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Characterization of Mannanase from a Novel Mannanase-Producing Bacterium

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ABSTRACT: Locust bean gum (LBG) was employed to screen mannanase-producing bacteria. The bacterium with highest mannanase ability was identified as *Paenibacillus cookii*. It revealed highest activity (6.67 U/mL) when cultivated in 0.1% LBG with 1.5% soytone and 0.5% tryptone after 4 days incubation at 27 °C. Its mannanase was purified to electrophoretical homogeneity after DEAE-Sepharose and Sephacryl S-100 separation. The purified mannanase, with an N-terminus of GLFGINAY, had pH and temperature optimum at 5.0 and 50 °C, respectively, and was stable at pH 5.0−7.0, ≤50 °C. It was strongly activated by β-mercaptoethanol, dithiothreitol, cysteine, and glutathione, but inhibited by Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, PMSF, iodoacetic acid, and EDTA. According to substrate specificity study, the purified mannanase had high specificity to LBG and konjac.

KEYWORDS: *mannanase, Paenibacillus cookii, LBG, konjac*

INTRODUCTION

Mannan is part of hemicelluloses in plant cell walls and widely distributed in nature as part of the hemicellulose fraction in hardwoods and softwoods. Polysaccharides in plant cell wall contain celluloses, hemicelluloses, and lignins. Hemicelluloses including mannan, xylan, galactan, and arabinan are not soluble in water or chelating agents, but in aqueous alkali.[1] They are also defined chemically as cell wall polysaccharides and structurally homologous to cellulose, because they have a backbone composed of 1,4-linked β-D-pyranosyl residues.[1] Within biomass, mannan and hemicellulose are situated between lignin and celluloses fibers. The mannan layer with its covalent linkage to lignin and its noncovalent interaction with celluloses is important in maintaining the integrity of celluloses in situ and in helping protect the fibers against degradation by cellulases.[2] It is the predominant hemicellulosic polysaccharide in softwoods from gymnosperms, but the minor hemicellulose in hardwood from angiosperms. Hardwood mannans are composed of β-1,4-linked mannopyranose and glucopyranose units, whereas softwood contains two different types of acetylated galactoglucomannans.[3]

Numerous screening methods exist for detecting mannanase activity in microorganisms. Solid fermentations providing rapid assays were useful for the direct measurement and isolation of mannanase-producing organisms from natural substrates and for the isolation of mutants. They are also used to screen cell-free extracts from microorganisms for mannan degrading enzyme activity.[1] Since the hemicellulases with multifaceted properties can be consequently used in a wide application, they have, therefore, been considered as key enzymes in biotechnology industry.[1] So far, most studies on hemicellulases have focused on those enzymes which can hydrolyze xylan, the primary constituent of hemicelluloses in grasses. However, enzymes that can hydrolyze mannan were neglected, even though mannan is also an abundant hemicellulose. Accordingly, the application of mannanase for the hydrolysis of β-D-1,4 mannopyranoside linkages in β-1,4 mannans is as important as that of xylanases. The use of mannanases in paper and pulp industries increased significantly with their discovery by Gubitz et al.[4] Since then, researchers worldwide have focused their attention on newer microbial isolates for the production of mannanases. The substrate used routinely for the study of mannanases is galactomannan from LBG (*Ceratonia siliqua*) with a mannose:galactose ratio of 3.5:1.[5] In addition, ivory nut (*Phytelephas macrocarpa*) mannan, an unbranched β-1,4-linked mannan homopolymer, and manno-oligosaccharides (manno-biose, mannotriose, mannotetraose, and mannopentose) can also be used as hydrolytic substrates.[1]

The main products obtained during the hydrolysis of mannan by mannanases are mannobiose and mannotriose. β-Mannanases from *Aspergillus tamari*,[6] *Trichoderma reesei*,[7] and *Aspergillus niger*[8] all produced mainly mannobiose and mannotriose and trace amounts of higher oligosaccharides. The pattern of galactomannan hydrolysis and the apparent subsite-binding requirements have been interpreted from the structural analysis by NMR of isolated hydrolysis products from glucomannan and galactomannan incubated with *Aspergillus niger* mannanase. A vast variety of bacteria, actinomycetes, yeasts, and fungi are known to be mannan degraders.[1] Among

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these bacteria, degradation is mostly confined to Gram-positive ones, including various *Bacillus* species and *Clostridia* species. The most mannolytic group among fungi belongs to genera *Aspergillus*, *Agaricus*, *Trichoderma*, *Sclerotium*.[1] Mannanases from several microorganisms have been purified and characterized.[1] However, the properties of purified mannanase from *Paenibacillus cookii* have not yet been reported. This study was to screen the mannanase-producing bacteria, and to further purify and characterize the mannanase from *Paenibacillus cookii*.

