PcXyl* intrinsic and α-secretion signal peptides. However, recombinant *P. pastoris* harboring *PcXyl* with intrinsic signal peptide did not produce rPcXyl according to both enzymatic assay and Western blot analysis (data not shown). Previously, we reported the expression of recombinant *P. chrysosporium* xylanase using its intrinsic signal peptide in *P. pastoris* (17). Thus, the effectiveness of the presence of intrinsic signal peptide in *P. pastoris* may depend on specific amino acid sites where the secretion system of *P. pastoris* can be detected and processed, which may not be present in *PcXyl*.

Among a total of 20 recombinant colonies, we selected a single colony with the greatest immunodetection significance by western blot, which further was used to produce rPcXyl enzyme. As shown in Figure S1, the enzyme activity of rPcXyl dramatically increased up to day 4 and reached a maximum of 26,141 U l^{-1}. Purified rPcXyl exhibited a specific activity of 1797 U mg^{-1}. The K_m and V_{max} of β-xylosidase activity of rPcXyl were 12.7 mM and 2812 U mg^{-1}, where the same kinetic parameters of α-L-arabinofuranosidase activity were found to be 12.6 mM and 492 U mg^{-1} for K_m and V_{max}, respectively. The molecular mass of purified rPcXyl was found to be around 83 kDa by determination using SDS-PAGE (Fig. 2). However, the molecular mass of endoH-treated rPcXyl shows to approximate 66 kDa, which is similar with the theoretical molecular mass of 65.6 kDa by calculating the number of amino acids present in the sequence. Therefore, the discrepancies between the predicted and recombinant enzymes seem to be due to glycosylation (17).

Effect of pH, temperature and sugar concentration on rPcXyl The optimum pH for maximum rPcXyl activity was found to be 5.0, and the optimum temperature was 45°C (Fig. 2A and B). The enzyme activity thereof rapidly decreased when the pH and temperature were, respectively, below 4.5 and 40°C or above 5.5 and 45°C.

The effect of various metal ions on the activity of rPcXyl was also investigated. As shown in Table 1, rPcXyl was almost completely inhibited by Cu^{2+} and Zn^{2+}. In additional, Ni^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+} decreased rPcXyl activity by 17.2%, 21.2%, 20.4%, and 47.6%, respectively. Ca^{2+} and K^{+} induced moderate inhibition, whereas $Mg^{2+}$and Fe^{3+} only slight affected rPcXyl activity. Due to the exhibition of arabinofuranosidase activity in the experiments below, the optimum pH, temperature and effect of metal ions on arabinofuranosidase were also studied. The results showed that optimum pH and temperature for the arabinofuranosidase activity of rPcXyl were 5.5 and 50°C, respectively (Fig. 3A and B). Similar to the effect on xylosidase activity, all metal ions exhibited an inhibitory effect on arabinofuranosidase activity, in which Zn^{2+}, Cu^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, and Fe^{2+} strongly inhibited arabinofuranosidase activity and Mg^{2+}, Ca^{2+}, K^{+}, and Fe^{3+} partially decreased rPcXyl activity (Table 1).

To assess the effect of sugar on rPcXyl, we examined the enzyme activity of rPcXyl in the presence of various concentrations of glucose, xylose and arabinose (Fig. 4A). Xylose and arabinose exerted a stronger effect on rPcXyl than glucose. These sugars increased enzyme activity by 151%, 126% and 123%, respectively, at concentrations of 5 mM. At the concentration of 10 mM, glucose still enhanced enzyme activity, while xylose and arabinose decreased rPcXyl activity. All three sugars inhibited rPcXyl at concentrations of 50 mM or above.

To decide of the effect of α-L-arabinofuranosidase substrate on xylosidase activity, rPcXyl was incubated with both substrates (pNPβX and pNPA) at different concentrations. As shown in Fig. 4B, pNPA supplementation decreased the enzyme activity. The enzyme activity was 51.9% in the presence of 5 mM pNPA, but dropped to 36.8%, 26.4% and 17.0% when pNPA concentration was 10 mM, 15, mM and 20 mM, respectively by compared to control that of without pNPA.

Substrate specific activity The hydrolysis activity of rPcXyl toward pNPG, pNPαX, pNPA, sugar beet arabinan, debranched arabinan, wheat arabinoxylan, birchwood xylan, and beechwood xylan are shown in Table 2. rPcXyl did not hydrolyze pNPG and pNPαX, but acted on pNPA. The specific activity of rPcXyl when using pNPA as a substrate was 197 U mg^{-1}. rPcXyl demonstrated slight hydrolysis on hemicelluloses substrates. Among those, rPcXyl efficiently degraded wheat arabinoxylan with a specific activity of $<$0.28 U mg^{-1}.

