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# Thermostable Xylanase from *Streptomyces thermocyaneoviolaceus* for Optimal Production of Xylooligosaccharides

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**Abstract** A thermo stable xylanase was purified from *Streptomyces thermocyaneoviolaceus* M049 for the production of xylooligosaccharides from xylan. The enzyme showed thermostability by maintaining 65% of remaining enzyme activity after 1 h heat treatment at 70°C. The molecular weight of the purified protein was 35 kDa in SDS-PAGE, and the optimal pH and temperature for the enzymatic activity were pH 5.0 and 60°C, respectively. N-terminal amino acid sequences of the purified xylanase, DTITSNQTGTHNGYF, were similar to StxII from *S. thermoviolaceus* and XlnB from *S. lividans*. Using those two genes, *stxII and xlnB* as probe DNA, a gene encoding xylanase, *xynB*, was cloned from genomic library of *S. thermocyaneoviolaceus* M049. The open reading frame of the *xynB* was composed of 1008 nucleotide sequences. Compared to N-terminal sequences from purified enzyme, it was proposed that the XynB contained a 40 amino acid long signal peptide to the N-terminus. For easy production and purification, a XynB overproduction strain was constructed using pET21a(+) and strain *E. coli* BLR(DE3). Consequently, the recombinant enzyme was tested for the production of xylooligosaccharides through TLC and HPLC analyses. © KSBB

*Keywords:* Streptomyces thermocyaneoviolaceus*, thermostable xylanase,* xynB*, Xylooligosaccharides*

## INTRODUCTION

Xylan is a polymer with a backbone chain of 1,4-linked β-D-xylopyranose that could be substituted with glucuronic acid and 4-*o*-methyl-D-glucuronic acid or L-arabinose, and it is the most abundant renewable hemicellulose comprising a major constituent of plant cell walls [1,2]. Xylan can be hydrolyzed to xylose through the enzymatic reactions of both endo-1,4-β-D-xylanase (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8), an enzyme degrading xylan backbone into xylooligosaccharides, and exo-β-D-xylosidase (1,4-β-D-xylan xylohydrolase; EC 3.2.1.37), an enzyme degrading xylooligosaccharides into the final product as xylose [3-8].

Xylanase has been used in various fields such as the food, animal feed, and the pulp or paper industry [9,10]. Xylooligosaccharides, the main xylan hydrolysates of endoxylanase, have been considered a very useful nutrient factor of human intestinal Bifidobacteria [11-15]. Bifidobacteria have been considered as useful probiotics, as they have been shown to lower incidence of food allergies [16] and delay proliferation of several tumor types [17]. Many xylanases have been purified and characterized from various microbial sources such as *Bacillus* species, fungi, *Streptomyces* species, and yeasts [18-22]. Generally, higher thermostability and broader pH stability of the enzyme are being demanded by the industrial requests.

In this study, we have purified, cloned, and overproduced a thermostable endo-xylanase from a strain, *S. thermocyaneoviolaceus* M049, for production of xylooligosaccharides from xylan.

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## MATERIALS AND METHODS

### Bacterial Strains, Plasmids, and Media

For screening thermostable xylanase, *S. thermocyaneo-*

*violaceus* KCCM 40049 was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea) and it was used as a wild type strain for purification and cloning of xylanase. The strain was cultured in XM media (xylan, 1.0%; yeast extract, 0.1%; bacto-peptone, 0.1%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.005%; $KH_2PO_4$, 0.05%; and $K_2HPO_4$, 0.2%) for 24 h at 50°C, 200 rpm. The optimal xylanase production was obtained as the strain was grown in WB media (wheat bran, 0.8%; yeast extract, 0.06%; bacto-peptone, 0.06%; $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.005%; $KH_2PO_4$, 0.05%; and $K_2HPO_4$, 0.2%) for 24 h at 50°C, 200 rpm.

The *E. coli* strains, DH5α and BLR(DE3) and its derivatives, were maintained and grown as described previously [20]. Bacteriophage λBlueSTAR (Novagen, Germany) was used as a vector for the construction of the DNA library. The *E. coli* BM 25.8 (*supE, thi,* Δ(*lac-proAB*) (*F'*, *traD36, proA*$^+$*B*$^+$, *lacI*$^q$*ZΔM15*), λ*imm434*(*kan*$^R$) P1(*cam*$^R$), and *hsdR* ($r_{K12}^-$ $m_{K12}^+$); Novagen, Germany) and the *E. coli* ER1647 (*F*$^-$, *fhuA2,* Δ (*lacZ*)*r1, supE44, recD1014, trp31, mcrA1272*:: Tn*10*(*tet*$^R$), *his-1, rpsL104*(*str*$^R$), *xyl7, mcl2, metB1,* Δ (*mcrC -mrr*)*102*:: Tn*10* (*tet*$^R$), and *hsdS* ($r_{K12}^-$ $m_{K12}^-$); Novagen, Germany) were used as a strain expressing *cre* recombinase (BM 25.8) or a λ-phage transpectable strain (ER1647). Plasmid pET21a(+) (Ap$^r$, *lacI*$^q$, and $P_{T7}$; Novagen, Germany) was used for the overexpression plasmid vector and plasmid pUC119 (Ap$^r$, $P_{lac}$, *lacZ*, and M13G) [23] was used for general subcloning.

