Journal of Microbiology and Biotechnology
Vol. 7. No. 6, 386-390, 1997

Purification and Characterization of Xylanase from *Bacillus* sp. Strain DSNC 101

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A xylanase from the *Bacillus* sp. strain DSNC 101, isolated from soil, was purified to homogeneity by anion-exchange and hydrophobic interaction chromatography followed by gel filtration chromatography. The enzyme cleaved xylan, but not carboxymethyl cellulose, Avicel, soluble starch, and pNPX. The main product of oat spelts xylan hydrolysates was xylobiose. The xylanase had a molecular weight of 25 kDa determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Optimum temperature and pH for the xylanase activity were 50°C and 6.0, respectively. K_m and V_{max} of the enzyme for oat spelts xylan were 12.5 mg of xylan/ml and 869.5 unit/mg of protein, respectively. Xylanase was completely inhibited by Hg, Cu, and *N*-bromosuccinimide, but was stimulated by Ca, Co, and Mg.

Xylan is an abundant biopolymer found in plant tissues as a major component of cell walls. It is a complex molecule composed of β-1,4-linked xylose chains with branches containing arabinose and 4-*O*-methylglucuronic acid. Biodegradation of xylan requires the action of several enzymes, among which xylanases(1,4-β-D-xylan xylanohydrolase [EC 3.2.1.8]) play a key role. Xylanases catalyze the hydrolysis of xylan which is useful feed stock for generating food and fuel (9). Xylanases have a potential application in paper and pulp industry for selective hydrolysis of hemicellulose (21). They can also be used to increase the digestibility of animal feed stock and in the baking and brewing industries (22). We isolated a xylanolytic bacterial strain, *Bacillus* sp. DSNC 101, from rice field soil. In this paper, we described the purification and characterization of a xylanase produced by this isolated bacterial strain.

MATERIALS AND METHODS

Isolation and Identification of Microbial Strain

A bacterial strain was isolated from soil under a stack of rice straw. The gathered soil was suspended in sterile distilled water. The suspension was plated on the screening medium contained 0.3% yeast extract, 1% oat spelts xylan, 2% agar and 0.1% Congo Red. Each colony which had xylanolytic activity appeared halo around the colony and the colony was transferred to the yeast-xylan broth and cultured at 40°C for 5 days and thereafter it was confirmed whether the culture supernatant had xylanolytic activity or not. The isolated xylanolytic bacteria was maintained in nutrient agar plate in order to preserve. The isolated bacterium was identified by morphological characteristics.

Cultivation of *Bacillus* sp. DSNC 101

Bacillus sp. DSNC 101 was cultivated at 40°C with an agitation at a rate of 200 rpm for 3 days in a 1-liter flask containing 200 ml of xylan medium consisted of 2.0% oat spelts xylan, 2.0% yeast extract, and 0.4% K_2HPO_4.

Enzyme Assay

Xylanase activity was assayed using 2.0% oat spelts xylan (Sigma Co. U.S.A.) as a substrate in 50 mM sodium acetate buffer (pH 6.0) at 50°C. The amount of reducing sugar produced from xylan as the result of this enzyme reaction was determined by Miller's dinitrosalicylic acid method (14). One unit of enzyme activity was defined as the amount of enzyme which catalyzed the production of 1 μmole of xylose per min under the conditions of this study.

Enzyme Purification

All the purification steps were carried out at 4°C. Culture supernatant obtained by centrifugation of culture broth was concentrated by ultrafiltration using membrane P-10 (Amicon Co.). The concentrate was adjusted to pH 7.6 with Tris-HCl buffer, and applied to a DEAE-Sephadex column, previously equilibrated with 20 mM Tris-HCl buffer (pH 7.6). Xylanase was eluted with a linear gradient of 0.0 to 0.5 M KCl in 20 mM Tris-HCl

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Key words: xylanase, *Bacillus* sp.

