J. Microbiol. Biotechnol. (2007), **17**(1), 29–36

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning, Characterization, and Expression of Xylanase A Gene from *Paenibacillus* sp. DG-22 in *Escherichia coli*

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Received: July 14, 2006
Accepted: August 28, 2006

Abstract The *xynA* gene encoding the xylanase A of *Paenibacillus* sp. DG-22 was isolated with a DNA probe obtained by PCR amplification, using degenerated primers deduced from the amino acid residues of the known N-terminal region of the purified enzyme and the conserved region in the family 11 xylanases. The positive clones were screened on the LB agar plates supplemented with xylan, by the Congo-red staining method. The *xynA* gene consists of a 630-bp open reading frame encoding a protein of 210 amino acids, and the XynA preprotein contains a 28-residues signal peptide whose cleavage yields a 182-residues mature protein of a calculated molecular weight of 20,000 Da and p*I* value of 8.77. The cloned DNA fragment also has another ORF of 873 nucleotides that showed 76% identity to the putative transcriptional activator of *Bacillus halodurans* C-125. Most of the xylanase activity was found in the periplasmic space of *E. coli*. The *xynA* gene was subcloned into pQE60 expression vector to fuse with six histidine-tag. The recombinant xylanase A was purified by heating and immobilized metal affinity chromatography. The optimum pH and temperature of the purified enzyme were 6.0 and 60°C, respectively. This histidine-tagged xylanase A was less thermostable than the native enzyme.

Key words: *Paenibacillus* sp. xylanase, cloning, expression, histidine-tag

Xylan is the major component of plant hemicellulose and the second most abundant renewable polysaccharide in nature [7]. It is a heterogeneous polysaccharide consisting of a main chain of β-1,4-linked D-xylose residues that often carry arabinosyl, acetyl, and glucuronosyl substituents [3, 30]. The complete hydrolysis of xylan requires the cooperative action of several enzymes such as endoxylanase, β-xylosidase, α-arabinosidase, and acetyl esterase. Among them, endo-β-1,4-xylanases (EC 3.2.1.8) are crucial enzymes since they initiate the degradation of xylan into short xylooligosaccharides [3].

Based on amino acid sequence similarities and hydrophobic cluster analysis, xylanases can be classified into two major groups of glycosyl hydrolases; family 10 and family 11 [9]. Family 10 contains xylanases of high molecular mass and acidic p*I* values, and family 11 is composed of low molecular weight endoxylanases that can be divided into alkaline p*I* and acidic p*I* xylanases based on the physicochemical properties of the enzymes [27]. Computer analysis of the family 11 xylanase sequences revealed that this group of enzymes shows a single domain and clear amino acid similarity, all having a common hold resembling a partly closed right hand. The catalytic residues are two conserved glutamate residues, which are located opposite to each other in an open active site cleft [24].

Microbial xylanases have attracted considerable research interest because of their potential industrial applications in the food, animal feed, and paper and pulp industries [2, 25, 28]. However, such applications generally prefer a thermostable and cellulase-free xylanase with broad pH and temperature optima [2, 28]. The pulp and paper industry is a primary target for the use of xylanases. The bio-bleaching of pulps would have environmental benefits, as the use of chlorine could be reduced by the use of xylanases during processing [2, 25, 28, 29].

Although many xylanase genes from various microorganisms have been cloned and their encoded enzymes have been isolated and characterized [4, 6, 11, 21, 24, 32], only a few reports on xylanases from *Paenibacillus* sp. have been published [12, 15]. *Paenibacillus* sp. DG-22, a moderately thermophilic bacterium isolated from timber yard soil, grows actively on xylan as a sole carbon source and does not have cellulase activity [16]. The bacterium secretes two types of xylanases, XynA and XynB, with molecular weights

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of 20 and 30 kDa, respectively, into the growth medium in the presence of xylan. We have purified these thermostable xylanases from the culture supernatant to homogeneity by ion-exchange and gel-filtration chromatographies. Both enzymes had similar pH and temperature optima, but showed different thermostabilities and modes of action [17]. In this study, we present the complete nucleotide sequence of a gene, *xynA*, encoding the xylanase A from *Paenibacillus* sp. DG-22. The cloned gene was over-expressed in *E. coli*, and biochemical characteristics of the recombinant protein were investigated.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

Paenibacillus sp. DG-22 was grown as described previously [17] and was used as the source of genomic DNA. *Escherichia coli* DH5α and *E. coli* M15 (pREP4) (Qiagen, Valencia, U.S.A.) were used as cloning and expression hosts, respectively. These strains were grown in LB medium, and the medium was supplemented with ampicillin (50 μg/ml) or kanamycin (50 μg/ml), when required. Plasmid pGEM-T Easy (Promega, Madison, U.S.A.) was used for cloning of PCR products. Plasmid pUC19 was used for cloning and sequencing of the *xynA* gene. Plasmid pQE60 (Qiagen) was used as an expression vector to construct a XynA containing a six histidine-tag at its carboxy-terminus.