### Purification of Xylanase

The culture filtrate of the *S. thermocyaneoviolaceus* was saturated with 50% ammonium sulfate and then the precipitated pellet was resuspended in a 50 mM Tris-HCl buffer (pH 8.6) and dialyzed against the same buffer three times over for 8 h. The dialyzed crude solution was loaded on a DEAE Sephadex A-50 ion exchange column pre-equilibrating with a 50 mM Tris-HCl buffer (pH 8.6). The bound proteins were eluted by a linear NaCl gradient (from 0.2 M to 0.6 M). Fractions containing xylanase activity were carefully selected and concentrated. For further purification, the concentrated sample was loaded on a Sephacryl S-200 HR gel filtration column and eluted with a 50 mM sodium phosphate buffer (pH 7.0). YM10 membrane (Amicon, Beverly, Mass, USA) was used for concentration of the crude enzyme solution. The protein concentration was determined by the absorbance at 280 nm or using the Bradford method [24] with bovine serum albumin (BSA) as a standard protein at 595 nm. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was conducted by the method described by Jung *et al* [25]. A low range molecular calibration kit (bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen, 24 kDa; and trypsin inhibitor, 20 kDa) (Sigma, USA) was used as a standard marker for the estimation of the molecular mass of purified xylanase.

### Xylanase Activity Assay

For the quantitative measurement of xylanase [β-1,4-D-xylan-xylanohydrolase (E.C.3.2.1.8)] activity, a 0.2 mL enzyme solution was incubated for 30 min at 65°C in a reaction mixture containing 100 mM sodium phosphate buffer (pH 7.0) and 1.0% xylan. After the reaction was stopped by the addition of dinitrosalicylic acid (DNS) and boiling for 10 min, the amount of reducing sugar was estimated from the optical density at 546 nm [26]. One unit of xylanase was defined as an amount of enzyme that produced one micromole of reducing sugar per minute.

An RBB xylan clearance zone test was used for screening *E. coli* clones producing the recombinant xylanase. The Bacterial strains were streaked and grew on LB agar plates with 0.1% 4-*o*-methyl-d-glucurono-D-xylan-remazol brilliant blue R (RBB)-xylan (Sigma, USA). Because the xylanase transforms blue colored RBB-xylan into a colorless product, clearance zone on RBB-xylan plates could be interpreted to xylanase activity.

Expression of recombinant xylanase was detected by the substrate-gel electrophoresis activity test (zymography), using a 10% SDS-PAGE gel and co-polymerizing 1% soluble birch wood xylan (Sigma). Briefly, protein samples were loaded onto the zymogram gel without treatment of reducing agents or heating. After electrophoresis, the gel was washed in renaturing buffer (2.5% of Triton X-100) overnight, and then incubated in a 50 mM phosphate buffer for 1 h at 50°C. The gel was stained with 1% Congo red solution (Sigma). Xylanase activity was detected by a clear band on a red background.

### Characterization of Xylanase

The optimal pH and pH stability range of xylanase activity were measured using the following buffers; 100 mM citrate phosphate buffer (pH 3.0~7.0), 100 mM sodium phosphate buffer (pH 6.0~8.0), 100 mM Tris-HCl buffer (pH 8.0~9.0), and 100 mM glycine-NaOH buffer (pH 9.0~10.5). For the assay to test pH stability, the enzyme was pretreated in each pH buffer for 24 h at 4°C. The pH was readjusted to 5.0 after pretreatment for the activity assay. In order to figure out optimal temperature, enzyme reactions were carried out at 5°C, differential temperature ranging from 50~80°C. For thermal stability of the xylanase, the enzyme was treated for 30 min at 50~80°C, and then the remaining activity was measured at 65°C The $K_m$ and $V_{max}$ values of the enzyme were determined using birch wood xylan as a substrate in a 50 mM citrate phosphate buffer (pH 5.5). Xylan binding assay was performed by measuring the remaining enzyme activity after incubating the enzyme with insoluble xylan or avicel in 50 mM citrate phosphate buffer (pH 5.5) for 1 h at 60°C. The N-terminal amino acid sequences were determined by the method of Hunkapiller and Hood [27], using Protein sequence 476 (ABI).

### Construction of DNA Library and DNA Analysis

General DNA manipulation was performed by the method of Sambrook [28], and the genomic DNA of *S. thermocyaneoviolaceus* was prepared by the method of Hopwood

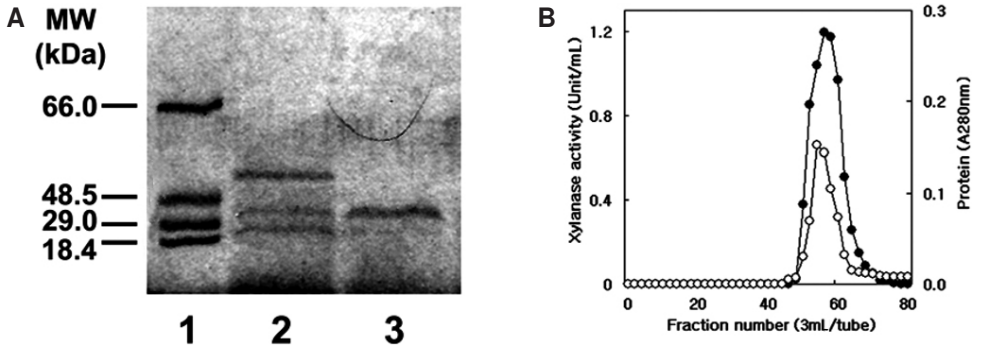


**Fig. 1.** Purified xylanase from *S. thermocyaneoviolaceus.* Panel A, 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis of crude enzyme and purified xylanase. Lane 1: serum albumin (66.0 kDa), fumarase (48.5 kDa), carbonic anhydrase (29.0 kDa), and β-lactoglobulin (18.4 kDa) as protein molecular weight standards; lane 2: crude enzyme after 50% ammonium sulfate treatment; lane 3: purified protein after gel filtration chromatography. Panel B, Sephacryl S-200 HR gel chromatography elution profile of xylanase during the purification. Gel was equilibrated with a 50 mM sodium phosphate buffer (pH 7.0), and eluted using the same buffer at a flow rate of 10 mL/h and 3 mL/tube fraction volume. ○-○, protein and ●-●, xylanase activity.