Hydrolysis of xylo-oligomer sugars and synergistic action with xylanase The mode of action of rPcXyl was analyzed using xylo-oligomers. As shown in Fig. 5A, rPcXyl degraded xylobiose to xylose and xylotriose to xylose and xylobiose. However, these xylo-oligomers were not completely degraded. On the other hand, a spot that may have corresponded to xylotetraose was observed in the reaction mixture of rPcXyl and xylopentaose.

Additionally, we also evaluated the efficiency of the synergistic action of rPcXyl and rPcXynC on birchwood xylan, beechwood xylan and arabinoxylan. rPcXyl released only 0.012 mg, 0.015 mg, and 0.019 mg reducing sugar per 5 mg of birchwood xylan, beechwood xylan and arabinoxylan, respectively (Table S1). The combination of rPcXyl and rPcXynC increased total sugar release by 0.146 mg, 0.123 mg, and 0.163 mg of these substrates compared with rPcXynC treatment (Table S1). Due to less xylosidase activity on xylan substrate, there was no observed hydrolysis product of birchwood xylan or beechwood xylan on TLC plate, while the combination of rPcXyl and rPcXynC increased xylose concentrations (Fig. 5B). This result indicated that the synergistic action of rPcXyl and rPcXyn enhanced the final hydrolysis product,