DNA Manipulation

Paenibacillus sp. DG-22 chromosomal DNA was isolated by the method of Marmur [18]. Plasmid DNAs and PCR products were purified using the plasmid DNA preparation kit and PCR purification kit (GeneralBiosystem, Seoul, Korea). Standard DNA manipulations and Southern hybridization were done as described by Sambrook *et al.* [22]. DNA transformation was performed by using the electroporation. All enzymes used were purchased from Promega. Oligonucleotides used for PCR were obtained from Bioneer (Cheongwon, Korea).

PCR Amplification and Southern Hybridization

As a first step in the cloning of the xylanase A gene from *Paenibacillus* sp. DG-22, the *xynA* gene fragment was amplified by PCR using the forward and reverse primers as follows: forward primer, 5'-GAYTAYTGGCARTAYTGGAC-3'; and reverse primer, 5'-ATRTCRTANGTNCCNCCRTCRCT-3' (where R, Y, and N indicate A/G, T/C, and A/T/C/G, respectively). The degenerate oligonucleotide primers were designed on the basis of the amino terminal sequence of XynA [17] and the highly conserved motif (SDGGTYDI) in the family 11 xylanases [24]. PCR conditions were an initial denaturation step at 95°C, 2 min, followed by 30 cycles of 95°C for 30 s, 50°C for 40 s, 72°C for 60 s, and a final extension step at 72°C for 5 min. The PCR product was cloned into pGEM-T Easy vector (Promega) and sequenced.

For Southern hybridization, the genomic DNA of *Paenibacillus* sp. DG-22 was completely digested with EcoRI, HindIII, or EcoRI plus HindIII, separated by 0.8% agarose gel electrophoresis and transferred to nylon membrane (Hybond-N, Amersham, England) by capillary transfer [22]. The partial *xynA* gene fragment cloned in the pGEM-T Easy vector was used as a probe. The labeling and hybridization were performed using the DIG DNA Labeling and Detection Kit (Roche, Mannheim, Germany).

Cloning and Sequencing

Chromosomal DNA of *Paenibacillus* sp. DG-22 was digested with EcoRI plus HindIII and electrophoresed on a 0.8% agarose gel, and the fragments of 3–4 kb fractions were excised from the gel and purified. These were ligated into the corresponding sites of pUC19 and transformed into *E. coli* DH5α to construct a *xynA*-enriched DNA library. Screening of the xylanase-positive clones was done on 0.5% birchwood xylan-LB agar plates by Congo red plate assay [31]. The colonies harboring xylanase activity showed clear zones on the plates. Recombinant DNAs were isolated from these clones and analyzed with various restriction enzymes to determine the size of the inserts. The nucleotide sequence of the insert was determined by automated sequencing using the dideoxynucleotide method [23]. Homology searches in the GenBank database were carried out by using the BLAST program [1]. Multiple sequence alignments were carried out with the CLUSTAL W program.

Expression and Purification of the Recombinant XynA

For expression and purification of the XynA in *E. coli*, the structural region of *xynA* without the coding sequence for its predicted 28-residues signal peptide sequence was amplified in a MyGenie32 Thermal Cycler (Bioneer), using the xylanase A gene cloned in pUC19 as a template. Two oligonucleotide primers were synthesized to contain NcoI and BglII recognition sites to facilitate cloning in-frame into the pQE60 expression vector (Qiagen). The forward primer 5'-GCAACCTCCCATGGCAGCGACAGACTAT-3' was designed to contain a NcoI site (underlined) and the reverse primer 5'-GCAGTTGACCTGAGATCTCACCGTTACG-3' was designed to contain a BglII site (underlined). PCR was performed for 30 cycles consisting of 95°C for 1 min, 50°C for 2 min, and 72°C for 3 min. The amplified DNA fragment was digested with NcoI and BglII, and was cloned into the corresponding sites of pQE60. The recombinant plasmid, pQE60-XynA, was used to transform into *E. coli* M15 (pREP4) and xylanase-producing recombinants were identified by the Congo red method [31]. The pQE60-XynA encodes a fusion protein

that consists of XynA plus a C-terminal tag with six histidines.