MATERIALS AND METHODS

Isolation of Mannanase-Producing Bacteria. The mannanase-producing bacterium was isolated from soil in northern Taiwan. Ten percent of soil samples suspended in sterile phosphate buffered saline (PBS, 10 mM potassium, 150 mM NaCl, pH 7.4) was inoculated and incubated in TSB medium with 0.1% LBG at 27 °C for 4 days. Ten percent of the resulting broth was inoculated to a medium (LBG broth, LBGB) containing 0.1% LBG, 1.5% soytone, 0.5% tryptone, 0.01% NaCl, 0.5% $(NH_4)_2SO_4$, 0.05% $MgSO_4{\cdot}7H_2O$, 0.20% KH_2PO_4, and 0.20% K_2HPO_4 and incubated at 27 °C for 4 days. The resulting broth was then spread on a LBG agar plate (LBGA, LBGB + 1.5% agar) containing 0.01% (w/v) Congo red and incubated at 27 °C for 4 days. The colonies with clear zone (indicating those with LBG hydrolyzing ability) on LBGA were selected and inoculated onto a fresh LBGA. After 4 days incubation at 27 °C, the colonies with biggest LBG hydrolyzing haloed ring were picked up and subjected to identification. The isolated strain was stored at −80 °C in LBGB with 20% (v/v) glycerol for further studies. LBGB was employed for further production of mannanase under aerobic incubation conditions at 27 °C with 150 rpm shaking.

Bacterial Identification. The sequence of 16S rDNA of the isolated strain was determined after genomic DNA extraction and PCR amplification as described by Yoon and Choi.[9] Comparison of the sequence with homologous strains in GenBank was performed using Basic Local Alignment Search Tool (BLAST).

Production of Mannanase. The isolated strain in LBGB was incubated under aerobic conditions at 27 °C with 150 rpm shaking. The mannanase activity and pH were monitored during incubation. The optimal condition was employed for further production of mannanase.

Determination of Protein Concentration. The concentration of soluble proteins was determined according to dye-binding method[10] using bovine serum albumin (BSA) as standard.

Enzyme Activity Analysis. After 4 days incubation at 27 °C, the broth was centrifuged at 15,000 rpm, 4 °C for 30 min to remove the cells. To 1.8 mL of LBG substrate buffer (0.5% LBG in 50 mM phosphate buffer, pH 7.0), 0.2 mL of the supernatant was added and reacted in a 50 °C water bath for 30 min. Three milliliters of dinitrosalicylic acid (DNS) was then added and heated at 100 °C for 5 min. After cooling to room temperature, the absorbance at 540 nm (A_{540}) was measured.[7] One unit (U) of mannanase activity was defined as the amount of enzyme that could hydrolyze LBG and release 1 μmol of mannose within 1 min reaction at 50 °C.[7]

Purification of Mannanase. After 4 days cultivation at 27 °C with shaking (150 rpm), the broth was centrifuged at 10000*g* for 30 min and then passed through a 0.45 μm membrane to remove cells. The resulting samples were then subjected to ammonium sulfate fractionation. The precipitated crude enzyme was dialyzed against 20 mM sodium phosphate buffer (pH 7.0, buffer A) overnight to remove the salt and then applied onto a DEAE-Sepharose Fast Flow column (2.6 cm × 30 cm) pre-equilibrated with buffer A. Fractions of 5.0 mL were collected at a flow rate of 0.5 mL/min by washing the column with buffer A, followed by a linear gradient of 0−500 mM of NaCl in buffer A. Fractions with mannanase activity were collected and concentrated by Amicon Ultrafiltration (cutoff: 5000 Da). Four milliliters of the resulting sample were chromatographed on Sephacryl S-100 HR (2.6 × 100 cm) which was pre-equilibrated with buffer A. Fractions corresponding to mannanase activity were pooled and stored at 4 °C for the following assays. All purification steps were carried out at 4 °C unless otherwise specified.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Electrophoresis was performed mainly according to Laemmli.[11] SDS−PAGE with a 12.5% polyacrylamide was performed using a Mini-Protein II system (Bio-Rad, USA). Broad molecular weight protein kit containing 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, and 200 kDa standard proteins was employed as marker.

