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Thermostable Xylanase Encoded by *xynA* of *Streptomyces thermocyaneoviolaceus*: Cloning, Purification, Characterization and Production of Xylooligosaccharides

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Abstract We have cloned a xylanase gene (*xynA*) from *Streptomyces thermocyaneoviolaceus*. The deduced amino acid sequences of the XynA, including the active site sequences of glycosyl hydrolase family 10, showed high sequence homology with several xylanases assigned in this category. The XynA was overexpressed under an IPTG inducible T7 promoter control in *E. coli* BLR(DE3). The overproduced enzymes were excreted into culture supernatants and periplasmic space. The purified XynA had an apparent molecular mass of near 54 kDa, which corresponds to the molecular mass calculated from its gene. The optimum pH and temperature of the purified XynA were determined to be 5.0 and 65°C, respectively. The XynA retained over 90% its activity after the heat treatment at 65°C for 30 min. The XynA was highly efficient in producing xylose (X1), xylobiose (X2), xylotriose (X3), and xylotetraose (X4) from xylan.

Key words: *Streptomyces thermocyaneoviolaceus*, xylanase, xylooligosaccharides

Hemicelluloses, the second most common polysaccharide in nature, account for about 20–25% of the total dry weight of higher land plants, and about 20% of agricultural waste such as rice straw or wheat straw [34, 20]. In recent years, the bioconversion of hemicelluloses 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 of consistency of beer [15, 42, 43, 45].

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Xylan is the most abundant of the hemicelluloses [8, 13]. The composition and structure of xylan varies according to their sources, however, all the xylans are heteropolysaccharides with homopolymeric backbone chains of 1,4-linked β-D-xylopyranose units [11]. The biodegradation of xylan is a complex process that requires coordinated action of several enzymes, among which xylanase (1,4-β-D-xylan xylanohydrolase), cleaving internal linkages on the β-1,4-xylose backbone, plays a key role. Microbial xylanases are preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction condition, negligible substrate loss and side-product formation. Many xylanase genes have been cloned from various sources such as *Bacillus* spp. [6, 9], fungi [21, 37], *Streptomyces* spp. [39–41], and yeasts [1, 17]. Furthermore, there are many potential applications for xylanases in industry and biotechnology [25].

Xylooligosaccharides are the major intermediates of xylan hydrolysis by xylanase and have received increasing attention as a growth-promoting factor for *Bifidus* bacteria in large intestine [30]. Specifically, xylooligosaccharides can be renewed and utilized as new functional food additives in dietary industries [22]. However, the study of the production of xylooligosaccharides has poorly been investigated, even though the hydrolysis of xylan has been extensively studied for the production of xylose monomer [3, 7]. The production of xylooligosaccharides and its technical development are becoming more and more important in terms of the industrial aspect of functional foods. Especially, thermostable enzymes are attractive candidates for some of these applications [14, 27]. We previously reported that a thermophilie *Streptomyces thermocyaneoviolaceus* produced a thermostable xylanase [26]. In this paper, we report cloning, purification and characterization of a thermostable xylanase from the *S. thermocyaneoviolaceus*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

Streptomyces thermocyaneoviolaceus KCCM 40049 was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea), and cultured in 100 ml of medium containing 0.3% yeast extract, 0.3% malt extract, 0.5% Bacto-peptone, and 1% dextrose (pH 6.5) at 50°C. Luria-Bertani (LB) containing ampicillin (100 μg/ml) or chloramphenicol (50 μg/ml) was used as the basal medium for the *Escherichia coli* strains, which were grown at 37°C. *E. coli* DH5α was used as a host for the plasmid amplification [10]; *E. coli* ER1647 was used for the λ phage transfection [44]; *E. coli* BM25.8 as a strain expressing *cre* recombinase [33]; and *E. coli* BLR(DE3) (Novagen Munich, Germany), having a copy of T7 RNA polymerase gene on chromosome, was used for the overexpression of the xylanase gene. Bacteriophage λBlueSTAR (Novagen) was used as a vector for construction of the gene library. Plasmid pET21a(+) (Novagen, Germany), containing the T7 promoter, was used for the expression vector. Plasmid pUC119 and pBluescriptII SK(+) were used for subcloning.

