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Bioproperties and Purification of Xylanase from *Bacillus* sp. YJ6

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To characterize the xylanase from *Bacillus* sp. YJ6, broth after 4 days incubation at 25 °C was collected and purified to electrophoretical homogeneity after Sephacryl S-100 HR chromatograph. About 3.5% recovery and 678.1 purification fold were achieved. The purified xylanase, with a M_w of 19 kDa, had an optimal pH and temperature at 5.0 and 50 °C, respectively, and was stable at pH 5.0−9.0 or <50 °C. It was inhibited by Cu^{2+}, Fe^{3+}, Hg^{2+}, phenylmethyl sulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-ethylmaleimide (NEM), and leupeptin but activated by K^+, Na^+, Co^{2+}, Mg^{2+}, β-mercaptoethanol (β-ME), and glutathione (GSH). The purified xylanase had high specificity to beechwood, birchwood, and oat spelt xylans. The DNA fragment encoding this xylanase, corresponding to 213 amino acids, exhibited about 95% homology with seven strains of *Bacillus* in the NCBI database.

KEYWORDS: ***Bacillus*** **sp.; xylanase; bioproperties; purification**

INTRODUCTION

Xylan, the major hemicellulose component of the plant cell wall in most plant species, is hydrolyzed by a repertoire of hydrolytic enzymes. It is a complex polysaccharide comprising a backbone of xylose residues linked by β-1,4-glycosidic bonds. Depending on the source, the xylopyranoside units are substituted with mainly acetyl, arabinosyl, and glucuronosyl residues. Hardwood xylan is typically 0-acetyl-4−0-methylglucuronoxylan with approximately 10% of the xylose units α-1,2-linked to a 4−0-methylglucuronic acid side chain and 70% of xylose residues acetylated at the C-2 or C-3 positions. Softwood xylans are commonly arabinoxylans in which 10% of the xylose units are substituted with α-2,3-linked arabinofuranose residues (Whistler and Richards, 1970; Biely, 1985). Microbial enzymes act cooperatively to convert xylan to its constituent simple sugars. These enzymes include β-1,4-endoxylanases (xylanases; EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55), which hydrolyzes arabinose side chains; α-glucuronidase (EC 3.2.1.131), which removes glucuronic acid side chains from xylose units; xylan esterases (EC 3.1.1.6), which release acetate groups; and finally xylosidase (EC 3.2.1.37), which hydrolyzes xylobiose to xylose (*1*). There is a considerable degree of synergy among these enzymes (*2*). Many xylanases do not cleave glycosidic bonds between xylose units that are substituted. Thus, side chains must be cleaved before the xylan backbone is completely hydrolyzed (*3*). Conversely, several accessory enzymes will only remove side chains from xylooligosaccharides. These enzymes therefore require xylanases to partially hydrolyze the plant structural polysaccharide, before side chains can be cleaved (*2*). Although the structure of xylan is more complex than that of cellulose and thus requires a large number of different enzymes to elicit efficient hydrolysis, the polysaccharide does not form tightly packed structures and is thus more accessible to hydrolytic enzymes. Consequently, the specific activity of xylanases is 2−3 orders of magnitude greater than that for cellulase hydrolysis of crystalline cellulose.

Most microorganisms capable of degrading cellulose and xylan synthesize a range of isoenzymes with very similar substrate specificities (*1*). However, xylan present in cell wall and also in the middle of lamella of plant cells is heterogeneous in structure and not degradable by a single enzyme but by a xylanolytic enzyme complex (*4*). Among these enzymes, endo-1,4-β-xylanase and β-D-xylosidase have been extensively studied. Others such as those containing acetylxylan esterase, arabinase, α-glucuronidase, ferulic acid esterase, and *p*-coumaric acidesterase were also discovered during recent years (*5*). Since xylan hydrolysates such as xylose and xylo-oligosaccharides can be good for animal feeds and pharmaceutical, chemical, and health food industries (*5*), many scientists are attracted to investigate xylanases (*6*).

