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CHARACTERIZATION OF *KRICT PX2* XYLANASE FROM THE *Paenibacillus* sp. HPL-002 FOR UTILIZATION OF PLANTS AS BIO-RESOURCES

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ABSTRACT

A new alkalophyllic endo-1, 4-beta-xylanase gene, *KRICT PX2* (GU967374) isolated from *Paenibacillus* sp. HPL-002 (KCTC11410BP) was expressed in *E. coli* and the biochemical properties of the purified enzyme was investigated. The specific activity of the purified xylanase was 51.26 μmol/min/mg proteins. And also, K_m and V_{max} values of the protein for birch wood xylan were verified to have 0.061 μM and 55.3 μmol/min/mg proteins, respectively. The optimum pH and temperature for the activity of the enzyme were pH 8~9 and 50°C, respectively, and also the activity were stably maintained at 40°C. Most metallic salts, ethylenediamine tetra-acetic acid, 2-mercaptoethanol, phenylmethane sulphonyl fluoride, and furfural have no impact on the enzyme activity at 1 mM. The simulated 3-D structure of this xylanase is similar to *Xyn*10B from *Paenibacillus barcinonensis*. Further research on the degradation of different-origin xylans and enzyme production will be necessary for the practical application.

Key words: Alkalophyllic xylanase, cloning, expression, *Paenibacillus* sp. HPL-002

INTRODUCTION

Xylan is a complex polysaccharide comprising a backbone of xylose residues linked by β-1, 4-glycosidic bonds with the main chain composed of β-xylopyranose residues. It is the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 15-30% and 7-12% of the total dry weight in hardwood from angiosperms and softwood from gymnosperms, respectively (Saha, 2003). Chemical hydrolysis of lignocelluloses results in hazardous byproducts such as phenolic compounds from lignin degradation, furan derivatives (furfural and HMF) from sugar degradation, and aliphatic acids (acetic acid, formic acid, and levulinic acid). They are considered to be fermentation inhibitors generated from chemical conversion of lignocellulosic biomass (Palmqvist and Hahn-Hagerdal, 2000). Consequently, the use of microbial enzymes which are specific in

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action for xylan hydrolysis has been accepted as an environmentally friendly option (Wong *et al.*, 1988; Biely *et al.*, 1997). Bioconversion of xylan has received much attention because of its practical applications in various agro-industrial processes: delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer. Currently, there is great interest in the utilization of xylose essential for cost-reductive conversion of lignocellulosic materials to fuel ethanol and other value-added fermentation products (Ragauskas *et al.*, 2006). Due to structural heterogeneity of xylan, complete degradation of this biopolymer requires synergistic action of different xylanolytic enzymes such as endo-xylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase, and esterase. Among them, endo-xylanase (1, 4-β-D-xylan xylohydrolase, EC 3.2.1.8) is considered as the most important one and it initiates the degradation of xylan into xylose and xylooligosaccharides of different sizes (Collins *et al.*, 2005). There are different types of xylanases varying in substrate specificities, primary sequences, folds and physicochemical properties and these are produced by a number of bacteria and fungi (Wong *et al.*, 1988; Howard, 2003). Owing to the increasing biotechnological importance of xylanases, much attention has been paid to discover new xylan-degrading enzymes, which can be applicable in various industrial processes (operating) at high temperature and broad range of pH condition. Thus, many attempts are being made to isolate new strains and to discover more relevant xylanases (Collins, 2005; Li *et al.*, 2009). Through the consecutive collection of microorganisms and screening for biomass degrading activity, we have recently isolated a strain of *Paenibacillus* sp. HPL-002 (Korean Collection for Type Culture: KCTC11410BP) from the old discarded mushroom farm located in Gara Mt., Geoje City, Gyeongsangnam-do, Korea (ROK), showing excellent xylanase activity.

In the present study, we cloned and expressed the *KRICT PX2* gene (GenBank accession code: GU967374) in *Escherichia coli* and examined some biochemical properties of the purified enzyme for applying to agronomical residues and weeds as a biomass resources.