[29]. DNA library was constructed using a λ-BlueSTAR *Bam*H I digested dephosphorylated arms kit (Novagen, Germany). At first, the genomic DNA of *S. thermocyaneoviolaceus* was purified and partially digested with a restriction endonuclease *Sau*3A I. About 12~20 kb of genomic DNA fragment was fractionated through the use of a sucrose density gradient [28]. The DNA fragments were linked to λBlueSTAR *Bam*H I digested dephosphorylated arms and packed into λ particle.

A DNA probe for plaque or southern hybridization was obtained by PCR reaction and oligonucleotide PCR primers were synthesized through Bioneer Co. (Korea). The DNA probe was labeled with ($\alpha$-$^{32}$Pd)CTP (Amersham Pharmacia Biotech., England) using a Promega labeling kit (Prime-a-Gene$^{R}$ Labeling System). Hybond -N$^{+}$ Nylon transfer membrane (Amersham Pharmacia Biotech., England) was used for the hybridization assay.

The Nucleotide sequence of the *xynB* gene was determined by the ALFexpress$^{TM}$ DNA Analysis System with ALFexpress$^{TM}$ AutoCycle$^{TM}$ Sequencing kit (Amersham Pharmacia Biotech., England) based on the dideoxy chain termination method of Sanger [30]. A homology search of nucleotide sequences or amino acid sequences was conducted by the BLAST program at http://www.ncbi.nlm.nih.gov/blast/Blast. cgi.

### TLC and HPLC Analysis of Xylan Hydrolysates

Thin layer chromatography (TLC) was performed on Silica gel 60 $F_{254}$ plate (Merck., USA) with developing solution (1-butanol, 2-propanol, water, acetic acid, and acetonitrile; 7:5:4:10:2, V/V). After the TLC reaction, the plate was sprayed in a sulfuric acid-methanol solution (1:9, V/V) containing 0.2% orcinol. The separated xylooligosaccharides pattern was detected after drying at 95°C for 5 min. High performance liquid chromatography (HPLC, Waters Model 600E) was carried out using a system equipped with Sugar-Pak I column (ϕ 6.5 × 300 mm, Waters, Co.) and detected by a Refractive Index Detector (Waters Model 410) using Ca-EDTA solution at 85°C with a flow rate of 0.5 mL/min. The standard xylooligosaccharides ($X_2$-$X_6$) were purchased from Suntory Co. (Japan).

## RESULTS AND DISCUSSION

### Purification of Xylanase from *S. thermocyaneoviolaceus* M049

A protein which produced xylooligosaccharides from xylan was purified by serial steps described in the materials and methods. Strong xylooligosaccharides production activity was detected from the 50% ammonium sulfate precipitated supernatant *S. thermocyaneoviolaceus* culture (Fig. 1A, lane 2). After further purification steps, the xylanase activity and protein elution profile of the gel-filtration chromatography were well correlated (Fig. 1B) and the SDS-PAGE showed a 35 kDa protein band (Fig. 1A, lane 3).

The optimal pH of the purified xylanase was pH 5.0 (Fig. 2A), and the enzyme was stable in the range of pH 4.5~10.5 after 24 h incubation at 4°C (Fig. 2B). The maximum enzymatic activity of the protein was obtained at 60°C (Fig. 2C) and the activity still remained at about 65% after 1 h incubation at 70°C (Fig. 2D). The high optimal temperature and thermostability with the broad range pH stability makes the enzyme useful for industrial application The higher optimal temperature and thermostability with the broad range of pH stability makes the enzyme useful for industrial application. The binding activity of both soluble xylan and avicel was confirmed by the binding assay (Figs. 2E and 2F). This result suggested that the purified xylanase has a cellulose binding domain in the protein structure, which is common as endoxylanase. The $K_m$ and $V_{max}$ of the enzyme were 10.88 mg/mL and 3.02 μmol/min, respectively.

The N-terminal amino acid sequences of purified xylanase were determined as DTITSNQTGTHNGYF which showed a