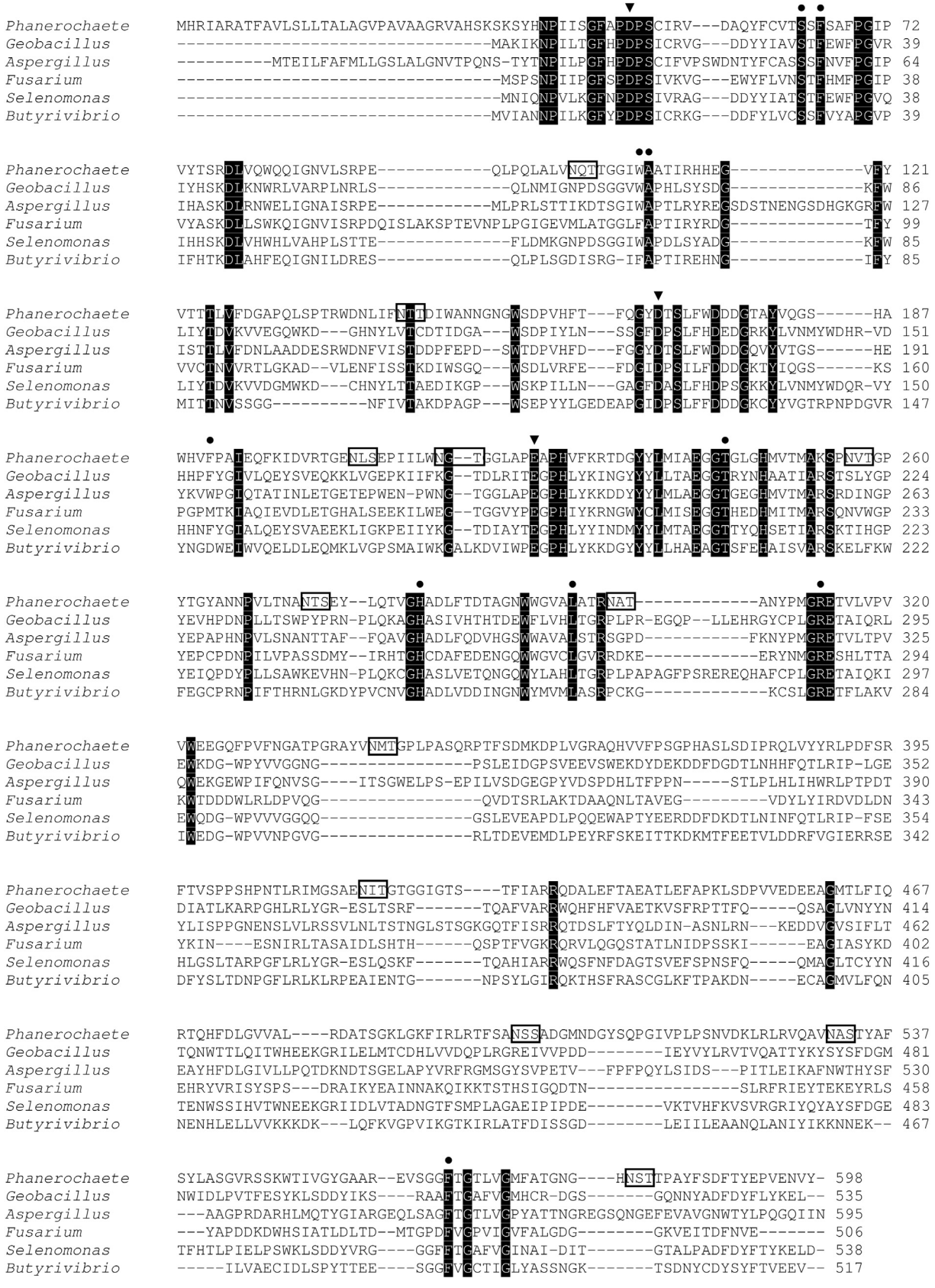

FIG. 1. The deduced amino acid sequence alignment of *P. chrysosporium* xylosidase/arabinofuranosidase (*PcXyl*) and other *Xyls* from *G. Stearothermophilus* (PDB: 2EXK_A), *Butyrivibrio fibrisolvens* (AAA63609), *Selenomonas ruminantium* (AAB97967), *Fusarium graminearum* (XP_391670) and *Aspergilus niger* (EIT77121) using the ClustalW2 tool. Identical amino acids are written in black letters. The Asp48, Asp169, and Glu223 conserved catalytic residues of xylosidases are showed as closed inverted triangles. Conserved residues that are essential for substrate recognition in xylosidases are marked by closed circles. The *N*-glycosylation sites (Asn−Xaa−Ser/Thr) are boxed.

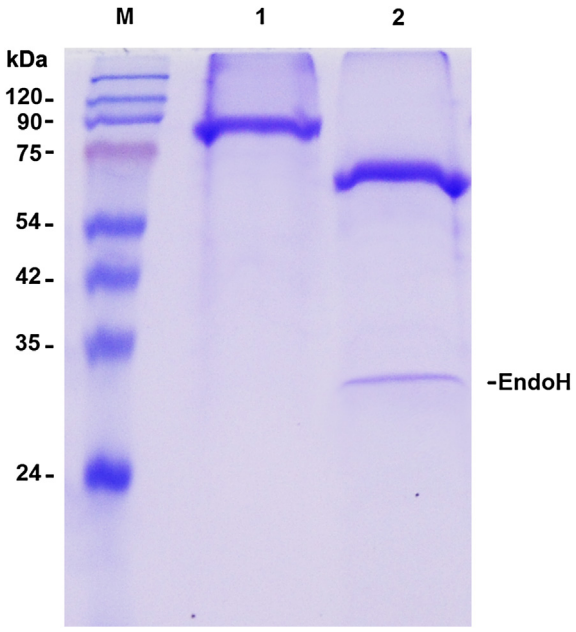

FIG. 2. SDS-PAGE analysis of purified recombinant xylosidase/arabinofuranosidase (rPcXyl). Lane M, molecular mass markers; lane 1, purified recombinant xylosidase/arabinofuranosidase (rPcXyl); lane 2, rPcXyl treated with EndoH.

suggesting that rPcXyl could be used to degrade xylan or other hemicelluloses substrates.

rPcXyl hydrolyzed to final product of xylose, but did not transferred xyloside from pNPβX to alkyl alcohol (Fig. 5C). This result indicated that rPcXyl performed hydrolysis reaction; however, there was no transglycosylation activity from this enzyme. Moreover, rPcXyl activity was significantly inhibited by alcohol, especially 2-propanol.

DISCUSSION

Xylanases and xylosidases are key enzymes in the degradation of xylan. *P. chrysosporium* is known to produce these enzymes in culture medium when using xylan substrate as a carbon source (24). However, the gene encoding for xylosidase has not yet been characterized. By following the putative xylosidase sequence, we demonstrated that this putative gene encodes for a protein that not only hydrolyzes xylo-oligomers, but also demonstrates significant arabinofuranosidase activity. rPcXyl is the first enzyme shown to exhibit both xylosidase and arabinofuranosidase functionality in

TABLE 1. Effect of metal ions on xylosidase and arabinofuranosidase activity of recombinant xylosidase/arabinofuranosidase (rPcXyl).

Additions	Relative activity (%)	
	Xylosidase	Arabinofuranosidase
None	100	100
Mg^{2+}	93	81.4
Zn^{2+}	0.3	0.1
Cu^{2+}	5.8	2.3
Ni^{2+}	17.2	14.5
Mn^{2+}	21.2	23.8
Ca^{2+}	76.2	73.8
Co^{2+}	20.4	16.9
K^{+}	73.7	70.1
Fe^{2+}	47.6	35.5
Fe^{3+}	90.4	91.8

rPcXyl was incubated in 100 μl of 50 mM sodium acetate buffer containing 10 mM of each ion at optimal pH and temperature for 1 h. The reaction was carried out by adding pNPβX or pNPA to a final concentration of 2.5 mM for 10 min. Data represent the means of three experiments and the values were averaged.