Effects of pH and Temperature. The effects of pH and temperature on mannanase activity were determined using LBG as substrate. The optimal pH was determined by measuring the activity of purified mannanases at pH 3.0−12.0 (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 9.0−12.0 using 20 mM sodium carbonate buffer), while the optimal temperature of purified mannanases in 20 mM sodium phosphate buffer (pH 7.5) was measured at temperatures from 10 to 90 °C on a 30 min assay according to Stalbrand et al.[7]

The pH stability was determined by incubating mannanases in various buffers with pH 3.0−12.0 (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 9.0−12.0 using 20 mM sodium carbonate buffer) at 37 °C for 30 min. The residual activity was determined at 50 °C on a 30 min assay according to Stalbrand et al.[7]

The thermal stability was determined by incubating mannanases in 20 mM sodium phosphate buffer (pH 7.5) at temperatures from 10° to 90 °C for 30 min. The residual activity was then determined at 50 °C on a 30 min assay according to Stalbrand et al.[7]

Effects of Metal Ion, Reducing Agent, or Inhibitor. Purified mannanases with various metal ions (Ag^+, K^+, Li^+, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}), reducing (thiol) agents such as dithiothreitol (DTT), glutathione (GSH), β-mercaptoethanol (β-Me), and other reagents or inhibitors such as iodoacetic acid (IAA), ethylene diamine tetraacetic acid (EDTA), leupeptin, *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (*p*CMB), phenylmethyl sulfonyl fluoride (PMSF), *N*-toysl-L-lysine chloromethyl ketone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), urea, etc. were incubated at 37 °C for 30 min. The residual activity was measured at 50 °C on a 30 min assay according to Stalbrand et al.[7]

Substrate Specificity. The hydrolytic ability against 0.5% LBG, carboxymethylcellulose, Birchwood xylan, mannan, konjac powder, and guar gum in 20 mM sodium phosphate buffer (pH 7.0) was determined at 50 °C on a 30 min assay according to Stalbrand et al.[7]

Determination of Kinetic Properties. The Michaelis constant (K_m) and maximum velocity (V_{max}) were determined in 20 mM sodium phosphate buffer (pH 7.0) containing 0.1−0.4 mg/mL substrates (LBG and konjac), after incubation with purified mannanase at pH 7.0 and 50 °C for 15 min. The data were plotted according to the Lineweaver−Burk method. Each data was an average of three independent experiments, and every experiment included three samples.

N-Terminal Amino Acid Sequence Analysis. Purified mannanases were subjected to SDS−PAGE analysis[11] and then electrotransferred onto polyvinylidene difluoride membrane (PVDF). Proteins were stained with Coomassie blue R-250 after electrophoresis. N-Terminal amino acid sequences were analyzed by Edman degradation[12] after being electrotransferred onto PVDF. Comparison of the sequence with homologous strains in GenBank was performed using Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was inferred from BLAST at National Center for Biotechnology Information (NCBI).

Statistical Analysis. One-way analysis of variance (ANOVA) was run using the Statistical Analysis System ([SAS/STAT], Release 8.0; Carry, NC, USA). Duncan's multiple range test was used to determine the significance of differences within treatments. For each treatment, three replicates were measured and the mean values were calculated. Values were considered to be significantly different when $P < 0.01$.

RESULTS AND DISCUSSION

Isolation of Mannanase-Producing Bacterium and the Production of Mannanase. The mannanase-producing bacteria, survived after 4 days incubation in a TSB medium with 0.1% LBG at 27 °C, were isolated from soil. After being transferred to LBGB, only 4 colonies had LBG degrading abilities among the total 21 colonies of these strains (data not shown). Strain with the highest hydrolytic activity was subjected to identification. According to the fatty acids analysis and 16S rDNA sequencing (data not shown), this strain was identified as *Paenibacillus cookii*, a catalase-positive, oxidase-positive, and endospore-forming Gram-negative bacterium. The alignment of 16S rDNA by BLAST from GenBank indicated about 99.0% homology with those from *Paenibacillus cookii*. According to API (analytical profile index) analysis and report by Logan et al.,[13] the morphology of the isolate is the most similar to that of *Paenibacillus cookii* LMG18419^T.