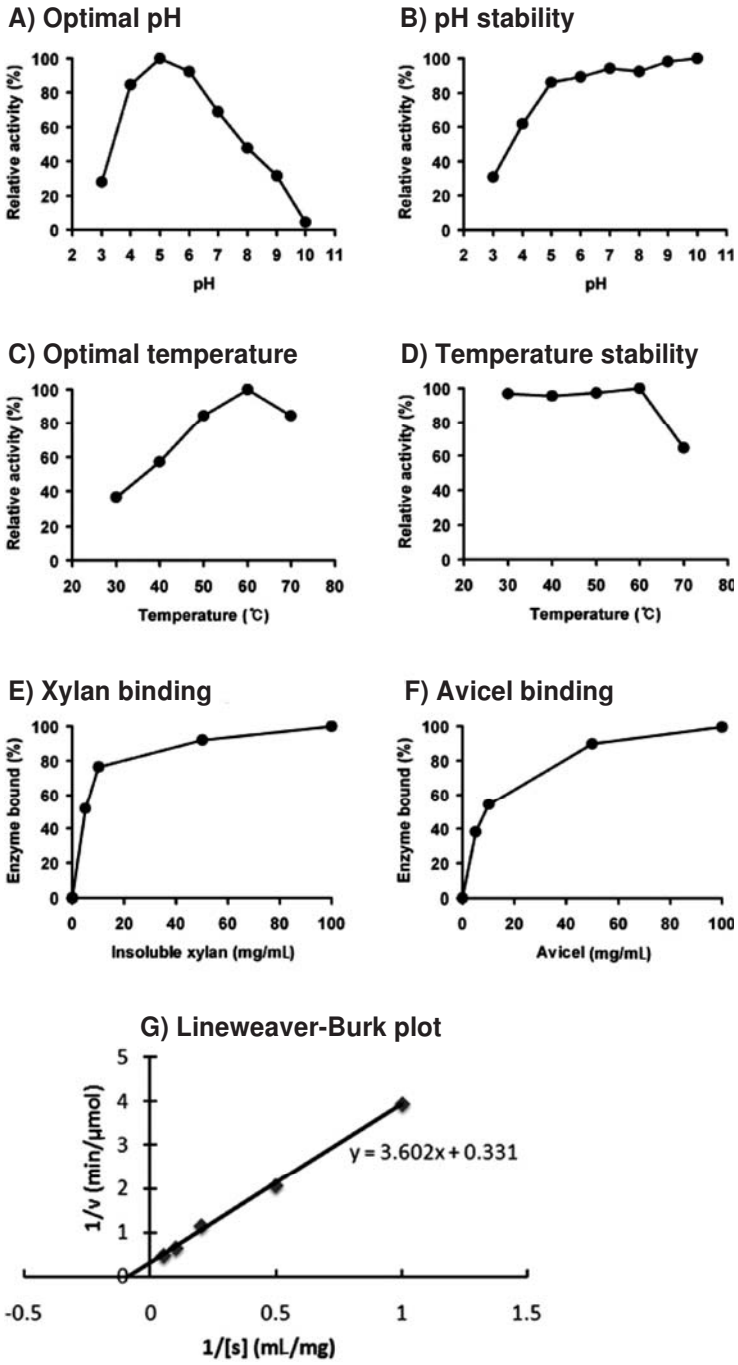


**Fig. 2.** Properties of purified xylanase from S. thermocyaneoviolaceus. Methods of xylanase assay, binding assay, and detail experimental procedure are described in materials and methods. Panel A, optimal pH for enzymatic activity. The enzyme activity was measured in the standard reaction mixture for 30 min at 50°C; Panel B, pH stability of purified xylanase. The purified enzyme was incubated at 4°C for 24 h in each pH buffer solution. The enzymatic activity was measured after adjusting the pH to 5.0; Panel C, optimal temperature for enzymatic activity. The enzyme activity was measured in each temperature for 30 min at pH 5.0; Panel D, thermal stability of the purified xylanase. The purified enzyme was incubated in pH 5.0 for 1 h at each temperature. After incubation, the remaining activity was measured at 65°C for 30 min; and Panel E-F, binding of xylanase to insoluble xylan and avicel. Binding experiments were run by adding 1 Unit/mL of enzyme to the indicated amount of insoluble xylan (panel E) or avicel (panel F) in 1 mL of 50 mM citrate-phosphate buffer (pH 5.5). Samples were incubated for 1 h at 60°C and then centrifuged at 15,000 rpm for 5 min. The amount of enzyme remaining in the supernatant was determined by a xylanase activity test; Panel G, Lineweaver-Burk plot of the enzyme. The activity was measured at 65°C for 30 min, pH 5.0.