MATERIALS AND METHODS

Selection and Identification of Bacterial strain

The xylan-overlaid plates were prepared by overlaying 0.7% molten agar containing 1% birchwood xylan (Fluka) over the solidified 1% agar containing 1X M9 minimal salts (Sigma) in a plastic Petri dishes with a diameter of 87 mm. The inoculated Petri dishes were incubated for 24 h at 37ºC, and stained with 0.1 % (wt/vol) Congo red (Aldrich) for 30 min and repeatedly washed with 1 M NaCl until the

transparent halo was appeared around colonies because of xylanase activity. The active bacterial isolate was identified by the 16S rRNA analysis with the two bacterial universal primers, 518F 5(5'-CCAGCAGCCGCGGTAATACG-3') and 800R (5'- TACCAG GGTATCTAATCC-3') (Lane, 1991), and the full sequence of 16S rRNA gene was analyzed and aligned with data in Ribosomal Database Project (http://rdp.cme.msu.edu) for the species identification. Image of this bacterium was taken by a scanning electron microscope (SEM 515, Philips). The identified bacterial strain was deposited to the Korean Collection for Type Culture (KCTC11410BP), KRIBB (Korean Research Institute of Bioscience and Biotechnology, Yuseong, Daejon 305-806, Korea).

Construction and Screening of a *Paenibacillus* sp. HPL-002 Gene Library

DNA fragments (around 5 kb) were collected and purified for library construction, and blunt-end repaired and dephosphorylated, then ligated into pCB31 plasmid vector (MACROGEN Co., Korea). The packaged library was electroporated into *Escherichia coli* DH10B cells according to the manufacturer's instructions. The total 1,152 clones of transformants were collected into twelve 96-well plates containing 200 µl LB broth (Difco) in each well. After incubation for 24 h at 37°C, the xylanase activity of each clone was screened by overlaid-xylan staining (Hwang, 2010) and DNS (3,5-dinitrosalicylic acid) assay (Miller, 1959), simultaneously. The xylanase active clone in each well appeared as dark brownish color due to the reaction between DNS and the reducing sugar ends of hydrolyzed product.

DNA Sequencing and Expression of Xylanase in *E. coli*

The most xylanase-active clone (01B3 clone, arbitrary named) was selected from the library screening, and the nucleotide sequence of the insert was determined by automated sequencing under BigDye^{TM} terminator cycling condition. The reacted product was purified using ethanol precipitation and run with Automatic Sequencer 3730xl (Applied Biosystems, Weiterstadt, Germany). The each PCR product was inserted into pSTV28 plasmid vector and transformed into *E. coli* JM109 (Takara Bio Inc.), and the xylanase activity of each transformant was examined with xylan-overlaid plate and DNS assay in liquid. The gene was inserted into the pIVEX GST fusion vector for the transformation into *E. coli* BL21 (Roche Applied Science) to produce the recombinant fusion protein.

Purification of Recombinant Xylanase

The transformed *E. coli* with GST-fused xylanase were grown overnight in 10 ml LB medium containing ampicillin (100 µg/ml) at 37^{o}C and 200 rpm in a shaking incubator. The culture was induced with 1 mM IPTG and incubated under the same conditions for 3 h

longer. The cells collected from the final washing process were treated with sonic disruptor (CosmoBio Co., LTD). After cell disruption, the lysate was centrifuged at 10,000xg for 20 min at 4°C, the supernatants was eluted through the GST binding resin column (Novagen, Madison WI, USA). All fractions were examined with Bradford's protein determination, PAGE analysis, and DNS assay. Active fractions were pooled and treated with Restriction Protease Factor Xa (Roche Applied Science), and eluted through p-aminobenzamidine-agarose column (Sigma-Aldrich) according to the manufacturer's instruction. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15% polyacrylamide was performed to separate each protein, and the protein fractions mixed with denaturing agent were boiled for 3 min and applied to the gel. Proteins were visualized by Coomassie brilliant blue R 250 staining. The protein concentration was determined with Bradford reagent (Sigma-Aldrich) assay using bovine serum albumin as a standard.