The histidine-tagged xylanase A protein was purified from *E. coli* M15 (pREP4) harboring pQE60-XynA. Cultures were grown at 37°C in 1 l of LB medium supplemented with ampicillin (50 μg/ml) and kanamycin (50 μg/ml), with shaking, to an optical density of 0.6 at 600 nm. After addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 1 mM, the culture was further incubated for 4 h at 37°C. The cells were harvested by centrifugation, washed, and resuspended in 20 mM phosphate buffer (pH 7.4). The cells were disrupted by sonication, and the lysate were centrifuged at 10,000 ×*g* for 30 min. Affinity chromatography on a Ni-NTA column (Qiagen) was performed according to the supplier's protocol. Active fractions were identified, combined, and desalted by dialysis against 50 mM sodium acetate buffer (pH 5.5). The purified XynA with 6× His-tag, was used for analysis of enzymatic properties.

Cell Fractionation

To determine the localization of the recombinant xylanase in *E. coli*, cells were fractionated using the osmotic shock method [20]. A culture of 50 ml was grown to late the exponential phase, and cells were harvested by centrifugation (6,000 ×*g* for 10 min), and the supernatant was stored at 4°C for later measurement of xylanase activity in extracellular fraction. The collected cells were resuspended in osmotic shock buffer (20% sucrose, 30 mM Tris-HCl, pH 8.0, 1 mM EDTA), and placed at room temperature for 10 min. After centrifugation at 12,000 ×*g* for 10 min at 4°C, the supernatant was discarded and the cell pellet was resuspended in ice-cold 5 mM $MgSO_4$. The supernatant containing the periplasmic fraction was collected after another centrifugation as described above. The cytoplasmic proteins were obtained by sonication of the cell pellet. Unlysed cells and cell debris were removed by centrifugation (16,000 ×*g* for 20 min at 4°C), and the supernatant containing the cytoplasmic proteins was stored at 4°C before measurement of the activity. Alkaline phosphatase was used as a marker enzyme for the periplasmic fraction.

Protein Analysis

Xylanase assay and protein determination were performed as described previously [17]. One unit of xylanase activity was defined as the amount of enzyme producing 1 μmol of reducing equivalents per min under the assay conditions. The alkaline phosphatase activity was assayed by measuring the amount of nitrophenol released from 4-nitrophenyl phosphate according to the method of Garen and Levinthal [8]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 15% running gel, and resolved proteins were visualized by staining with Coomassie Brilliant Blue R250 (Sigma, St. Louis, U.S.A.).

Enzymatic Properties

The effect of pH on the activity of the recombinant enzyme was investigated using xylanase assay in McIlvaine buffer (0.2 M Na_2HPO_4-0.1 M citric acid) from pH 3.0 to 7.5 at 60°C. The effect of temperature on the activity was estimated by incubating the purified enzyme with 1% (w/v) birchwood xylan in McIlvaine buffer (pH 6.0) at different temperatures in the range of 40°C to 80°C. The thermostability of the purified xylanase were monitored by preincubating the enzyme in the absence of substrate at 50°C, 55°C, and 60°C. After various times, aliquots were withdrawn and the residual activities were measured under the standard assay conditions.

Nucleotide Sequence Accession Number

The nucleotide sequences for *xynA* and *xynR* have been deposited in the GenBank database under accession numbers DQ869568 and DQ869569, respectively.

RESULTS AND DISCUSSUION

PCR Cloning and Southern Hybridization

Paenibacillus sp. DG-22 secretes two types of xylanases into the growth medium in the presence of xylan [17]. These xylanases were designated XynA (gene *xynA*) and XynB (gene *xynB*), respectively, in accordance with the suggestions of Henrissat *et al.* [10]. Previous N-terminal