Although acid hydrolysis is usually employed in xylan processing, it easily forms toxic compounds. Therefore, efficient enzymatic processes are highly beneficial and might be an alternative method for xylan hydrolysis (*5*). Xylanases are considered to be able to effectively hydrolyze xylan, the principal type of hemicellulose containing a linear polymer of β-D-xylopyranosyl units linked by (1−4) glycoside bonds (*7*). Some bacteria, actinomycetes, yeasts, and fungi have been found to have xylan-digestion abilities (*8*). A novel xylanase and mannanase-producing bacterium, *Bacillus* sp. YJ6, has been isolated in our laboratory.

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It revealed high mannan and xylan hydrolytic abilities. This study aimed to determine the bioproperties of xylanase from *Bacillus* sp. YJ6, which consequently provides some references or clues for the further study of its applications.

MATERIALS AND METHODS

Microorganism and Culture. *Bacillus* sp. YJ6 was cultivated in a medium containing 2% skim milk, 1% rice straw, 0.5% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.01% NaCl. The viable cell counts (CFU/mL), xylanase activity, and pH were monitored during incubation. After 4 days incubation at 25 °C with 150 rpm shaking, the culture broth was centrifuged at 8000 × *g* for 30 min and then passed through a 0.45 μm membrane to remove cells. The resulting sample was used for xylanase purification.

Enzyme Activity Assay. Xylanase activity was determined by incubation of 1.8 mL of 1% birchwood xylan in 50 mM phosphate buffer (pH 7.0) with 0.2 mL of the appropriate concentration of enzyme at 50 °C (*9*). After a 30 min reaction, 3 mL of dinitrosalicylic acid (DNS) was added, and the mixture was boiled in a water bath for 5 min to stop the reaction. The resulting sample was then cooled to room temperature, and the absorbance at 540 nm (A_{540}) was measured by the DNS method using xylose as standard (*10*). One unit of xylanase activity was defined as the amount of enzyme that could hydrolyze birchwood xylan and release 1 μmol of xylose within 1 min reaction at 50 °C.

Determination of Protein Concentration. Protein concentrations were determined by following the dye binding method (*11*) using bovine serum albumin as standard and incubation of 0.4 mL of diluted dye in 50 mM phosphate buffer (pH 7.0) with 0.1 mL of the appropriate concentration of enzyme at room temperature. After a 5 min reaction, the absorbance of the resulting sample at 570 nm (A_{570}) was measured.

Purification of Xylanase. After 30 min centrifugation at 8000 × *g* and filtration through a 0.45 μm membrane, crude xylanase in the culture broth was precipitated by 40−60% saturation of ammonium sulfate. After 15 min centrifugation at 8000 × *g*, the precipitate was dissolved in 20 mM phosphate buffer (pH 7.0, buffer A) and dialyzed against the same buffer at 5 °C overnight. The resulting crude enzyme was eluted by CM-Sepharose Fast Flow (2.6 × 30 cm^2) with a linear gradient of 100−500 mM NaCl in buffer A at a flow rate of 0.5 mL/min. Fractions with xylanase activity were collected and concentrated by ultrafiltration with a 5000 Da cutoff membrane. The concentrated crude enzymes were then eluted on Sephacryl S-100 HR (1.6 × 100 cm^2) with buffer A at a flow rate of 0.25 mL/min. Fractions with xylanase activity were collected and subjected to enzyme activity assay and electrophoresis (SDS−PAGE). The mentioned procedures were performed in a walk-in cool room (temperature < 5 °C).

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Electrophoresis was performed mainly according to Laemmli (*12*). To the purified enzyme, an equal volume of sample buffer (0.05% bromophenol blue, 5% β-mercaptoethanol (β-ME), 10% glycerol, and 2% SDS in 0.25 M Tris−HCl buffer (pH 6.8) was added, and the mixture was boiled at 100 °C for 5 min. The resulting sample was then subjected to SDS−PAGE (resolving gel, 12.5%; stacking gel, 4%) by using a Mini-Protein II system (Bio-Rad). Electrophoresis was performed at room temperature for 1.5 h with a 100 V.

