J. Microbiol. Biotechnol. (2009), **19**(12), 1514–1519
doi: 10.4014/jmb.0907.07004
First published online 6 November 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Cloning of the *Bacillus subtilis* AMX-4 Xylanase Gene and Characterization of the Gene Product

Yoon, Ki-Hong*

Department of Food Science and Biotechnology, Woosong University, Daejeon 300-718, Korea

Received: July 7, 2009 / Accepted: July 25, 2009

A gene encoding the xylanase of *Bacillus subtilis* AMX-4 isolated from soil was cloned into *Escherichia coli* and the gene product was purified from the cell-free extract of the recombinant strain. The gene, designated *xylA*, consisted of 639 nucleotides encoding a polypeptide of 213 residues. The deduced amino acid sequence was highly homologous to those of xylanases belonging to glycosyl hydrolase family 11. The molecular mass of the purified xylanase was 23 kDa as estimated by SDS–PAGE. The enzyme had a pH optimum of 6.0–7.0 and a temperature optimum of 50–55°C. Xylanase activity was significantly inhibited by 5 mM Cu^{2+} and 5 mM Mn^{2+}, and noticeably enhanced by 5 mM Fe^{2+}. The enzyme was active on xylans including arabinoxylan, birchwood xylan, and oat spelt xylan, but it did not exhibit activity toward carboxymethylcellulose or *p*-nitrophenyl-β-xylopyranoside. The predominant products resulting from xylan and xylooligosaccharide hydrolysis were xylobiose and xylotriose. The enzyme could hydrolyze xylooligosaccharides larger than xylotriose.

Keywords: *Bacillus subtilis* AMX-4, xylanase, characterization, recombinant *Escherichia coli*

Xylan, a major component of hemicellulose found in large amounts in plant cell walls, is a highly branched β-1,4-linked D-xylose polymer with substituents that include acetyl, arabinosyl, and glucuronyl groups. Complete degradation of xylan requires the action of several types of enzymes such as endo-β-1,4-xylanase, β-xylosidase, α-arabinofuranosidase, α-glucuronidase, and acetylxylan esterase. Among these enzymes, endoxylanase and β-xylosidase play the most important roles in the degradation of xylan. Endoxylanases (xylanases), which catalyze the random hydrolysis of the β-D-1,4-xylopyranosyl linkages within the backbone of heteroxylans from oat spelt, birchwood, beechwood, and wheat flour, are enzymes useful in the food, feed, paper, pulp, and biorefinery industries [23]. The most important activity of xylanase, which is characterized as a hemicellulase along with glucanase and mannanase, is the saccharification of the recyclable plant resource hemicellulose into a carbon source that is readily metabolized by living organisms.

So far, numerous xylanases and their genes have been identified in bacteria and fungi. Bacterial xylanase genes have been obtained and characterized in many strains, including members of *Bacillus* [22]. Fungal xylanase genes have been reported mostly in filamentous fungi [18]. Since the production of xylanase by these fungi was higher than that by bacterial strains, commercial production of the enzyme has been carried out utilizing strains of *Trichoderma* [11].

On the basis of amino acid similarity, almost all xylanases produced by *Bacillus* strains have been shown to belong to either glycosyl hydrolase (GH) family 10 or 11, whereas xylanase V of *B. subtilis* 168 was reported to be a GH5 enzyme [12]. *Bacillus* sp. AMX-4, which had been previously isolated from soil, produced cellulase-free xylanase at different levels according to the presence of additional carbon sources in the culture medium [24]. In order to investigate the physicochemical properties of this enzyme, the *Bacillus* sp. AMX-4 gene encoding xylanase was cloned into *Escherichia coli* and the enzyme was purified from the recombinant strain in this work.