Purification of Mannanase from *Paenibacillus cookii*. Mannanase was purified by ammonium sulfate precipitation, DEAE-Sepharose Fast Flow and Sephacryl S-100 HR chromatography. After ammonium sulfate precipitation at 40−60% saturation and checking for mannanase activity, the resulting sample was dialyzed and applied on DEAE-Sepharose Fast Flow chromatography (Figure 1). Fractions with

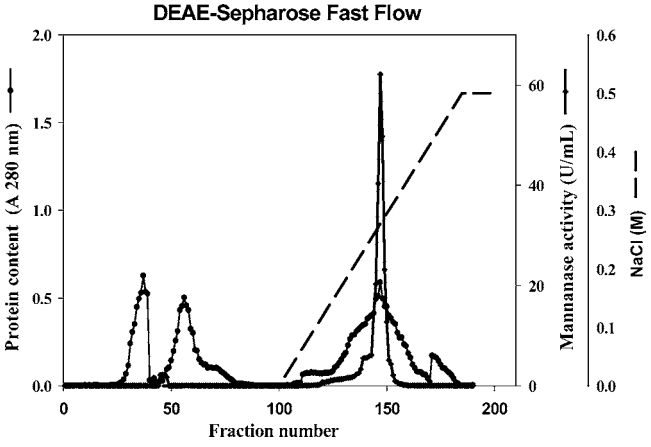

Figure 1. Elution profile of *P. cookii* mannanase on DEAE-Sepharose Fast Flow. (The column was washed with 20 mM phosphate buffer and eluted by 0−0.5 M NaCl gradient in 20 mM phosphate buffer, pH 7.0. Fractions of 5 mL were collected at a flow rate of 0.5 mL/min.)

mannanase activity were collected, concentrated, and further purified by Sephacryl S-100 HR column twice to electrophoretical homogeneity (Figures 2 and 3). A specific activity of 635.4 units/mg, 6.4% recovery, and 90.2-fold purification was achieved at this stage (Table 1).

Molecular Mass (*M*). According to SDS−PAGE (Figure 3), the molecular mass (*M*) of purified mannanase was estimated to be 68.0 kDa, which was similar to that from *Clostridium thermocellum* (70 kDa),[14] but lower than that from *Thermotoga maritime* (76.9 kDa),[15,16] and greater than that from *Paenibacillus* sp. BME-14 (53 kDa).[17]

Effects of pH. Purified mannanase had an optimal pH at 5.0 and was stable at pH 5.0−7.0 (Figure 4A). According to the previous studies, mannanases from bacteria, actinomycetes, and fungi had optimal pH from acidic to neutral,[18] such as *Bacillus* sp. SA-22 (6.5),[19] *Paenibacillus* sp. BME-14 (4.5),[17] and *Paenibacillus curdlanolyticus* (4.0).[20] According to the pH

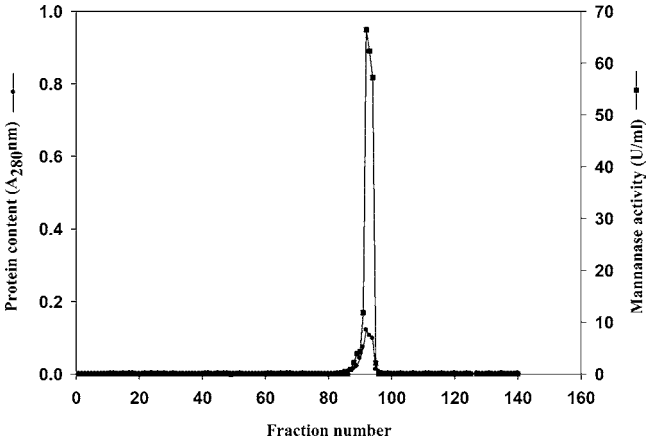

Figure 2. Elution profile of *P. cookii* mannanase on the second Sephacryl S-100 HR chromatography. (The column was washed with 20 mM phosphate buffer, pH 7.0. Fractions of 2 mL were collected at a flow rate of 0.25 mL/min.)