N-Terminal Amino Acid Sequence Analysis. Purified xylanase was analyzed with SDS−PAGE. The xylanase band was electrotransferred onto polyvinylidene difluoride membrane (PVDF). Proteins were stained with Coomassie blue R-250 after electrophoresis. N-terminal amino acid sequences were determined by Edman degradation (*13*) and performed using an Applied Biosystems Procise sequencer.

Effects of pH and Temperature. The effects of pH and temperature on xylanase activity were determined using birchwood xylan as substrate. The optimal pH was determined by measuring the activity of purified xylanase at pH 3.0−11.0 (pH 3.0−6.0 using 20 mM citrate buffer, pH 6.0−8.0 using 20 mM sodium phosphate buffer, pH 8.0−9.0 using 20 mM Tris-HCl buffer, and pH 8.0−11.0 using 20 mM sodium carbonate buffer), while the optimal temperature for purified xylanase in 20 mM sodium phosphate buffer (pH 7.0) was measured at temperatures from 10 to 90 °C in a 30 min assay according to Bailey et al. (*9*).

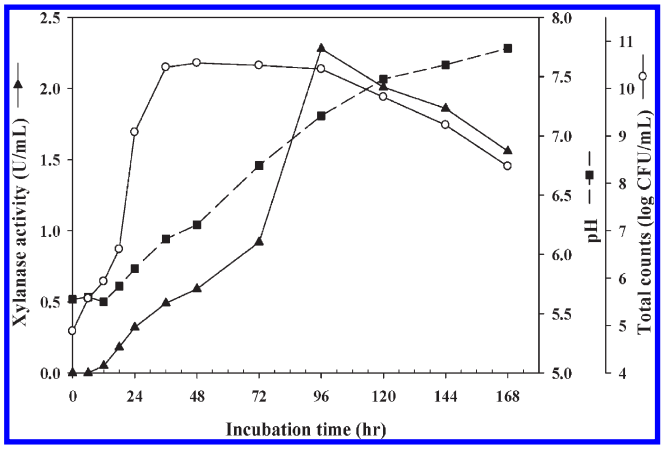

Figure 1. Changes in pH, growth curve, and xylanase activity of *Bacillus* sp. YJ6 during 7 days incubation at 37 °C.

The pH stability was determined by incubating xylanase in various buffers with pH 3.0−11.0 at 37 °C for 30 min. The residual activity was determined at 50 °C in a 30 min assay according to Bailey et al. (*9*).

The thermal stability was determined by incubating xylanase in 20 mM sodium phosphate buffer (pH 7.0) at temperatures ranging from 10 to 90 °C for 30 min. The residual activity was then determined at 50 °C in a 30 min assay according to Bailey et al. (*9*).

Effects of Metal Ion, Reducing Agent, or Inhibitor. Purified xylanase in 20 mM Tris-HCl buffer (pH 7.0) with various metal ions (K^+, Li^+, Na^+, NH_4^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Sr^{2+}, Zn^{2+}, Fe^{3+}), reducing (or thiol) agents (cysteine, dithiothreitol (DTT), glutathione (GSH), β-mercaptoethanol (β-Me)), and other reagents or inhibitors such as ethylene diamine tetraacetic acid (EDTA), leupeptin, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonyl fluoride (PMSF), *N*-toyl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and urea were incubated at 37 °C for 30 min. The residual activity was measured in a 30 min assay according to Bailey et al. (*9*).

Substrate Specificity. The hydrolytic abilities against 1% birchwood xylan, oat spelt xylan, beechwood xylan, 4-*o*-methylglucuronoxylan, pectin, Avicel, and carboxymethylcellulose (CMC) in 20 mM phosphate buffer (pH 7.0) were determined to evaluate the substrate specificity of purified xylanase. The xylanase activity was measured in a 30 min assay according to Bailey et al. (*9*).