Properties of Recombinant Xylanase

Xylanase activity was measured according to the method as previously reported (Hwang, 2010), using 50 µl of 1% (w/v) solution of birchwood xylan (Fluka) and 200 mM of each pH buffer incubated with 30 µl of an appropriately diluted enzyme (3.3 mg/ml) for 20 min at different temperatures. The released reducing sugars were assayed using the DNS method. One unit of xylanase activity was defined as the amount of the enzyme that liberated reducing sugar ends equivalent to 1 µmol of xylose per minute under the assay conditions. The optimal temperature and pH condition for the xylanase activity of recombinant *KRICT PX2* protein were examined in 96-well micro plates with DNS assay at various temperatures (ranging from 10 to 80ºC) and pH conditions (ranging from pH 2 to 12). The effect of birchwood xylan concentration on xylanase activity was evaluated under optimal assay conditions. Xylanase activity was measured with DNS assay at 540 nm as described above. Effect of metallic ions and other chemicals on the xylanase activity of *KRICT PX2* protein was studied as described above at pH 9 with addition of 1 mM NaCl, LiCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $CuSO_4$, $ZnSO_4$, $FeCl_3$, $CsCl_2$, ethylenediamine tetra-acetic acid (EDTA), 2-mercaptoethanol (2-ME), dithiothreitol (DTT), phenylmethane sulphonyl fluoride (PMSF), acetate, and furfural, respectively.

TLC Analysis of Hydrolytic Products

The hydrolyzed products of xylan was analyzed by the thin-layer chromatography (TLC) using silica gel plates 60 F 254 (Merck KGaA, Germany) as reported previously (Hwang, 2010). Aliquots (0.2 ml) of the samples were collected at 0, 15, 30, 60, 120, 240, and 480 min of the incubation period. After immediate boiling of each sample,

10 µl of each aliquot with xylo-oligomer standard and enzyme blank was spotted on a TLC plates. The plates were subsequently developed with acetonitrile:water (80:20, v/v). After elution for 2 h, the resultant plate was sprayed with a staining solution (1% diphenylamine and aniline in acetone, and 10% phosphoric acid), and heated for 10 minutes at 120°C in an oven to visualize the xylo-oligomers prior to take photographs. A xylo-oligosaccharide mixture was consisted of xylose, xylobiose, xylotriose, xylotetrose, and xylopentose (Wako Chemical and Megazyme).

Nucleotide Sequence Analysis and Simulation of 3D Structure

Nucleotide and deduced amino acid sequences were analyzed with CLC Free Workbench, Ver. 3.2.1 (CLC bio A/S, www.clcbio.com). Related sequences were obtained from database searches (SwissPort and GenBank). The genome sequence of *KRICT PX2* was submitted to GenBank, and assigned as Accession Number GU967374. The biomolecular 3D structure of *KRICT PX2* xylanase was predicted with a deduced amino acid sequence as homology model structure compared to Xyn10B (PDB ID: 3emz) from *Paenibacillus barcinonensis* with Discovery Studio 2.5 (accelrys®).

RESULTS

Screening and Selection of The Bacterial Strain Degrading Xylan

From the screening of the natural bacteria, the strain of HPL-002 exhibited the highest xylanolytic activity (Figure 1A and B). Therefore, this strain was selected for further identification at the molecular level. Its full sequence of 16S rRNA gene was amplified and analyzed through a RDP tool, SeqMatch with data set involving both type and non-type strains, both environmental (uncultured) sequences and isolates, and near-full-length sequences (>1,200 bases) showing good quality sequences against total RDP data. Alignment of the 1,234 bases of 16S rRNA gene showed that it was very close to 16S rRNA genes of bacteria from genus *Paenibacillus* with similarity score range of 0.904~0.963. From this result, HPL-002 was identified as *Paenibacillus* sp., and the deposit number of KCTC 11365BP was obtained from Korean Collection for Type Culture, KRIBB. The bacterial cells were Gram-positive (data not shown) with rod shape of 1.1~1.5 x 2.5~3.0 µm in size without any flagella, and ellipsoidal spores were formed in swollen sporangia (Figure 1C). The photographs were taken after Congo red (0.1%) staining and repetitive washing with 1 M NaCl. Scanning Electron Microscope (SEM) photograph (C) of the isolated *Paenibacillus* sp. HPL-002.

