Characterization, Gene Cloning, and Heterologous Expression of β-Mannanase from a Thermophilic *Bacillus subtilis*

Pijug Summpunn[1,2], Suttidarak Chaijan[2], Duangnate Isarangkul[2], Suthep Wiyakrutta[2], and Vithaya Meevootisom[2]*

[1]*Department of Biotechnology*, [2]*Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand*

Bacillus subtilis **BCC41051 producing a thermostable β-mannanase was isolated from soybean meal-enriched soil and was unexpectedly found to be thermophilic in nature. The extracellular β-mannanase (ManA) produced was hydrophilic, as it was not precipitated even with ammonium sulfate at 80% saturation. The estimated molecular weight of ManA was 38.0 kDa by SDS-PAGE with a pI value of 5.3. Optimal pH and temperature for mannan-hydrolyzing activity was 7.0 and 60°C, respectively. The enzyme was stable over a pH range of 5.0-11.5, and at temperatures of up to 60°C for 30 min, with more than 80% of its activity retained. ManA was strongly inhibited by Hg^{2+} (1 mM), but was sensitive to other divalent ions to a lesser degree. The gene of ManA encoded a protein of 362 amino acid residues, with the first 26 residues identified as a signal peptide. High expression of recombinant ManA was achieved in both *Escherichia coli* BL21 (DE3) (415.18 U/ml) and *B. megaterium* UNcat (359 U/ml).**

***Keywords*: β-mannanase, *Bacillus subtilis*, characterization, gene expression, thermophilic**

Mannans, important components of hemicellulose in plant cell walls, are classified into four subfamilies: linear mannan, glucomannan, galactoglucomannan, and galactomannan (de O. Petkowicz *et al.*, 2001). Structurally, both linear mannan and galactomannan consist of β-1,4-linked mannose residues attached to a different extent to a single α-1,6-linked D-galactosyl residue (5% or less and 20-100%, respectively) (Politz *et al.*, 2000; de O. Petkowicz *et al.*, 2001; de Vries, 2003); while glucomannan is composed of a combination of β-1,4-linked mannose and β-1,4-linked glucose residues, alternating in a random order. The last type of mannan, galactoglucomannan, is glucomannan with α-1,6-linked D-galactopyranose attached to both D-glucosyl and D-mannosyl units (Moreira and Filho, 2008).

In nature, linear mannans (β-mannans) are mostly found in non-leguminous plants such as *Aloe vera*, ivory nuts, and coffee beans (Moreira and Filho, 2008). Glucomannans and galactoglucomannans are major components of hemicellulose in softwood and hardwood (de Vries, 2003) while galactomannans are usually found as storage polymers in seeds of leguminous plants (Lee *et al.*, 2005).

Cereal grains, such as wheat and corn, and soybean meal are the major ingredients of poultry and swine feeds. These raw materials contain varying amounts of mannans, some of which cannot be digested by monogastric animals (Choct and Annison, 1992). Moreover, these undigested constituents have an adverse effect on the digestibility of other nutrients by increasing viscosity and inhibiting gut enzymes necessary for digestion (Langhout *et al.*, 2000). Addition of enzymes, including β-mannanase, into the feed has increased feed utilization and improved the digestion of monogastric animals

(Jackson *et al.*, 1999).

β-mannanase (1,4-β-D-mannan mannanosidase, EC 3.2.1.78) is an endohydrolase, which randomly hydrolyzes β-1,4-linkages of mannan-based polysaccharides. Several genes encoding β-mannanases from plants, fungi and bacteria, including *Bacillus subtilis*, have been cloned and their encoded enzymes have been purified and characterized (Moreira and Filho, 2008). These enzymes have potential applications in food and feed industries, as well as in other industrial processes such as viscosity reduction in coffee manufacturing, extraction of oils from leguminous seeds, and bleaching of pulp wood (Heck *et al.*, 2005). Of these mannanases, none has been reported from a thermophilic *B. subtilis*. Therefore in this report we describe the cloning and heterologous expression in *Escherichia coli* and *Bacillus megaterium* of a β-mannanase from thermophilic *B. subtilis* MUSc-1 isolated from soybean meal-enriched soil in Thailand.

Materials and Methods

Chemicals, media, enzymes, and bacterial strains
Locust bean gum (LBG) was purchased from Sigma (USA). Luria-Bertani (LB) medium was obtained from Oxoid (UK). Chemicals and reagents used were of analytical or biotechnological grade from Sigma and Merck (Germany). *Pfu* DNA polymerase was purchased from Promega (USA), and restriction endonucleases (*Bam*HI, *Nde*I, *Nru*I, and *Xho*I) and T4 DNA ligase were acquired from New England Biolabs (USA). *E. coli* DH5α and BL21 (DE3) were purchased from Novagen (USA). *E. coli* BL21 (DE3) and *B. megaterium* UNcat (Panbangred *et al.*, 2000) were used as Gram-negative and Gram-positive expression hosts, respectively.

* For correspondence. E-mail: scvmv@mahidol.ac.th; Tel: +66-2201-5671; Fax: +66-2644-5411

Isolation and identification of mannan-degrading microorganisms

Mannanase-producing microorganisms were screened from soil, soybean meal-enriched soil, and compost and manure samples obtained from various places in Bangkok, Thailand. For primary screening, 0.5 g of each fresh soil sample was suspended in 4.5 ml of sterile normal saline solution and serially tenfold diluted to a final 10^{-4} dilution. Aliquots (100 μl) of the diluted samples were spread on screening plates containing GLM medium (0.6% locust bean gum, 0.34% KH_2PO_4, 0.355% $Na_2HPO_4·2H_2O$, 0.02% $MgSO_4·7H_2O$, 0.01% NH_4Cl, 0.001% $CaCl_2·2H_2O$, and 1.5% agar). GLM plates were incubated at 50℃ for 1-2 days to allow bacterial growth and generation of clear hydrolytic zones. Positive strains were selected and purified by repeated streaking on GLM medium. Each of these purified strains was inoculated into 20 ml of GLM broth and incubated at 50℃ with shaking (180 rpm) for 2 days. Culture was harvested by centrifugation at 10,000×g at 4℃ for 10 min, and the supernatant was incubated at 50℃ for 20 min before assaying for mannan-degrading enzyme activity. The bacterial strain whose extract showed the best enzyme activity was selected for enzyme production, purification and characterization. The selected strain was identified by standard biochemical tests (Gerhardt *et al.*, 1994) and phylogenetic analysis based on 16S rRNA gene sequence data and it was deposited in the BIOTEC Culture Collection (BCC), Thailand.