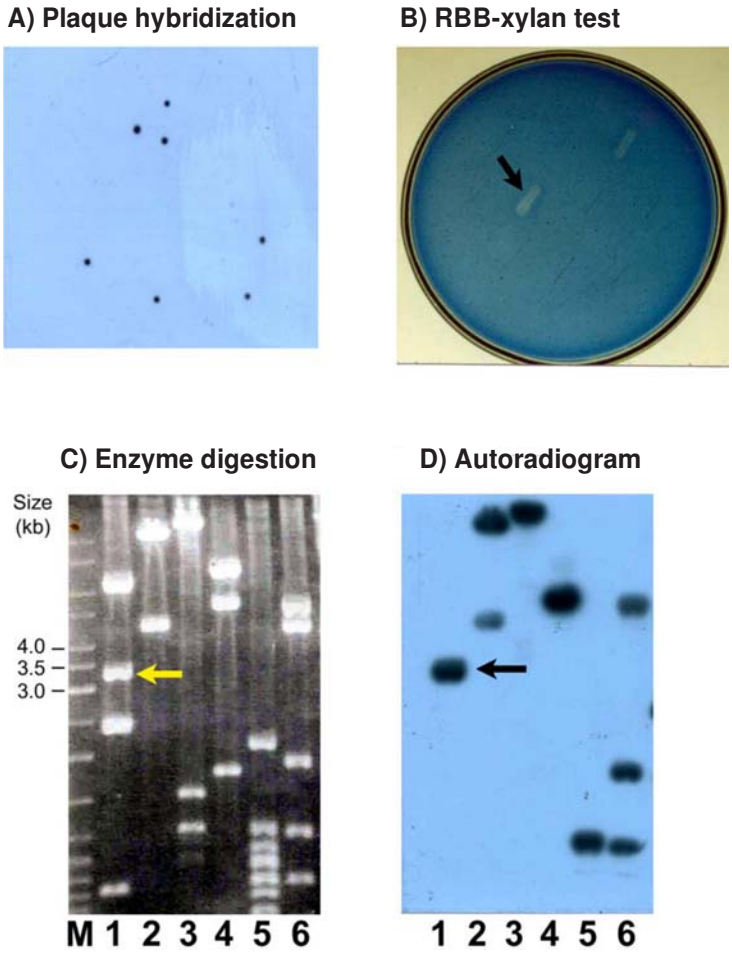


**Fig. 3.** Cloning procedure of xylanase gene from *S. thermocyaneoviolaceus*. The detail cloning and subcloning methods and procedures are described in materials and methods. Panel A, an autoradiogram of plaque hybridizaiton result. Positive clones containing putative xylanase genes from the genomic DNA library in λ phage from *S. thermocyaneoviolaceus* were screened by plaque hybridization with the 32P-labelled xlnA gene from the *S. lividans* as probe DNA. Panel B, selection of xylanase producing *E. coli* transformants. LB-amphicillin agar plate with 0.1% RBB-xylan and 1mM IPTG was patched with *E. coli* transformants selected by plaque hybridization. Colonies degrading RBB-xylan are surrounded by clearing zones. The plates were photographed after 24 h of incubation at 37°C. An arrow indicated that a selected transformant harboring 12 kb insert DNA. Panel C and D, a southern hybridization result for subcloning of the xylanase gene. The plasmid DNA selected from the RBB-xylan test was digested with various restriction enzymes and hybridized with the 32P-labelled probe DNA An agarose gel electrophoresis picture (C) and its autoradiogram after hybridization (D) is shown. Lane M, DNA size standard marker; lanes 1, 1 μg of *Bam*H I digest; lanes 2, 1 μg of *Bgl* II digest; lanes 3, 1 μg of *Kpn* I digest; lanes 4, 1 μg of *Pst* I digest; lanes 5, 1 μg of *Sac* II digest; and lanes 6, 1 μg of *Sal* I digest. Arrows indicates a 3.3 kb DNA fragment for subcloning.

100% identity with XlnA from *S. lividans* and Stx II from *S. thermoviolaceus* OPC-520 [31], 92% identity with XynA from *Clostridium thermocellum*, 77% identity with XynD from *Cellulomonas fimi*, 75% identity with XynY from *Bacillus* sp., and 71% identity with CgxB from *Chaetomium gracile*.

## Gene Cloning of the Xylanase

A gene for the purified xylanase was cloned from the *S. thermocyaneoviolaceus* genomic DNA library. Plaque hybridization was performed using an *xln*A gene from *S. lividans,* which has 100% matching identity with the purified xylananase in N-terminal 15 amino acid sequences (Fig. 3A). Positive signaling plaques were subcloned to plasmid DNA by a *cre-loxP* mediated auto-subcloning system of the λBlueSTAR kit. Subcloned plasmids were transformed into *E. coli* DH5α, and a clone producing xylanase was selected by the RBB-xylan clearance zone test as described in materials and methods. A plasmid, named pSMB8 later, containing about a 12 kb DNA fragment was selected by displaying a clear zone near colonies (Fig. 3B). Through the restriction

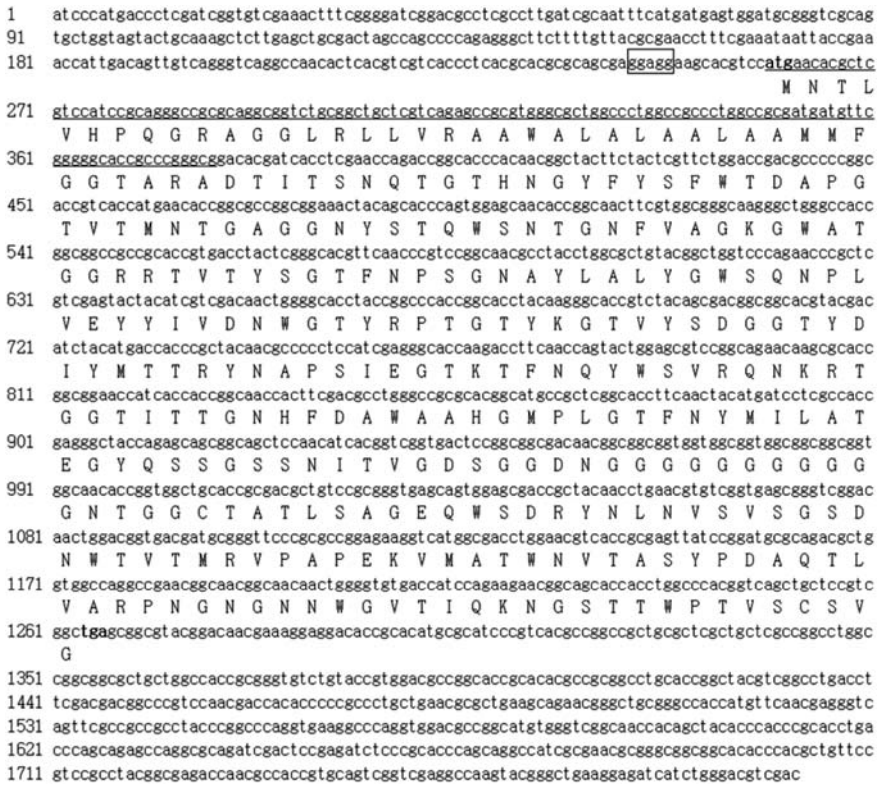

**Fig. 4.** Nucleotide sequences and deduced amino acid sequences of *xyn*B of *S. thermocyaneoviolaceus*. The deduced amino acid sequences are shown below the nucleotide sequences and start or stop codon for XynB are indicated in bold. The putative Shine-Dalgarno region is displayed within the box and the signal peptide sequences are underlined.

enzyme digestion experiment and southern hybridization selection, a DNA fragment of approximately 3.3 kb was selected as a minimal size xylanase gene subclone (Figs. 3C and 3D). The 3.3 kb DNA was sequenced with at least 5 times overlapping in both directions to avoid technical error.

As shown in Fig. 4, a 1008-bp long open reading frame was designated as cloned xylanase and the sequence was registered in GenBank (ac. no. AF194025). The putative ribosomal binding site was shown at 7 bp upstream from the ATG start codon. A putative signal peptide including 40 amino acids was found by comparing the N-terminal sequences of the purified enzyme. Comparing the nucleotide sequences of other xylanase genes using the Blast program on the NCBI website, the xylanase gene shared homologies with 99% of the Stx II gene from *S. thermoviolaceus*, 86% of the *xln*B gene from *S. lividans*, and 87% of the *xyn*D gene from *Cellulomonas fimi*. The gene from *S. thermocyaneoviolaceus* was named as *xyn*B. The G+C content of the *xyn*B were 67.66% and the molar ratio of third base in each codon was 94.35%. The pattern of G+C contents was in general range of *Streptomyces* species. A conservative motif of family 11 group xylanase activity sites, PLVEYYIVDNW, was found in the translated sequences [32].