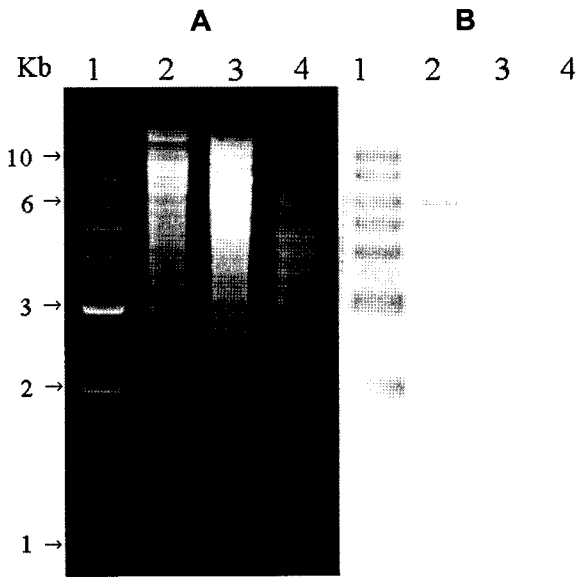

Fig. 1. Southern hybridization analysis of *Paenibacillus* sp. DG-22 genomic DNA digests with a DIG-labeled specific probe. **A.** Agarose gel (0.8%) stained with ethidium bromide. **B.** Blot hybridized with the DIG-labeled xylanase A gene fragment. Lane 1, molecular size markers (1-kb ladder, Bioneer); lanes 2, 3, and 4, *Paenibacillus* sp. DG-22 genomic DNA digested with EcoRI, HindIII, and EcoRI plus HindIII, respectively.

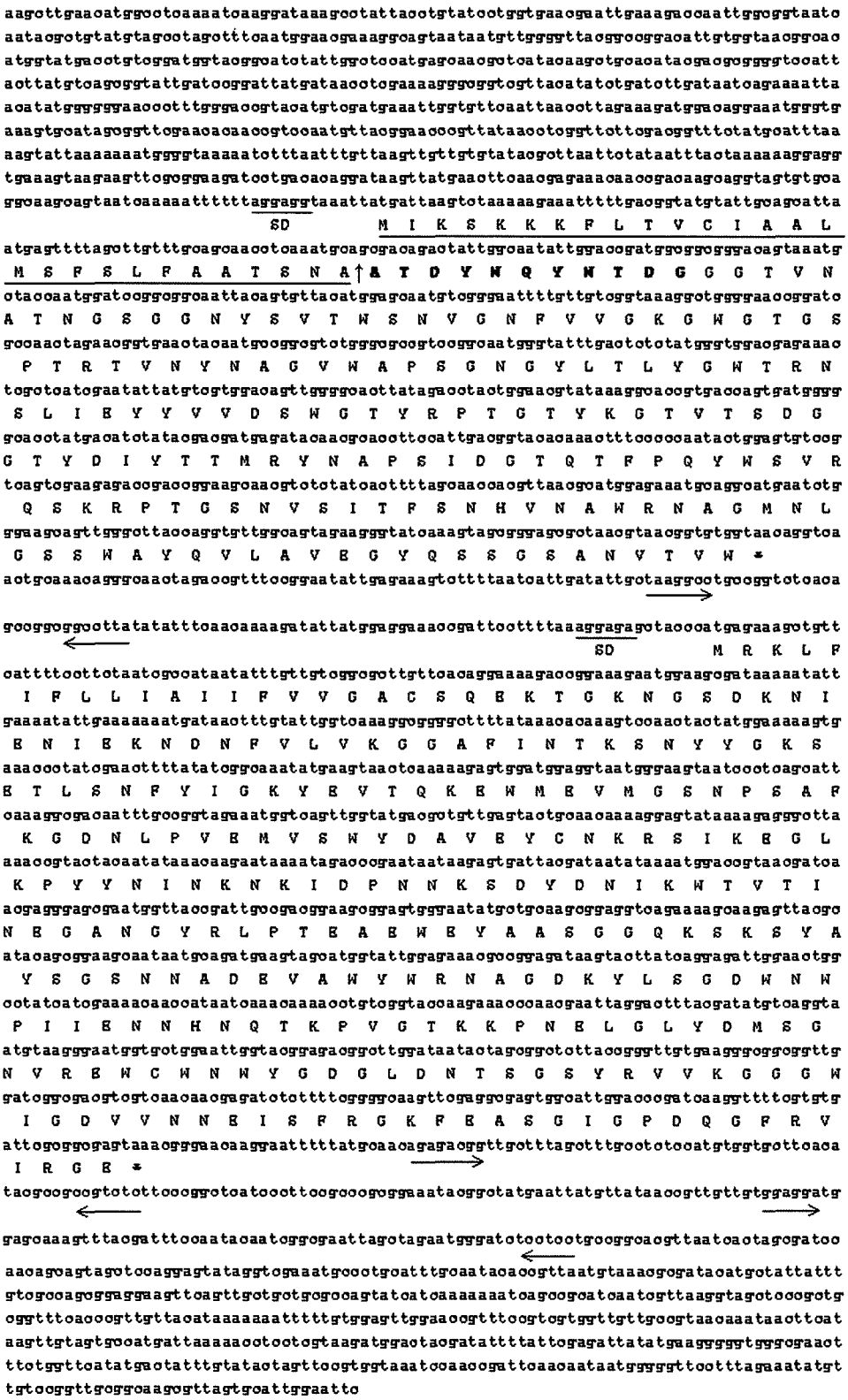

Fig. 2. Nucleotide sequences of the *Paenibacillus* sp. DG-22 *xynA* and *xynR* genes and their flanking regions and deduced amino acid sequences.