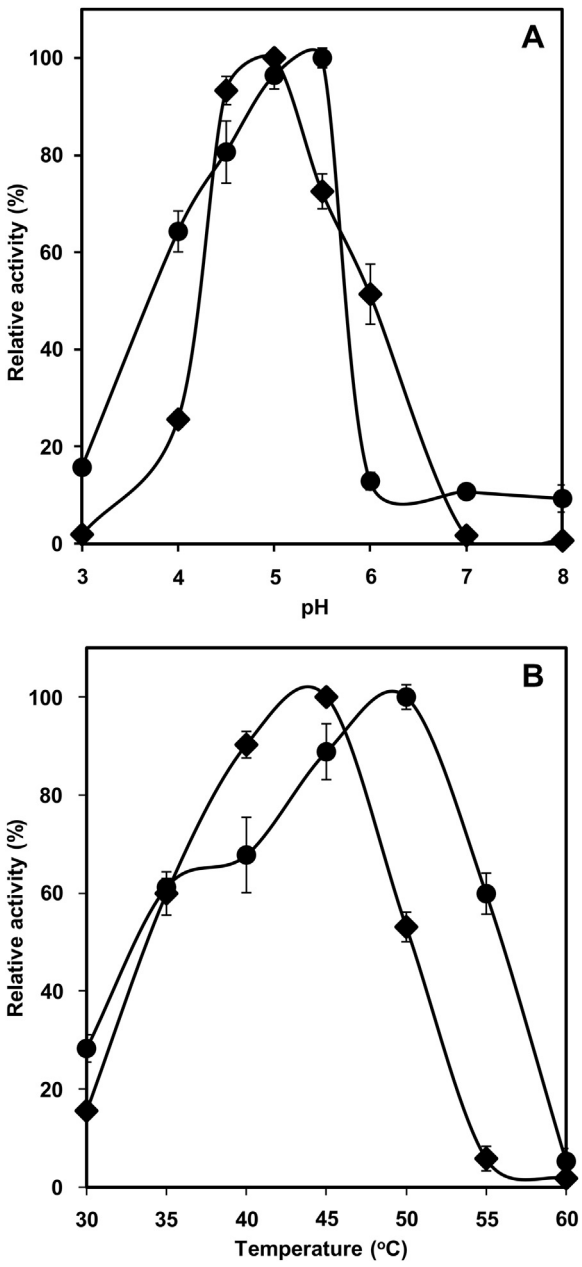

FIG. 3. Effect of pH (A) and temperature (B) on recombinant xylosidase and arabinofuranosidase activity. The reactions were performed in 100 μl of 50 mM sodium acetate buffer, containing 2.5 mM of pNPβX or pNPA, at an appropriate pH and temperature for 10 min. Diamonds and circles represent the relative of xylosidase and arabinofuranosidase activities, respectively at appropriate pHs and temperatures. Data represent the means of three experiments, and error bars represent means ± standard errors.

P. chrysosporium fungus. Unlike bifunctional xylosidase/arabinofuranosidase enzymes from bacteria, there is limited literature on the characterization of these enzymes in fungal sources (7). As observed in this study, the highest similarity in the amino acid sequences of *PcXyl* and characterized xylosidases/arabinofuranosidases was 33%. Moreover, *PcXyl* was not related to xylosidase/arabinofuranosidase enzymes from rumen metagenome, *P. purpurogenum*, *P. woosongensis*, *C. stercorarium*, and *T. ethanolicus*. These results may suggest that *PcXyl* is a novel xylosidase/arabinofuranosidase among family 43 glycoside hydrolases.

The optimum pH and temperature of rPcXyl toward pNPβX and pNPA were found to be around 5.0 and 45°C. These are in accordance with most bifunctional xylosidases/arabinofuranosidases,