Amino acid sequences of the XynB showed high similarity to Stx II from *S. thermoviolaceus* OPC-520 [33]. Only two amino acids are different between Stx II (V34 in signal sequence and D147 in mature sequence) and XynB (F34 and Y147). While Stx II had low avicel-binding affinity, XynB had high avicel-binding affinity similar to that of xylan in this study despite only one amino acid difference in mature sequence (Figs. 2E and 2F). Further study should be necessary to elucidate this important characteristic of XynB compared to Stx II.

## Overexpression of XynB

For industrial scale production of the xylanase, a recombinant *E. coli* strain producing the xylanase was constructed. The 1008 base paired *xyn*B structural gene was introduced to the *Nde*I-*Eco*RI site in pET21a(+) vector by the general restriction enzyme engineered PCR and ligation method.

The *xyn*B containing recombinant plasmid, named pEMB10, was transformed to *E. coli* BLR(DE3) and the overproduction of the recombinant xylanase was examined. The main enzymatic activity of the recombinant xylanase was found in the supernatant. Therefore, it can be assumed that the signal peptide of the XynB had been worked successfully into the *E. coli* strain. As shown in Fig. 5, the overproduced xylanase bands were observed both in SDS-PAGE and zymogram assay (Fig. 5A, lane 2 and 3, respectively). The expressed recombinant xylanase displayed the same level of enzyme thermostability, pH stability, optimal enzymatic reac-

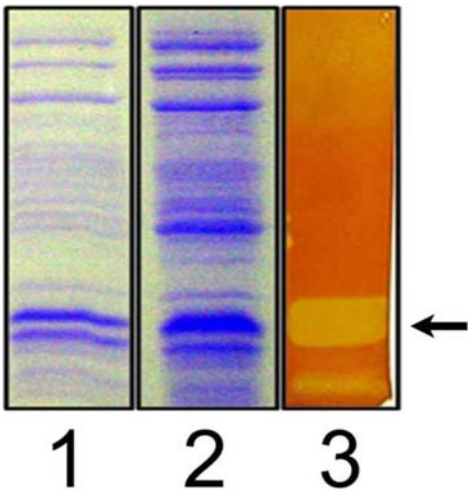


**Fig. 5.** Overexpression of xylanase (XynB) in recombinant strain *E. coli* BLR(DE3)/pEMB10. A ten percent SDS-polyacrylamide gel was used for protein bands from the supernatant of recombinant *E. coli.* 10% gel co-polymerized with 1% soluble Birchwood xylan was used for zymogram to detect active xylanase bands. Lane 1, SDS-PAGE analysis using the crude supernatant of *E. coli* BLR(DE3)/pET21a(+); lane 2, SDS-PAGE analysis using the crude supernatant of *E. coli* BLR(DE3)/pEMB10; and lane 3, zymogram assay using the crude supernatants of *E. coli* BLR(DE3)/pEMB10.

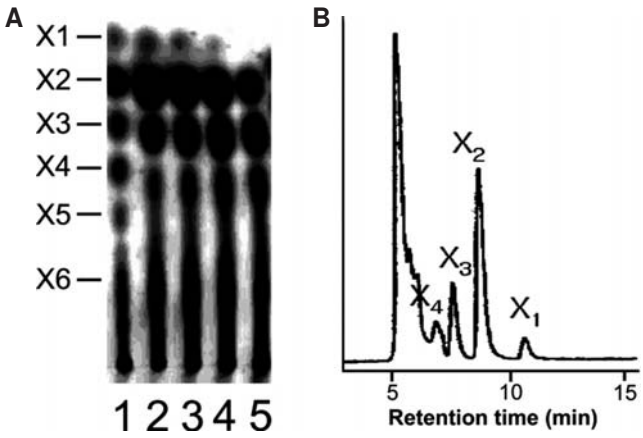


**Fig. 6.** Analysis of xylooligosaccharides produced by recombinant xylanase from Birchwood xylan. Panel A, thin layer chromatography result of the xylan hydrolysates by recombinant xylanase. The reaction mixture was composed of 10% Birchwood xylan in a 50 mM citrate phosphate buffer (pH 6.0) with indicated amount of recombinant enzyme. The reaction was done at 60°C for 1 h. Lane 1, xylooligosaccharides standard (Suntory xylooligomer 20P), oligomeric state of the standard xylooligomer was labeled as X1-X6; lane 2, 40 Unit/mL of xylanase; lane 3, 20 Unit/mL of xylanase; lane 4, 10 Unit/mL of xylanase; and lane 5, 5 Unit/mL of xylanase. Panel B, high performance liquid chromatography (HPLC) analysis of the xylan hydrolysates by recombinant xylanase. One hundred gram of Birchwood xylan in 1 L of 50 mM sodium phosphate buffer (pH 6.0) was incubated at 60°C for 24 h with *E. coli* crude extract containing 2,000 units of recombinant enzyme. The oligomeric state of each peak was determined by comparing it with the retention time of the xylooligomer standard marker. Detail TLC and HPLC methods and procedures were described in materials and methods.

tion temperature, and optimal pH (data now shown).

### Production of Xylooligosaccharides by Recombinant Xylanase

For the confirmation of xylooligosaccharides production by the recombinant xylanase from *E. coli* BLR(DE3)/pEMB10, xylan hydrolyzation assay was carried out with various amounts of recombinant enzyme and 10% (w/v) of birch wood xylan in a 50 mM sodium phosphate buffer (pH 6.0). After incubating at 60°C for 1 h, the xylan hydrolysates were analyzed by thin layer chromatography (TLC) and/or high performance liquid chromatography (HPLC). Five units per milliliter of recombinant xylanase could convert xylan into xylooligosaccharides within 1 h, proficiently (Fig. 6A, lane 5).