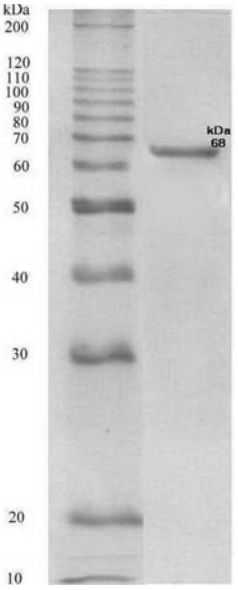

Figure 3. SDS−PAGE of the purified mannanase from *Paenibacillus cookii*.

stability of purified mannanase in this study, it was similar to that of mannanases obtained from *Bacillus* strains (5.0−7.0),[21,22] but slightly lower than that from *Aspergillus* sp. (6.0−7.5).[23]

Effects of Temperature. Purified mannanase had an optimal temperature at 50 °C (Figure 4B) which was similar to that from *Bacillus* sp. M50[24] and *Bacillus subtilis* B36[25] and lower than that from *Bacillus stearothermophilus* (60 °C)[26] and *Paenibacillus* sp. BME-14 (60 °C).[17] However, it was higher than that from *Cellulomonas fimi* (42 °C)[27] and *Paenibacillus polymyxa* (37 °C).[28] The purified mannanase had a TC_{50} (temperature for 50% activity decrease after 30 min incubation) at 62 °C and was stable at ≤40 °C. The thermal stability of purified mannanase was similar to those from *Paenibacillus polymyxa* (<50 °C),[28] *Bacillus* sp. M50 (<50 °C)[24] and *Bacillus subtilis* WY34 (<50 °C).[29]

Effect of Inhibitors and Other Reagents. Compared with sample without chemicals added, IAA, PMSF, leupeptin, urea, NEM, EDTA, PCMB, TLCK, and TPCK inhibited the

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Table 1. Summary of the Purification of Mannanase from *Paenibacillus cookii*

procedure	total act. (U)	total protein (mg)	sp act. (U/mg)	purifn (fold)	yield (%)
crude enzyme	2562.8	363.8	7.0	1.0	100.0
ammonium sulfate fractionation (40−60%)	1979.5	91.3	21.7	3.1	77.2
DEAE-Sepharose	1207.2	12.7	94.9	13.5	47.1
1st Sephacryl S-100 HR	494.8	1.1	434.0	61.6	19.3
2nd Sephacryl S-100 HR	163.9	0.3	635.4	90.2	6.4

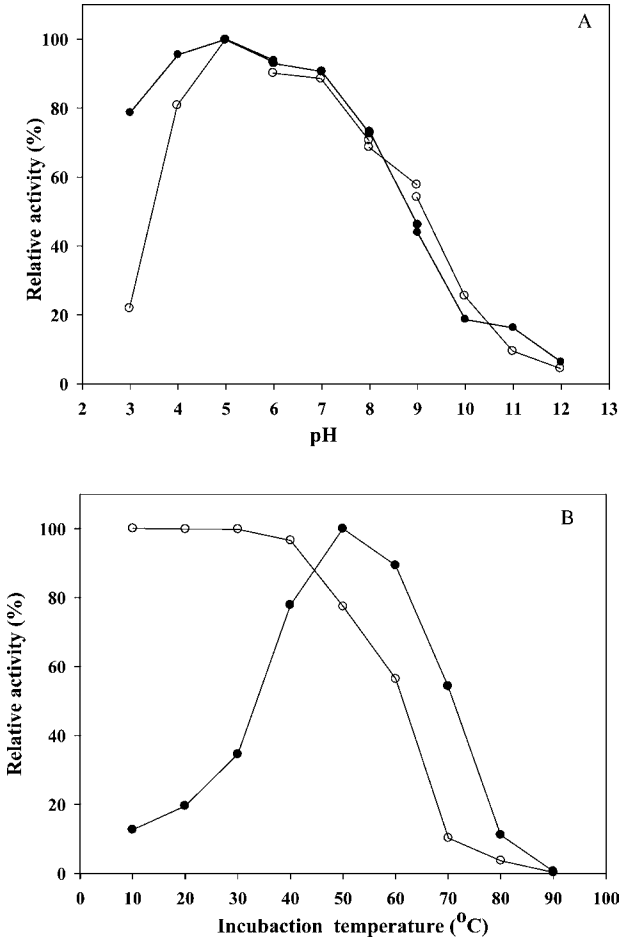

Figure 4. Effect of pH (A) and temperature (B) on mannanase (●, optimal; ○, stability).

purified mannanase activity ($p < 0.01$), while 0.5 mM PCMB, TLCK, TPCK, leupeptin, and GSH did not affect its activity (Table 2). According to Jiang et al.,[29] the mannanase from *Bacillus subtilis* WY34 was also inhibited by EDTA. Since DTT, glutathione, cysteine, and β-Me could recover −SH group on the protein molecules, their activation on the purified mannanase activity (Table 2) suggests that the active site of purified mannanase contains −SH group.