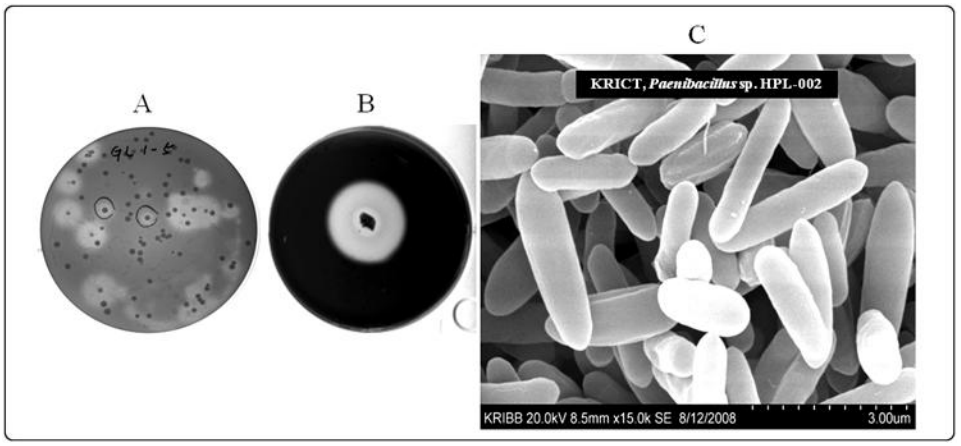


Figure 1. Screening of xylan degrading bacteria (A), and isolation of *Paenibacillus* sp. HPL-002 (B) in the xylan-overlaid plates.

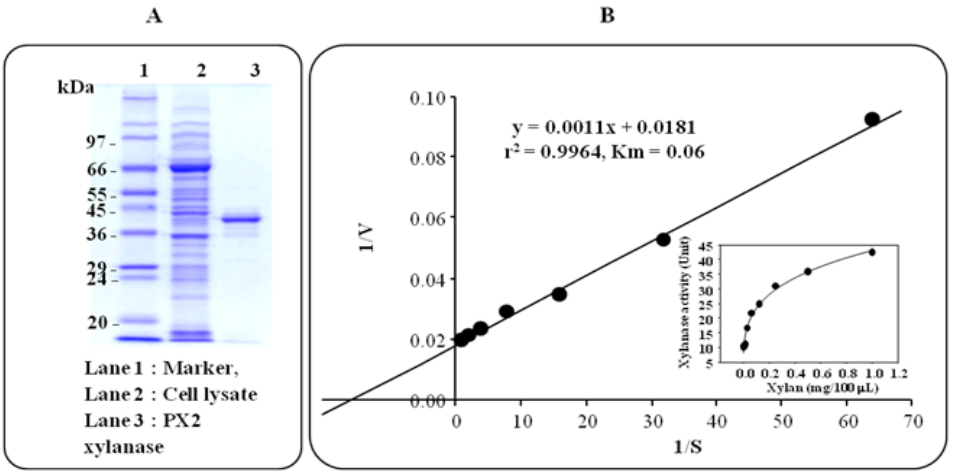


Figure 2. SDS PAGE analysis of the purified *KRICT PX2* xylanase (A, MW 38.4, lane 3) from cell lysate (lane 2) over-expressed with GST-fused xylanase in *E. coli* BL21 (64.4 kDa, fused with GST 26 kDa and *KRICT PX2* 38.4 kDa), and molecular weight markers (lane 1), and Lineweaver-Burk double reciprocal analysis of *KRICT PX2* xylanase with birchwood xylan as substrate.

Purification and Characterization of Recombinant Xylanase

DNS assay and overlaid xylan analysis of the *Paenibacillus* sp. strain HPL-002 genomic DNA library led to the isolation of single clone, arbitrary named as 01B3 (the first plate, well number of B3) with great xylanase activity. The sequence analysis of the insert in 01B3 clone exhibited that this insert has total insert size of 4,180 bps, and further ORF Finder analysis revealed that it is constructed with 7 ORFs. The final preparation of *KRICT PX2* xylanase gave a single band on SDS-PAGE (Figure 2-A). The molecular weight (MW) of the protein was estimated to be 38.4 kDa with the deduced amino acid sequence of the gene. The purified xylanase was appeared slightly over the 36 kDa MW markers. And also, the GST-fused *KRICT PX2* protein calculated as MW 64.4 kDa was observed just below the 66 kDa MW markers.