Construction of Genomic Library and Cloning of *xynA* Gene

Chromosomal DNA from *S. thermocyaneoviolaceus* was prepared by the method of Hopwood [16]. The *xlnA* gene of *Streptomyces lividans* was used as the probe DNA for the cloning of xylanase genes [29, 38, 41]. The probes were radio-labeled with [α-^{32}P]dCTP with the Rediprime DNA labeling system (Amersham-Pharmacia Biotech., England) according to the manufacturer's instructions. The chromosomal DNA of *S. thermocyaneoviolaceus* was partially digested with Sau3AI to yield fragments with an average size of 9 to 15 kb. These fragments were ligated in the λBlueSTAR phage, which had been completely digested with BamHI and dephosphorylated with an alkaline phosphatase. *In vitro* packaging and infection into *E. coli* ER1647 were carried out according to the recommendations of the manufacturer (Novagen, Germany). Recombinant clones were stored at −80°C. The colony hybridization of the *S. thermocyaneoviolaceus* DNA library was performed, as described by Sambrook *et al.* [35], using a Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech., England). The positive clones were subcloned into pBluescriptII SK(+) and transformed into competent cells of *E. coli* BM25.8 (Novagen).

Sequencing and DNA Analysis

Plasmid DNA was prepared according to standard method, and the inserted DNA was digested with several restriction endonucleases for the construction of a restriction map. For Southern-blot analysis, restriction fragments were transferred onto a Hybond-N$^+$ nylon membrane (Amersham-Pharmacia Biotech., England) and hybridized with the probe DNA, *xlnA* gene of *S. lividans*. Restriction fragments hybridized to the probe DNA were cloned into pUC119, and the insert nucleotides were sequenced by the dideoxy-chain-termination method [36]. The sequence data was analyzed with the PCGENE software package (IntelliGenetics Inc., Mountain View, CA, U.S.A.). The nucleotide and amino acid sequences were scanned against the databases available at the NCBI of the NIH through the BLAST server. The hydrophobicity of the xylanase was analyzed through the Institute Pasteur server.

Construction of Expression Plasmids and Purification

In order to overexpress XynA, the *xynA* gene of *S. thermocyaneoviolaceus* was subcloned by the polymerase chain reaction (PCR) between the NdeI and EcoRI sites of pET21a(+). Two oligonucleotides were used to generate the unique NdeI and EcoRI sites: (sense) 5'-GGGGTACCATATGGGCTCTCACGCCCTTCC-3' and (antisense) 5'-CGGAATTCAGGAGACGGTC CAGCGC-3'. The PCR products were digested and ligated into the expression vector pET21a(+), and the ligated plasmids were introduced into *E. coli* BLR(DE3). A transformant forming clear zone around the colony on 0.1% RBB-xylan agar medium (LB agar medium containing 100 μg/ml of ampicillin, 1 mM of IPTG, and 0.1% RBB-xylan; Sigma-Aldrich Co., MO, U.S.A.) was selected. Plasmid DNA was prepared from the candidate transformant, and the inserted DNA was confirmed by nucleotide sequence.

E. coli BLR(DE3) harboring recombinant plasmid, pEMA144, was grown overnight at 37°C in LB media containing 100 μg/ml of ampicillin. After overnight culture, cells were diluted 50-fold into fresh medium and grown to A_{600} of 0.8, at which point XynA expression was induced by the addition of 0.4 mM IPTG and incubation for an additional 4 h.