Materials and Methods

Chemicals, Enzymes, Bacterial Strain, and Media

Oat spelt xylan, birchwood xylan, locust bean gum, carboxymethylcellulose (CMC) and *para*-nitrophenyl-β-xylopyranoside were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Xylooligosaccharides and arabinoxylan were obtained from Megazyme (Wicklow, Ireland), and the bacterial medium was from Difco (Detroit, MI, U.S.A.). Restriction endonucleases were obtained from Boehringer Mannheim

*Corresponding author
Phone: +82-42-630-9742; Fax: +82-42-636-2676;
E-mail: ykh@wsu.ac.kr

(Mannheim, Germany), and T4 DNA ligase was from Solgent Co. (Deajeon, Korea). All chemicals and enzymes were used as recommended by their manufacturers. *E. coli* XL-1 blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *relA*1 *thi lac*$^-$ F'[*proAB*$^+$ *lacI*^{q} *lacZ* M15 Tn10(*tet*^{r})]) was used as a host for the recombinant plasmid. pUC19 was used as a vector for constructing recombinant plasmid pMX4 carrying the *B. subtilis* AMX-4 xylanase gene. Recombinant *E. coli* (pMX4) was cultured at 37°C in LB broth (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter, pH 7.0) supplemented with ampicillin (50 μg/ml).

DNA Manipulation, DNA Sequencing, and Computer Analysis

Chromosomal DNA was isolated from *B. subtilis* AMX-4 cells grown exponentially in LB medium using a genomic DNA prep kit from Solgent Co. (Deajeon, Korea), and then partially digested with Sau3AI. DNA fragments ranging in size from 2 to 10 kb were isolated from an agarose gel and introduced into the dephosphorylated BamHI site of pUC19. The ligation mixture was transformed into *E. coli* XL-1 blue by the electroporation method. Restriction endonuclease-generated DNA fragments of *B. subtilis* DNA were subcloned into pUC19. The nucleotide sequences of the fragments were determined with a DNA sequencer (ABI Prism 377, Perkin Elmer Co., Foster, CA, U.S.A.). DNA and protein sequences were analyzed using the DNASIS program (Hitachi Software Engineering, Japan).

Xylanase Purification

Recombinant *E. coli* (pMX4) was used for the production of *B. subtilis* AMX-4 xylanase. The recombinant strain was grown overnight at 37°C in LB medium supplemented with ampicillin (50 μg/ml). Cells were collected by centrifugation at 6,000 ×*g* for 15 min, washed once, resuspended in 50 mM Tris-HCl buffer (pH 8.0), and then disrupted by sonication with a Branson Sonifier. The resulting cell debris was removed by centrifugation at 10,000 ×*g* for 30 min. The cell-free extract was precipitated with 30–70% saturated ammonium sulfate and then dialyzed against the same buffer. Crude samples were run through a DEAE-Sepharose column equilibrated with the same buffer, and unbound proteins were concentrated by ultrafiltration and dialyzed against 50 mM Tris-HCl buffer (pH 8.0) containing 1.0 M $(NH_4)_2SO_4$. The sample was then applied to a phenyl-Sepharose CL-4B column and proteins were eluted with a linear $(NH_4)_2SO_4$ gradient (1.0 to 0 M). Active fractions were concentrated and then dialyzed against 20 mM sodium phosphate buffer (pH 6.0). Purified xylanase was concentrated by ultrafiltation and analyzed by SDS–PAGE.

Enzyme Assay

Xylanase activity was determined by measuring the amount of reducing sugars liberated during the hydrolysis of oat spelt xylan by the dinitrosalicylic acid method [15]. The standard assay reaction mixture consisted of 0.5% (w/v) of polysaccharide substrates supplemented with 50 mM sodium citrate buffer (pH 6.0) and enzyme to make a final volume of 0.3 ml. The reaction mixture was incubated at 50°C for 15 min. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmole of reducing sugar per minute.

Effects of pH, Temperature, and Various Reagents on Enzyme Activity

The effect of pH on the reaction rate was determined by measuring xylanase activity at different pH values at 50°C using 50 mM sodium citrate (pH 4.0 to 6.0), sodium phosphate (pH 6.0 to 8.0), and KCl-borate (pH 8.0 to 9.0) buffers. The activity of the purified enzyme was also assayed with 50 mM sodium citrate buffer (pH 6.0) at various temperatures ranging from 30 to 70°C. To investigate the effects of chemicals on enzyme activity, xylanase activity was measured following the addition of several different reagents into the standard reaction mixture to make a final concentration of 5 mM.