Determination of DNA Sequence. Total genomic DNA was extracted from *Bacillus* sp. YJ6 according to Ausubel et al. (*14*). Cloning was performed according to the guideline of Sambrook et al. (*15*). Polymerase chain reaction (PCR) was employed to amplify xylanase DNA with 33 cycles (30 s denaturation at 95 °C, 30 s annealing at 58 °C, and 45 s extension at 72 °C) and then 10 min DNA thermal cycler at 72 °C (2720 Thermal Cycler; Applied Biosystems, Foster, CA). Primers were designed according to the alignment between N-terminal amino acids of purified xylanase and functional sequences from the NCBI Database (http://www.ncbi.nlm.nih.gov/) and used for PCR. The forward primer was 5′-(ATG TTT AAG TTT AAA AAG AAT TTC TTA GTT GGA TTA TCG G)-3′, while the reverse primer was 5′-(*TTA* CCA CAC TGT TAC GTT AGA ACT TCC)-3′. The underlined sequence is the start codon, while the *italic* sequence is the stop codon. After amplification, the PCR product was ligated with pGEM-T easy vector and transformed into the cloning host, *Escherichia coli* Top 10F′, according to Hanahan and Meselson (*16*). After blue/white selection, plasmid from transformed *E. coli* was extracted and prepared for DNA sequencing using T7 and SP6 as sequencing primers.

RESULTS AND DISCUSSION

Enzyme Production and Purification. As indicated in **Figure 1**, an increase in pH was observed during 5 days cultivation, while maximum xylanase was obtained after 4 days incubation at 25 °C. During the early 1.5 days incubation, the cells exponentially

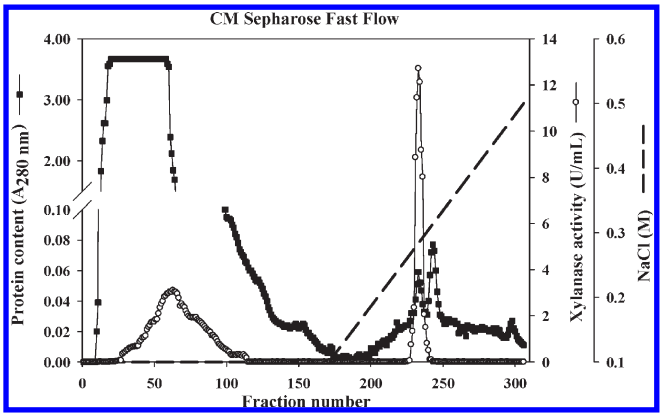

Figure 2. Elution profile of *Bacillus* sp. YJ6 xylanase on CM-Sepharose Fast Flow.

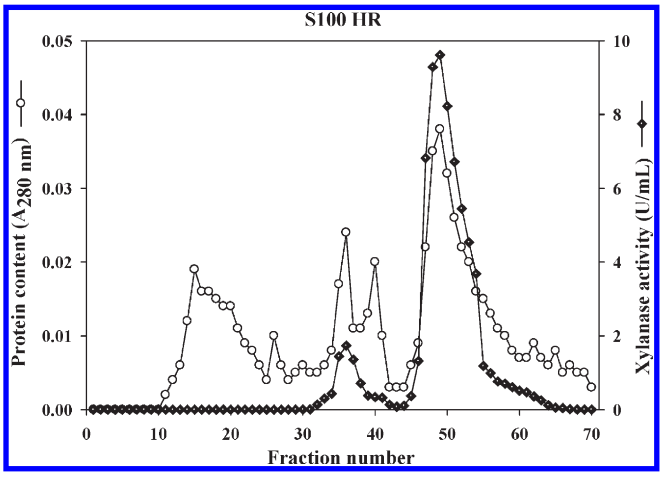

Figure 3. Elution profile of *Bacillus* sp. YJ6 xylanase on the Sephacryl S-100 HR chromatography.

increased (exponential phase). However, they grew steadily during the prolonged 4 day incubation, suggesting that the enzyme was produced during 1.5−4 days cultivation. Accordingly, broth from *Bacillus* sp. YJ6 after 4 days cultivation at 25 °C was collected for xylanase purification and characterization.