The supernatant of the culture broth was collected by centrifugation and concentrated using an ultrafiltration kit with stirred Amicon 8050 (Amicon, Beverly, MA, U.S.A.) and YM10 membrane (Amicon). The concentrated enzyme suspensions were then applied to a Superose 12 HR 10/30 column operated with a FPLC system (Amersham-Pharmacia Biotech., Buckingham-Shire, England). The column was equilibrated with a column buffer (50 mM Tris-HCl, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, pH 8.0) and loaded with 3 ml volume of the enzyme preparations. The xylanase was eluted with the same buffer at a flow rate of 0.2 ml/min. The eluted proteins were detected by monitoring at A_{280}, and each fraction was tested by a standard xylanase activity assay method. Finally, the fractions with xylanase activity were concentrated using an ultrafiltration kit as described above and stored at 4°C for later use. The crude extracts and each fraction through the purification procedures were collected and analyzed by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were stained with Coomassie brilliant blue R-250 (CBB; Sigma-Aldrich Co., St. Louis, MO, USA). To detect xylanase in gel, a sample was resolved by standard SDS-PAGE containing 1% birchwood xylan (Sigma-Aldrich Co.). Then, the gel was incubated for 4 h at 50°C and stained with 1% Congo red (Sigma-Aldrich Co.).

Measurement of Xylanase Activity

The standard assay for xylanase activity was done by incubating the enzyme at 50°C for 15 min, with 1% (w/v) birchwood xylan as a substrate, in 0.05 M citrate phosphate buffer, pH 5.5 [5]. The reducing sugars released were detected by the dinitrosalicylic acid method of Miller [28]. One unit of enzyme activity was defined as the amount that releases 1 μmol reducing sugar per min. All activity measurements were done in triplicate. The protein concentration was measured with the Bradford method [4].

Characteristics of Xylanase

The optimum temperature for the enzyme activity was determined by measuring the xylanase activity at 50, 55, 60, 65, 70, 75 and 80°C. Thermostability was measured by preincubating the enzyme at 50, 55, 60, 65, 70, 75 and 80°C for 1 h. Samples were taken at intervals and residual xylanase activity was measured. The optimum pH was determined by measuring the xylanase activity at 50°C, using 0.1 M citrate phosphate buffer for the range of pH 3 to 5 and 0.1 M sodium phosphate buffer for pH 6 to 9. The pH was monitored before and after the reaction, however, no significant pH changes due to the reaction were observed. All assays were done in triplicate.

Preparation of Subcellular Fractions

Periplasmic and cytoplasmic fractions were prepared by the conventional osmotic shock method [32]. The cells were collected by low-speed centrifugation (4,000 ×g, 4°C, 10 min), resuspended in 0.033 M Tris-HCl (pH 7.4) containing 40% sucrose, and incubated at 20°C for 10 min. After the low-speed centrifugation for 10 min, the supernatant was saved as the periplasmic fraction. The pellets were resuspended in 0.05 M citrate phosphate (pH 5.5) and disrupted by two passages through a French press (Aminco, U.S.A.) at 18,000 psi. Centrifugation (10,000 ×g, 4°C) was carried out for 15 min to remove cell debris, and the supernatant was collected as the cytoplasmic fraction.

TLC and HPLC Analysis of Xylooligosaccharides Produced by Xylanase

The digestion of xylan by the xylanase was carried out at 60°C for 12 h in 0.05 M citrate phosphate buffer (pH 5.5). Then, trichloroacetic acid solution was added to quench the reaction, and the precipitated protein was removed by centrifugation. After centrifugation, the supernatant was analyzed for xylooligosaccharides produced by the enzymatic hydrolysis of xylan. The sample was applied onto a silica gel thin layer chromatography plate (Silica gel 60 F_{254}; Merck, U.S.A.) and developed in a solvent system, containing 1-buthanol:2-propanol:water:acetic acid:acetonitrile (7:5:4:10:2, v/v). After separation, the plate was dried in air. The detection was carried out by spraying the plate with 0.2% orcinol, which is solublized in sulfuric acid and methanol solution (1:9, w/v), and then dried at 95°C for 5 min. The identification of the xylooligosaccharides was achieved by comparing the R_f values with those of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), and xylopentaose (X5) standard marker, purchased from the Megazyme Co. (England).