Production and purification of β-mannanase

B. subtilis BCC41051 was inoculated in 200 ml of GLM medium and incubated with shaking (180 rpm) at 50℃ for 36 h. Culture supernatant was collected as described above, and subsequently concentrated and desalted using a Minitan™ Ultrafiltration System (Minitan® 4/PK 5,000 NMWL filter plates, low binding regenerated cellulose; Millipore, USA) and Centricon Plus-20 centrifugal filters with MW cut off at 10,000 (Millipore). The concentrated enzyme solution was purified by anion-exchange chromatography using DEAE- Sepharose CL-4B (Sigma, USA) with a linear gradient of 0-0.5 M NaCl in 25 mM Tris-HCl (pH 7.5). Fractions with β-mannanase activity were pooled and concentrated as described above. The pooled enzyme solution was adjusted to 1 M $(NH_4)_2SO_4$, applied onto a Phenyl Sepharose CL-4B column (GE Healthcare, USA), and then eluted with a linear salt gradient from 1.0-0 M $(NH_4)_2SO_4$ in 25 mM Tris-HCl (pH 7.5). Fractions with β-mannanase activity were pooled, concentrated and further purified by gel-filtration chromatography (Superdex 200 HR 10/30; Amersham Pharmacia Biotech, USA) using 25 mM Tris-HCl (pH 7) as elution buffer. Enzyme purity and molecular mass were determined by SDS-PAGE (Laemmli, 1970).

Assay of β-mannanase and protein

β-mannanase activity was assayed by measuring the amount of reducing sugars released from LBG using the dinitrosalicylic acid (DNS) method (Sengupta *et al.*, 2000). A 0.1 ml aliquot of enzyme sample was mixed with 0.9 ml of 100 mM sodium phosphate buffer (pH 6.8) containing 0.5% LBG and incubated at 42℃ for 20 min. The amount of reducing sugar liberated was estimated by adding 3 ml of DNS reagent and boiling for 5 min. After cooling to room temperature, absorbance at 540 nm was measured (BioSpec-1601; Shimadzu, Japan). D-mannose was used as the standard. One unit of mannanase activity was defined as the amount of the enzyme required to liberate 1 μmol of reducing sugar (mannose) per min under the assay condition. Protein content was determined by using the Bradford method (Bradford, 1976).

Characterization of β-mannanase

The pH activity profile of β-mannanase was determined by measuring the enzyme activity at 42℃ (Richards, 1970) in a pH range of 3.5-11.5, using 50 mM of citrate-NaOH buffer (pH 3.5-6.0), sodium phosphate buffer (pH 6.0-8.0), glycine-NaOH buffer (pH 8.0-10.0) and 3-[cyclohexylamino]-1-propanesulfonic acid buffer (CAPS, pH 10.0-11.5). The effect of pH on β-mannanase stability was determined by incubating the enzyme in buffers of various pH at 37℃ for 30 min before assaying at pH 7.0. Optimal temperature of enzyme activity was determined by assaying the enzyme activity in 50 mM sodium phosphate buffer (pH 7.0) at various temperatures ranging from 25-85℃. In addition, thermal stability of the enzyme was determined by pre-incubation at various temperatures (25-85℃) in 50 mM sodium phosphate buffer (pH 7.0) for 30 min. The effects of chemicals (1 mM of $CaCl_2$, $CoCl_2$, $CuSO_4$, $FeSO_4$, $HgCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, or $NiSO_4$) on enzyme stability were investigated by pre-incubating the enzyme with each of the chemicals in 50 mM sodium phosphate buffer (pH 7.0) at room temperature for 24 h.

Cloning of *manA* gene

Washed cells from 10 ml overnight culture of the selected bacterium in LB broth were suspended in 5 ml of GET buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) and incubated with lysozyme (0.25 mg/ml final concentration) at 37℃ for 1 h. Then 100 μl of proteinase K (10 mg/ml) was added and the solution further incubated for 1 h. One mililiter of 10% SDS was added and the mixture was again incubated at 37℃ for 1 h. DNA was extracted with phenol/ chloroform:isoamyl alcohol and precipitated with chilled absolute ethanol/sodium acetate (Sambrook and Russell, 2001). The DNA was used as a template for PCR amplification of gene encoding β-mannanase using primers SM001 (5′-CTGTGTCGCCTGTGAATCC-3′) and SM002 (5′-CTGCCGTTTGCTGTTTGC-3′), based on the conserved sequences of *B. subtilis* β-mannanases, and *Pfu* DNA polymerase with the following thermal cycling conditions: 95℃ for 5 min; 30 cycles of 95℃ for 30 sec, 60℃ for 30 sec, 72℃ for 1 min; and 72℃ for 10 min. The amplicon was directly sequenced using BigDye™ Terminator cycling and an automatic sequencer 3730xl (Macrogen, Korea). To amplify the full length of gene encoding β-mannanase, two additional primers, SM003 (5′-GGAGAATTTACAATAGAAGG-3′) and SM004 (5′-GGGTTAGGCGATATGAATGC-3′), were designed from the known sequence of the *B. subtilis* β-mannanase gene (GenBank accession number NC-000964). PCR was carried out as follows: 95℃ for 5 min; 30 cycles of 95℃ for 30 sec, 55℃ for 30 sec, 72℃ for 3 min; and 72℃ for 10 min. Two additional primers, SM005 (5′-CAC CGATTACAAACGATCAG-3′) and SM006 (5′-CCGTTCATTTCAT GCAGC-3′), were also used as sequencing primers to confirm the DNA sequence. The DNA sequences were assembled to obtain the complete sequence. Nucleotide and deduced amino acid sequences were analyzed using Gene Runner, EasyGene 1.0 server (http://www.cbs.dtu.dk), and Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html), as well as programs provided by the National Center for Biotechnology Information (NCBI) server (Altschul *et al.*, 1997).