buffer (pH 7.6). The active fractions from the DEAE-Sephadex column were pooled and concentrated. The concentrated enzyme solution after ammonium sulfate was added to 1.5 M was applied to a HPLC (Waters Co.) Phenyl column (Shodex HIC PH-814 for HPLC, 0.8×7.5 cm) which had been equilibrated with 20 mM Tris-HCl buffer containing 1.5 M ammonium sulfate. Xylanase was eluted with a linear gradient of 1.5 M to 0.0 M ammonium sulfate. The active fractions from the HPLC Phenyl column were pooled, concentrated, and applied to a HPLC gel filtration column (Protein-Pak 300SW for HPLC, 0.78×30 cm) pre-equilibrated with 20 mM HEPES buffer (pH 7.0) containing 0.1 M KCl. Protein concentration was determined by Lowry's method (13) with bovine serum albumin as a standard.

Thin Layer Chromatography (TLC)

The sugars released by enzymatic hydrolysis of xylan were separated by ascending TLC aluminium sheet (Silica gel $60F_{254}$, Merck Co.) in the solventing system of butanol : ethanol : water (5 : 3 : 2) (4). After the separation, the sheet was sprayed with a silver nitrate/sodium hydroxide solution [solution A (0.5% silver nitrate in acetone)/solution B (0.5 M sodium hydroxide in ethanol)] and dried at room temperature for 30 min.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was carried out in 12% polyacrylamide gel containing 0.1% SDS under 30 mA constant current condition (12). After the run, proteins were stained with Coomassie Brilliant Blue R-250.

pH Effect on Xylanase Activity

To determined pH stability of the enzyme, 50 μl of enzyme solution was added to 350 μl of 0.1 M sodium citrate (pH 3~4), sodium acetate (pH 4~6), sodium phosphate (pH 6~7.5), Tris-HCl (pH 7.5~9.0), and sodium glycine (pH 9~11) buffers. After 1-h standing at room temperature, the residual xylanase activity was assayed using 70 μl of each solution in the same way as described above. To find the optimum pH of the enzyme, the xylanase activities were checked at various pHs.

Temperature Effect on Xylanase Activity

The optimum temperature of the enzyme activity was determined by assaying the xylanase activities at various temperatures. The temperature effect on the stability of the enzyme was tested as follows. After enzyme solutions were heat-treated at various temperatures for one hour, the residual activity of the solution was assayed.

Amino Acid Composition Analysis and N-Terminal Amino Acid Sequencing

The amino acid analysis of the enzyme was done by Pico-Tag method (Waters Co.) after hydrolysis in constant boiling 6N HCl (Sigma Co.) at 110°C for 24 h. PITC-derivatized free amino acids were applied to a Pico-Tag Free Amino Acid Analysis column (3.9×300 mm). The N-terminal sequence of protein was determined by Edman degradation in an Applied Biosystems model 476A Protein/Peptide sequencer (Applied Biosystems Ins., CA, U.S.A.).

RESULTS AND DISCUSSION

Identification of Bacterial Strain

The isolate DSNC 101 was a endospore-forming, motile, gram-positive rod and aerobic bacterium, and it was named *Bacillus* sp. DSNC 101.

Purification of the Xylanase

Bacillus sp. DSNC 101 was grown in xylan broth composed of 2.0% yeast extract, 2.0% oat spelts xylan, and 0.4% potassium phosphate (dibasic), pH 8.0 at 40°C for 3 days. The supernatant of the culture was concentrated by ultrafiltration, adjusted to pH 7.6 with Tris-HCl buffer, and applied to a DEAE-Sephadex column. Xylanase was eluted with linear gradient of KCl. A few proteins, at a negligible concentration and without xylanase activity, copulated with the enzyme. These contaminants were removed after two chromatography steps with HPLC Phenyl column and HPLC gel column. The purified enzyme was homogeneous as judged by SDS-PAGE (Fig. 1), and the estimated molecular weight of the enzyme was 25,000