High performance liquid chromatography (HPLC) analysis was used for quantitative analysis of the xylooligosaccharides. A Waters Sugar-Pak column (6.5× 300 mm) was used for analysis. The column was eluted with water containing 50 ppm Ca-EDTA for 20 min at a flow rate of 1 ml/min. All chromatographic procedures were performed at 85°C. The HPLC effluent was detected by a refractive index detector (Waters Model 410), using 10 ppm ribose as an internal standard.

RESULTS AND DISCUSSION

Cloning of a Xylanase Gene from *S. thermocyaneoviolaceus*

The heterologous hybridization method has widely been used as an efficient cloning technique. To clone a xylanase gene from *S. thermocyaneoviolaceus*, we used the *xlnA* of *S. lividans* as a probe. The genomic DNA library of *S. thermocyaneoviolaceus* was constructed using the λBlueSTAR phage and infected into *E. coli* ER1647. Three positive clones were detected among 1,000 plaques by colony hybridization, when the *xlnA* was used as the probe DNA.

The positive clones were subcloned into pBluescriptII SK(+) and transformed into competent cells of *E. coli* BM25.8 expressing *cre* recombinase. As a result, an inset fragment of 15 kb containing a xylanase gene was obtained and designated as pSMA4. Then, the pSMA4 was digested by various DNA restriction endonucleases, and the digested fragment was analyzed by Southern hybridization as described in Materials and Methods. Finally, a 2.7 kb DNA fragment containing a xylanase gene was isolated from pSMA4 digested with *Sph*I and cloned into pUC119. The resulting plasmid was named pUMA27. The inserted fragments were sequenced as described in Materials and Methods.

Some xylanase activity was observed, when *E. coli* DH5α containing pUMA27 was cultured at 37°C in L-broth, which means that the cloned gene from *S. thermocyaneoviolaceus* could be expressed in *E. coli* by their own promoters (data not shown).