During the CM Sepharose FF chromatography process, most contaminants were excluded (**Figure 2**). Fractions with xylanase activity were further purified to electrophoretic homogeneity by S-100 HR chromatography (**Figures 3** and **4**). At this stage, a specific activity of 1436.0 U/mg and 678-fold purification were achieved (**Table 1**).

Molecular Mass (*M*) and N-Terminal Amino Acid Sequence. The molecular mass (*M*) of purified xylanase was estimated to be 19.0 kDa by SDS−PAGE (**Figure 4**). It was similar to that from *Bacillus amyloliquefaciens* (*17*), but lower than those from *Bacillus* sp. (20.7 kDa) (*18*), *Bacillus* sp. K-1 (23 kDa) (*19*), and *Bacillus* sp. AR-009 (23 kDa) (*20*). The N-terminal amino acids were ASTDYWQN. From comparison of the N-terminal sequence with those from other species in GenBank, the purified xylanase had 95% homology with those from seven different strains of *Bacillus* species (data not shown). This N-terminal sequence was, therefore, employed to design the primer for DNA amplification from *Bacillus* sp. YJ6.

Effect of pH and Temperature. The purified xylanase had an optimal pH and temperature of 5.0 and 50 °C, respectively, and was stable at pH 5.0−9.0 and < 50 °C (**Figures 5** and **6**). Its optimal temperature (50 °C) was lower than that from some

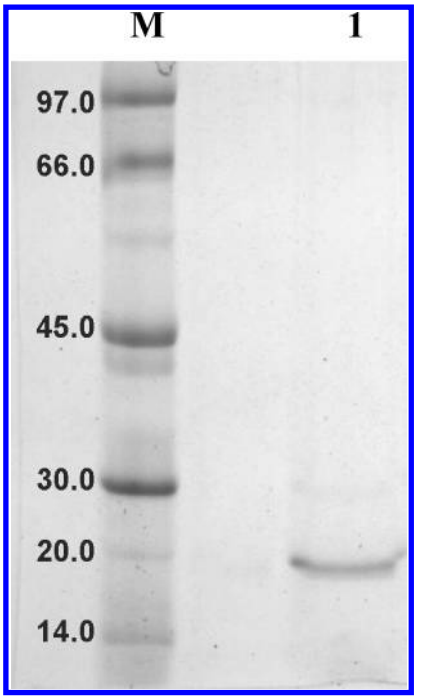

Figure 4. SDS−PAGE of purified xylanase from *Bacillus* sp. YJ6: M, marker; I, purified after passing through Sephacryl S-100 HR.

Table 1. Summary of Purification of Xylanase from *Bacillus* sp. YJ6

procedure	total activity (U)	total protein (mg)	specific activity (U/mg)	purification (fold)	yield (%)
crude enzyme	3600	1700.0	2.1	1	100.0
fraction of ammonium sulfate (40−60%)	2381	290.67	8.2	3.9	66.2
CM Sepharose Fast Flow	418	1.46	287.1	135.6	11.6
S-100 HR	125	0.09	1436.0	678.1	3.5

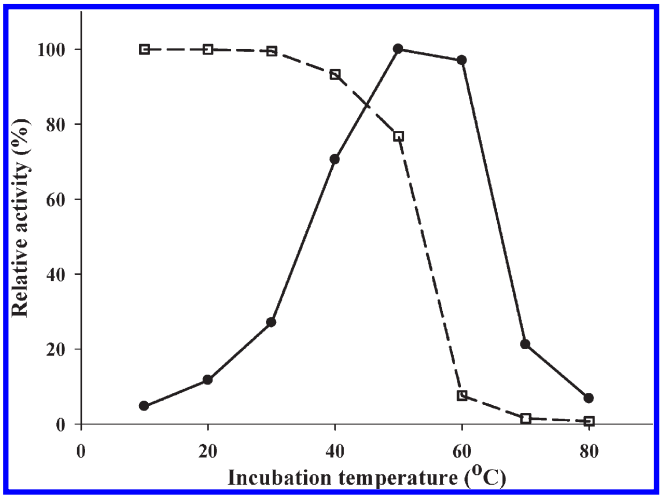

Figure 5. Effect of temperature on xylanase. (●, optimal; □, stability).