For phylogenetic analysis, amino acid sequences of *Bacillus* mannanases were collected from GenBank and aligned by CLUSTAL X software (version 1.81) (Thompson *et al.*, 1997) using Gonnet series matrices as multiple alignment parameters, and gap opening and extension parameters as default values. Insertions or deletions were removed from the data sets before submitting to reconstruct the maximum likelihood tree using the RAxML web server (Stamatakis *et*

al., 2008).

DNA manipulation and expression of β-mannanase in *E. coli* BL21 (DE3)

The open reading frame of gene encoding β-mannanase was amplified by PCR using primers Man-CHF (5′-GTACGCCATATGTTTAAGAAACATACGATCTCTTTGC-3′) [*Nde*I site underlined] and Man-CHR (5′-GTACGCCTCGAGTTCAACGATTGGCGTTAAAGAATC-3′) [*Xho*I site underlined] and bacterial genomic DNA as a template, with the following thermal cycling conditions: 95°C for 5 min; 30 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 2 min; and 72°C for 10 min. The amplicon was digested with *Nde*I and *Xho*I, and then ligated into the corresponding restriction sites of pET24b(+), resulting in recombinant pEManAhis encoding a C-terminal hexahistidine-tagged β-mannanase. Moreover, the *manA* gene had been modified to convert the TTG start codon to ATG and the GGGGAG SD sequence to AAGGAG. pEManAhis was transfected into *E. coli* BL21 (DE3), and transformants were cultured in LB medium supplemented with 30 μg/ml of kanamycin at 37°C with shaking (200 rpm). When absorbance of 0.5 (measured at 600 nm) was reached, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added (0.4 mM final concentration) and the culture was incubated at 25°C for 24 h. Cells were harvested at various time points and analyzed for protein content (by SDS-PAGE) and β-mannanase activity.

Purification of recombinant β-mannanase

After 4 h of expression in *E. coli* BL21 (DE3), cells were harvested and washed with 20 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0), and resuspended in the same buffer before being lysed by sonication (9.9 sec on and 9.9 sec off for 6 min) on ice (VibraCell™; Sonics & Materials, USA). Cell debris and insoluble aggregates were sedimented at 17,000×g for 30 min and the supernatant was loaded onto an immobilized Co^{2+} affinity column (BD Talon™; BD Biosciences, USA). Proteins were eluted using a linear gradient of 0-80 mM imidazole in 20 mM Tris-HCl buffer containing 100 mM NaCl (pH 8.0). Fractions with β-mannanase activity were collected, and purity and molecular mass of the enzyme were determined by SDS-PAGE.

Expression of β-mannanase in *Bacillus megaterium* UNcat

An *E. coli-Bacillus* shuttle and expression vector, pXb, modified from pWH1520 (MoBiTec, Germany) was constructed as follows. A *Bam*HI site was introduced into pWH1520 by long-PCR using primers PWH_1 (5′-CGGGATCCCCCTTTGATTTAAGTG-3′) and PWH_2 (5′-GTACGGATCAATTCGAGC-3′). The amplicon then was self-ligated by blunt-end ligation to generate pXb. The β-mannanase gene was amplified from genomic DNA using the primers Man_F1 (5′-GTACGCGGATCCGACAAATGTTTAAGAAACATACGATC-3′) [*Bam*HI site underlined], and Man_R1 (5′-CTGATTCATTCAACGA
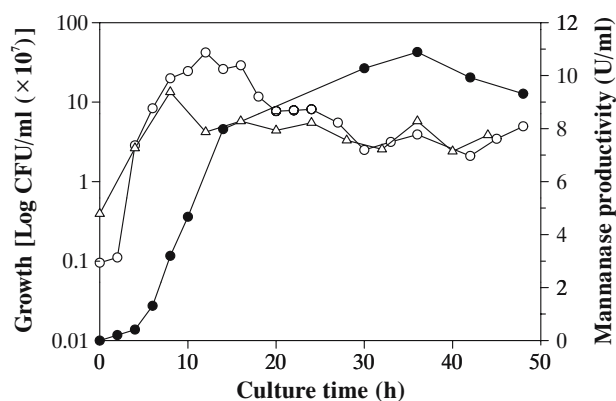

Fig. 1. Growth curves of *B. subtilis* BCC41051 and extracellular mannanase production profile. *B. subtilis* BCC41051 was grown in GLM medium with shaking (180 rpm) at 37°C (open triangle) and at 50°C (open circle). ManA activity (closed circle) was measured in culture medium of *B. subtilis* BCC41051 grown in GLM medium at 50°C.

TTGG-3′). The amplicon containing the modified *manA* gene in which the TTG start codon had been changed to ATG was digested with *Bam*HI and ligated into *Bam*HI/*Nru*I-linearlized pXb. The resulting recombinant plasmid, pXManA, was transformed into *B. megaterium* UNcat using the protoplast transformation method (Chang and Cohen, 1979). Transformed *B. megaterium* UNcat was cultured at 30°C with shaking (200 rpm). At OD_{600} of 0.3, expression of β-mannanase was induced with 0.5% (w/v) xylose for 24 h. Cells were sedimented and extracellular β-mannanase activity was measured.

N-terminal peptide sequencing

In order to determine the cleavage site between the signal peptide and mature sequence of ManA, the N-terminal sequences of the mature enzyme purified from its native host, and from the recombinant *E. coli* BL21 (DE3) harboring pEManAhis, were sequenced by Edman degradation (Biomolecular Research Facility, University of Newcastle, Australia).

Nucleotide sequence accession number

The nucleotide sequences of the *manA* gene and the 16S rRNA gene of *B. subtilis* BCC41051 were deposited in the GenBank under accession numbers GU982918 and GU982919, respectively.