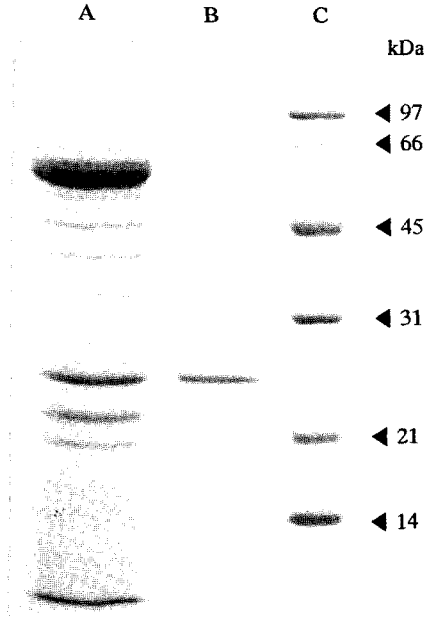

Fig. 1. SDS-PAGE of purified xylanase.
Samples were analyzed in 12% polyacrylamide gel. Lane A, concentrated culture supernatant; lane B, purified xylanase; lane C, molecular weight standards: phosphorylase (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa). The amount of purified xylanase loaded in the gel was 2 μg for protein staining.

daltons. The molecular weight of the purified xylanase was similar to those of several other *Bacillus* sp. xylanases (1, 16, 23).

Effect of Substrate Concentration on Enzyme Activity

Reaction velocity of the hydrolysis of xylan from oat spelts at various concentrations was measured, and the Michaels constant (K_m) was estimated from Lineweaver-Burk plot. The K_m value from oat spelts xylan was 12.5 mg/ml and the maximal velocity (V_{max}) for oat spelts xylan was calculated to be 869.5 unit/mg of protein. The K_m was found to be higher than those of *Thermomonospora fusca* (8) and *Bacillus* sp. strain 41M-1 (15) xylanases, but was similar to that of *Bacillus* sp. strain BP-23 xylanase A (3). The V_{max} was higher than those of *Thermomonospora fusca* xylanase and *Bacillus* sp. strain BP-23 xylanase A, but was lower than that of *Bacillus* sp. strain 41M-1 xylanase.

Effects of pH and Temperature on Enzyme Activity

The xylanase activities were measured at various pHs from pH 3.0 to 11.0 using several buffer solutions described in Materials and Methods. The enzyme had an optimum pH of 6.0 as shown in Fig. 2. However, the enzyme also showed a high level of activity at alkaline pH (60% and 40% activity at pH 9.0 and 10.0, respectively), and it was fairly stable at pH values ranging from 5.0 to 10.0. The activity and stability of the purified xylanase at alkaline pH were similar to those of several alkalophilic *Bacillus* sp. xylanases (6, 15, 19).

The xylanase activities were measured at various temperatures ranging from 30 to 70°C. The optimum temperature was found to be 50°C as shown in Fig. 3. The enzyme remained stable after incubation at 50°C in pH 6.0 buffer for 1 h. The study of thermostability showed that the enzyme was highly stable at temperatures up to 40°C. After 24 h of incubation at this temperature and at pH 6 or 7, the enzyme retained 95% activity. Incubation at temperatures over 50°C rapidly inactivated the enzyme; for instance, the half-life of the enzyme activity was 1 h at 55°C.