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1    cccgggagtcggcgagcgaaccaagtcggcctgaacgcggtgttgacaggggattcggcaggagacctttcctgtcctcgtccgtcttcg
91   gccctgtctcgccgggagcgccgcggctgcgcgacgtaccgcctcggcagaaatgatcttcgaaagtttcgaaagaaaaccggaaagatt
181  ctccgtccagcgggttgacgagacaccgtcaagtctcaatcatcctgtcggggaaaccgatcctgcaccgagccgcgcaaccatgtcggt
271  cccgagcacccgtgccgcacgacagagccgcgtacgaagcacccgcgtcatgccgtggtacgtcagtgaggtccacaccacggcagtggc
361  cggtgcgcatgtgcagcccgggggtccgggggccgacccgtccgtccgccgtggaagcggacggtgtctccccccgcgcctcgctcgttc
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                                M  G  S  H  A  L  P  R  P  A  L  R  Q  R  I  R  G  G  C  G  R
541  ggccgcggcgtcctcggtctgggcgccacgctgagcacaccgccgaccgcgcacgccgccgagagcacgctcggtgcggccgcggctcag
     G  R  G  V  L  G  L  G  A  T  L  S  T  P  P  T  A  H  A  A  E  S  T  L  G  A  A  A  A  Q
631  agcggccgtiacttcggcaccgccatcgccgcgggcaggctgagcgactcgacgtacacgtcgatcgccagccgcgagttcaacatggtg
     S  G  R  Y  F  G  T  A  I  A  A  G  R  L  S  D  S  T  Y  T  S  I  A  S  R  E  F  N  M  V
721  acggccgagaacgagatgaagatcgacgccaccgaaccgcagcgcgggcagttcgacttctccgccggcgaccgcgtctacaactgggcg
     T  A  E  N  E  M  K  I  D  A  T  E  P  Q  R  G  Q  F  D  F  S  A  G  D  R  V  Y  N  W  A
811  gtgcagaacggcaaagaggtgcgcggccacacccctggcctggcactcccagcagccgtactggatgcagagcctgagcggcagtgacctg
     V  Q  N  G  K  E  V  R  G  H  T  L  A  W  H  S  Q  Q  P  Y  W  M  Q  S  L  S  G  S  D  L
901  cgccaggcgatgatcgaccacatcaacggcgtgatgaaccactacaagggcaagatcgcccagtgggacgtcgtgaacgaggcctttcgag
     R  Q  A  M  I  D  H  I  N  G  V  M  N  H  Y  K  G  K  I  A  Q  W  D  V  V  N  E  A  F  E
991  gacgggaactcgggagcccggcgcgactccaacctgcagcgcaccggcaacgactggatcgaggtcgcctccgcaccgcgcgcgccgcc
     D  G  N  S  G  A  R  R  D  S  N  L  Q  R  T  G  N  D  W  I  E  V  A  F  R  T  A  R  A  A
1081 gacccgtccgccaagctctgctacaacgactacaacatcgagaactggacctgggccaagacccaggccgtgtacaacatggtccgcgac
     D  P  S  A  K  L  C  Y  N  D  Y  N  I  E  N  W  T  W  A  K  T  Q  A  V  Y  N  M  V  R  D
1171 ttcaagcagcgcggcgtaccgatcgactgcgtcggcttccagtcgcacttcaacagcggcagcccgtacgacagcaacttccgcaccacc
     F  K  Q  R  G  V  P  I  D  C  V  G  F  Q  S  H  F  N  S  G  S  P  Y  D  S  N  F  R  T  T
1261 ctgcagagcttcgcggcgctcggtgtcgacgtggccatcaccgaactcgacatccagggtgcctcgcccacgacgtacgccaacgtggtc
     L  Q  S  F  A  A  L  G  V  D  V  A  I  T  E  L  D  I  Q  G  A  S  P  T  T  Y  A  N  V  V
1351 aacgactgcctggccgtctcgcgctgcctgggcatcaccgtctggggtgtgcgcgacaccgactcctggcggtcgggtgacaccccgctg
     N  D  C  L  A  V  S  R  C  L  G  I  T  V  W  G  V  R  D  T  D  S  W  R  S  G  D  T  P  L
1441 ctgttcaacggcgacggcagcaagaagcctgcctactccgccgttctcgacgcgctcaacggcggctcctccggcgaacctccggaggac
     L  F  N  G  D  G  S  K  K  P  A  Y  S  A  V  L  D  A  L  N  G  G  S  S  G  E  P  P  E  D
1531 ggcgggtccggacagatcaagaacgccgcgtcgggccggtgcctcgacgtctccggtgccggcacgccgacggcacagcagtccagctc
     G  G  S  G  Q  I  K  N  A  A  S  G  R  C  L  D  V  S  G  A  G  T  A  D  G  T  A  V  Q  L
1621 tatgactgccacggcggcaccaaccagcagtggacctacaccgacgccggcgagttcagggtctacggcaacaagtgcctggacgccggc
     Y  D  C  H  G  G  T  N  Q  Q  W  T  Y  T  D  A  G  E  F  R  V  Y  G  N  K  C  L  D  A  G
1711 ggcaccggaaatggaacgcgtgttcaaatttacagctgctgggcggcgacaaccagaagtggcgcgtgaactccgacggcaccatcgtc
     G  T  G  N  G  T  R  V  Q  F  Y  S  C  W  G  G  D  N  Q  K  W  R  V  N  S  D  G  T  I  V
1801 ggtgtccagtcgggcctgtgcctggacgccgccggcacgggcaacagcaccccggtccagctctacacctgctcgtacgccgacaaccag
     G  V  Q  S  G  L  C  L  D  A  A  G  T  G  N  S  T  P  V  Q  L  Y  T  C  S  Y  A  D  N  Q
1891 cgctggaccgtctcctgacacggcatgc
     R  W  T  V  S
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of *Streptomyces thermocyaneoviolaceus* XynA.
Amino acids are shown below the codons. The putative Shine-Dalgarno (SD) sequence is represented as a box, and the start and stop codons are given in bold. The signal peptide sequence is underlined, and the N-terminal residues of the purified XynA are double underlined.