other *Bacillus* strains such as 60 °C for *Bacillus* sp. K-1 (*19*), 70 °C for *Bacillus subtilis* CCMI 966 (*21*) and 80 °C for *Bacillus amyloliquefaciens* (*17*). The thermal stability of purified xylanase was similar to those from *Bacillus amyloliquefaciens* (*17*) and *Bacillus* sp. K-1 (*19*) but lower than that from *Bacillus* sp. GRE7 (60−80 °C) (*22*).

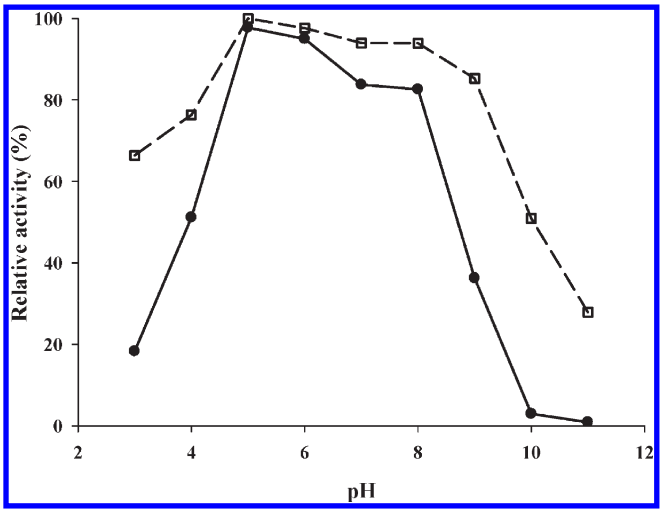

Figure 6. Effect of pH on xylanase (●, optimal; □, stability).

Table 2. Effect of Metal Ions and Chemicals on Xylanase Activity (Relative Activity %)

	concentration (mM)		
metal ions	1.0	5.0	10.0
none	100.0	100.0	100.0
K^+	111.4	122.3	125.9
Li^+	100.3	111.0	113.2
Na^+	110.5	114.4	129.9
NH_4^+	109.2	112.7	115.0
Ca^{2+}	96.2	102.4	103.2
Cd^{2+}	95.3	88.7	83.9
Co^{2+}	104.0	118.7	130.0
Cu^{2+}	58.6	4.01	0.0
Fe^{2+}	99.6	96.5	91.1
Hg^{2+}	30.9	1.6	0.0
Mg^{2+}	114.1	134.7	144.4
Ni^{2+}	97.5	102.1	109.6
Sr^{2+}	107.7	116.0	118.2
Zn^{2+}	109.2	110.4	113.5
Fe^{3+}	95.8	77.4	74.6
	concentration (mM)		
chemicals	0.5	1.0	2.0
cysteine	98.8	98.3	100.2
DTT	95.3	99.3	103.5
EDTA	91.9	87.0	80.7
GSH	110.4	112.1	116.6
leupeptin	83.1	80.9	73.9
β-ME	135.3	145.0	147.6
NEM	88.8	82.4	73.4
PMSF	89.1	82.2	75.9
TLCK	97.0	92.0	89.6
TPCK	75.3	70.5	68.5
urea	92.7	91.8	88.8

According to previous studies, the optimal pH of most of xylanases from *Bacillus* strains was 6.0 (*21*, *23*, *24*), except for those from *Bacillus* sp. K-1 (5.5) (*19*) and *Bacillus amyloliquefacienats* (6.8−7.0) (*17*). The pH stability of purified xylanase (pH 5.0−9.0) was almost the same as that from *Bacillus* sp. SPS-0 (*24*), but narrower than those from *Bacillus circulans* D1 (pH 5.5−10.5) (*25*) and *Bacillus* sp. GRE7 (pH 5.0−11.0) (*22*).