Analysis of Hydrolysis Products

Reaction mixtures containing xylooligosaccharides or xylans were incubated at 50°C for a time sufficient to ensure complete hydrolysis, and then boiled for 10 min and centrifuged. Hydrolysis products in the supernatant were analyzed by thin-layer chromatography. The supernatant was spotted on a thin-layer plate precoated with silica gel (Merck, Whitehouse Station, NJ, U.S.A.), and developed at room temperature with 1-propanol, nitromethane, and water [7:2:1 (v/v)]. For detection of carbohydrates, a mixture consisting of 0.5 ml of *p*-anisaldehyde, 0.5 ml of concentrated H_2SO_4, and a few drops of glacial acetate in 9 ml of 95% ethanol was used as a spray reagent, and the plate was incubated at 121°C for 5 min.

Results and Discussion

Cloning and Nucleotide Sequencing of *B. subtilis* AMX-4 Xylanase

A *B. subtilis* AMX-4 genomic library was constructed in *E. coli* XL-1 Blue using pUC19 as a cloning vector. Approximately 3,000 transformants were transferred to LB agar plates supplemented with 0.5% oat spelt xylan. After overnight incubation, a single *E. coli* clone capable of hydrolyzing xylan was obtained by scoring the clear zone around a colony. Restriction analysis of the recombinant plasmid pMX4 isolated from this clone indicated that it harbors a 3.3-kb insertion fragment (data not shown).

The sequence of 2,269 bp of the 3.3-kb insert in pMX4 was determined (GenBank Accession No. GQ334372). The deduced xylanase amino acid sequence yielded an open reading frame of 639 nucleotides encoding a protein of 213 residues with an estimated molecular mass of 23,253 Da and a pI of 9.27. The xylanase gene (*xylA*) is followed by *orf*1 (nt positions 811–2,046), which encodes a protein of 411 amino acids showing 90% identity to cytochrome P450 of *B. subtilis* (GenBank Accession No. NP_39055) (data not shown). These genes occupy adjacent positions on the *B. subtilis* AMX-4 chromosome, but they are distantly located on the *B. subtilis* 168 chromosome. The presumed initiation codon ATG of *xylA* was identified according to a putative ribosome-binding site (GGAGG) located 6 bp upstream. In addition, *xylA* has a total G/C content of 41.9%, and the G/C content is 34.8% at the third base of the codon.

Comparison of *B. subtilis* Xylanase with Other Xylanases

When the deduced amino acid sequence of AMX-4 xylanase was compared with the sequences of other xylanases in the NCBI database using the BLAST search program, it

showed high homology with those from *Bacillus* strains belonging to GH family 11: *B. amyloliquefaciens* 5582 (GenBank Accession No. ACH69967) (identity: 100%); *Paenibacillus macerans* (AAZ17386) and *B. subtilis* BD403 (AAZ17392) (identity: 96%); and *B. licheniformis* (AAZ17387), *Brevibacillus brevis* (AAZ17389), *Pectobacterium carotovorum* (ABW87792), *B. cereus* (AAZ17391), and *B. pumilus* (AAZ17390) (identity: 95%). These xylansases have a single catalytic module, whereas GH11 xylanases from *Clostridium thermocellum* DSM 4150 (ZP_03150748), *Ruminococcus albus* 7 (AAA85198), *Neocallimastix frontalis* (AAN07081), and *Bacillus* sp. 41M-1 (BAA82316) are composed of at least two modules with high molecular weight.

A stretch of 28 residues in the N-terminus of the predicted amino acid sequence has properties similar to typical signal peptides in *Bacillus* sp., which consist of positively charged amino acids followed by several hydrophobic residues. Comparisons of this N-terminal region with the cleavage sites of other signal peptides revealed that it is identical to a typical signal peptidase processing site (A-X-A) [21].

