CLONING, EXPRESSION, AND CHARACTERIZATION OF AN ALKALOPHILLIC ENDO-1,4-BETA-XYLANASE FROM *PAENIBACILLUS* SP. HPL-002

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The biochemical properties of a purified enzyme of a new alkalophillic endo-1,4-beta-xylanase gene, *KRICT PX2* (GU967374), which was isolated from *Paenibacillus* sp. HPL-002 (KCTC11410BP) and expressed in *E. coli*, were investigated. The specific activity of the purified xylanase was 51.26 μmol/min/mg proteins. The K_m and V_{max} values of the protein for birch wood xylan were also 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, the activity was 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's activity at 1 mM. The simulated 3-D structure of this xylanase is similar to Xyn10B from *Paenibacillus barcinonensis*. Further research on the degradation of different-origin xylans and enzyme production will be necessary for practical applications.

Keywords: Alkalophyllic xylanase; Cloning; Expression; Paenibacillus sp. HPL-002

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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 second most abundant biopolymer, after cellulose, and the most common hemicellulosic polysaccharide in cell walls of land plants, representing up to 15 to 30 percent and seven to 12 percent of the total dry weight in hardwood from angiosperms and softwood from gymnosperms, respectively (Saha 2003).

Chemical hydrolysis of lignocelluloses results in hazardous byproducts, phenolic compounds from lignin degradation, furan derivatives (furfural and HMF) from sugar degradation, and aliphatic acids (acetic acid, formic acid, and levulinic acid); these byproducts from chemical conversion of lignocellulosic biomass are considered to be fermentation inhibitors (Palmqvist and Hahn-Hagerdal 2000). Also, the use of microbial enzymes specific for xylan hydrolysis has been accepted as an environmentally friendly option (Wong et al. 1988; Biely et al. 1997).

Bioconversion with xylan-degrading enzymes has received much attention because of its practical applications in various agro-industrial processes, such as efficient conversion of hemicellulosic biomass to fuels and chemicals, delignification of paper pulp, digestibility enhancement of animal feedstock, clarification of juices, and improvement in the consistency of beer (Beg et al. 2001). Also, xylanases that degrade, or help to degrade, xylan are of great interest to the utilization of xylose; they are 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 these, endo-xylanase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.8) is considered to be 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 et al. 2003). Xylan-degrading enzymes of micro-organisms are potentially important in various industrial processes at a high temperature and broad range of pH conditions. Owing to the increasing biotechnological importance of xylanases, interest in industrially applicable xylanases has markedly increased. Thus, many attempts are being made to isolate new strains and to discover more relevant xylanases (Collins et al. 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.

EXPERIMENTAL

Selection and Identification of Bacterial Strain

A strain of *Paenibacillus* sp. HPL-002 was isolated from a decaying wood sample from an old discarded mushroom farm by an overlaid-xylan staining technique, followed by culture purification on tryptic soy agar (TSA, Difco) media. The Petri dishes with active colonies showing transparent halos after Congo red staining were photographed, and the colonies were collected, cultured, then stored at -80°C in 10 percent glycerol. The active bacterial isolate was identified by the 16S rRNA analysis with the two bacterial universal primers, 530F 5(5'-TTAATTAAGATGTCTACCGAAATTCCGT-3') and 1492R-1 (5'-GGATCCCCATGTCTACCGAAATTC-3') (Lane 1991), and the full sequence of the 16S rRNA gene was analyzed and aligned with data in the Ribosomal Database Project (http://rdp.cme.msu.edu) for the species identification. An image of this bacterium was taken by a scanning electron microscope (SEM 515, Philips). The identified bacterial strain was deposited in 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

The genomic DNA of *Paenibacillus* sp. HPL-002 was obtained with a genomic-DNA isolation kit (Promega) and size-fractionated in a 0.5 percent low-melting-point agarose gel after fragmentation by shearing with a nebulizer (Invitrogen). 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 transformants were selected on a LB agar plate supplemented with kanamycin; a total of 1,152 clones were collected in twelve 96-well plates containing 200 μL LB broth (Difco) in each well. After incubation for 24 hours at 37°C, 25 μL glycerol (Sigma) was added to each well, mixed, and stored at -80°C. Xylanase activity of each clone was screened by overlaid-xylan staining (Hwang et al 2010) and DNS (3,5-dinitrosalicylic acid) assay (Miller 1959), simultaneously.