Sequence and Characteristics of the Gene Product

We have determined the nucleotide sequence of the cloned xylanase gene and designated it as *xynA*. The nucleotide sequences of *xynA* were deposited with accession no. AF194024 in GenBank. The 1,918 bp of insert DNA in pUMA27 is shown in Fig. 1. According to the deduced amino acid sequences of XynA, the protein consists of 476 amino acids with a calculated molecular mass of 51,125 Da and a calculated pI of 5.79. A putative ribosomal binding site is found at 8 bp upstream from the ATG translational initiation codon. The frequencies of A, T, G, and C at the third position of codons of the *xynA* gene are 16 A, 24 T, 296 C, and 141 G, respectively. In the *xynA* gene, cytosine and guanine are highly preferred at the third position of codons. The GC content of the *xynA* is 67.6%.

Hydropathy patterns of the deduced amino acids revealed that there is a hydrophobic region near the N-terminus (data not shown). In addition, the N-terminal sequences of purified XynA are AESTLGAAAA. These results indicate that the hydrophobic region of the N-terminus is the signal peptide, which consists of 40 amino acids that were split between 40th and 41st Ala residues (Fig. 1).

In *E. coli* DH5α/pUMA27, xylanase activity was observed mostly in culture supernatants (28.0%) and the periplasm (67.6%) from the beginning of the stationary phase (Fig. 2).

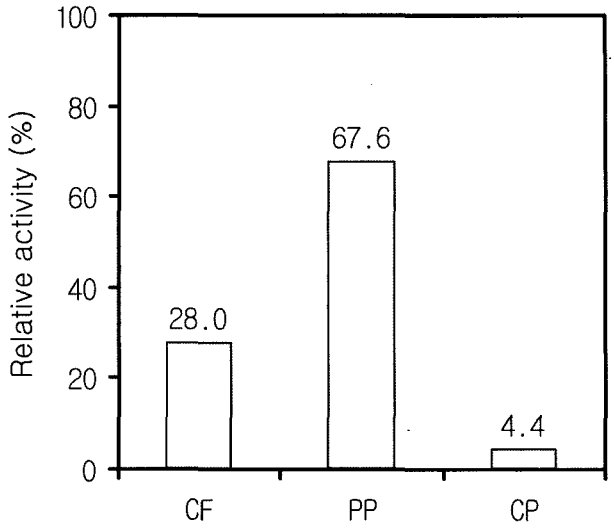

Fig. 2. The localization of XynA in *Escherichia coli* DH5α/pUMA27.
CF, culture filtrate; PP, periplasm; CP, cytoplasm.

Activity was also found in the cytoplasm, however, it represented only a small percentage of the total activity (Fig. 2). On the other hand, no xylanase activity was observed in the culture supernatants or the periplasm fraction, when we truncated the signal peptide sequences (data not shown). These results suggest that the signal peptide of XynA acts as a transmembrane signal in *E. coli* secretion system (Fig. 2). This property could be beneficial in industrial application, because the recombinant enzyme could be used directly after proper osmotic shock without any high-cost purification step.