Effects of Metals, Reducing Agents and Inhibitors. Purified xylanase was strongly inhibited by Cu^{2+} and Hg^{2+} and moderately inhibited by Fe^{3+}, PMSF, TPCK, NEM, and leupeptin but highly activated by K^+, Na^+, Co^{2+}, Mg^{2+}, β-ME and slightly activated by NH_4^+, Sr^{2+}, Zn^{2+}, and glutathione (GSH) (**Table 2**). These phenomena suggested that the active site of purified xylanase contained a SH group. These results are similar to those from *Bacillus stearothermophilus* T-6 (*26*), *Bacillus amyloliquefaciens* (*17*) and *Bacillus* sp. SPS-0 (*24*). According to Cho and Bai (*27*), Hg^{2+} and Cu^{2+} could inhibit the xylanase from *Bacillus* sp. DSNC 101, but Ca^{2+}, Co^{2+}, and Mg^{2+} activated it. The data from the inhibitor study further confirmed that the active site of purified xylanase contained cysteine. Although the xylanase from *Staphylococcus* sp. SG-13 was not affected by PMSF (*28*), it was inhibited in this study (**Table 2**). Xylanases from *Bacillus amyloliquefaciens* (*17*), *Bacillus* sp. SPS-0 (*24*), or *Bacillus* sp. JB-99 (*29*) were activated by reducing agents such as DTT, β-ME, and cysteine, suggesting that cysteine was in or near the active site of xylanase (*30*).

Table 3. Substrate Specificity of the Purified Xylanase

substrate	relative activity (%)
birchwood xylan	100.0
beechwood xylan	114.5
oat spelt xylan	86.5
4-*o*-methylglucuronoxylan	39.3
CMC	0.0
avecil	0.0
pectin	0.0

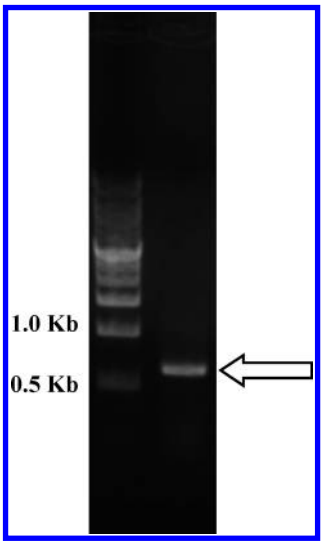

Figure 7. DNA fragment encoding xylanase amplified from genomic DNA of *Bacillus* sp. YJ6.

Substrate Specificity. The purified xylanase revealed higher hydrolytic activity on xylans from birchwood, beechwood, and oat spelts, compared with that on 4-*o*-methylglucuronoxylan, CMC, avecil, and pectin (**Table 3**). This phenomenon indicated that the substrate binding domain of xylanase had very high affinity for xylans from softwood (birchwood and beechwood) and hardwood (oat spelt). This might be due to the differences in xylan polymer structures. The binding of xylanase to xylans from birchwood, beechwood, and oat spelt might be due to reactive group exposure on the surface that can much more easily bind even the nonproteins. A specific binding on oat spelt xylan was also found in that from *Streptomyces chattanoogensis* CECT 3336 (*31*). The substrate specificity of the purified xylanase revealed high hydrolytic activities on various xylans, and it was considered as an endo-1,4-β-xylanase.