Effect of Metal Ions. Compared to the sample without metal added, Cu^{2+} and Zn^{2+} and 10 mM Hg^{2+} and Fe^{3+} completely inhibited, while Li^{+}, Ni^{2+}, and Fe^{2+} moderately inhibited, the purified mannanase activity ($p < 0.01$) (Table 3). Cd^{2+} and Mg^{2+} did not affect the activity, however, Ag^{+}, K^{+}, Ca^{2+}, Co^{2+}, and Mn^{2+} greatly activated the purified mannanase ($p < 0.01$). The mannanase from *Bacillus subtilis* WY34 was also inhibited by Li^{+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}.[29] It is known that the heavy metal ions can interact with −SH to form mercaptide, or with imidazole and carboxyl groups. The metal ion effects further confirm that the active site of the purified mannanase contains SH group.

Table 2. Effect of Various Inhibitors and Reductants on Mannanase Activity (%) from *Paenibacillus cookii*

chemicals[a]	0.5 mM	1.0 mM	2.0 mM
none	100	100	100
IAA	89.5 ± 1.5*[b]	54.1 ± 3.3*	30.1 ± 4.1*
PCMB	99.3 ± 0.8	96.3 ± 1.6*	92.8 ± 2.2*
PMSF	93.9 ± 1.3*	77.8 ± 2.6*	40.1 ± 3.7*
TLCK	98.3 ± 1.7	94.9 ± 1.4*	92.8 ± 1.4*
TPCK	97.4 ± 1.1	89.7 ± 1.5*	87.7 ± 1.2*
leupeptin	99.4 ± 1.6	88.9 ± 1.6*	64.1 ± 3.0*
urea	91.2 ± 1.1*	78.3 ± 1.8*	65.2 ± 2.8*
NEM	96.8 ± 0.4*	87.7 ± 0.8*	76.8 ± 2.1*
EDTA	93.9 ± 0.9*	75.1 ± 1.5*	52.2 ± 1.9*
DTT	106.8 ± 1.3*	110.0 ± 1.2*	125.4 ± 2.0*
GSH	101.7 ± 1.0	115.6 ± 1.7*	134.7 ± 1.6*
cysteine	104.1 ± 1.2*	109.4 ± 1.4*	124.4 ± 3.0*
β-Me	147.6 ± 3.1*	170.9 ± 3.3*	209.1 ± 4.7*

[a]IAA: iodoacetic acid. PCMB: *p*-chloromercuribenzoate. PMSF: phenylmethylsulfonyl fluoride. TLCK: *N*-tosyl-L-lysine chloromethyl ketone. TPCK: *N*-tosyl-L-phenylalanine chloromethyl ketone. NEM: *N*-ethylmaleimide. EDTA: ethylenediamine tetraacetic acid. DTT: dithiothreitol. GSH: glutathione. β-Me: β-mercaptoethanol. [b]Values with an asterisk are significantly different ($p < 0.01$), compared with the sample without chemicals added.

Table 3. Effect of Metal Ions on the Mannanase Relative Activity (%) from *Paenibacillus cookii*