The enzyme activity was measured with various concentrations of birchwood xylan in 50 mM citric buffer (pH 9.0) at 50°C. The optimal pH was the pH in which the enzyme displayed its maximal activity, which was considered 100% activity. The optimal pH for *KRICT PX2* xylanase activity was determined at pH 9.0, which was considered 100% activity, and retaining about 98% of its activity at pH 8.0, and about 90% of its activity at pH 7 and pH 10, respectively (Figure 3A). The optimal temperature for *KRICT PX2* xylanase activity was determined at 50°C, which was considered 100% activity, and retaining about 75% of its activity at 40 and 60°C (Fig. 3B). The *KRICT PX2* xylanase was very stable for one hour at 40°C, however, the activity of *KRICT PX2* xylanase was sharply decreased at 50°C conditions after incubation for 10 min (data not shown). Kinetic analysis of this xylanase with birchwood xylan as substrate (Figure 2) was performed at 50(C in pH 9.0. The specific activity of the purified KRICT PX2 xylanase was 51.26 μmoles/min/mg proteins. And also, Km and Vmax values of the protein for birch wood xylan were determined as 0.061 μM and 55.3 μmol/min/mg proteins, respectively. Most salts, such as NaCl, LiCl, KCl, NH4Cl, CaCl2, MgCl2, MnCl2, CuSO4, ZnSO4, FeCl3, CsCl2 did not significantly change the enzyme activity at 1 mM. And also, 1 mM of ethylenediamine tetra-acetic acid, 2-mercaptoethanol, phenylmethanesulphonyl fluoride, and furfural were not effective on the enzyme activity (Table-1). The hydrolysis products of birchwood xylan were analyzed by TLC. As shown in Fig. 4, the products hydrolyzed by the KRICT PX2 xylanase were mainly xylobiose and much smaller amount of xylose and xylopentose. Especially, large amount of xylobiose was continuously produced from the start of the reaction.

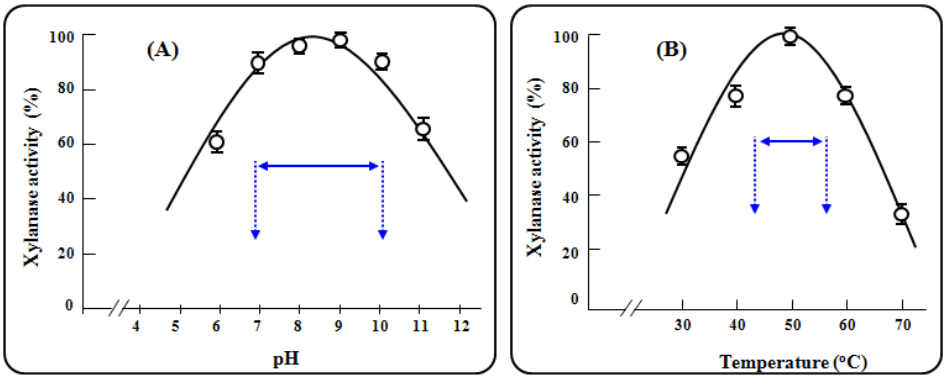


Figure 3. Xylanase activity of *KRICT PX2* at various pH (A) and temperature (B) conditions. The enzyme activity was assayed at 50°C in 50 mM citric buffer (pH 2~6.5), phosphate buffer (pH 7~9), and glycine buffer (pH 9.5~12), and also assayed at different temperature in 50 mM citric buffer (pH 5.5) and glycine buffer (pH 9.5), respectively.