Results

Isolation and identification of mannanase-producing bacterium

Eighty bacterial strains were isolated. Of these the selected

Table 1. Summary of purification of β-mannanase from *B. subtilis* BCC41051

| Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	800	137.5	4653	34	1.00	100
Minitan™ ultrafiltration	60	13.8	3394	247	7.3	73
Centrifugal ultrafiltration	8	9.1	3292	361	10.7	71
DEAE-Sepharose CL-6B	1.2	0.7	1412	2002	59.1	30
Phenyl agarose CL-4B	1.2	0.2	565	3167	93.5	12
Superdex 200 HR 10/30	0.7	0.04	137	3169	93.6	3
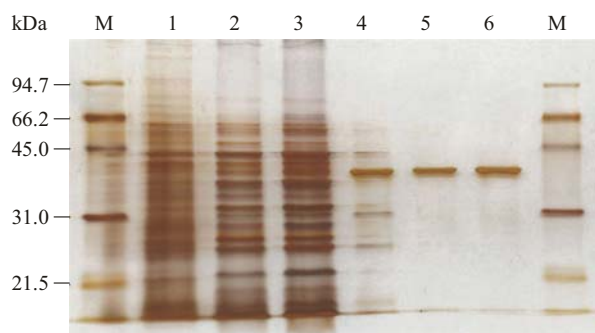
Fig. 2. SDS-PAGE of ManA from *B. subtilis* BCC41051. Protein samples were obtained from the purification steps described in Table 1. Lanes: M, standard protein molecular weight marker; 1, crude ManA enzyme; 2-6, ManA after Minitan™ ultrafiltration, centrifugal ultrafiltration, DEAE Sepharose CL-6B chromatography, phenyl agarose CL-4B chromatography, and Superdex 200HR 10/30 chromatography, respectively.

strain was from soybean meal-enriched soil at the Faculty of Science, Mahidol University and was found to produce a thermostable mannanase in a high yield. It was designated MUSc-1 and was later deposited in the BIOTEC Culture Collection (BCC), Thailand with the code number BCC41051. The BCC41051 formed medium-sized, smooth, circular, white and opaque colonies on GLM agar. The organism was a motile, Gram-positive, rod-shaped, aerobic endospore-forming bacterium. An ellipsoidal-shaped endospore was formed in the central position of the cell after 48 h cultivation. Based on biochemical properties, the strain BCC41051 was identified as a *B. subtilis* and named *B. subtilis* BCC41051. In addition, its 16S rDNA sequence was 99-100% identical with those of *B. subtilis* type strains.

Production, purification, and characterization of BCC 41051 β-mannanase

Growth of *B. subtilis* BCC41051 was determined by measuring viable counts at various times of incubation at 37°C and 50°C. β-Mannanase activity in culture medium reached a maximum level of 10.9 U/ml after cultivation at 50°C for 36 h (Fig. 1). The enzyme in the culture medium was concentrated and partially purified by ultrafiltration, which facilitated the removal of salt and small impurity molecules. After the first ultrafiltration, the enzyme was purified by 7.3-fold with an activity yield of 73% (Table 1). The enzyme was subjected to further purification using centrifugal filtration, ion exchange chromatography, hydrophobic-affinity chromatography and gel filtration. β-mannanase was purified 94-fold with a final yield of 3% and specific activity of 3,169 U/mg. The purification process and purity of the enzyme were monitored by SDS-PAGE and silver staining, showing a single polypeptide chain of 38.0 kDa (Fig. 2). The purified β-mannanase was tested for exo-β-mannosidase and α-galactosidase activity by incubating with *p*-nitrophenyl-β-D-mannopyranoside and *p*-nitrophenyl-α-D-galactopyranoside, respectively. Lack of hydrolysis, as evidenced by no increase in absorbance at 420 nm (data not shown), indicated that the purified enzyme contained no exo-β-mannosidase or α-galactosidase but only endo-β-mannanase activity.

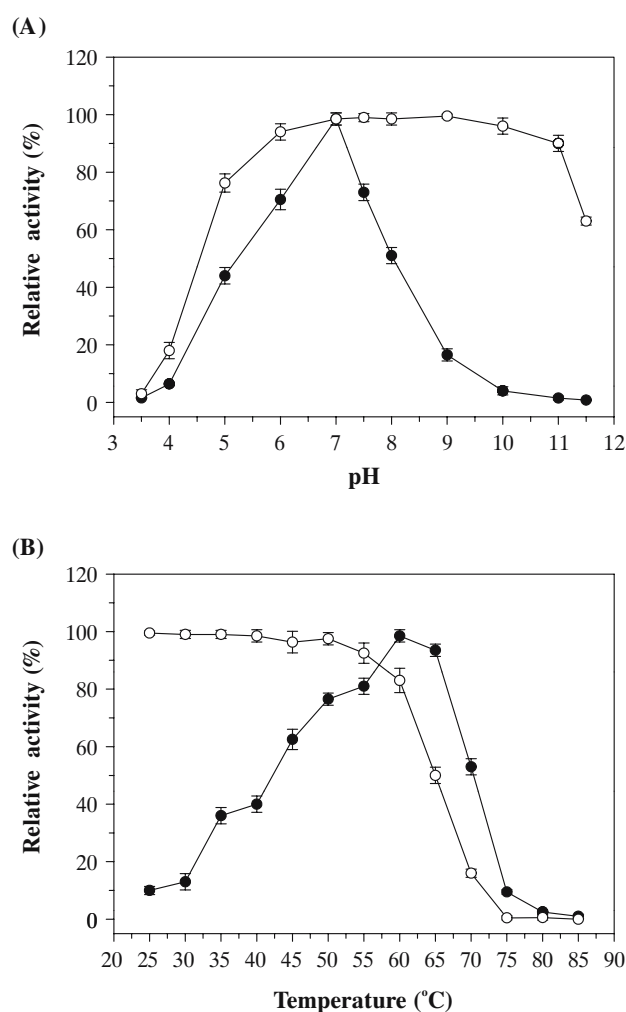Enzyme pI was estimated to be 5.3, using ampholytes over

Fig. 3. pH and temperature profiles of ManA. (A) A pH-activity profile (closed symbol) was obtained by measuring mannanase activity at 42°C in the range of pH 3.5-11.5. The effect of pH on enzyme stability (open symbol) was determined at pH 7.0 and 42°C after incubating in various pH buffers for 30 min at 37°C. (B) A temperature-activity profile (closed symbol) was obtained by measuring mannanase activity at pH 7.0 and at temperatures from 25 to 85°C. Thermal stability (open symbol) of the enzyme was determined at pH 7.0 and 42°C after incubating at various temperatures at pH 7.0 for 30 min.