According to the sequence-based classification method of glycosyl hydrolase [12], the XynA was assigned to glycosyl hydrolase family 10, which includes the amino acid sequence, GVDVAITELDI, corresponding to the xylanase active site of family 10. Also, the RICIN domain consensus sequence, a putative carbohydrate-binding domain formed from presumed gene triplication, was found on the C-terminal domain of XynA (data not shown). A search for sequence similarity between XynA and other proteins was conducted using the standard protein-protein BLAST, and XynA showed high sequence homology to several family 10 xylanases: The XynA of *S. thermocyaneoviolaceus* showed the highest similarity with that of *S. thermoviolaceus*: 93% identity [40]. The xylanase A of *S. lividans* [38] and *S. coelicolor* [2] also showed high similarity throughout the entire sequence: 81% identity and 87% similarity, respectively. Endo-1,4-β-Xylanase of *S. olivaceoviridis* [19] showed high sequence similarity, except for the N-terminus signal peptide region: 86% identity and 90% similarity. Other family 10 xylanases also showed similar levels of identity and similarity to XynA, but not to the entire sequence (data not shown).

Expression and Purification of XynA

The first step in the purification was the construction of the expression vector. The PCR product of the *xynA* gene with NdeI and EcoI Rrestriction sites was ligated into the IPTG-inducible expression vector pET21a(+) which was digested with the same enzymes. The ligated plasmid, pEMA144, was then introduced into *E. coli* BLR(DE3), and the successive subclones were selected as described in Materials and Methods. To overproduce the XynA protein, 0.4 mM IPTG was added at A_{600} of 0.8 and incubated for an additional 4 h. Overproduction or purification of the enzyme was monitored by CBB staining of SDS-PAGE gels (Fig. 3). We observed an increase in the intensity of the 54 kDa band, corresponding to the molecular mass calculated from its gene, after IPTG induction (Fig. 3, lane 3). Also, the zymogram assay revealed that the major band with the same molecular weight in induced samples was *S. thermocyaneoviolaceus* xylanase, even though minor bands were also observed (Fig. 3, lane 4). It is highly likely that the minor bands were originated from *E. coli* xylanase. A similar result was also observed in the expression of *Paenibacillus* sp. [18], leading us to conclude that the 54 kDa band represents XynA. In order to test whether the substrate xylan affects the mobility of the active enzyme, the crude enzyme was boiled for 2 min in the presence of 10 mM β-mercaptoethanol, however, no difference in mobility was observed (data not shown).

The overproduced xylanase was purified using the fast performance liquid chromatography (FPLC) mediated gel filtration with a Superose 12 HR column, as described in Materials and Methods. The purified xylanase band had an apparent molecular mass of near 54 kDa, as shown in the crude enzyme fraction (Fig. 3, lane 1). We obtained 0.08 mg of the purified protein from about 1.6 mg of crude extract (data not shown).

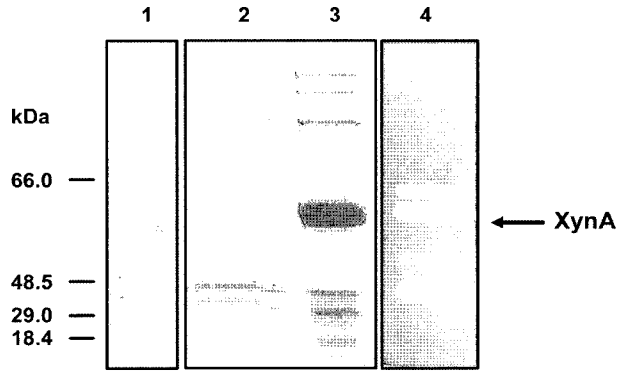

Fig. 3. Expression and purification of XynA.
Cells were cultured and harvested as described in Materials and Methods. The gel of lane 4 contained 1% soluble xylan. After electrophoresis, the gel was incubated at 30°C for 15 min and stained by 1% Congo red. Lane 1, purified XynA; lane 2, crude supernatants of *E. coli* BLR(DE3)/pET21a(+); lane 3, crude supernatants of *E. coli* BLR(DE3)/pEMA144; lane 4, zymogram assay of lane 3 sample.