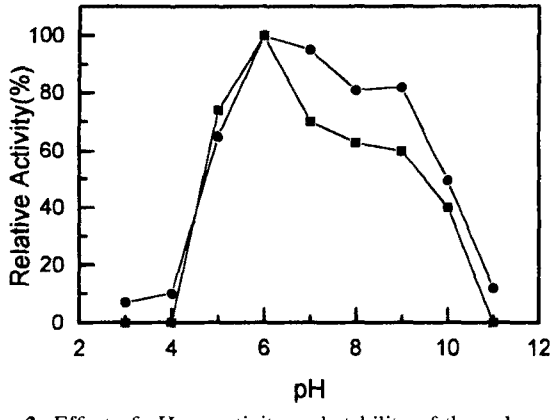

Fig. 2. Effect of pH on activity and stability of the xylanase.
The enzyme activity was assayed at various pHs (■) and the residual activity was measured after incubation of the enzyme at various pHs for 1 h (●). The following buffers were used: 50 mM sodium citrate (pH 3.0 to 4.0), 50 mM sodium acetate (pH 4.0 to 6.0), 50 mM sodium phosphate (pH 6.0 to 8.0), 50 mM Tris-HCl (pH 8.0 to 9.0), and 50 mM sodium glycine (pH 9.0 to 11.0).

Effect of Metal ions and Inhibitor

The metal ion effects on the xylanase activity was examined by testing its remaining activity (Table 1). It has been reported that many bacterial xylanases were inhibited strongly by Hg (2, 3, 11, 15, 18) and some were inhibited weakly also by Cu (2, 11, 18). The xylanase of *Bacillus* sp. DSNC 101 was completely inhibited by Cu and Hg at a concentration of 5 mM. Zn also led to a strong inhibition of xylanase (38% of the remaining activity). Mn and EDTA had a relatively small degree of inhibition, while Ca, Co, and Mg had a strong stimulatory

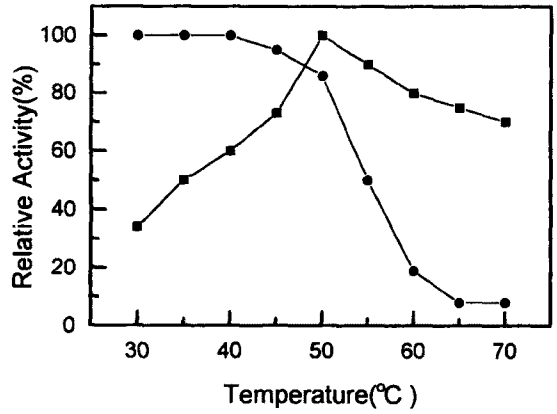

Fig. 3. Effect of temperature on activity and stability of the xylanase.
The enzyme activity was assayed at various temperatures (■) and the residual activity was measured after incubation of the enzyme at various temperatures for 1 h (●).

Table 1. Effect of metal ions and inhibitor on the xylanase activity.

Metal ions and inhibitor (1 mM)	Relative activity (%)
None	100
$MnSO_4$	87
$FeSO_4$	95
$MgCl_2$	146
$ZnSO_4$	38
$CaCl_2$	187
$HgCl_2$	0
$CuSO_4$	6
$CoCl_2$	178
EDTA	71
N-bromosuccinimide	0

effect on activity. A stimulatory effect of Co on xylanase activity had already been reported (2). Xylanase was also completely inhibited by 1 mM *N*-bromosuccinimide, and this inhibition caused by tryptophan modifier (10, 20) indicates that there is an involvement of tryptophan residues in the active site of the enzyme. And the inhibition is similar to what has been reported for several xylanases (5, 15, 25).

Thin-layer Chromatography (TLC) Analysis of Xylanolytic Products

The hydrolysis of xylan was carried out with 1.0% substrate at 50°C and pH 6.0. Samples were removed at intervals during incubation and analyzed (Fig. 4). The predominant products of xylan hydrolysates were xylobiose, xylotriose, and higher oligosaccharides, but a small quantity of xylose was also produced, indicating that the enzyme was an endoxylanase. The substrate specificity of the xylanase was examined by testing its ability to hydrolyze various substrates at 1.0%. The xylanase of *Bacillus* sp. DSNC 101 did not show activity on carboxymethyl cellulose, Avicel, soluble starch, dextran, *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-xylopyranoside, or *p*-nitrophenyl-α-L-arabinofuranoside (data not shown). The products of oat spelts xylan hydrolysis were analyzed with silica gel TLC. The xylanase acted on neither crystalline cellulose nor carboxymethyl cellulose, suggesting a possible application of the enzyme to biobleaching processes.