Table 2. Effect of metal ions on β-mannanase stability

Chemicals	Relative activity (%)
None	100
CuSO$_4$	71
NiCl$_2$	61
CoCl$_2$	50
MgCl$_2$	47
NiSO$_4$	46
MnCl$_2$	34
CaCl$_2$	32
FeSO$_4$	16
HgCl$_2$	5

Residual enzyme activity was measured after treatment with 1 mM of each chemical for 24 h.
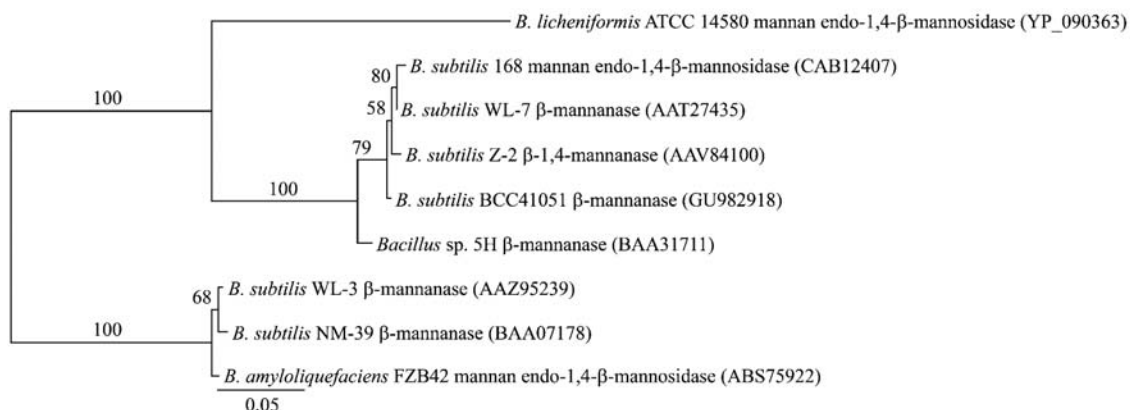
Fig. 4. Maximum-likelihood phylogenetic tree of *Bacillus* mannanases. Amino acid sequence alignments were performed by CLUSTAL X, and the maximum-likelihood tree was generated using RAxML web servers. Scale bar (0.05) indicates substitutions per amino acid position.
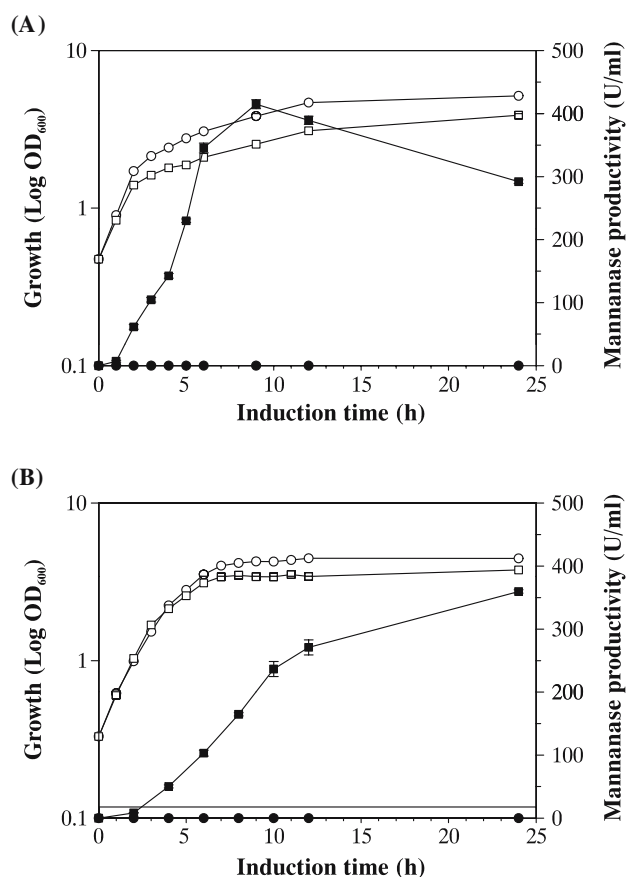

Fig. 5. Production of recombinant β-mannanase by *E. coli* BL21 (DE3) and *B. megaterium* UNcat and their growth. (A) Recombinant β-mannanase activity in cell extract of *E. coli* BL21 (DE3) harboring pET24b(+) (closed circle) and recombinant pEManAhis (closed square), and growth of *E. coli* BL21 (DE3) harboring pET24b(+) (open circle) and pEManAhis (open square) in LB medium at 25°C with IPTG induction. (B) β-mannanase activity in culture medium of *B. megaterium* UNcat harboring recombinant pXb (closed circle) and pXManA (closed square), and growth of *B. megaterium* UNcat harboring pXb (open circle) and pXManA (open square) in LB medium at 30°C with xylose induction.

a pH range of 5.0-8.0 (data not shown). Optimum enzyme activity was at pH 7.0 and was stable over a pH range of 5-11, with >76% of maximum activity retained after incubation at 37°C for 30 min (Fig. 3A). Enzyme activity was completely inactivated at <pH 3.5, but >63% of the maximum activity was retained after exposure to pH 11.5 at 37°C for 30 min. Activity of ManA increased with increasing temperature up to 60°C, with >82% of activity being retained after exposure to this temperature for 30 min; but activity was completely and irreversibly lost after heating at 75°C for 30 min (Fig. 3B). ManA activity was strongly inhibited by 1 mM Hg^{2+}, and partially (to varying extents) by other divalent ions tested (Table 2).

Cloning and nucleotide sequence of *manA* gene encoding BCC41051 mannanase

Primers SM001 and SM002 were designed to amplify a partial region of the gene encoding β-mannanase from *B. subtilis* BCC41051 genomic DNA. The expected 820-bp amplicon was obtained and directly sequenced. Other primers, SM003 and SM004, were designed to amplify the full length of the gene, and primers SM005 and SM006 were also designed to use as sequencing primers. The assembled 1,153-bp DNA sequence revealed an open reading frame (ORF) of 1,089 nucleotides encoding 362 amino acids of a protein with an estimated molecular mass of 40.9 kDa. The putative start codon of *manA* was identified as TTG, as seen in gene encoding β-mannanase of *B. subtilis* 168 (AL009126), *B. subtilis* WL-7 (AY601725), and *B. subtilis* Z-2 (AY827489); the termination codon was TGA. The putative ribosome binding (SD) site (GGGGAG) was located 6-bp upstream of the start codon. A BLAST search of the deduced amino acid sequence against other mannanases in the NCBI database showed that the predicted amino acid sequence had high identity to mannanase from *B. subtilis* WL-7 (99%) (AAT27435), *B. subtilis* Z-2 (99%) (AAV84100), *B. subtilis* str. 168 (98%) (CAB12407), *B. subtilis* 5H (97%) (BAA31711), *B. subtilis* NM-39 (74%) (BAA07178), and *B. subtilis* WL-3 (74%) (AAZ95239) (Fig. 4).