Physicochemical Properties of the Purified Xylanase

Although the amino acid sequence of AMX-4 xylanase was identical to that of *B. amyloliquefaciens* 5582 xylanase, the properties of the latter protein have not yet been characterized. Therefore, AMX-4 xylanase was purified by means of column chromatography on DEAE and phenyl-Sepharose from the cell-free extract of recombinant *E. coli* cells (pMX4) as described in the Materials and Methods section. Since the xylanase was not bound to the DEAE-Sepharose column equilibrated with 50 mM Tris-HCl buffer (pH 8.0), purified xylanase was obtained by phenyl-Sepharose column chromatography of the unbound proteins. When subjected to SDS–PAGE, the purified enzyme showed a single protein band corresponding to a molecular mass of 23 kDa (Fig. 1), which agrees with that of the pre-protein predicted by the AMX-4 *xylA* nucleotide sequence, whereas the mature xylanase is expected to have a molecular mass of 20 kDa. In addition, pI is predicted to be 9.27 for the pre-protein but 8.33 for the mature xylanase, suggesting that the enzyme in the cell-free extract of the recombinant strain was not bound to the DEAE-Sepharose equilibrated with 50 mM Tris buffer (pH 8.0). Therefore, the purified xylanase is assumed to be in the premature form containing a leader peptide corresponding to the 28 amino acid residues in the N-terminus, although their sequence was not determined.

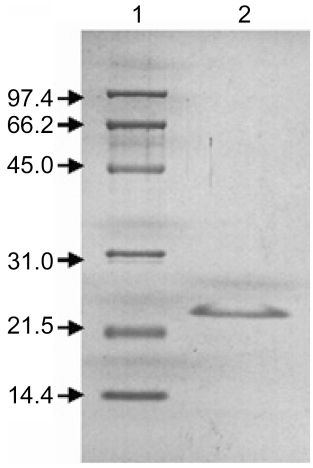

Fig. 1. SDS–PAGE of xylanase purified from recombinant *E. coli*. Lane 1, molecular mass markers; lane 2, purified enzyme. Molecular size is shown in kilodaltons on the left side of the gel.

Xylanases from *B. firmus* (xyn11A) [6], *Bacillus* sp. BP-7 [8], *Bacillus* sp. NCL 87-6-10 (XylA) [1], *Bacillus* sp. K-1 [20], *B. circulans* BL53 [9], and *B. subtilis* [3] were reported to have low molecular mass, similar to AMX-4 xylanase. In particular, *B. licheniformis* 77-2 [7] produces xylanase-1 with a molecular mass of 17 kDa.

Purified AMX-4 xylanase exhibited the highest activity on oat spelt xylan at 50–55°C (Fig. 2) and pH 6.0–7.0, and greater than 90% of its maximal activity between pH 5.0 and 7.0 (Fig. 3). Many purified *Bacillus* xylanases, including those from strains such as *B. pumilus* MK001 [13], *B. subtilis* B10 [10], *Bacillus* sp. BP-7 [8], *B. pumilus* PLS [17], *B. circulans* Teri-42 [19], *Bacillus* sp. SSP-34 [22], *Bacillus* sp. K-1 [20], *Bacillus* sp. BP-23 [4], *Bacillus* sp. 41M-1 [16], and *B. subtilis* [3] were reported to have optimal temperatures between 50°C and 60°C and to exhibit maximal activity at pH 5.0–7.0 [3, 4, 8, 10, 13, 17, 19, 20], 6.0–8.0 [22], or 9.0 [16]. In a previous report, the maximal xylanase activity in the culture supernatant of *Bacillus* sp. AMX-4 was detected at 50°C and pH 6.0 [24].