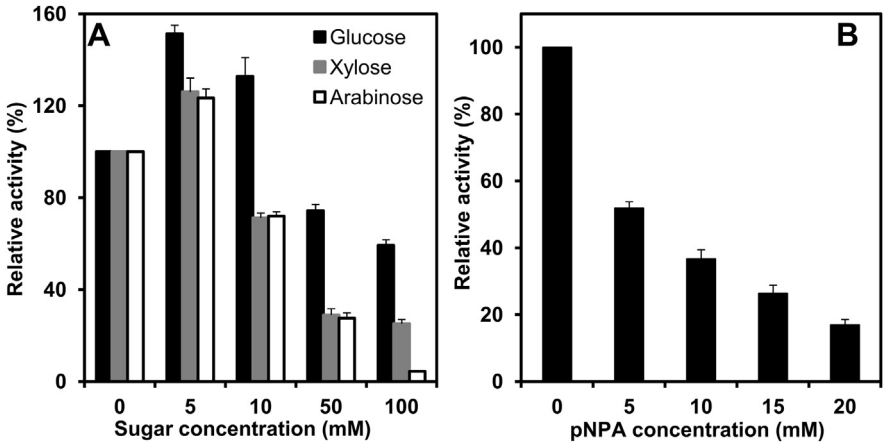

FIG. 4. (A) Effect of sugar concentration on the xylosidase activity of recombinant xylosidase/arabinofuranosidase (rPcXyl). Sugar concentrations of 5, 10, 50 and 100 mM were investigated. rPcXyl was incubated with respective concentrations of each sugar in 100 μl of 50 mM sodium acetate buffer for 1 h at 45°C. Reactions were carried out by adding pNPβX to a final concentration of 2.5 mM for 10 min. The residual activity of a control in the absence of sugar was fixed as 100%, and sample residual activity was compared with the control. Data represent the means of three experiments, and error bars represent means ± standard errors. (B) Inhibition of pNPA on xylosidase activity of rPcXyl. The competitive inhibition of pPNA on rPcXyl was assayed in 100 μl of 50 mM sodium acetate buffer containing 5 mM of pNPβX, the range pNPA concentration of 5, 10, 15 and 20 mM were added into reaction following by 10 min incubation at 45°C. The residual activity was measured as standard method. Data represent the means of three experiments, and error bars represent means ± standard errors.

where the optimum pH and temperature were reported to be around 5.0–7.0 and 45–65°C, respectively. rPcXyl was almost completely inhibited by Zn^{2+} and Cu^{2+}; however, Zn^{2+} did not strongly affect the xylosidases/arabinofuranosidases of *P. woosongensis* (5) and *Caulobacter crescentus* (25) in which the residual activities were decreased to only 83% and 77%, respectively. The retained enzyme activity of *C. crescentus* was 20% in the presence of Zn^{2+}. Additionally, Mn^{2+} enhanced *P. woosongensis* xylosidase and arabinofuranosidase activity up to 187% and 133%, respectively, but decreased rPcXyl xylosidase and arabinofuranosidase activity to 21.2 and 23.8%. The xylosidase K_m value of rPcXyl is similar to *P. purpurogenum* bifunctional α-L-arabinfuranosidase/β-xylosidase, while arbinofuranosidase K_m of rPcXyl is higher than that of enzyme (7). In comparison to other fungi α-L-arabinfuranosidase/β-xylosidase from *P. purpurogenum* (7) and *F. graminearum* (21), rPcXyl exhibited lower specific activity. The lower specific activity may due to hyperglycosylated of rPcXyl, where prevents the reorganization of enzyme and substrate.

Xylose and arabinose initially inhibited the xylosidase/arabinofuranosidase activities of rPcXyl as well as enzymes from *P. woosongensis* (5) and *S. ruminantium* (20) at a concentration of 10 mM. Glucose demonstrated less inhibition of rPcXyl activity than xylose and arabinose, which is similar to a previous report by Kim and Yoon (5). Interestingly, low concentrations of these sugars enhanced rPcXyl xylosidase and arabinofuranosidase activity, but seemed not to affect the activity of enzymes from *P. woosongensis* (5) and *S. ruminantium* (20) seemed to be not changed. Xylo-oligomers and arabino-oligomers did not increase the enzyme activity of rPcXyl, except for xylotriose. The hydrolysis by xylosidase is initiated by recognizing the hydrogen bonds of oxygen atoms of xylose substrate and specific amino acids of enzyme active sites, which release single units of sugar from the non-reducing end of xylo-oligomers (18). Thereby, low concentrations of sugar may boost enzyme–substrate interactions by activating hydrogen bonds; however, increasing sugar concentrations may also enhance competition that would prevent recognition between enzyme and substrate.

TABLE 2. Substrate specific activity of recombinant xylosidase/arabinofuranosidase (rPcXyl).

Substrates	Activity (U mg^{-1})
p-Nitrophenyl-β-D-xylopyranoside	1797
p-Nitrophenyl-α-L-arabinofuranoside	197
p-Nitrophenyl-α-D-xylopyranoside	nd
4-Nitrophenyl-β-D-glucopyranoside	nd
Birchwood xylan	<0.03
Beechwood xylan	<0.1
Sugar beet arabinan	<0.08
Debranched arabinana	<0.18
Wheat arabinoxylan	<0.28

The specific activity of rPcXyl was assessed by incubating of 10 μg of purified rPcXyl with 2.5 mM of pNPβX, pNPA, pNPαX, and pNPG or 0.5% of birchwood xylan, beechwood xylan, sugar beet arabinan, debranched arabinan, and wheat arabinoxylan in 50 mM sodium acetate buffer at 45°C for 10 min. The release of pNP reducing sugar was measured at 410 nm wavelength with a spectrometer, while reducing sugar release was determined by DNSA assay. One unit of xylosidase activity was defined as the release of 1 nmol of pNP or 1 μmol of reducing sugar per minute. nd: Not detectable.

Because the bifuntionality of xylosidase is due to the spatial similarities between D-xylopyranose and L-arabinofuranose, there is likely that both substrates are recognized and catalyzed by the same enzyme domain. Moreover, the α-L-arabinofuranosidase specific activity of rPcXyl was 9 times lower than that of β-xylosidase specific activity (Table 2). Thus, we examined where α-L-arabinofuranosidase substrate decreases enzyme activity or not. As a result, enzyme activity decreased to 52% at same concentration of both pPNA and pNPβX, and increasing pNPA concentration reduced enzyme activity (Fig. 4B). This result indicated the inhibition of pPNA on enzyme activity is due to the competition on catalyzed domain of enzyme.