The N-terminal amino acid sequence of the mature protein of ManA was HTVSPVNPNAQQTTKTVMNW, identical to the deduced amino acid residues 27-46 translated from the
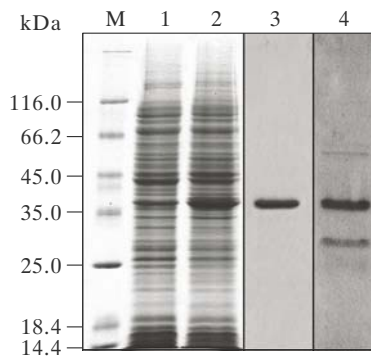
Fig. 6. SDS-PAGE of recombinant β-mannanase expressed in *E. coli* BL21 (DE3) and *B. megaterium* UNcat. Lanes: M, standard protein molecular weight markers; 1 and 2, proteins of recombinant *E. coli* BL21 (DE3) harboring pET24b(+) and recombinant pEManAhis, respectively; 3, purified ManA from extract of *E. coli* BL21 (DE3) harboring recombinant pEManAhis; 4, ManA from culture medium of *B. megaterium* UNcat harboring pXManA.

ORF; this indicated that the first 26 amino acids constituted the signal peptide.

Expression of *B. subtilis* BCC41051 β-mannanase in *E. coli* BL21 (DE3) and *B. megaterium* UNcat

For heterologous expression in *E. coli*, the coding region of *manA* was amplified using primers Man-CHF and Man-CHR and then ligated into pET24b(+), yielding recombinant pEManAhis encoding β-mannanase fused to a hexahistidine tag at the C-terminus. pEManAhis was used to transform *E. coli* BL21 (DE3), and gene encoding β-mannanase expression was induced by IPTG. The highest β-mannanase activity in cell extract of 415.18 U/ml was obtained after 9 h of induction at 25°C (Fig. 5), whereas no activity was detected in cells transformed with pET24b(+) (negative control). The recombinant enzyme was purified to homogeneity by cobalt affinity chromatography (Fig. 6) with a specific activity of 3,965 U/mg, similar to that of the purified enzyme from *B. subtilis* BCC41051 (3,169 U/mg).

For expression in *B. megaterium* UNcat, the *manA* ORF was amplified using primers Man_F1 and Man_R1 and then ligated into the modified *E. coli-Bacillus* shuttle and expression vector, pXb. The resulting recombinant pXManA was used to transform *B. megaterium* UNcat. After induction expression of β-mannanase with xylose (Fig. 6), enzyme activity in culture medium was 359 U/ml after 24 h of induction while no enzyme activity was detected when *B. megaterium* UNcat was transformed with pXb (negative control).

Discussion

In this study, screening of microbes for thermostable mannan-degrading enzymes was performed by raising the growing temperature to 50°C on a screening medium supplemented with LBG as the sole carbon source. Based on growth at this elevated temperature and production of a large mannan hydrolysis zone on the screening plate, *B. subtilis* BCC41051 was isolated. The ability of *B. subtilis* BCC41051 to grow better

at 50°C than at 37°C (Fig. 1) indicated that it is thermophilic rather than thermotolerant, as are *B. subtilis* KU-1 (Zakaria *et al.*, 1998), *Bacillus* sp. JAMB-750 (Hatada *et al.*, 2005), *Bacillus* sp. MSJ-5 (Zhang *et al.*, 2009) and *Bacillus circulans* CGMCC1554 (Yang *et al.*, 2009), whose growing temperatures are in the range of 30-37°C.

The highest β-mannanase accumulation in culture medium of BCC41051 was obtained when the bacteria was grown at 50°C for 36 h. In contrast to other mannanases from *B. subtilis* strains NM-39 (Mendoza *et al.*, 1994), KU-1 (Zakaria *et al.*, 1998), and WL-3 (Yoon *et al.*, 2008), and *Bacillus* sp. MSJ-5 (Zhang *et al.*, 2009), our enzyme could not be precipitated even with ammonium sulfate at 80% saturation, suggesting that the enzyme is hydrophilic. Thus, ultrafiltration was employed to concentrate and desalt the enzyme in the culture medium before purification to homogeneity using a series of anion-exchange, hydrophobic-affinity and gel-filtration chromatographies. The purified β-mannanase (ManA) had a molecular mass of 38.0 kDa as determined by SDS-PAGE. Cloning and sequencing of the whole gene revealed an ORF of 362 amino acids with an estimated molecular mass of 40.9 kDa. These data indicated that ManA is a monomeric protein similar to mannanases from other organisms (Mendoza *et al.*, 1994; Zakaria *et al.*, 1998; Jiang *et al.*, 2006; Yoon *et al.*, 2008; Zhang *et al.*, 2009), except that from *Bacillus stearothermophilus* which is produced as a dimer (Talbot and Sygusch, 1990).

Heterologous expression in *E. coli* of recombinant ManA fused to a hexahistidine tag at the C-terminus allowed single-step cobalt affinity purification. This resulted in an enzyme with essentially the same specific activity as purified ManA, indicating that the presence of C-terminal hexahistidine residues did not compromise enzyme activity. Moreover, the N-terminal sequence of the mature recombinant ManA was identical to that of the wild-type enzyme, suggesting that *E. coli* BL21 (DE3) could correctly process the signal peptide of recombinant ManA.