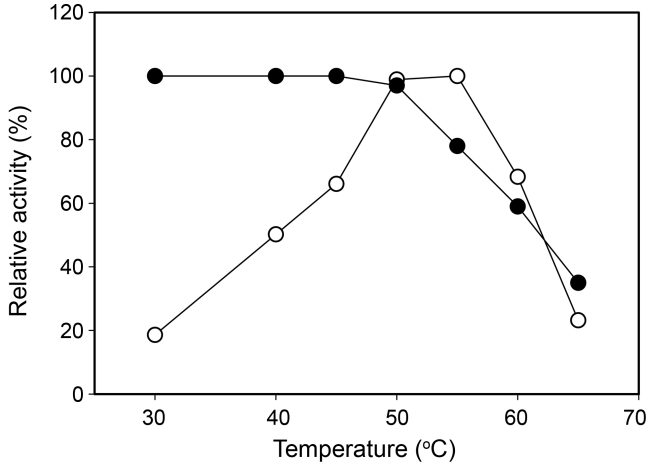

Fig. 2. Temperature optimum and thermostability of the purified xylanase.

The enzyme reaction was performed at various temperatures (open circles) for 15 min in 50 mM sodium citrate buffer (pH 6.0). Thermostability (closed circles) was determined by measuring the residual activity after preincubation for 1 h at different temperatures.

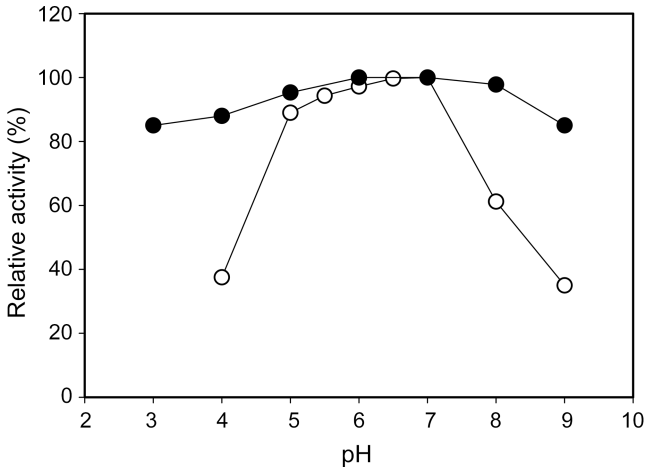

Fig. 3. pH optimum and stability of the purified xylanase.
The following buffers (50 mM) were used: sodium citrate (pH 3–6), sodium phosphate (pH 6–8), and KCl-borate (pH 8–9). Xylanase activity (open circles) was assayed at various pHs at 50°C. pH stability (closed circles) was determined by measuring the residual activity after preincubation for 1 h at different pHs at 4°C.

The thermostability and pH stability of purified AMX-4 xylanase were also examined by measuring residual activity after preincubating at various temperatures (30 to 55°C) or pHs (3.0 to 9.0) without substrate. The enzyme retained 70% of its activity at 50°C after 1 h of incubation, but its stability rapidly decreased at temperature above 50°C (Fig. 2). AMX-4 xylanase was stable for 1 h at pH 3.0 to 9.0. Notably, the enzyme retained more than 85% of its activity after 4 h at pH between 3.0 and 9.0 (Fig. 3). *B. subtilis* B10 xylanase was also reported to be stable for 30 min at pH 4.0 to 9.0 in the absence of substrate [10].

Effects of Metal Ions and Other Reagents on Enzyme Activity

The effects of various reagents including metal salts, EDTA, and SDS on AMX-4 xylanase activity were investigated (Table 1). Activity was noticeably enhanced by Fe^{2+} as in the case of *Bacillus* sp. K-1 xylanase, whereas the activity of both enzymes was inhibited by Mn^{2+} [1]. In addition, the xylanases of both *B. circulans* BL53 [9] and *Bacillus* sp. BP-7 [8] were inhibited by Mn^{2+}, but *Bacillus* sp. SPS-0 xylanase was stimulated by Mn^{2+} [2]. AMX-4 xylanase activity was seriously inhibited by Cu^{2+} and slightly inhibited by Ca^{2+}, EDTA, and SDS. *B. circulans* BL53 xylanase was also inhibited by Cu^{2+} and Ca^{2+}, but Ca^{2+} increased the activity of *Bacillus* sp. K-1 xylanase.