For larger scale application, 100 g of Birchwood xylan in 1 L of a 50 mM sodium phosphate buffer (pH 6.0) was incubated at 60°C for 24 h with *E. coli* crude extract containing 2,000 units of recombinant enzyme. After incubation, the composition of xylose and xylooligosaccharides (xylobiose to xylohexose) were calculated from the HPLC flowchart (Fig. 6B). Total xylooligosaccharides were approximately 65.0 g from 100g of birch wood xylan. As well, 4.3 g of monosaccharide, xylose was produced. The xylooligosaccharides producing value by recombinant xylanase was far above those of the wild type crude enzyme extract [34].

Xylanase from *Aspergillus oryzae* MTCC 5154 produce around 89.5% xylooligosaccharides in the hydrolysate of 1% birch wood xylan at 24 h of reaction [35]. However the hydrolysate contained around 12% of xylose which is not desirable product for the industrial purpose. Akpinar *et al*. also tried to produce xylooligosaccharides using enzymatic process [36]. They used commercial xylanase, Veron 191, produced from *A. niger* (AB Enzymes, Darmstadt, Germany). They succeed to produce 53% xylooligosaccharide from 2% xylan solution at the optimal hydrolysis conditions: 40°C, pH 5.4, and 24 h incubation. Both the yield and incubation time is not desirable to industrial scale production. Moreover, it is not clear whether the enzymes from two *Aspergillus* spp. could hydrolyze an industrial scale of xylan concentration, which is over 10%. In a previous report [33], we claimed the crude enzymes from *S. thermocyaneoviolaceus* produced 58.5% xylooligosaccharides from 10% xylan solution within a 12 h period. Compared to crude enzyme of *S. thermocyaneoviolaceus*, recombinant XynB not only produced a better yield of the xylooligosaccharides but also better quality. Recombinant XynB could produce over 50% of xylooligomers within only 1 h incubation with less enzyme amount and or volume. Moreover, the recombinant enzyme produced a maximum

4.3% of monomer (xylose) even after 24 h of incubation at 6°C with 2,000 units, 100~200 fold more enzyme than working conditions (compare Figs. 6A to 6B).

In conclusion, the thermostable xylanase from *S. thermocyaneoviolaceus* can be expected to be an advantageous enzyme for the production of xylooligosaccharides in various industrial processes.

## CONCLUSION

A thermostable xylanase purified from *S. thermocyaneoviolaceus* had suitable properties for the production of xylooligosaccharides from the wood xylan. For industrial scale production of the xylanase, a recombinant *E. coli* strain producing the xylanase was constructed. The expressed recombinant xylanase displayed the same level of enzyme thermostability, pH stability, optimal enzymatic reaction temperature, and optimal pH. The enzyme production level of the recombinant strain was far higher than the wild type strain (52 Unit/mL from recombinant strain versus 12 Unit/mL from *S. thermocyaneoviolaceus*). The recombinant enzyme or crude extract from the recombinant strain had an efficient capability for production of xylooligosaccharides from the wood xylan. In conclusion, the thermostable xylanase from *S. thermocyaneoviolaceus* can be expected to be an advantageous enzyme for the production of xylooligosaccharides in various industrial processes.

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## REFERENCES

1. Kulkarni, N., E. A. Shendy, and M. Rao (1999) Molecular and biotechnological aspects of xylanase. *FEMS Microbiol. Rev.* 23: 411-456.
2. Liab, K., P. Azadi, R. Collins, J. Tolan, J. Kim, and K. Eriksson (2000) Relationships between activities of xylanases and xylan structures. *Enzym. Microb. Technol.* 27: 89-94.
3. Biely, P. (1985) Microbial xylanolytic systems. *Trends Biotechnol.* 3: 286-290.
4. Jeya, M., S. Thiagarajan , Jung-Kul Lee , and P. Gunasekaran (2009) Identification of new GH 10 and GH 11 xylanase genes from *Aspergillus versicolor* MKU3 by genome-walking PCR. *Biotechnol. Bioprocess Eng.* 14: 13-19.
5. Dekker, R. F. H. and G. N. Richards (1976) Hemicellulases: their occurrence, purification, properties, and mode of action. *Adv. Carbohydr. Chem. Biochem.* 32: 277-352.
6. Frederick, M. M., C. H. Kiang, J. R. Frederick, and P. J. Reilly (1985) Purification and characterization of endoxylanases from *Aspergillus niger.* I. Two isozymes active on xylan backbones near branch points. *Biotechnol. Bioeng*. 27: 525-532.
7. Irwin, D., E. D. Jung, and D. B. Wilson (1994) Characterization and sequence of a Thermomonospora fusca xylanase. *Appl. Environ. Microbiol.* 60: 763-770.
8. Reilly, P. J. (1981) Xylanase: structure and function in trends in the biology of fermentations for fuels and chemicals. *Basic Life Sci.* 18: 111-129.
9. Wong, K. K. Y. and J. N. Saddler (1993) *Applications of hemicellulases in the food, feed and pulp, and paper industries. Hemicellulose and Hemicellulases.* pp. 127-143. Portland Press, London, UK.
10. Kim, K. C., S. W. Kim, M. J. Kim, and S. J. Kim (2005) Sacchrification of foodwastes using cellulolytic and amylolytic enzymes from *Trichoderma harzianum* FJ1 and its kinetics. *Biotechnol. Bioprocess Eng*. 10: 52-59.
11. Chen C., J. L. Chen, and T.Y. Lin (1997) Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. *Enzym. Microb. Technol.* 21: 91-96.
12. Jeong, K. J., I. Y. Park, M. S. Kim, and S. C. Kim (1998) High-level expression of an endoxylanase gene from *Bacillus* sp. in *Bacillus subtilis* DB104 for the production of xylobiose from xylan. *Appl. Microbiol. Biotechnol.* 50: 113-118.
13. Jiang, Q., W. Deng., Y. P. Zhu., L. T. Li., Y. J. Sheng, and K. Hayashi (2004) The recombinant xylanase B of *Thermotoga maritima* is highly xylan specific and produces exclusively xylobiose from xylans, a unique character for industrial applications. *J. Mol. Catal. B: Enzymatic* 27: 207-213.
14. Vázquez, M. J., J. L. Alonso., H. Domínguez, and J. C. Parajó (2000) Xylooligosaccharides: manufacture and applications. *Trends Food Sci. Technol.* 11: 387-393.
15. Yuan, Q. P., H. Zhang, Z. M. Qian, and X. J. Yang (2004) Pilot-plant production of xylo-oligosaccharides from corncob by steaming, enzymatic hydrolysis, and nonofiltration. *J. Chem. Technol. Biot.* 79: 1073-1079.
16. Björkstén, B., E. Sepp, K. Julge, T. Voor, and M. Mikelsaar (2001) Allergy development and the intestinal microflora during the first year of life. *J. Allergy and Clinical Immunology*. 108: 516-520.
17. Guarner, F. and J. R. Malagelada (2003) Gut flora in health and disease. *The Lancet* 361: 512-519.
18. Basaran, P., Y. D. Hang, N. Bsaran, and R. W. Worobo (2001) Cloning and heterologous expression of xylanase from Pichia stipitis in *Escherichia coli*. *J. Appl. Microbiol.* 90: 248-255.
19. Cho, S. G. and Y. J. Choi (1995) Nucleotide sequence analysis of an endo-xylanase gene(xynA) from *Bacillus stearothermophilus*. *J. Microbiol. Biotechnol.* 5: 117-124.
20. Choi, J. H., O. S. Lee, J. H. Shin, Y. Y. Kwak, Y. M. Kim, and I. K. Rhee (2006) Thermostable xylanase encoded by xynA of *Streptomyces thermocyaneoviolaceus*: cloning, purification, characterization, and production of