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™ terminator cycling conditions. Open reading frames (ORFs) from the sequence data of the clone 01B3 were predicted using the ORF Finder (NCBI), taking ATG, GTG, and TTG as possible starting codones. Also, homology searches for the resulting seven ORFs were carried out by using the BLAST program in the GenBank database.

All seven sets of primers fully-covering each ORF (ORF clone ID: SLX1-O1 ~ SLX1-O7) were designed, and amplified to construct transformants. 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 most active xylanase sub-clone inserted with ORF5 was selected, and the insert, which was modified with detaching ribosomal binding site (RBS) from ORF5 sequence, was designated as *KRICT PX2*. The gene, *KRICT PX2* 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°C and 200 rpm in a shaking incubator. After re-inoculation, the culture was induced with 1 mM IPTG and incubated under the same conditions for 3 hours longer. The cells collected from the final washing process were resuspended in the lysis buffer (pH 7.0, 200 mM Tris-HCl, 10 mM NaCl, 10 mM β-mercaptoethanol, 1 mM EDTA), and treated with sonic disruptor (CosmoBio Co., LTD). After cell disruption, the supernatants were 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 the Restriction Protease Factor Xa (Roche Applied Science), and eluted through the *p*-aminobenzamidine-agarose column (Sigma-Aldrich), according to the manufacturer's instruction. The eluate was precipitated with 70 percent ammonium sulfate, solubilized in phosphate-buffered saline, and dialyzed to concentrate at 4°C with a dialysis membrane (Spectra/Por CE, MWCO 10,000), then the protein content was determined prior storage at –20°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 15 percent polyacrylamide was performed to separate each protein, and stained with Coomassie brilliant blue R 250.

Properties of Recombinant Xylanase

Xylanase activity was measured according to the method as previously reported (Hwang et al. 2010), using 50 µL of 1 percent (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 10 min at different temperatures. 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 conditions 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. Diluted enzyme solution (100 µg protein in 30 µL) was incubated with 0.5 mL of various concentrations (0 to 10 mg/mL) of xylan in 50 mM glycine buffer (pH 9.0) at 50°C for 10 minutes.

The kinetic parameters (Michaelis-Menten constant, K_m, and maximal reaction velocity, V_{max}) were estimated by linear regression from double-reciprocal plots. The effect of metallic ions and other chemicals on the xylanase activity of KRICT PX2 protein was studied, as described above, at pH 9 with the 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 hydrolysis of birchwood xylan was carried out in a 10 mL conical flask containing 5 mL glycine buffer (pH 9.0, 50 mM) with 1 percent xylan and enzyme solution (160 µg protein/mL) for 8 hours at 37°C, with a stirring rate of 200 rpm in a shaking incubator. Aliquots (0.2 mL) of the samples were collected at 0, 15, 30, 60, 120, 240, and 480 minutes of the incubation period and immediately boiled. 10 µL of each aliquot, xylo-oligomer standard mixture (xylose, xylobiose, xylotriose, xylotetrose, and xylopentose from Wako Chemical and Megazyme), and enzyme blank was spotted on silica gel plates 60 F 254 (Merck KGaA, Germany), then the plate developed with acetonitrile: water (80:20, v/v), as previously reported (Hwang et al. 2010). After elution for 2 hours, the resultant plate was sprayed with a staining solution (1 percent diphenylamine and 1 percent aniline in 9 ml acetone, and mixed with 1 ml phosphoric

acid just before use), and heated for 10 minutes at 120°C in an oven to visualize the xylo-oligomers prior to taking photographs.

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 a homology model structure comparing to Xyn10B (PDB ID: 3emz) from *Paenibacillus barcinonensis* with Discovery Studio 2.5 (accelrys®).