Table 1. Effects of metal ions and other reagents on the xylanase activity.

Effector (5 mM)	Relative activity (%)
None	100.0
NaCl	99.3
KCl	96.3
$MgCl_2$	98.0
$MnCl_2$	77.6
$CaCl_2$	92.5
$CuCl_2$	50.8
$FeCl_2$	127.5
EDTA	93.2
SDS	95.3

Substrate Specificity

The purified xylanase was assayed with various substrates to investigate its specificity. When polysaccharides or synthetic substrate derivatives were used as substrates, enzyme activity was determined by measuring the amount of released reducing sugars or *para*-nitrophenol, respectively. As shown in Table 2, when wheat flour arabinoxylan was used as the substrate, the largest quantity of reducing sugars was liberated. The enzyme was active toward xylans of wheat flour, birchwood, and oat spelt in descending order of activity.

AMX-4 xylanase was not active toward other polysaccharides including CMC, locust bean gum, lichenan, laminarin, and Avicel or toward synthetic substrate derivatives such as *p*NP-β-mannoside, *p*NP-β-cellobioside, and *p*NP-β-xyloside, indicating that it can hydrolyze only β-1,4-xylosidic linkages and has no β-xylosidase activity. On the other hand, the xylanase of alkalophilic *Bacillus* SSP-34 has been reported to hydrolyze the CMC substrate with low efficiency in addition to hydrolyzing xylan [22]. Since AMX-4 xylanase had no cellulase activity and was very stable at a broad range of pHs, it is assumed to have potential utility in the pulp and paper industries.

Xylan Hydrolysis Patterns

The final oat spelt and birchwood xylan hydrolysates generated by purified AMX-4 xylanase were analyzed by thin-layer chromatography (Fig. 4). Xylotriose and xylobiose were the main products of xylan hydrolysis, whereas no xylose was detected among the two hydrolysates. Additionally, xylotetraose and higher oligosaccharides were detected in

Table 2. Substrate specificity of the purified xylanase.

Substrates	Relative activity (%)
Arabinoxylan	100
Birchwood xylan	83
Oat spelt xylan	70
Lichenan	ND
Laminarin	ND
Locust bean gum	ND
Carboxymethylcellulose	ND
*p*NP-β-xyloside	ND

ND, not detected.

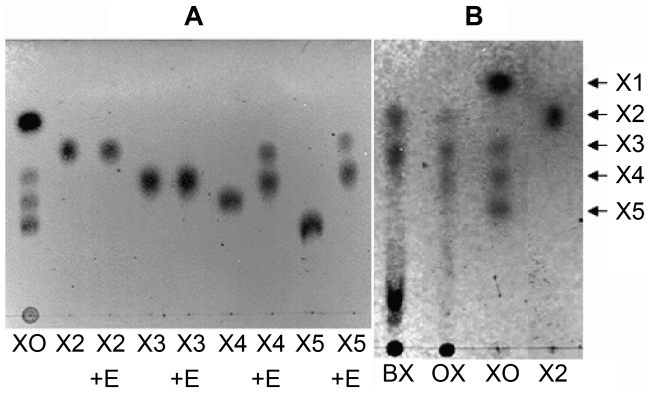

Fig. 4. Thin-layer chromatogram of β-1,4-linked xylooligosaccharide (**A**) and xylan (**B**) hydrolysis products generated by the purified xylanase.