A protein BLAST search of the deduced amino acid sequence of *manA* revealed ManA having high identity to mannanases from *B. subtilis* strains WL-7, Z-2, 168, 5H, NM-39, and WL-3, which belong to the glycosyl hydrolase family 26 (GH26). Its signal peptide of 26 amino acids is the same length as that of *B. subtilis* strains WL-7 (AAT27435), Z-2 (AAV84100), 168 (CAB12407), and 5H (Khanongnuch *et al.*, 1999), although a signal peptide of 24 amino acids has been identified, viz. mannanases of *B. subtilis* strains NM-39 (Mendoza *et al.*, 1995) and WL-3 (AAZ95239). The start codon (TTG) is also identical to that of *B. subtilis* strains WL-7 (AY601725), Z-2 (AY827489), and 168 (AL009126), but the canonical ATG start codon is also used (Mendoza *et al.*, 1995; Khanongnuch *et al.*, 1999).

The pI value of ManA was 5.3, higher than that of *B. subtilis* NM-39 (4.8) (Mendoza *et al.*, 1994), *B. subtilis* KU-1 (4.5) (Zakaria *et al.*, 1998) and *Bacillus* sp. N16-5 (4.3) (Ma *et al.*, 2004). The optimum pH (7.0) of ManA activity is similar to that of mannanases from *B. subtilis* 5H (Khanongnuch *et al.*, 1998), *B. subtilis* KU-1 (Zakaria *et al.*, 1998) and *B. licheniformis* (Zhang *et al.*, 2000). However, other *Bacillus* mannanases have maximal activity in pH ranges of 5.0-6.0 (Mendoza *et al.*, 1994; Jiang *et al.*, 2006; Yoon *et al.*, 2008; Zhang *et al.*, 2009) and 9.0-10.0 (Ma *et al.*, 2004; Hatada *et al.*, 2005).

ManA was stable over a wide pH range compared with the other mannanases mentioned above and this is a good property for use in animal feed. ManA was strongly inhibited by Hg^{2+}, but was more sensitive to other divalent ions (Fe^{2+}, Ca^{2+}, Mg^{2+}, and Co^{2+}) as compared to those reported for other mannanases from *Bacillus* sp. (Mendoza *et al.*, 1994; Ma *et al.*, 2004; Yang *et al.*, 2009; Zhang *et al.*, 2009). Thus, the differences in divalent ion inhibition pattern, high hydrophilicity and broad pH stability are the distinguishing characteristics of this enzyme in comparison with other *Bacillus* mannanases.

It is worth noting that heterologous expression of ManA in *B. megaterium* UNcat produced a 33-fold increase in enzyme activity in culture medium compared with that from *B. subtilis* BCC41051. This high expression of recombinant β-mannanase was also obtained in *E. coli* BL21 (DE3) (38-fold). This could be attributed to strong induction of the transfected gene from *xylA* (xylose isomerase) promoter in *B. megaterium* (Rygus and Hillen, 1991; Rygus *et al.*, 1991) and *T7-lac* promoter in *E. coli* (Studier *et al.*, 1990). Also, higher expression could be due to the change of the start codon from TTG to ATG, as seen with expression of *B. subtilis* Z-2 (Zhang *et al.*, 2006) and of *Bacillus* sp. OxB-1 phenylacetaldoxime dehydratase (Kato and Asano, 2003) in *E. coli*. The high expression should facilitate future scale-up preparation of ManA for practical applications. It is possible that mannanase in the culture could be used directly as an enzyme source without further purification.

Purified ManA showed the highest activity at 60°C, and with greater than 80% of the activity remaining after 30 min incubation at 60°C. This property is similar to most *Bacillus* mannanases reported, except that of *B. subtilis* 168 which has an optimum temperature of 37°C (el-Helow and Khattab, 1996).

In summary, a thermostable β-mannanase (ManA) was produced by the thermophilic *B. subtilis* BCC41051 when cultured at 50°C for 36 h. ManA was highly hydrophilic in nature. It showed differences in the inhibition profiles of divalent ions for Fe^{2+}, Ca^{2+}, Co^{2+}, and Mg^{2+} from most *Bacillus* mannanases. Its stability over a broad pH range from 5-11.5 was significantly different from other mannanases reported. Heterologous expression in *E. coli* and *B. megaterium* of the recombinant ManA fused to a hexahistidine tag at the C-terminus resulted in a high yield of the enzyme, with essentially the same specific activity as purified ManA and with ease of purification. The yield with *E. coli* was 415 U/ml and that with *B. megaterium* was 359 U/ml, with a 38- and a 33-fold increase, respectively, over that produced by *B. subtilis* BCC41051 (10.9 U/ml). The ManA secreted by *B. megaterium* was of relatively high purity and could be used directly as an enzyme source without further purification.

Acknowledgements

References

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.

Chang, S. and S.N. Cohen. 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. *Mol. Gen. Genet.* 168, 111-115.

Choct, M. and G. Annison. 1992. The inhibition of nutrient digestion by wheat pentosans. *Br. J. Nutr.* 67, 123-132.

de O. Petkowicz, C.L., F. Reicher, H. Chanzy, F.R. Taravel, and R. Vuong. 2001. Linear mannan in the endosperm of *Schizolobium amazonicum*. *Carbohydr. Polym.* 44, 107-112.

de Vries, R.P. 2003. Regulation of *Aspergillus* genes encoding plant cell wall polysaccharide-degrading enzymes; relevance for industrial production. *Appl. Microbiol. Biotechnol.* 61, 10-20.

el-Helow, E.R. and A.A. Khattab. 1996. The development of a *Bacillus subtilis* 168 culture condition for enhanced and accelerated β-mannanase production. *Acta Microbiol. Immunol. Hung.* 43, 289-299.

Gerhardt, P., R.G.E. Murray, W.A. Wood, and N.R. Krieg. 1994. Methods for General and Molecular Bacteriology. Am. Soc. Microbiol., Washington, D.C., USA.

Hatada, Y., N. Takeda, K. Hirasawa, Y. Ohta, R. Usami, Y. Yoshida, W.D. Grant, S. Ito, and K. Horikoshi. 2005. Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus* sp. strain JAMB-750, its expression in *Bacillus subtilis* and characterization of the recombinant enzyme. *Extremophiles* 9, 497-500.

Heck, J.X., L.H. de Barros Soares, and M.A.Z. Ayub. 2005. Optimization of xylanase and mannanase production by *Bacillus circulans* strain BL53 on solid-state cultivation. *Enzym. Microb. Technol.* 37, 417-423.