Table-1. Effect of metallic ions and other chemicals on the xylanase activity of *KRICT PX2* protein

Additives (1 mM)	Relative activity (%)	Additives (1 mM)	Relative activity (%)
None	100	$CuSO_4$	101
NaCl	95	$ZnSO_4$	99
LiCl	101	$FeCl_3$	103
KCl	104	EDTA	98
NH_4Cl	110	2-ME	115
$CaCl_2$	116	DTT	115
$MgCl_2$	108	PMSF	107
$MnCl_2$	84	Acetate	82
$CsCl_2$	81	Furfural	97

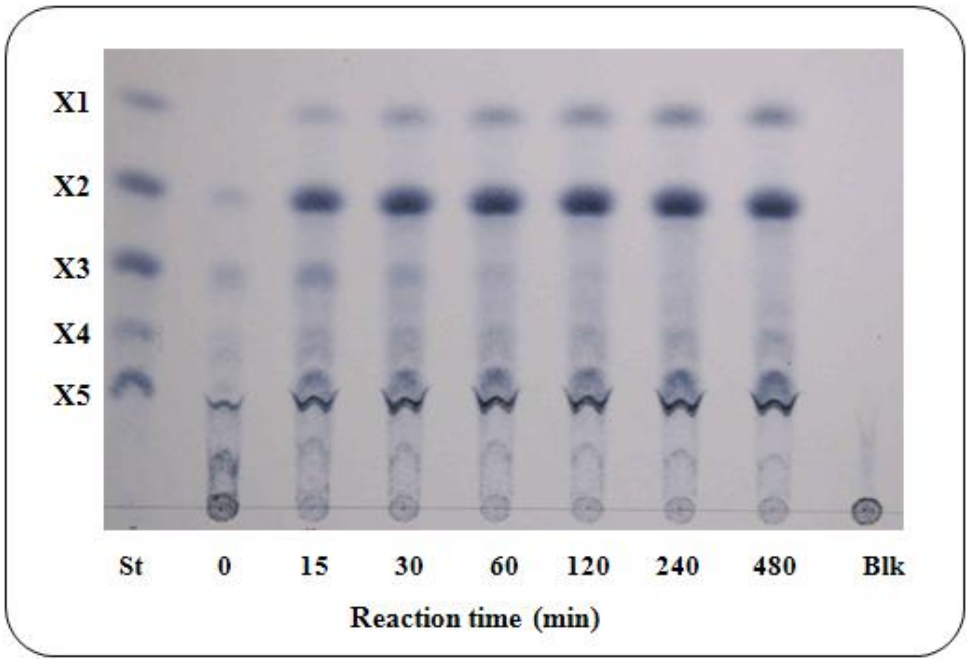
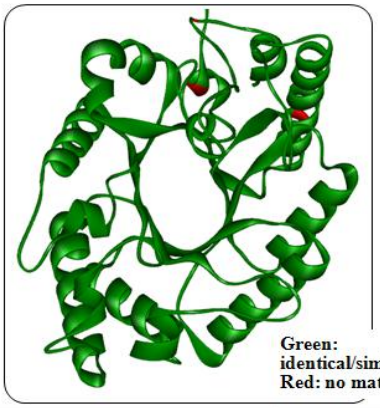


Figure 4. TLC analysis of the products after hydrolysis of birch wood xylan (0.5%) by *KRICT PX2* xylanase (160 ug/ml) for each reaction time of 0, 15, 30, 60, 120, 240, and 480 min in 50mM glycine buffer (pH 9.0) at 40°C. St: xylo-oligomer standards, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetrose, X5: xylopentose, respectively. Blk: blank without xylanase.

Sequence Analysis and 3D Model Structure

The complete nucleotide sequence of the plasmid harboring 999bp insert of ORF5 isolated from 01B3 clone was compared with related amino acid sequences obtained from database (Swiss Port and Gen Bank). The deduced amino acid sequence of *KRICT PX2* (GU967374) was aligned with closely-related xylanases, and turned out to be very much correlated with family 10 glycosyl hydralase (GH10) with many common conserved regions and similar essential amino acids for the catalytic activity as xylanase. And also, the simulated 3D structure of *KRICT PX2* appeared to have the typical (α/β) 8 barrel fold structure of family GH 10 xylanase (Fig. 5).