Reaction mixtures containing the purified xylanase and xylooligosaccharides or xylans in 50 mM sodium citrate buffer (pH 6.0) were incubated for 4 h at 50°C. XO in panels **A** and **B** stands for standard xylooligosaccharides including xylose, xylotriose, xylotetraose, and xylopentaose. In panel **A**, X1 to X5 represent xylose to xylopentaose; E, purified xylanase. In panel **B**, BX and OX stand for hydrolysates of birchwood xylan and oat spelt xylan, respectively.

the hydrolysate of oat spelt xylan and birchwood xylan, respectively. Many xylanases hydrolyzed xylans from oat spelt, birchwood, beechwood, and wheat flour to yield xylose and xylooligosacharides. Whether xylose is produced by xylan hydrolysis depends on the origin of the xylanase; whereas xylanases from *Bacillus* sp. NCL 87-6-10 (XylA) [1], *Bacillus* sp. SPS-0 [2], and *Bacillus* sp. BP-23 [5] produced xylose, those from *Bacillus* (NCL 87-6-10) XylC [1], *Bacillus* sp. 41M-1 [16], and *Bacillus* sp. K-1 [20] did not.

The action of xylanase on xylooligosaccharides was also investigated. The enzyme was active on xylopentaose and xylotetraose but not on xylotriose or xylobiose. Xylobiose and xylotriose were the final products released separately from xylotetraose and xylopentaose by the purified enzyme. Xylose was not released from either xylotetraose or xylopentaose. The xylan and xylooligosaccharide hydrolysis patterns of AMX-4 xylanase were similar to those of *Bacillus* sp. 41M-1 xylanase J [16]; neither enzyme could hydrolyze xylooligosaccharides with three or fewer xylose units. However, xylanases from *B. licheniformis* [14] and *Bacillus* sp. SPS-0 [2] hydrolyzed xylooligosaccharides with two or more xylose units, and both xylanase B and xylanase C of *Bacillus* sp. BP-23 [4, 5] hydrolyzed xylooligosaccharides with three or more xylose units.

REFERENCES

1. Balakrishnan, H., B. Kamal Kumar, M. Dutta-Choudhury, and M. V. Rele. 2002. Characterization of alkaline thermoactive cellulase-free xylanases from alkalophilic *Bacillus* (NCL 87-6-10). *J. Biochem. Mol. Biol. Biophys.* **6:** 325–334.
2. Bataillon, M., A. Nunes Cardinali, N. Castillon, and F. Duchiron. 2000. Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. *Enzyme Microb. Technol.* **26:** 187–192.
3. Bernier, R. Jr., H. Driguez, and M. Desrochers. 1983. Molecular cloning of a *Bacillus subtilis* xylanase gene in *Escherichia coli*. *Gene* **26:** 59–65.
4. Blanco, A., P. Díaz, J. Martínez, O. Lopez, C. Soler, and F. I. Pastor. 1996. Cloning of a *Bacillus* sp. BP-23 gene encoding a xylanase with high activity against aryl xylosides. *FEMS Microbiol. Lett.* **137:** 285–290.
5. Blanco, A., T. Vidal, J. F. Colom, and F. I. Pastor. 1995. Purification and properties of xylanase A from alkali-tolerant *Bacillus* sp. strain BP-23. *Appl. Environ. Microbiol.* **61:** 4468–4470.
6. Chang, P., W. S. Tsai, C. L. Tsai, and M. J. Tseng. 2004. Cloning and characterization of two thermostable xylanases from an alkaliphilic *Bacillus firmus*. *Biochem. Biophys. Res. Commun.* **319:** 1017–1025.
7. Damiano, V. B., R. Ward, E. Gomes, H. F. Alves-Prado, and R. Da Silva. 2006. Purification and characterization of two xylanases from alkalophilic and thermophilic *Bacillus licheniformis* 77-2. *Appl. Biochem. Biotechnol.* **129–132:** 289–302.
8. Gallardo, O., P. Diaz, and F. I. Pastor. 2004. Cloning and characterization of xylanase A from the strain *Bacillus* sp. BP-7: Comparison with alkaline pI–low molecular weight xylanases of family 11. *Curr. Microbiol.* **48:** 276–279.
9. Heck, J. X., S. H. Flores, P. F. Hertz, and M. A. Ayub. 2006. Statistical optimization of thermo-tolerant xylanase activity from Amazon isolated *Bacillus circulans* on solid-state cultivation. *Bioresour. Technol.* **97:** 1902–1906.
10. Huang, J., G. Wang, and L. Xiao. 2006. Cloning, sequencing and expression of the xylanase gene from a *Bacillus subtilis* strain B10 in *Escherichia coli*. *Bioresour. Technol.* **97:** 802–808.
11. Huitron, C., R. Perez, A. E. Sanchez, P. Lappe, and L. Rocha Zavaleta. 2008. Agricultural waste from the tequila industry as substrate for the production of commercially important enzymes. *J. Environ. Biol.* **29:** 37–41.
12. John, F. J. St., J. D. Rice, and J. F. Preston. 2006. Characterization of XynC from *Bacillus subtilis* subsp. *subtilis* strain 168 and analysis of its role in depolymerization of glucuronoxylan. *J. Bacteriol.* **188:** 8617–8626.
13. Kapoor, M. and R. C. Kuhad. 2007. Immobilization of xylanase from *Bacillus pumilus* strain MK001 and its application in production of xylo-oligosaccharides. *Appl. Biochem. Biotechnol.* **142:** 125–138.
14. Liu, M. Q. and G. F. Liu. 2008. Expression of recombinant *Bacillus licheniformis* xylanase A in *Pichia pastoris* and xylooligosaccharides released from xylans by it. *Protein Expr. Purif.* **57:** 101–107.
15. Miller, M. L., R. Blum, W. E. Glennon, and A. L. Burton. 1960. Measurement of carboxymethylcellulase activity. *Anal. Biochem.* **2:** 127–132.
16. Nakamura, S., K. Wakabayashi, R. Nakai, R. Aono, and K. Horikoshi. 1993. Purification and some properties of an alkaline xylanase from alkaliphilic *Bacillus* sp. strain 41M-1. *Appl. Environ. Microbiol.* **59:** 2311–2316.