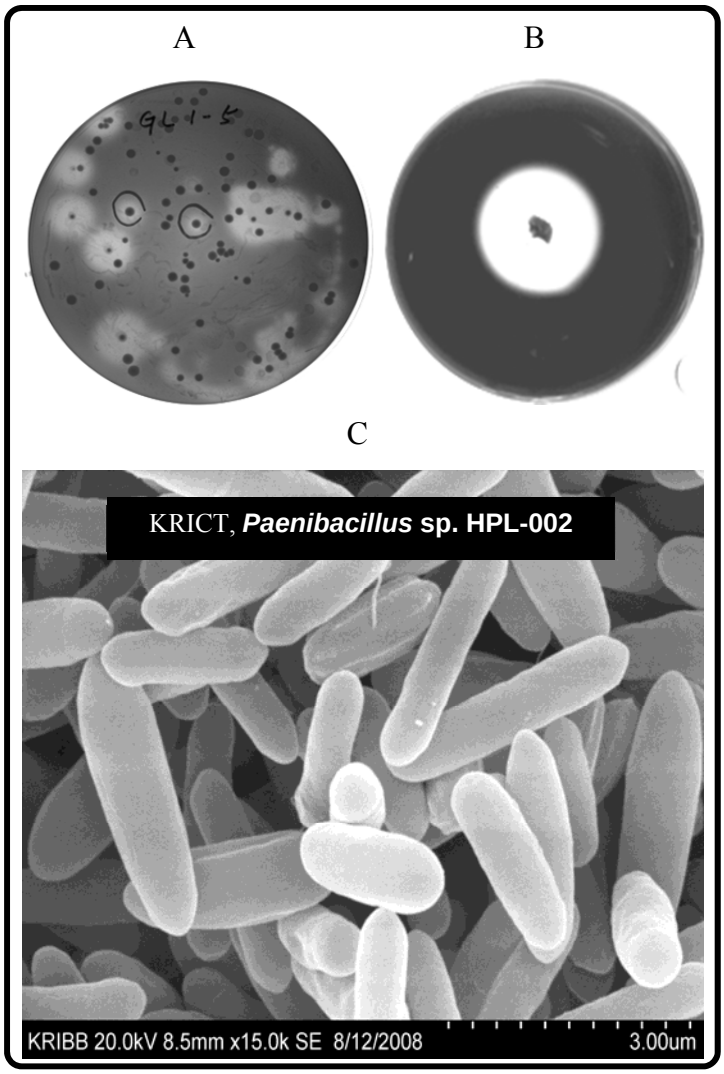


Fig. 1. Screening of xylan degrading bacteria (A), and isolation of *Paenibacillus* sp. HPL-002 (B) in the xylan-overlaid plates. The photographs were taken after Congo red (0.1%) staining and repetitive washing with 1 M NaCl. Scanning Electron Microscope (SEM) photograph (C) of the isolated *Paenibacillus* sp. HPL-002.

RESULTS AND DISCUSSION

Screening and Isolation of the Xylanase Gene

Based on the screening of the bacteria for their ability to hydrolyze xylan, the strain HPL-002 exhibited high xylanolytic activity (Figs. 1A and B). Therefore, this strain was selected for further identification at the molecular level. Its full sequence of the 16S rRNA gene was amplified and analyzed through a RDP tool. 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* (Table 1) with a similarity score range of 0.904 to 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 (Fig. 1C).

Table 1. Sequence Match Results from the Ribosomal Database Project with 16S rRNA Sequence from *Paenibacilus* sp. HPL-002

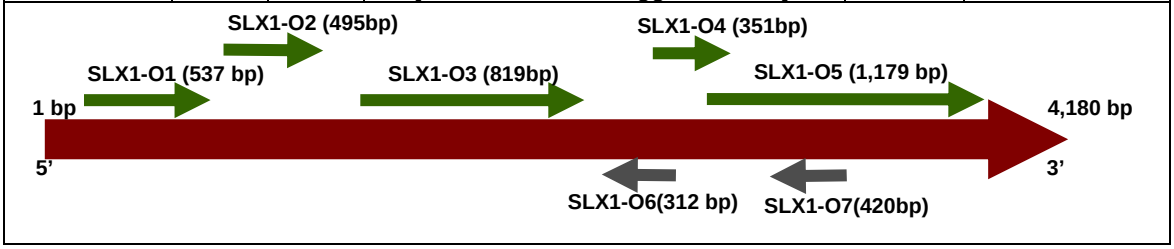
Bacterial species	Similarity score*	Gene size (bases)	Accession No. (GenBank)
Paenibacillus pabuli	0.904	1399	AB045094
Paenibacillus barcinonensis (T)	0.929	1429	AJ716019
Paenibacillus sp. HC1	0.911	1434	AB198337
Paenibacillus sp. 6M01	0.905	1391	AM162345
Paenibacillus sp. Enf35	0.904	1373	DQ339607
Paenibacillus barcinonensis; J3-48	0.948	1331	DQ363432
Paenibacillus sp. GPTSA21	0.952	1405	DQ854978
Paenibacillus amylolyticus; PCL1756	0.934	1388	DQ313379
Paenibacillus barcinonensis; JSC_SF93	0.955	1348	DQ870733
Paenibacillus sp. MH74	0.928	1311	EU182900
Paenibacillus sp. SZ002	0.951	1385	EU256397
Paenibacillus pabuli; 4RS-1a	0.905	1348	EU379277
Paenibacillus sp. MOLA 507	0.905	1359	AM990732
Paenibacillus sp. B3a	0.906	1425	EU558285
Paenibacillus sp. B21a	0.904	1421	EU558288
Paenibacillus sp. WPCB056	0.939	1347	FJ006879
Paenibacillus sp. WPCB158	0.963	1372	FJ006910

* These are the number of (unique) 7-base oligomers shared between *Paenibacillus* sp. HPL-002 sequence and a given RDP sequence divided by number of unique oligos in *Paenibacillus* sp. HPL-002 sequence.