Template structure	Resolution	description 1	description 2	length	Identity	Similarity
3emz	2.08	Crystal structure of xylanase Xyn10B from *Paenibacillus barcinonensis*	intracellular xylanase	331	90%	96%

Figure 5. Xylanase *KRICT PX2* homology model structure.

DISCUSSION

Biomass is originally photosynthesized organic materials from inorganic compounds such as CO_2, minerals, water and solar energy and is continuously produced by plants and microorganisms in any places on the earth surface. Cellulosic materials, such as grass and woods, are not easily digested by human enzymes (Gilbert and Hazelwood, 1993). Therefore, human cannot degrade such recalcitrant biomass but some microorganisms can do by their enzymes, cellulases, xylanases, and chitinases (Kunio *et al.*, 2005). Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall.

Xylanases have attracted considerable research interest because of their potential industrial applications to improve the quality and texture of bakery products, reduce the amount of chlorine required for bleaching of paper pulp, and increase the quality of poultry diet (Beg *et al.*, 2001). Recently, xylanase has been refocused in fuel alcohol industry to convert xylan-rich agricultural residues such as corn stover, wheat straw, and sugar cane bagasse and energy crops such as switch grass into fermentable sugars (Dhiman *et al.*, 2008).

Characterization experiments with the purified *KRICT PX2* xylanase revealed a great deal of information regarding the biochemical nature of this xylanase presenting a possibility for practical application of this xylanase. One of the interesting properties of this enzyme is that the optimal temperature of 45~60^{o}C and alkalophillic pH range of 7~10 with maximal peaks at pH 9.0. The

optimal temperature for *KRICT PX2* xylanase activity was 50°C, and very stable for more than one hour at 40°C. Gallardo *et al.* (2003) described the pH dependence of xylanase (CAA07074) from *Paenibacillus barcinonensis*. Its optimum pH is 5.5 and shows more than 75% of maximum activity from pH 5 to 10, and still active at pH 12 with optimum temperature of 50°C. However, the activity was lost completely after incubation for 15 minutes at 50°C as like *KRICT PX2*.

From the amino acid sequence analysis and alignment with other microbial xylanase, it was confirmed that *KRICT PX2* xylanase belongs to the GH10 family with many highly conserved regions and almost similar essential amino acids for the catalytic activity (Marchler-Bauer, 2009). Xylanase *KRICT PX2* model structure is highly homologous to Xyn10B from *Paenibacillus barcinonensis*. Xyn10B is highly homologous to six xylanases of the GH10 family (XynX from A. caviae, XynA2 from *Bacillus stearothermophilus* T-6, XyaA from *Bacillus* sp. N137, Xyn2 from *B. stearothermophilus* 21, XynA from *Thermobacillus xylanilyticus*, and XynA from *Caldicellulosiruptor saccharolyticus*), and also they do not exhibit a signal peptide sequence similar to Xyn10B (Gallardo *et al.*, 2010). This signal peptide-less xylanases form a distinctive group of enzymes that cluster separately from the rest of GH10 xylanases and seem to constitute a new type of xylanases.

The degradation profile of birchwood xylan by *KRICT PX2* xylanase monitored by the TLC analysis revealed that the major product is xylobiose with smaller amount of xylose, xylopentose, and some longer xylo-oligomers. Interestingly, the amount of xylose and xylopentose is consistently increasing in proportion to reaction time. This indicates that *KRICT PX2* is an endo-type xylanase with similar catalytic property with most GH10 xylanases (Ducros *et al.*, 2000; Cheng *et al.*, 2009). Furthermore, the TLC separation of product from the birchwood xylan degradation by *KRICT PX2* xylanase revealed that the produced xylo-oligomers have somewhat smaller numbers of xylose residues than other GH10 xylanases previously reported.

Considering many advantages of moderate optimum temperature, thermostability, alkalophillic pH range, good xylanolytic efficiency. *KRICT PX2* xylanase may provide a candidate for future biocatalyst development. To maximize the efficient utilization of xylan by the xylanase, *KRICT PX2* isolated from *Paenibacillus* sp. strain HPL-002 supports its further development and genetic exploitation for long-term goal to convert lignocellulosic weeds or agricultural residue biomass to alternative fuels and bio-based products.

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