The total hydrolysis products of xylan substrates are not much increased by the combination of rPcXyl and rPcXynC (Table S1), but TLC analysis clearly showed that xylose concentration was increased from xylo-oligomers by synergistic action of rPcXyl and rPcXynC, indicating the main function of rPcXyl is to hydrolyze short xylo-oligomer to xylose (Fig. 5B). rPcXyl displayed less activity on xylan substrates, similar to most reports on β-xylosidase (11). The crystal structure of *G. stearothermophilus* β-xylosidase was shown to comprise a loop related to the active site that restricts the length of xylose units that can enter its active site (18). Thus, xylan, which is composed of a long chain of xylose, may not be able to link to β-xylosidases such as rPcXyl. As shown in this study, rPcXyl exhibits both function xylosidase and arabinofuranosidase activity that may boost the efficiency of biomass degradation by enhancing

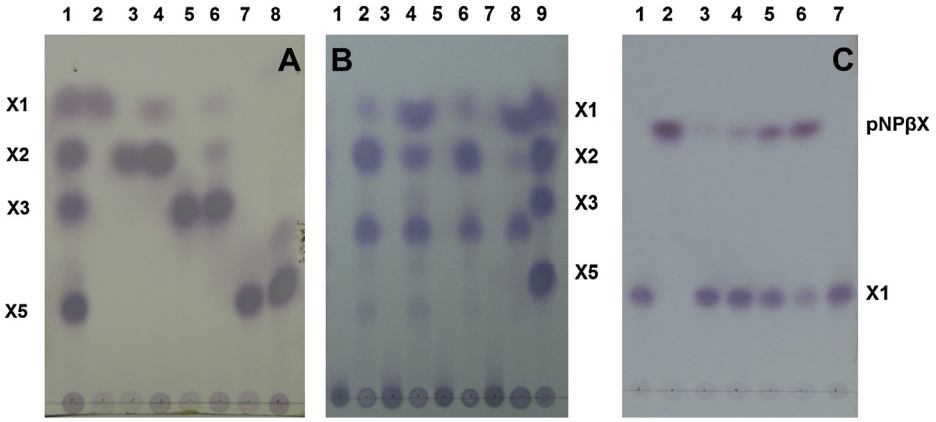

FIG. 5. Thin layer chromatogram of sugars produced during hydrolysis. (A) Xylo-oligomers hydrolysis products: lane 1, xylo-oligomers; lanes 2, 3, 5 and 7 represent xylose (X1), xylobiose (X2), xylotriose (X3) and xylopentaose (X5), respectively; lanes 4, 6, 8 represent the xylobiose, xylotriose and xylopentaose degradation by recombinant xylosidase/arabinofuranosidase (rPcXyl). Xylobiose, xylotriose or xylopentaose at concentrations of 5 μmol, 3.3 μmol or 2 μmol, respectively, were incubated with 50 U of rPcXyl for 10 min at 45°C in 100 μl of 50 mM sodium acetate buffer at pH 5.0. The reaction was terminated by heating at 100°C for 5 min, after which 1 μl of reaction products were taken and subjected on TLC silica gel. The hydrolysis products were separated and visualized on TLC silica gel. (B) Xylan hydrolysis products: lanes 1 and 5 represent beechwood and birchwood xylans; lanes 2 and 6 represent the hydrolysis of beechwood and birchwood xylans by recombinant xylanase C (rPcXynC); lanes 3 and 7 represent the hydrolysis of beechwood and birchwood xylans by rPcXyl; lanes 4 and 8 represent the hydrolysis of beechwood and birchwood xylans by the combination of rPcXyl and rPcXynC; lane 9 represents the xylo-oligomers of xylose (X1), xylobiose (X2), xylotriose (X3) and xylopentaose (X5). rPcXyl and rPcXynC at concentrations of 40 U and 0.5 U were incubated with 0.5% substrate in 100 μl of 50 mM sodium acetate at pH 5.0 and 45°C for 20 h. The reaction was terminated by heating at 100°C for 5 min, after which 1 μl of reaction products were taken and subjected on TLC silica gel. The hydrolysis products were separated and visualized on TLC silica gel. (C) pNPβX hydrolysis products: lane 1 and 7 represent xylose (X1); lane 2 represents pNPβX; lane 3, 4, 5, and 6 represent the hydrolysis of pNPβX by rPcXyl without alcohol, ethanol, methanol, and 2-propanol, respectively. The transglycosylation ability of rPcXyl was determined with pNPβX and various alcohols (ethanol, methanol, and 2-propanol). rPcxyl was incubated with 10% of each alcohol in 100 μl of 50 mM sodium acetate at pH 4.5 containing 5 mM pNPβX. The reaction was carried out at 45°C for 10 min, after which 1 μl of reaction products were taken and subjected on TLC silica gel. The hydrolysis products were separated and visualized on TLC silica gel.

the synergistic action of xylose and arabinose sugars. Accordingly, rPcXyl increased xylose concentrations in xylan hydrolysis when combined with rPcXynC, offering great promise as a candidate for which to degrade hemicelluloses biomass via synergistic action.