17. Nuyens, F., W. H. van Zyl, D. Iserentant, H. Verachtert, and C. Michiels. 2001. Heterologous expression of the *Bacillus pumilus* endo-β-xylanase (*xynA*) gene in the yeast *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **56:** 431–434.
18. Ogasawara, W., Y. Shida, T. Furukawa, R. Shimada, S. Nakagawa, M. Kawamura, *et al*. 2006. Cloning, functional expression and promoter analysis of xylanase III gene from *Trichoderma reesei*. *Appl. Microbiol. Biotechnol.* **72:** 995–1003.
19. Qureshy, A. F., L. A. Khan, and S. Khanna. 2000. Expression of *Bacillus circulans* Teri-42 xylanase gene in *Bacillus subtilis*. *Enzyme Microb. Technol.* **27:** 227–233.
20. Ratanakhanokchai, K., K. L. Kyu, and M. Tanticharoen. 1999. Purification and properties of a xylan-binding endoxylanase from alkaliphilic *Bacillus* sp. strain K-1. *Appl. Environ. Microbiol.* **65:** 694–697.
21. Sakakibara, Y., K. Tsutsumi, K. Nakamura, and K. Yamane. 1993. Structural requirements of *Bacillus subtilis* α-amylase signal peptide for efficient processing: *In vivo* pulse-chase experiments with mutant signal peptides. *J. Bacteriol*. **175:** 4203–4212.
22. Subramaniyan, S. and P. Prema. 2000. Cellulase-free xylanases from *Bacillus* and other microorganisms. *FEMS Microbiol. Lett.* **183:** 1–7.
23. Subramaniyan, S. and P. Prema. 2002. Biotechnology of microbial xylanases: Enzymology, molecular biology, and application. *Crit. Rev. Biotechnol.* **22:** 33–64.
24. Yoon, K.-H., S. J. Seol, H.-C. Cho, M. S. Lee, J. H. Choi, and K. H. Cho. 2002. Isolation and enzyme production of a xylanase-producing strain, *Bacillus* sp. AMX-4. *Kor. J. Microbiol. Biotechnol*. **30:** 123–128.