The locations of the putative ribosome-binding site (SD), and the 28-amino acid putative signal peptide are underlined. The vertical arrow indicates the cleavage site. The N-terminal amino acid sequence of the mature enzyme obtained by Edman degradation is shown in bold type. The nucleotide sequences forming the inverted repeats are indicated by antiparallel arrows. The translational stop codons are indicated by asterisks (*).

analysis of the purified XynA suggested that the amino acid sequence of XynA has high homology with those of family 11 bacterial xylanases [17]. Based on the identified N-terminal sequence of XynA and the highly conserved motif (SDGGTYDI) in the family 11 xylanases [24], the forward and reverse degenerated primers were synthesized and used for partial *xynA* gene cloning by PCR with genomic DNA of *Paenibacillus* sp. DG-22 as the template. The PCR product of 300 bp was amplified and subsequently cloned into pGEM-T Easy vector and sequenced. As expected, the deduced amino acid sequence of this fragment corresponded to the N-terminal amino acid sequence determined by Edman degradation. Southern blot analysis of restriction enzyme-digested *Paenibacillus* sp. DG-22 genomic DNA using the partial *xynA* DNA fragment as a probe indicated that double digestion with EcoRI and HindIII gives a single band of adequate size (~3 kb) for cloning (Fig. 1). To reduce the number of recombinants to be selected, the fragments of 3–4 kb fractions were excised from the gel and ligated into the corresponding sites of pUC19.

Cloning and Sequence Analysis

A *xynA* gene-enriched genomic library was constructed as described above. Of 1,000 transformants screened by the Congo red method, ten positive clones that showed clear halos of xylan hydrolysis were obtained. A positive clone that had the highest xylanase activity was selected for further characterization. A recombinant plasmid, named pXA8, was isolated from this clone and found to contain a 3.1-kb DNA fragment. The complete nucleotide sequence of the 3.1-kb insert was determined and analyzed by using the online computer program (Compute pI/Mw, ExPASy).

Translation of the nucleotide sequence revealed two open reading frames (ORFs) (Fig. 2). The first ORF of 630 bp initiated from nucleotide ATG, encoding a polypeptide of 210 amino acid residues, with a calculated molecular mass of 23,004 Da and a p*I* of 9.36. A potential ribosome-binding site (SD) sequence, 5'-AGGAGG-3', was found 7 bp upstream of the translation start codon (ATG) and it was the same as the consensus sequence, 5'-AGGAGG-3', of *Bacillus subtilis* [5]. The TAA stop codon was followed by an inverted repeat that probably constitutes a transcription terminator. The nucleotide sequence corresponding to a typical signal peptide was present at the 5' end of the open reading frame. The putative signal peptide contained 28 amino acid residues, rich in hydrophobic residues. Comparison with the N-terminal amino acid sequence of the purified mature xylanase (boldface in Fig. 2) confirmed the signal peptide cleavage site (indicated by vertical arrow in Fig. 2) between the Ala28 and Ala29 residues. The mature polypeptide consisted of 182 amino acid residues with a calculated mass of 20,000 Da, which is consistent with experimental data previously obtained from the purified enzyme [17]. The cloned enzyme seems to belong to the type of "alkaline p*I*-low molecular weight xylanases" that are ubiquitous among *Bacillus* species [24, 27].

Downstream of the *xynA* gene, a second ORF was found, which was carried on the same strand and had the same direction of transcription. The second ORF of 873 bp encoded a polypeptide of 291 amino acids with a calculated molecular mass of 33,059 Da and an isoelectric point of 6.08. This polypeptide was designated XynR (gene *xynR*). The putative initiation codon ATG was preceded 8 bp by a potential ribosome binding sequence, 5'-AGGAGA-3'. The TAA stop codon was followed by two inverted repeats.