Characterization of the Purified XynA

Several characteristics of the purified xylanase were investigated. The optimum temperature and pH were determined by incubating the enzyme at different temperatures and pH values according to the standard assay. The optimum temperature of the purified xylanase was 65°C (data not shown). Thermal stability was also investigated by preincubating the enzyme for 1 h at different temperatures. The XynA retained over 90% of its activity after treatment at 65°C for 30 min (data not shown).

The purified xylanase was most active at pH 5.0 and over 90% of its activity were kept at from pH 4.0 to 8.0 (data not shown). Some characteristics, including molecular mass, optimum temperature and optimum pH, were not found to be significantly different between the native xylanse from *S. thermocyaneoviolaceus* and the recombinant (data not shown).

Production of Xylooligosaccharides

The TLC analysis of the enzymatic hydrolysate of xylan showed that the recombinant xylanase were very useful in the production of xylooligosaccharides from xylan. The recombinant xylanase produced xylose, xylobiose, xylotriose, and xylotetraose from xylan (Fig. 4). Also, the HPLC analysis of the hydrolysates revealed the relative composition of the xylooligosaccharides. The hydrolysates produced by the recombinant xylanase consisted of 14% xylose, 25%

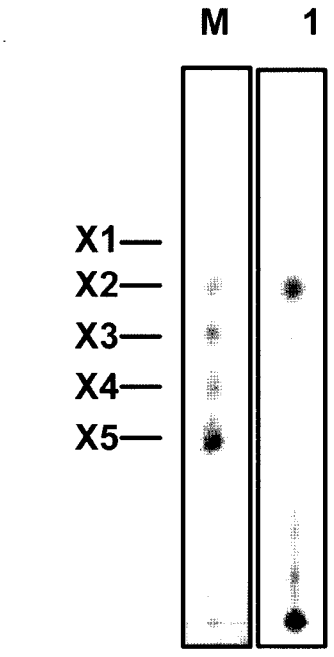

Fig. 4. TLC analysis of the xylan hydrolysates produced by XynA.
The reaction mixture consisted of 10% birchwood xylan in 0.05 M citrate phosphate buffer (pH 6.0) with 10 U/ml of XynA. The reaction was terminated by boiling for 10 min after incubation for 1 h at 60°C. M, standard marker (X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose); lane 1, xylooligosaccharides produced by the enzyme.

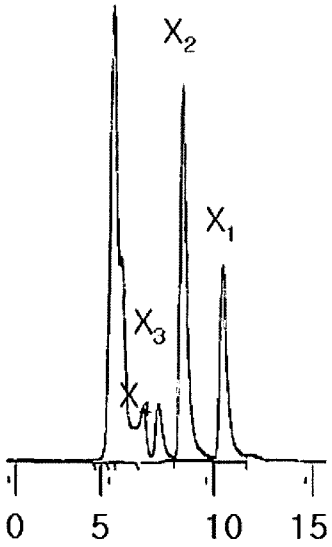

Fig. 5. HPLC analysis of the xylan hydrolysates produced by XynA.

The reaction condition was same as described in Fig. 4. Each peak was determined by comparing the retention times of the standard marker.

xylobiose, 4% xylotriose, 3% xylotetraose, and the rest was either xylooligosaccharides or xylan (Fig. 5). There are a number of reports about xylan-degrading xylanase produced by fungi and bacteria. The production of xylotetraose from xylan by *Aeromonas caviae* [24], of xylose and xylooligosaccharides (X2 to X6) by *Paenibacillus* sp. [18], and of xylose and xylooligosaccharides (X2 to X3) by *Clostridium thermocellum* [23] has also been reported. Among these, the high production of xylose and xylobiose by the xylanase from *S. thermocyaneoviolaceus* appears to be encouraging, because these two products are high value of sugars in food industry.

The results presented in this paper indicate that the recombinant xylanase is an attractive candidate for industrial application because of its excretive property into culture supernatant, thermostability, and production of several xylooligosaccharides.

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