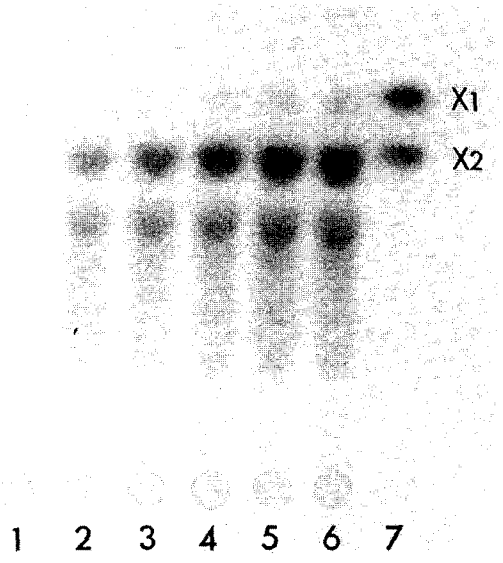

Fig. 4. Thin layer chromatogram of the hydrolysates of xylan with purified *Bacillus* sp. DSNC 101 xylanase.
Enzyme reaction was done at 50°C for 120 min. The hydrolysis products were analyzed at different reaction time. Lane 1, 0 min; lane 2, 10 min; lane 3, 30 min; lane 4, 60 min; lane 5, 90 min; lane 6, 120 min; lane 7, standards (X_1: xylose, X_2: xylobiose).

Amino Acid Composition and N-Terminal Amino Acid Sequence

The amino acid composition of *Bacillus* sp. DSNC 101 xylanase is shown in Table 2. The table shows that the values of Gly and Asx (Asp+Asn) of amino acids were relatively high, while those of cysteine and tryptophan were very low. The sequences of the first 10 amino acid residues of *Bacillus* sp. DSNC 101 xylanase were identical to those of the 28th~37th amino acid residues of *Bacillus pumilus* xylanase (7). On the other hand, they were identical only 60% to those of 60th~69th amino acid residues of *Clostridium acetobutylicum* P262 xylanase (24) (Fig. 5).

Although xylanase of *Bacillus* sp. DSNC 101 was similar to that of *B. pumilus* in molecular mass and N-terminal sequence, they showed different characteristics from each other in hydrolysis products of oat spelts xylan and optimum temperature for their activities. The xylanase of *B. pumilus* has been reported to produce xylooligosaccharides, but not xylose from oat spelts xylan, and the optimum temperature to be 40°C (7, 17). On the other hand, the *Bacillus* sp. DSNC 101 xylanase was found be to produce xylose as well as xylooligosaccharides

Table 2. Amino acid composition of *Bacillus* sp. DSNC 101 xylanase.

Amino acid	Composition (mol%)
Asx	11.64
Thr	7.52
Ser	7.02
Glx	7.61
Pro	2.81
Gly	12.43
Ala	7.43
Cys	0.05
Val	4.39
Met	2.83
Ile	5.69
Leu	5.20
Tyr	7.10
Phe	5.87
Lys	5.53
His	2.23
Trp	0.18
Arg	4.48

Asx, Asp+Asn; Glx, Glu+Gln.

Bacillus sp. DSNC 101	1-R T I T N N E M G N-10
Bacillus pumilus	28-R T I T N N E M G N-27
Clostridium acetobutylicum P262	60-K T I T S N E I G V-69

Fig. 5. Alignment of the N-terminal sequence of the xylanase of *Bacillus* sp. DSNC 101 with the N-terminal sequences of XynA from *B. pumilus* and XynB from *C. acetobutylicum* P262.
Identical amino acids are boxed.

from oat spelts xylan, and its optimum temperature for the activities to be 50°C.

Acknowledgement

This paper was supported by Non Directed Research Funds of the Korea Research Foundation, 1996.

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(Received August 23, 1997)