xylooligosaccharides. *J. Microbiol. Biotechnol.* 16: 57-63.
21. Kimura, T., H. Suzuki, H. Furuhashi, T. Aburatani, K. Morimoto, S. Karita, K. Sakka, and K. ohmiya (2000) Molecular cloning, overexpression, and purification of a major xylanase from *Aspergillus oryzae. Biosci. Biotechnol. Biochem.* 64: 2734-2738.
22. Srivastava, R., S. S. Ali, and B. S. Srivastara (1991) Cloning of xylanase gene of *Streptomyces flavogriseus* in *Escherichia coli* and becteriophage λ-induced lysis for the release of cloned enzyme. *FEMS Microbiol. Lett.* 78: 201-206.
23. Vieira, J. and J. Messing (1987) Production of single-stranded plasmid DNA. *Methods Enzymol.* 153: 3-11.
24. Bradford, M. M. (1976) A rapid and sensitive method for the quantitification of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248-254.
25. Jung Y. J., J. S. Yoo, Y. S. Lee, I. H. Park, S. H. Kim , S. C. Lee, M. Yasuda, S. Y. Chung, and Y. L. Choi (2007) Purification and characterization of thermostable β-1,3-1,4 glucanase from *Bacillus* sp. A8-8. *Biotechnol. Bioprocess Eng.* 12: 265-270.
26. Miller, G. L. (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 31: 426-428.
27. Hunkapiller, M. W. and L. E. Hood (1983) Analysis of phenylthiohydantoins by ultrasensitive gradient high-performance liquid chromatography. *Methods Enzymol.* 91: 486-493.
28. Sambrook, J., E. F. Fritch, and T. Maniatis (1989) *Molecular cloning*: *A Laboratory Manual.* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
29. Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Shrempf (1985) *Genetic Manipulation of Streptomyces: A Laboratory Manual.* The John Innes Foundation, Norwich, England.
30. Sanger, F., S. Nicklen, and A. R. Coulson (1977) DNA sequencing with chain - terminating inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463-5467.
31. Tsujibo, H., K. Miyamoto, T. Kuda, K. Minami, T. Sakamoto, R. Hasegawa, and Y. Inamori (1992) Purification, properties and partial amino acid sequence of thermostable xylanases *Streptomyces thermoviolaceus* OPC-520. *Appl. Environ. Microbiol.* 58: 371-375.
32. Henrissat, B. and A. Bairoch (1993) Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316: 695-696.
33. Tsujibo, H., T. Ohtsuki, T. Iio, I. Yamazaki, K. Miyamoto, M. Sugiyama, and Y. Inamori (1997) Cloning and sequence analysis of genes encoding xylanases and acetyl xylan esterase from *Streptomyces thermoviolaceus* OPC-520. *Appl. Environ. Microbiol.* 63: 661-664.
34. Lee, O. S., C. S. Choi, J. H. Choi, G. J. Joo, and I. K. Rhee (2001) Production of xylooligosaccharides with thermostable xylanase from *Streptomyces thermocyaneoviolaceus. Kor. J. Appl. Microbiol. Biotechnol.* 29: 221-226.
35. Aachary, A. A. and S. G. Prapulla (2008) Corncob-induced *endo*-1,4-β-D-xylanase of *Aspergillus oryzae* MTCC 5154: Production and characterization of xylobiose from glucuronoxylan. *J. Agric. Food Chem.* 56: 3981-3988.
36. Akpinar, O., O. Ak, A. Kavas, U. Bakir, and L. Yilmaz (2007) Enzymatic production of xylooligosaccharides from cotton stalks. *J. Agric. Food Chem.* 55: 5544-5551.