A DNS assay and an overlaid xylan analysis of the *Paenibacillus* sp. strain HPL-002 genomic DNA library, led to the isolation of a single clone, arbitrarily named as 01B3 (the first plate, well number of B3) with greatest xylanase activity among 7 clones (data not shown). This insert has total insert size of 4,180 bps, and was constructed with 7 ORFs. Protein Blast searches of each ORFs, SLX1-O5 were close to that of endo-1,4-beta-xylanase of *Paenibacillus barcinonensis* (GenBank Accession No. CAA07174), exo-beta-1,4-xylanase of *Aeromonas punctata* (GenBank Accession No. BAA31551), and Endo-1,4-beta-xylanase of *Paenibacillus* sp. JDR-2 (GenBank Accession No. ZP_02849901) had 90, 85, and 62 percent amino acid identities, respectively (Table 2). Also, SLX1-O5 has 83 percent identity with the gene encoding endo-1,4-beta-xylanase of xylanase B (GenBank Accession No. AJ006646) with Blast N analysis result. DNS assay with each cell lysate of the sub-clones inserted with each ORF revealed that only SLX1-O5 clone has active xylanase activity among 7 sub-clones.

Table 2. Blast Analysis Results and Schematic Representation of each ORF from 01B3 Clone Insert in GenBank

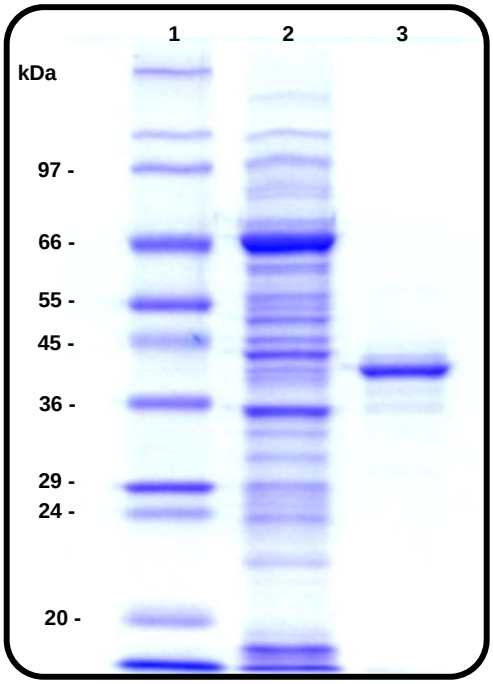
ORF clone ID	Length		Blast P result	Identities (%)	GenBank Ref.
	bp	aa			
SLX1-O1	537	179	GCN5-related N-acetyltransferase [*Paenibacillus* sp. JDR-2]	43	ZP_02850197
SLX1-O2	495	165	Hypothetical protein [*Bacillus* sp. KSM-K16]	58	YP_174594
SLX1-O3	819	273	Aminoglycoside phosphotransferase [*Geobacillus* sp.]	50	ZP_03039448
SLX1-O4	351	117	Hypothetical protein [*Paenibacillus* sp. JDR-2]	57	ZP_02845404
SLX1-O5	1,179	393	Endo-1,4-beta-xylanase [*Paenibacillus barcinonensis*]	90	CAA07174
			Exo-beta-1,4-xylanase [*Aeromonas punctata*]	85	BAA31551
			Endo-1,4-beta-xylanase [*Paenibacillus* sp. JDR-2]	62	ZP_02849901
SLX1-O6	420	140	Hypothetical protein [*Oryza sativa*]	36	EAY99446
SLX1-O7	312	104	Hypothetical protein [*Caenorhabditis briggsae* AF16]	38	XP_001676163



Purification and Characterization of Recombinant Xylanase

The final preparation of KRICT PX2 xylanase gave a major single band on SDS-PAGE (Fig. 2), with a molecular weight (MW) of 38.4 kDa, which appeared slightly over the 36 kDa MW marker (Fig. 2, lane 3). Also, the GST-fused KRICT PX2 protein of MW

64.4 kDa was observed just below the 66 kDa MW marker (Fig. 2, lane 2). The optimal pH, showing maximal xylanase activity, was measured at a pH of 9.0, which was considered 100 percent activity, and retaining about 98 percent of its activity at a pH of 8.0, and about 90 percent of its activity at a pH of 7 and a pH of 10, respectively (Fig. 3A). The optimal temperature for the KRICT PX2 xylanase activity appeared to be 50°C (Fig. 3B). The KRICT PX2 xylanase was very stable for one hour at 40°C; however, the activity of the KRICT PX2 xylanase sharply decreased at 50°C after incubation for 10 minutes (Fig. 4).