metal ions[a]	1.0 mM	5.0 mM	10.0 mM
none	100.0	100.0	100.0
Ag^{+}	118.1 ± 0.5*[b]	144.0 ± 1.8*	150.3 ± 1.9*
K^{+}	113.3 ± 0.8*	121.8 ± 1.1*	124.4 ± 1.3*
Li^{+}	33.8 ± 0.1*	27.2 ± 2.1*	19.6 ± 1.6*
Ca^{2+}	104.7 ± 0.8	124.4 ± 1.2*	151.3 ± 2.7*
Cd^{2+}	100.3 ± 0.4	104.1 ± 1.1	115.5 ± 1.3*
Co^{2+}	122.8 ± 0.9*	150.3 ± 2.2*	178.8 ± 3.5*
Cu^{2+}	17.4 ± 1.3*	0.0 ± 0.5*	0.0 ± 0.6*
Hg^{2+}	29.1 ± 1.6*	10.4 ± 1.3*	0.0 ± 0.8*
Fe^{2+}	92.4 ± 0.4*	75.3 ± 1.8*	71.8 ± 2.7*
Fe^{3+}	63.9 ± 1.6*	14.8 ± 1.4*	0.0 ± 0.4*
Mg^{2+}	100.5 ± 0.5	100. ± 0.6	105.1 ± 0.1*
Mn^{2+}	105.1 ± 0.8*	165.8 ± 3.1*	227.2 ± 3.1*
Ni^{2+}	51.3 ± 1.2*	50.2 ± 2.3*	50.0 ± 1.6*
Zn^{2+}	39.2 ± 1.4*	0.0 ± 0.5*	0.0 ± 0.7*

[a]The counterion of the metals is chloride. [b]Values with an asterisk are significantly different ($p < 0.01$), compared with the sample without metals added.

Substrate Specificity. Among the substrates including LBG (galactomannan), carboxymethylcellulose, birchwood xylan, mannan, konjac powder (glucomannan), and guar gum, the purified mannanase revealed the highest activity against

LBG. Adjusting purified mannanase activity on LBG to 100%, it is shown that it was 70.3% active on konjac powder ($p < 0.01$). However, it showed relatively low activity on mannan (17.6%), cellulose (11.9%), birchwood xylan (4.7%), CMC (2.6%), and guar gum (1.6%) ($p < 0.01$) (Table 4). LBG (with a mannose to galactose ratio of 3.5) is a galactomannan similar to guar gum consisting of a (1→4)-linked β-D-mannopyranose backbone with branch points from their 6-positions linked to α-D-galactose (that is, 1→6-linked α-D-galactopyranose).[30] Konjac powder (with a mannose to glucose ratio of 3) is a glucomannan consisting of a (1→4)-linked β-D-mannopyranose, a water-soluble dietary fiber consisting of mannose and glucose sugars. High hydrolysis ability on LBG and konjac suggests that the purified mannanase prefers to hydrolyze (1→4)-linked β-D-mannogalactose and (1→4)-linked β-D-mannoglucose. However, our purified mannanase has less hydrolytic ability against mannan (17.6%). These phenomena are similar to those from *Paenibacillus polymyxa*[28] and *Bacillus subtilis* WY34,[29] which had high activity against LBG. However, it had very low hydrolytic ability on guar gum, which is also a galactomannan. This might be due to the guar gum with a linear chain of β-1,4-linked mannose residues to which galactose residues are 1,6-linked at every second mannose, forming short side branches and lower mannose/galactose ratio (2.0) than that in LBG (3.5).[1] The lower mannanase activity toward guar gum as substrate compared with mannanase activity assayed in the presence of LBG supports the hypothesis that the enzyme activity is limited by the number of branched α-galactose residues. The same reduction of activity (14 to 27% of maximal activity) has also been noted for other mannanases, using a galactomannan substrate possessing the same percentage of galactose residues as guar gum.[1]

Table 4. Substrate Specificity of the Purified Mannanase from *Paenibacillus cookii*

substrate	rel act. (%)
LBG	100.0
cellulose	11.90 ± 0.11*[a]
guar gum	1.62 ± 0.06*
CMC	2.63 ± 0.14*
konjac powder	70.31 ± 0.18*
mannan	17.64 ± 0.10*
xylan	4.73 ± 0.05*

[a]Values with an asterisk are significantly different ($p < 0.01$), compared with LBG.

Kinetic Parameters. The kinetics (V_{max} and K_m) of low viscosity LBG and konjac hydrolysis by the purified mannanase were calculated from Lineweaver–Burk double reciprocal plots (Figure 5A,B). The K_m and V_{max} values for the purified mannanase on LBG and konjac were 0.0095 and 0.0136 mg/mL, and 556 and 484 U/min/mg, respectively. Higher K_m value of konjac than LBG suggested the higher affinity of LBG to the purified mannanase, which was highly in accordance with the result of substrate specificity (Table 4).