Jackson, M.E., D.W. Fodge, and H.Y. Hsiao. 1999. Effects of β-mannanase in corn-soybean meal diets on laying hen performance. *Poult. Sci.* 78, 1737-1741.

Jiang, Z., Y. Wei, D. Li, L. Li, P. Chai, and I. Kusakabe. 2006. High-level production, purification and characterization of a thermostable β-mannanase from the newly isolated *Bacillus subtilis* WY34. *Carbohydr. Polym.* 66, 88-96.

Kato, Y. and Y. Asano. 2003. High-level expression of a novel FMN-dependent heme-containing lyase, phenylacetaldoxime dehydratase of *Bacillus* sp. strain OxB-1, in heterologous hosts. *Protein Expr. Purif.* 28, 131-139.

Khanongnuch, C., K. Asada, H. Tsuruga, T. Ooi, S. Kinoshita, and S. Lumyong. 1998. β-Mannanase and xylanase of *Bacillus subtilis* 5H active for bleaching of crude pulp. *J. Ferment. Bioeng.* 86, 461-466.

Khanongnuch, C., T. Ooi, and S. Kinoshita. 1999. Cloning and nucleotide sequence of β-mannanase and cellulase genes from *Bacillus* sp. 5H. *World J. Microbiol. Biotechnol.* 15, 249-258.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

Langhout, D.J., J.B. Schutte, J. de Jong, H. Sloetjes, M.W. Verstegen, and S. Tamminga. 2000. Effect of viscosity on digestion of nutrients in conventional and germ-free chicks. *Br. J. Nutr.* 83, 533-540.

Lee, J.T., S. Connor-Appleton, C.A. Bailey, and A.L. Cartwright. 2005. Effects of guar meal by-product with and without β-mannanase Hemicell on broiler performance. *Poult. Sci.* 84, 1261-1267.

Ma, Y., Y. Xue, Y. Dou, Z. Xu, W. Tao, and P. Zhou. 2004. Characterization and gene cloning of a novel β-mannanase from alkaliphilic *Bacillus* sp. N16-5. *Extremophiles* 8, 447-454.

Mendoza, N.S., M. Arai, T. Kawaguchi, T. Yoshida, and L.M. Joson. 1994. Purification and properties of mannanase from *Bacillus*

subtilis. *World J. Microbiol. Biotechnol.* 10, 551-555.

Mendoza, N.S., M. Arai, K. Sugimoto, M. Ueda, T. Kawaguchi, and L.M. Joson. 1995. Cloning and sequencing of β-mannanase gene from *Bacillus subtilis* NM-39. *Biochim. Biophys. Acta.* 1243, 552-554.

Moreira, L.R. and E.X. Filho. 2008. An overview of mannan structure and mannan-degrading enzyme systems. *Appl. Microbiol. Biotechnol.* 79, 165-178.

Panbangred, W., K. Weeradechapon, S. Udomvaraphant, K. Fujiyama, and V. Meevootisom. 2000. High expression of the penicillin G acylase gene (*pac*) from *Bacillus megaterium* UN1 in its own *pac* minus mutant. *J. Appl. Microbiol.* 89, 152-157.

Politz, O., M. Krah, K.K. Thomsen, and R. Borriss. 2000. A highly thermostable endo-(1,4)-β-mannanase from the marine bacterium *Rhodothermus marinus*. *Appl. Microbiol. Biotechnol.* 53, 715-721.

Richards, S.A. 1970. The role of hypothalamic temperature in the control of panting in the chicken exposed to heat. *J. Physiol.* 211, 341-358.

Rygus, T. and W. Hillen. 1991. Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Appl. Microbiol. Biotechnol.* 35, 594-599.

Rygus, T., A. Scheler, R. Allmansberger, and W. Hillen. 1991. Molecular cloning, structure, promoters and regulatory elements for transcription of the *Bacillus megaterium* encoded regulon for xylose utilization. *Arch. Microbiol.* 155, 535-542.

Sambrook, J. and D.W. Russell. 2001. Molecular cloning: a laboratory manual, 3rd (ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Sengupta, S., M.L. Jana, D. Sengupta, and A.K. Naskar. 2000. A note on the estimation of microbial glycosidase activities by dinitrosalicylic acid reagent. *Appl. Microbiol. Biotechnol.* 53, 732-735.

Stamatakis, A., P. Hoover, and J. Rougemont. 2008. A rapid boot-strap algorithm for the RAxML web servers. *Syst. Biol.* 57, 758-771.

Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* 185, 60-89.

Talbot, G. and J. Sygusch. 1990. Purification and characterization of thermostable β-mannanase and alpha-galactosidase from *Bacillus stearothermophilus*. *Appl. Environ. Microbiol.* 56, 3505-3510.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25, 4876-4882.

Yang, P., Y. Li, Y. Wang, K. Meng, H. Luo, T. Yuan, Y. Bai, Z. Zhan, and B. Yao. 2009. A novel β-mannanase with high specific activity from *Bacillus circulans* CGMCC1554: gene cloning, expression and enzymatic characterization. *Appl. Biochem. Biotechnol.* 159, 85-94.

Yoon, K.H., S. Chung, and B.L. Lim. 2008. Characterization of the *Bacillus subtilis* WL-3 mannanase from a recombinant *Escherichia coli*. *J. Microbiol.* 46, 344-349.

Zakaria, M.M., S. Yamamoto, and T. Yagi. 1998. Purification and characterization of an endo-1,4-β-mannanase from *Bacillus subtilis* KU-1. *FEMS Microbiol. Lett.* 158, 25-31.

Zhang, M., X.L. Chen, Z.H. Zhang, C.Y. Sun, L.L. Chen, H.L. He, B.C. Zhou, and Y.Z. Zhang. 2009. Purification and functional characterization of endo-β-mannanase MAN5 and its application in oligosaccharide production from konjac flour. *Appl. Microbiol. Biotechnol.* 83, 865-873.

Zhang, J., Z. He, and K. Hu. 2000. Purification and characterization of β-mannanase from *Bacillus licheniformis* for industrial use. *Biotechnol. Lett.* 22, 1375-1378.

Zhang, Q., X. Yan, L. Zhang, and W. Tang. 2006. Cloning, sequence analysis, and heterologous expression of a β-mannanase gene from *Bacillus subtilis* Z-2. *Mol. Biol.* 40, 368-374.