Step	Protein (mg/ml)	Specific Activity (U/mg protein)	Purification (fold)
Cell lysate	2.95	10.82	1
KRICT PX2	0.47	51.26	4.7

Fig. 2. SDS PAGE analysis of the purified KRICT PX2 xylanase (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).

Kinetic analysis of this xylanase with birchwood xylan was performed at 50°C in pH 9.0 (Fig. 3). The specific activity of the KRICT PX2 xylanase was 51.26 μmoles/min/mg proteins. Also, K_m and V_{max} values of the protein for birch wood xylan were determined as 0.061 μM and 55.3 μmol/min/mg proteins, respectively (Fig. 4).

Most salts, such as NaCl, LiCl, KCl, NH_4Cl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_3$, and $CsCl_2$, did not significantly change the enzyme activity at 1 mM. Also, 1 mM of ethylenediamine tetra-acetic acid, 2-mercaptoethanol, phenylmethanesulphonyl fluoride, and furfural were not effective on the enzyme activity (Table 3). As shown in Fig. 5, the

products of hydrolysis by the KRICT PX2 xylanase were mainly xylobiose and much smaller amounts of xylose and xylopentose. The amount of xylobiose especially continued to increase during the entire reaction.

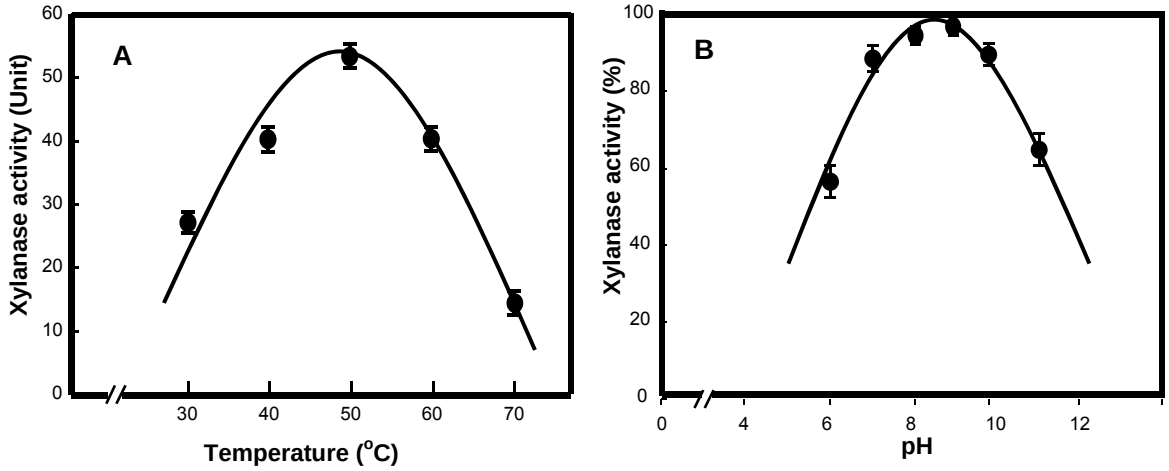


Fig. 3. The xylanase activity of *KRICT PX2* at various temperature (A) and pH (B) conditions. The enzyme activity was assayed at 50°C in 50 mM citric buffer (pH 2~6.5), phosphate buffer (pH 7~9), and glycine buffer (pH 9.5~12), and also assayed at different temperature in 50 mM citric buffer (pH 5.5),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