Molecular Characterization of Xylanase. The DNA fragment encoding xylanase was amplified from genomic DNA by PCR (**Figure 7**) and cloned into pGEM-T Easy cloning vector. The full

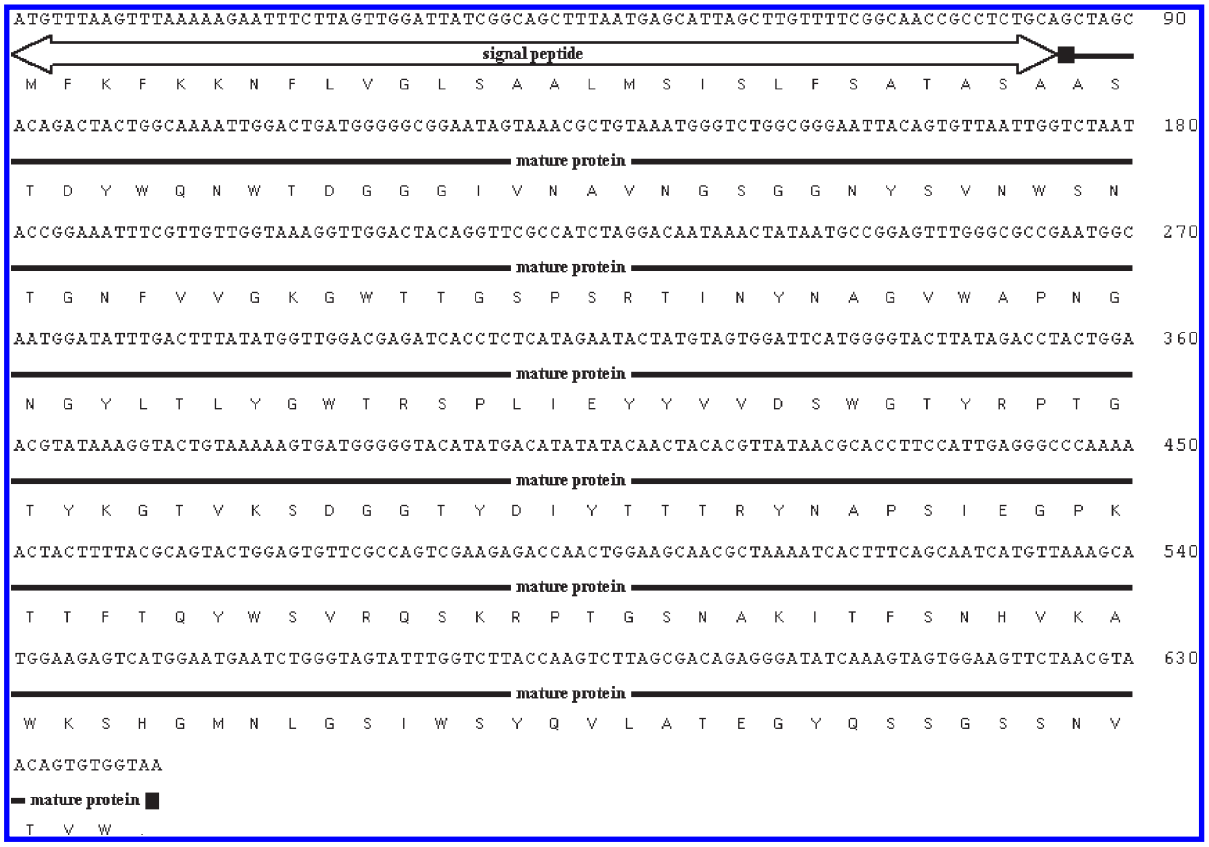

Figure 8. The full DNA sequence of xylanase amplified from *Bacillus* sp. YJ6.

DNA of xylanase (642 bp) (**Figure 8**) was confirmed by screening and sequencing. Compared with those from different microorganisms in GenBank using BLAST, it had very high homology (> 95%) to those from seven strains of *Bacillus* sp. (data not shown) but was different from those produced by *Aspergillus* sp. and *Streptomyces* sp. It was, therefore, considered that the xylanase was from a novel *Bacillus* sp.

From the results obtained in this study, this novel *Bacillus* sp. could utilize natural wastes such as rice straw as a substrate for growth and produce high levels of xylanase. This phenomenon highly benefits industrial application. The further optimization of the commercial scale production of xylanase using this strain is ongoing currently.

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