Comparison of Deduced Amino Acid Sequences

On the basis of sequence homology and hydrophobicity, xylanases have been classified into family 10 and 11 glycosyl hydrolases [9]. The deduced amino acid sequence of XynA was used to search for the homologous sequences in the BLAST database. This sequence showed extensive homology to xylanases belonging to family 11 glycosyl hydrolases. The highest homology (83% identity) was with endoxylanase A of *Bacillus stearothermophilus* (GenBank accession no. U15985) [6]. XynA from *Paenibacillus* sp. DG-22 also had much sequence similarity with xylanases from *Aeromonas caviae* ME-1 (82% identity, D32065) [14], *Paenibacillus* sp. KCTC8848P (82% identity, AF195421) [15], *B. subtilis* (82% identity, M36648) [21], *B. circulans* (82% identity, X07723) [32], and *B. halodurans* C-125 (81% identity, AY170624) [26]. The alignments of these homologous sequences are given in Fig. 3. Ko *et al.* [13] reported that two glutamate residues of the *Bacillus pullulans* xylanase, which also belongs to family 11 xylanase, were catalytic residues on the basis of their three-dimensional structure and site-directed mutagenesis. The crystal structures of the four family 11 xylanases known so far revealed that they consist of two β-sheets and one α-helix and have been described to resemble a partly closed right hand. The catalytic residues are two conserved glutamate residues, which are located opposite to each other in an open active site cleft [24]. The catalytic mechanism is thought to resemble that of the widely studied enzyme lysozyme. The role of one glutamate is to act as an acid/base catalyst, whereas the other is a nucleophile and stabilizes the reaction intermediate [27]. The two glutamic acid residues Glu104 and Glu197 are also conserved in XynA of *Paenibacillus* sp. DG-22 (boldface in Fig. 3).

A search of the BLAST database found that the deduced amino acid sequence of XynR had 76% identity with a putative transcriptional regulator, which enhances xylanase production from *B. halodurans* C-125 [26] (data not shown). Miyazaki *et al.* [19] showed that regulation of the *xynABD* xylanase gene cluster in the ruminal anaerobe *Prevotella bryantii* $B_1$4 involves a multidomain regulatory protein XynR, which is related to two-component regulatory proteins.

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Pdg  MIKSKKKFLTVCIAALMSFSLFAATSNAA-TDYWQYWTDGG-TVNATNGSGGNYSVTWSN 58
Psp  MFKSSKKLLTVVLAASMSFGFFASTSNAA-TDYWQNWTDGGGTVNAVNGSGGNYSVTWKN 59
Aca  MFKFGKKLMTVVLAASMSFGVFAATSSAA-TDYWQNWTDGGGTVNAVNGSGGNYSVSWQN 59
Bst  M-KLKKKMLTLLLTASMSFGLFGATSSAA-TDYWQYWTDGGGMVNAVNGPGGNYSVTWQN 58
Bsu  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
Bha  MFKFVTKVLTVVIAATISFCLSAVFASAN--TYWQYWTDGGGTVNATNGPGGNYSVTWRD 58
Bcl  MFKFKKNFLVGLSAALMSISLFSATASAASTDYWQNWTDGGGIVNAVNGSGGNYSVNWSN 60
          * *          * *          *    *** ***** *** ** ******* *

Pdg  VGNFVVGKGWGTGSPTRTVNYNAGVWAPSGNGYLTLYGWTRNSLIEYYVVDSWGTYRPTG 118
Psp  SGNFVVGKGWTTGSPDRTINYNAGVWAPSGNGYLALYGWTRNSLIEYYVVDSWGTYRPTG 119
Aca  TGNFVVGKGWTYGTPNRVVNYNAGVPAPSGNGYLTFYGWTRNALIEYYVVDSWGTYRPTG 119
Bst  TGNFVVGKGWTVGSPNRVINYNAGIWEPSGNGYLTLYGWTRNALIEYYVVDSWGTYRPTG 118
Bsu  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
Bha  TGNFVVGKGWEIGSPNRTIHYNAGVWEPSGNGYLTLYGWTRNQLIEYYVVDNWGTYRPTG 118
Bcl  TGNFVVGKGWTTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTG 120
          ********** * * *  ****   * *****  *****  ******** ********

Pdg  TYKGTVTSDGGTYDIYTTMRYNAPSIDG-TQTFPQYWSVRQSKRPTGSNVSITFSNHVNA 177
Psp  TYKGTVTSDGGTYDIYTTMRYDAPSIEGGQKTTFIQYWSVRQTKRPTGGNSTITFSNHVKA 179
Aca  TYKGTVNSDGGTYDIYTTMRYNAPSIDG-TQTFPQYWSVRQSKRPTGVNSTITFSNHVNA 178
Bst  NYKGTVNSDGGTYDIYTTMRYNAPSIDG-TQTFQQPWSVRQSKRPTGSNVSITFSNHVNA 177
Bsu  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNAIITFSNHVNA 180
Bha  THRGTVVSDGGTYDIYTTMRYNAPSIDG-TQTFQQPWSVRQSKRPTGNNVSITFSNHVNA 177
Bcl  TYKGTVKSDGGTYDIYTTTRYNAPSIDGDRTTFTQYWSVRQSKRPTGSNAIITFTNHVNA 180
        *** ********** ** **** *   ** * ***** ***** *  *** *** *