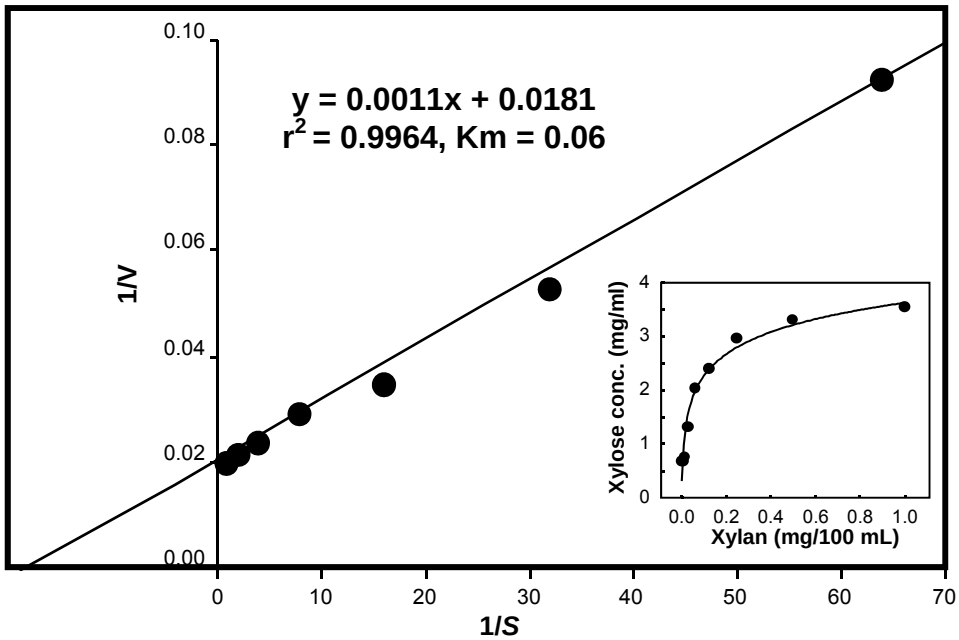


Fig. 4. Lineweaver-Burk double reciprocal analysis of *KRICT PX2* xylanase with birchwood xylan as the substrate. The enzyme activity was measured with various concentrations of birchwood xylan in 50 mM phosphate buffer (pH 9.0) at 50°C.

Table 3. 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
NH4Cl	110	2-Mercaptoethanol	115
$CaCl_2$	116	Dithiothreitol	115
$MgCl_2$	108	PMSF**	107
$MnCl_2$	84	Acetate	82
$CsCl_2$	81	Furfural	97
*EDTA: ethylenediamine tetra-acetic acid; **PMSF: phenylmethane sulphonyl fluoride			

The KRICT PX2 xylanase from the *Paenibacillus* sp. strain HPL-002 seems to be an extracellular enzyme. There is some evidence supporting this assumption. First, the xylan-degrading activity was observed to have a significantly larger-sized halo in the xylan-overlaid agar plate. When the gene was transformed to *E. coli*, its activity was monitored in both detection methods using the xylan-overlaid agar plate and the DNS assay of culture media, respectively. This character of the xylanase is different from intracellular or subcellular xylanases, such as Xylanase B from *Paenibacillus* sp. BP-23 and Xylanase 5 from *Paenibacillus* sp. strain W-61 (Yasuko et al. 2003), but similar to extracellular XynA1 xylanase from *Paenibacillus* sp. Strain JDR-2. Extracellular xylanases are considered important from an industrial viewpoint, as they ease the extraction procedure during mass production with a simple and cost-effective bioprocess (Gupta et al. 2009).

One of the interesting properties of this enzyme is that the optimal temperature range is 45 to 60°C and the alkalophillic pH range is 7 to 10, with maximal peak at pH 9.0. The optimal temperature for KRICT PX2 xylanase activity was 50°C, and the activity was very stable for more than one hour at 40°C (Fig. 5). 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 percent of the maximum activity from pH 5 to 10, and it is still active at pH 12 with an optimum temperature of 50°C. However, the activity is lost completely after incubation for 15 minutes at 50°C, as like KRICT PX2.

Many alkaline-active xylanases have been reported from many bacteria species with various optimum temperature conditions (Subramaniyan et al. 2000; Gallardo et al. 2003; Chang et al. 2004). The alkaline-stable xylanase has many advantages in many industrial applications.