For the mannanase, kinetic comparisons on galactomannans are difficult since polymeric chains of mannose substituted with galactose, which are used as substrates, vary with the length and degree of substitution and are a function of the preparation method and the source of galactomannan.[31] K_m values for different galactomannan substrates have been determined for the *C. saccharolyticum* mannanase and reported to be 0.127 mg/mL for LBG. However, the V_{max} of the mannanases from *T. neapolitana* and *C. saccharolyticum* against LBG and guar gum was almost similar, 3.8 U/mg.[31]

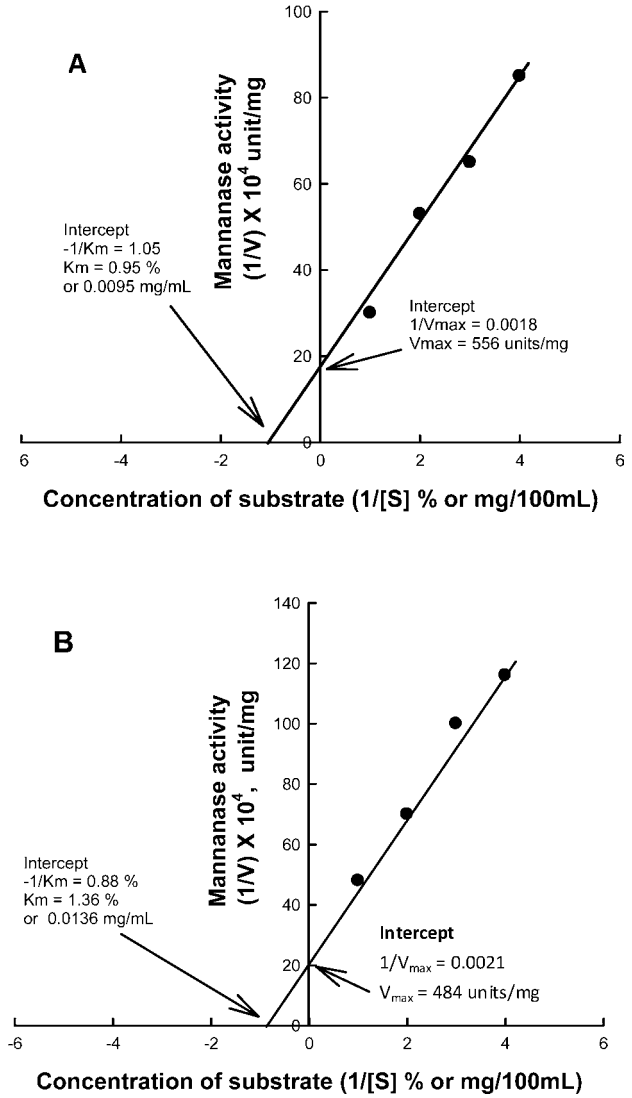

Figure 5. Lineweaver–Burk double reciprocal plot of the LBG (A) and konjac (B) substrate concentration effect on the purified mannanase hydrolysis rate. The K_m and V_{max} were calculated from the Lineweaver–Burke plot.

N-Terminal Amino Acid Sequence Analysis. The N-terminal amino acid sequence of mannanase was GLFGINAY (Table 5). Compared with those from other species, 6 amino

Table 5. Comparison of N-Terminal Amino Acid Sequences of Mannanases from *Paenibacillus cookii* with Other Species

microbial source	N-terminal amino acid sequence							
Paenibacillus cookii	G	L	F	G	I	N	A	Y
Lycopersicon esculentum	Y	I	N	G	F	N	A	Y
Coffea arabica	Y	L	N	G	F	N	A	Y
Aspergillus sulphureus	Y	I	A	G	T	N	A	Y
Trichoderma reesei	Y	F	A	G	I	N	C	Y
Lactuca sativa	Y	I	N	G	F	N	A	Y
Thermotoga maritima	K	L	L	S	I	E	T	V

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acids were similar to that from *Coffea arabica*,[32] and 5 were similar to those from *Lycopersicon esculentum*,[33] *Aspergillus sulphureus*,[34] and *Lactuca sativa*.[35]

Further study on the structure and function of the purified mannanase from this novel strain of *Paenibacillus cookii* including the determination of the active site by site directed mutagenesis and cloning of the mannanase gene are necessary to elucidate the reaction mechanism of this enzyme and to accelerate its industrial application.

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Notes

The authors declare no competing financial interest.

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