There are two basic types of glycoside hydrolases: retaining and inverting. Retaining enzymes can perform both hydrolysis and transglycosylation reactions, whereas inverting enzymes can perform only hydrolysis (2). rPcXyl hydrolyzed pNPβX to xylose but did not transferred xyloside to alkyl alcohol (Fig. 5C), indicating rPcXyl catalyze the hydrolysis via inverting mechanism which is found in xylosidases from glycoside hydrolase family 43 (3). Thus, it is proposed that *PcXyl* is belonging to glycoside hydrolase family 43.

In previous studies, numerous bifunctional xylosidase/arabinofuranoside enzymes have been characterized; moreover, the genes that encode for these enzymes have also been cloned and expressed. However, the expression host of these genes in previous studies was *E. coli*, and consequently, the enzymes were produced as intracellular enzymes (4–6,19–23,25,26). Compared to intracellular enzymes, extracellular enzymes offer more advantages in industry, compared to intracellular enzymes, which must undergo a cell destroying step that encumbers their isolation on an industrial scale. Furthermore, purification of a single protein from the native extracellular protein matrix of wild type microorganisms without contamination is also very complicated and may be disadvantageous at the industrial level. Therefore, the secretion of rPcXyl into culture medium could potentially be produced on a large scale with relatively greater ease.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2013.02.004.

ACKNOWLEDGMENTS

This research was supported by the Technology Development Program for Agriculture and Forestry, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea. This research was also supported in part by research funds of Chonbuk National University in 2012, and Korea Research Council of Fundamental Science & Technology (Joint Degree and Research Center for Biorefinery). We would like to thank the Research Institute of Bioindustry at Chonbuk National University for kindly providing the facilities for which to conduct this research.

References

1. **Shallom, D. and Shoham, Y.:** Microbial hemicellulases, Curr. Opin. Microbiol., **6**, 219–228 (2003).
2. **Juturu, V. and Wu, J. C.:** Microbial xylanases: engineering, production and industrial applications, Biotechnol. Adv., **30**, 1219–1227 (2012).
3. **Shao, W., Xue, Y., Wu, A., Kataeva, I., Pei, J., Wu, H., and Wiegel, J.:** Characterization of a novel beta-xylosidase, XylC, from *Thermoanaerobacterium saccharolyticum* JW/SL-YS485, Appl. Environ. Microbiol., **77**, 719–726 (2011).
4. **Wagschal, K., Heng, C., Lee, C. C., and Wong, D. W. S.:** Biochemical characterization of a novel dual-function arabinofuranosidase/xylosidase isolated from a compost starter mixture, Appl. Microbiol. Biotechnol., **81**, 855–863 (2009).
5. **Kim, Y. A. and Yoon, K.-H.:** Characterization of a *Paenibacillus woosongensis* beta-xylosidase alpha-arabinofuranosidase produced by recombinant *Escherichia coli*, J. Microbiol. Biotechnol., **20**, 1711–1716 (2010).
6. **Mai, V., Wiegel, J., and Lorenz, W. W.:** Cloning, sequencing, and characterization of the bifunctional xylosidase–arabinosidase from the anaerobic thermophile *Thermoanaerobacter ethanolicus*, Gene, **247**, 137–143 (2000).
7. **Ravanal, M. C., Callegari, E., and Eyzaguirre, J.:** Novel bifunctional alpha-l-Arabinofuranosidase Xylobiohydrolase (ABF3) from *Penicillium purpurogenum*, Appl. Environ. Microbiol., **76**, 5247–5253 (2010).
8. **Raweesri, P., Riangrungrojana, P., and Pinphanichakarn, P.:** α-l-Arabinofuranosidase from *Streptomyces* sp. PC22: purification, characterization and its synergistic action with xylanolytic enzymes in the degradation of xylan and agricultural residues, Bioresour. Technol., **99**, 8981–8986 (2008).
9. **Kambourova, M., Mandeva, R., Fiume, I., Maurelli, L., Rossi, M., and Morana, A.:** Hydrolysis of xylan at high temperature by co-action of the xylanase from *Anoxybacillus flavithermus* BC and the β-xylosidase/α-arabinosidase from *Sulfolobus solfataricus* Oα, J. Appl. Microbiol., **102**, 1586–1593 (2007).
10. **Qing, Q. and Wyman, C. E.:** Supplementation with xylanase and beta-xylosidase to reduce xylo-oligomer and xylan inhibition of enzymatic hydrolysis of cellulose and pretreated corn stover, Biotechnol. Biofuels, **4**, 18 (2011).
11. **Gírio, F. M., Fonseca, C., Carvalheiro, F., Duarte, L. C., Marques, S., and Bogel-Łukasik, R.:** Hemicelluloses for fuel ethanol: a review, Bioresour. Technol., **101**, 4775–4800 (2010).