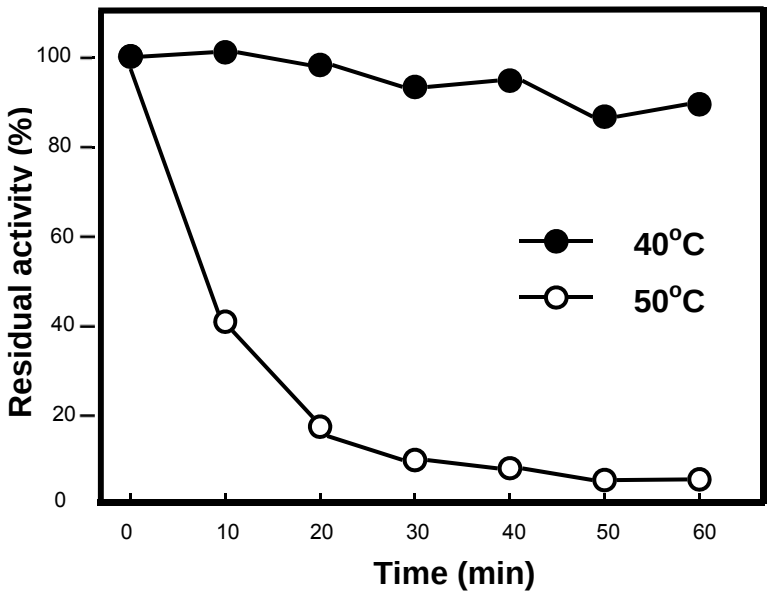


Fig. 5. Thermal stability of *KRICT PX2* xylanase. The enzyme was maintained for 1 hour at 40 and 50°C, and residual activity was measured at 10 minute intervals under standard assay conditions.

To save time and costs during the xylan-related process, desirable properties of xylanases include stability and activity at high temperature and alkaline pH (Palackal et al. 2004; Dhiman et al. 2008). From this perspective, KRICT PX2 xylanase is attractive for further development and application through the modification by rational design of proteins or by directed molecular evolution of proteins (Gallardo et al. 2010), which are ongoing in our further studies.

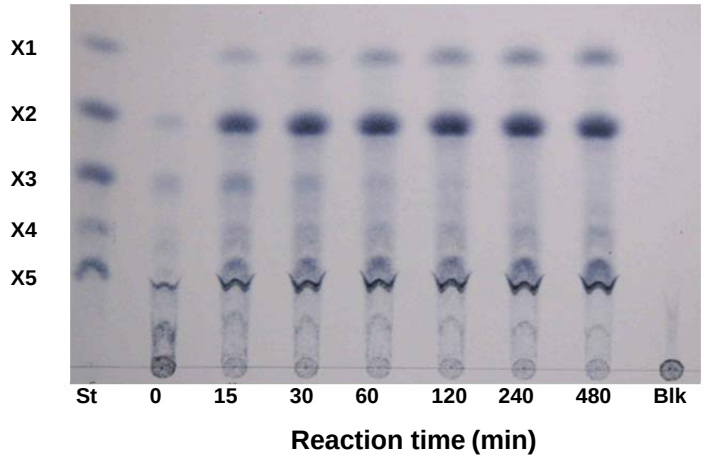


Fig. 6. TLC analysis of the products after hydrolysis of birch wood xylan (0.5%) by KRICT PX2 xylanase (160 ug/mL) for each reaction time of 0, 15, 30, 60, 120, 240, and 480 minutes in a 50mM glycine buffer (pH 9.0) at 40°C. St: xylo-oligomer standards, X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetrose, X5: xylopentose, respectively. Blk: blank without xylanase

The degradation profile of birchwood xylan by KRICT PX2 xylanase, monitored through the TLC analysis, revealed that the major product was xylobiose with smaller amounts of xylose, xylopentose, and some longer xylo-oligomers (Fig. 6). Interestingly, the amount of xylose and xylopentose was consistently increasing in proportion to the reaction time. This indicates that KRICT PX2 is an endo-type xylanase with similar catalytic property of 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 many other GH10 xylanases.

Sequence 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 searches from SwissPort and GenBank. The deduced amino acid sequence of *KRICT PX2* (GU967374) was aligned with closely-related xylanases (Fig. 7), 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. Also, the simulated 3D structure of KRICT PX2 appeared to have the typical $(\alpha/\beta)_8$ barrel fold structure of family GH 10 xylanase (Fig. 8)