Pdg  WRNAGMNLGSSWAYQVLAVEGYQSSGSANVTVW 210
Psp  WARQGMHLGNNWSYQVLATEGYQSSGSSNVTVW 212
Aca  WPSKGMYLGNSWSYQVMATEGYQSSGNANVTVW 211
Bst  WRSKGMNLGSSWAYQVLATEGYQSSGRSNVTVW 210
Bsu  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW 213
Bha  WRNAGMNLGSSWSYQVLATEGYQSSGRSNVTVW 210
Bcl  WKSHGMNLGSNWAYQVMATEGYQSSGSSNVTVW 213
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Fig. 3. Alignment of the amino acid sequence of the *Paenibacillus* sp. DG-22 XynA with those of family 11 xylanases of bacterial origin.

Amino acid numbers begin with the start codon. Gaps (-) were introduced during alignment. Identical amino acids among the xylanases are marked by asterisks (*). The Glu residues corresponding to Glu 104 and 197 of *Paenibacillus* sp. DG-22 XynA, essential to the catalytic activity, are in boldface. Abbreviations: Pdg, *Paenibacillus* sp. DG-22 (this study); Psp, *Paenibacillus* sp. KCTC8848P; Aca, *Aeromonas caviae* ME-1; Bst, *Bacillus stearothermophilus*; Bsu, *B. subtilis*; Bha, *B. halodurans* C-125; Bci, *B. circulans*.

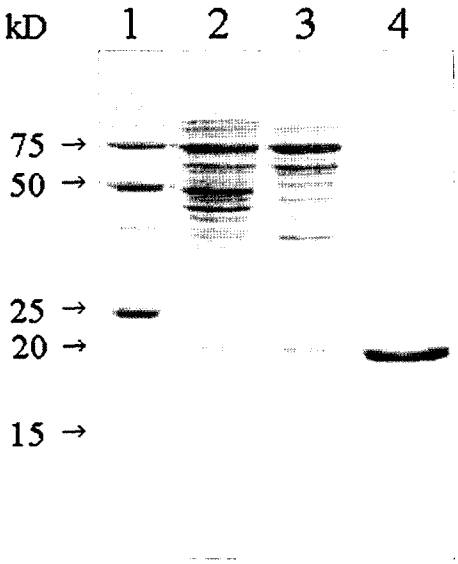

Fig. 4. SDS-PAGE analysis of recombinant xylanase A purified from *E. coli* (pQE60-XynA).

Lane 1, molecular mass markers (Bio-Rad); lane 2, crude extract of *E. coli* (pQE60-XynA) (4 μg); lane 3, heat-treated crude extract (4 μg); lane 4, after Ni-NTA agarose affinity chromatography (0.4 μg).

Expression and Purification of the Recombinant Xylanase

To facilitate purification by affinity chromatography, we expressed an XynA fusion protein with a C-terminal six histidine-tag (XynA-H_6) in *E. coli*. The *Paenibacillus* sp. DG-22 *xynA* gene without the coding sequence for its predicted signal peptide was cloned into the expression vector pQE60 in-frame, resulting in the plasmid pQE60-XynA. The expressed XynA-H_6 was purified in two steps; heat treatment and immobilized metal affinity chromatography (IMAC) (Table 1). Cells from 1 l culture were subjected to enzyme purification, and crude extract was prepared by sonication. Heat treatment of the cell extract at 60°C for 5 min increased the specific activity 1.6-fold with a recovery yield of 82%. Although this step was not an efficient procedure, it was necessary to purify the enzyme to homogeneity. Final purification was performed by IMAC with a Ni-NTA column (Qiagen). The endoxylanase was purified about 72-fold in specific activity with a recovery yield of 71%. The final preparation gave a single band with a molecular mass of 20 kDa on the SDS-PAGE gel (Fig. 4).

Localization of Recombinant Xylanase in *E. coli*

In order to investigate the localization of cloned xylanase A in *E. coli*, subcellular fractions from *E. coli* cells harboring pXA8 and pQE60-XynA were isolated and assayed for xylanase activities. In the clone containing pXA8, the majority of the xylanase activity (65%) was found in the periplasm, whereas 20% was associated with cytoplasmic fractions (Table 2). The periplasmic location of the enzyme suggests that the signal peptide of xylanase A from *Paenibacillus* sp. DG-22 is recognized and the protein is processed by *E. coli* cells. As expected, all of the xylanase activity was obtained from the cellular fraction after cell disruption in the *E. coli* cells harboring pQE60-XynA, which has no signal peptide sequence (data not shown).