12. **Toivari, M. H., Nygård, Y., Penttilä, M., Ruohonen, L., and Wiebe, M. G.:** Microbial D-xylonate production, Appl. Microbiol. Biotechnol., **96**, 1–8 (2012).
13. **Saha, B. C. and Bothast, R. J.:** Production of 2,3-butanediol by newly isolated *Enterobacter cloacae*, Appl. Microbiol. Biotechnol., **52**, 321–326 (1999).
14. **Tien, M. and Kirk, T. K.:** Lignin peroxidase of *Phanerochaete chrysosporium*, Methods Enzymol., **161**, 238–249 (1988).
15. **Vasu, P., Bauer, S., and Savary, B. J.:** Cloning and expression of hemicellulases from *Aspergillus nidulans* in *Pichia pastoris*, 3rd ed., pp. 433–450, in: Recombinant gene expression: reviews and protocols. Methods in molecular biology, vol. 824. Humana Press, New York (2012).
16. **Sengupta, S., Jana, M. L., Sengupta, D., and Naskar, A. K.:** A note on the estimation of microbial glycosidase activities by dinitrosalicylic acid reagent, Appl. Microbiol. Biotechnol., **53**, 732–735 (2000).
17. **Huy, N. D., Kim, S. W., and Park, S. M.:** Heterologous expression of endo-1,4-beta-xylanaseC from *Phanerochaete chrysosporium* in *Pichia pastoris*, J. Biosci. Bioeng., **111**, 654–657 (2011).
18. **Brüx, C., Ben-David, A., Shallom-Shezifi, D., Leon, M., Niefind, K., Shoham, G., Shoham, Y., and Schomburg, D.:** The structure of an inverting GH43 beta-xylosidase from *Geobacillus stearothermophilus* with its substrate reveals the role of the three catalytic residues, J. Mol. Biol., **359**, 97–109 (2006).
19. **Utt, E. A., Eddy, C. K., Keshav, K. F., and Ingram, L. O.:** Sequencing and expression of the *Butyrivibrio fibrisolvens* xylB gene encoding a novel bifunctional protein with beta-D-xylosidase and alpha-L-arabinofuranosidase activities, Appl. Environ. Microbiol., **57**, 1227–3124 (1991).
20. **Whitehead, T. R. and Cotta, M. A.:** Identification of a broad-specificity xylosidase/arabinosidase important for xylooligosaccharide fermentation by the ruminal anaerobe *Selenomonas ruminantium* GA192, Curr. Microbiol., **43**, 293–298 (2001).
21. **Carapito, R., Carapito, C., Jeltsch, J.-M., and Phalip, V.:** Efficient hydrolysis of hemicellulose by a *Fusarium graminearum* xylanase blend produced at high levels in *Escherichia coli*, Bioresour. Technol., **100**, 845–850 (2009).
22. **Zhou, J., Bao, L., Chang, L., Zhou, Y., and Lu, H.:** Biochemical and kinetic characterization of GH43 β-D-xylosidase/α-L-arabinofuranosidase and GH30 α-L-arabinofuranosidase/β-D-xylosidase from rumen metagenome, J. Ind. Microbiol. Biotechnol., **39**, 143–152 (2012).
23. **Sakka, K., Yoshikawa, K., Kojima, Y., Karita, S.-i., Ohmiya, K., and Shimada, K.:** Nucleotide sequence of the *Clostridium stercorarium* xylA gene encoding a bifunctional protein with β-D-xylosidase and α-L-arabinofuranosidase activities, and properties of the translated product, J. Microbiol. Biotechnol., **57**, 268–272 (1993).
24. **Dobozi, M. S., Szakács, G., and Bruschi, C. V.:** Xylanase activity of *Phanerochaete chrysosporium*, Appl. Environ. Microbiol., **58**, 3466–3471 (1992).
25. **Graciano, L., Corrêa, J. M., Gandra, R. F., Seixas, F. A. V., Kadowaki, M. K., Sampaio, S. C., Silva, J. L.d. C., Osaku, C. A., and Simão, R.d. C. G.:** The cloning, expression, purification, characterization and modeled structure of *Caulobacter crescentus* β-xylosidase I, World J. Microbiol. Biotechnol., **28**, 2879–2888 (2012).
26. **Morana, A., Paris, O., Maurelli, L., Rossi, M., and Cannio, R.:** Gene cloning and expression in *Escherichia coli* of a bi-functional β-D-xylosidase/α-L-arabinosidase from *Sulfolobus solfataricus* involved in xylan degradation, Extremophiles, **11**, 123–132 (2007).