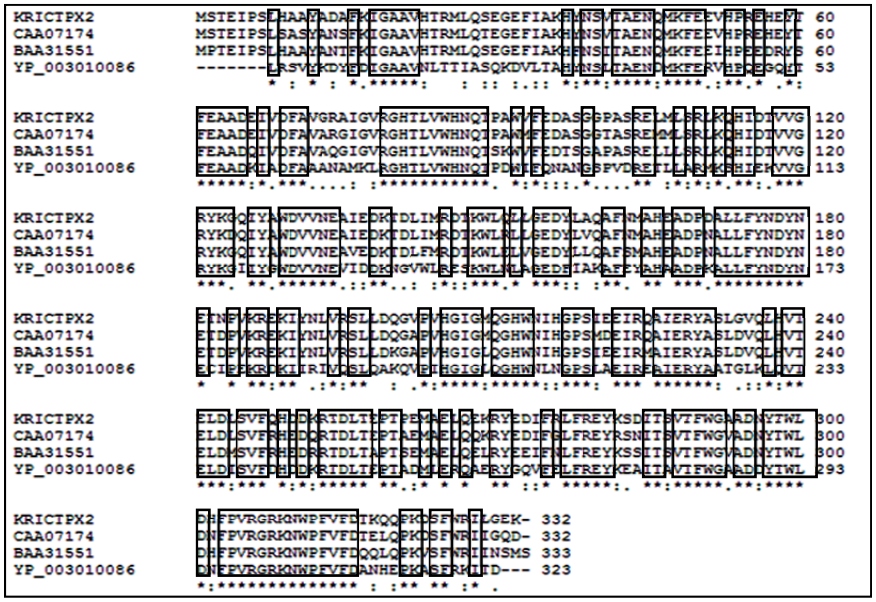


Fig. 7. Multiple alignment of *KRICT PX2* with endo-1,4-beta-xylanase [*Paenibacillus barcinonensis*, CAA 07174], exo-beta-1,4-xylanase [*Aeromonas punctata*, BAA31551], and endo-1,4-betaxylanase [*Paenibacillus sp*. JDR-2, YP_003010086] by using CLUSTAL W (1.82) multiple sequence alignment program.

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 et al. 2009).

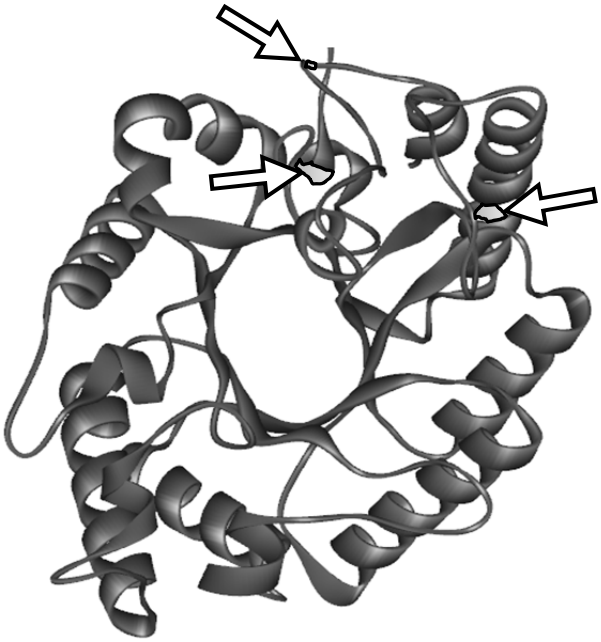


Fig. 8. Xylanase KRICT PX2 homology model structure (Dark: identical/similarity region, Bright: no match, indicated by 3 arrows) simulated with the crystal structure of xylanase Xyn10B from *Paenibacillus barcinonensis* as a template showing the identity and similarity of 90 and 96 percent with 331 amino acids, respectively.

Xylanase KRICT PX2 model structure (Fig. 8) is highly homologous to Xyn10B from *Paenibacillus barcinonensis*. Xyn10B is highly homologous to other 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 they also do not exhibit signal peptide sequences similar to Xyn10B (Marchler-Bauer et al. 2009). These xylanases without signal peptide 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 general property of GH10 xylanases is their relatively high molecular weight with low *p*I values and an $(\alpha/\beta)_8$ barrel fold, typical to this family, attacking two unsubstituted xylopyranosyl residues between the branches, and possessing multiple smaller substrate binding sites with various catalytic activities such as aryl-β-D-xylosidases (Chow et al. 2007). These enzymes are highly active on short xylo-oligosaccharides, thereby indicating small substrate binding sites. Many hydrolysis studies have also shown that most GH10 xylanases can attack the xylosidic linkage on the nonreducing end of a substituted residue or 1,3- β bond, but can only cleave at the third xylosidic linkage after a substituted residue, and the second after a 1,3-β bond (Biely et al. 1997). As result of this substrate selectiveness, the xylo-oligomer produced by GH10 xylanases hydrolysis usually has small number of xylose residues, generally smaller than hexamer.

CONCLUSIONS

Considering its many advantages, including a moderate optimum temperature, thermostability, an alkalophillic pH range, and 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 a long-term goal to convert lignocellulosic biomass to alternative fuels and bio-based products.

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