Table 1. Summary of purification steps of the recombinant xylanase from *E. coli* M15 (pREP4) harboring pQE60-XynA.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Crude extract	20.9	157.8	7.5	1.0	100
Heat treatment	10.6	130.6	12.3	1.6	82.8
IMAC	0.2	112.4	562.0	74.9	71.2

Table 2. Distribution of cloned xylanase in *E. coli* (pXA8).

	Percent of total activity	
Compartment	Xylanase	Alkaline phosphatase
Extracellular fluid	14.8	5.5
Periplasm	65.0	78.1
Cytoplasm	20.2	16.4

Properties of the Recombinant Xylanase

The biochemical properties of histidine-tagged xylanase A (XynA-H_6) was investigated. The activities of XynA-H_6 at various pH values were measured by using birchwood xylan as the substrate. The reaction pHs were adjusted to 3.0–7.5 with McIlvaine buffer. The cloned xylanase showed enzyme activity over a broad pH range of 5–7 at 60°C (Fig. 5A). The pH optimum was at 6.0. The activity of the cloned enzyme was also measured at different temperatures (Fig. 5B). The optimal reaction temperature of XynA-H_6 was at 60°C and still retained more than 90% activity at 65°C. At 70°C, the enzymatic activity dropped dramatically. The effects of pH and temperature on activity of the purified XynA-H_6 from *E. coli* were similar to those of the native enzyme. The thermostability of the cloned enzyme was investigated by preincubating the purified enzyme without the substrate at 50°C, 55°C, and 60°C for designated time periods and then analyzing the xylanase activity under the standard assay conditions (Fig. 5C). The thermostability experiment indicated that in the absence of substrate, recombinant XynA-H_6 retained only 33% of its initial activity after 2 h of preincubation at 50°C. At 55°C, the half-life of XynA-H_6 was about 35 min. XynA-H_6 was unstable at temperatures above 60°C. Therefore the histidine-tagged xylanase A was less thermostable than the native enzyme, which was stable for 2 h at 60°C [17]. This result suggested that the histidine tail is responsible for the reduced thermostability of XynA-H_6. Addition of a six histidine-tag at the C-terminus might induce tiny changes in the tertiary structure, reducing the thermostability of the enzyme.

The results presented here suggest that this cloned xylanase could be a model system for gene expression, secretion, and protein engineering studies. Large-scale production of xylanase from *E. coli* will also be useful for industrial applications.

Acknowledgments

This work was supported by the research program of Dongguk University.

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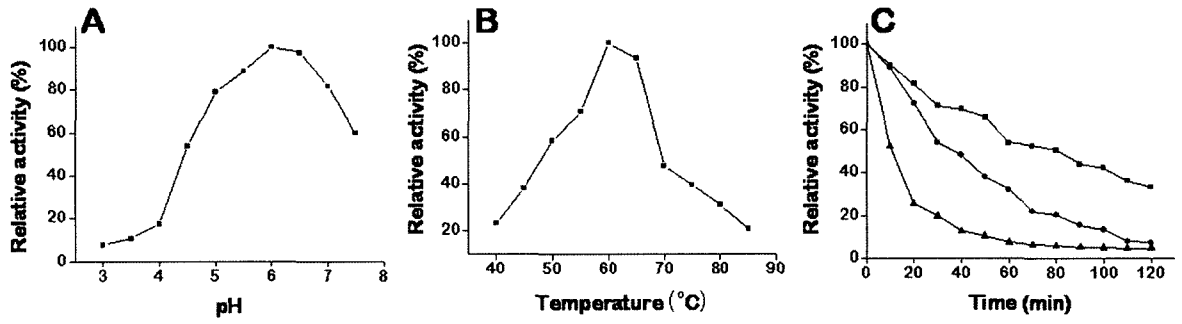

Fig. 5. Effects of pH (**A**) and temperature (**B**) on the activity of purified recombinant xylanase (XynA-H_6) from *E. coli* (pQE60-XynA). The activities at the optimal pH and the optimal temperature were defined as 100%. **C**. Thermostability of purified XynA-H_6 at pH 6.0 in the absence of xylan.
Residual activity was monitored at various times after incubation at 50°C (■), 55°C (●), and 60°C (▲). The initial activity was defined as 100%.

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