# A β-Mannanase from *Bacillus subtilis* B36: Purification, Properties, Sequencing, Gene Cloning and Expression in *Escherichia coli*

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MANB36, a secrete endo-β-1,4-D-mannanase produced by *Bacillus subtilis* B36, was purified to homogeneity from a culture supernatant and characterized. The optimum pH value for the mannanase activity of MANB36 is 6.4 and the optimum temperature is 50 °C. The enzyme activity of MANB36 is remarkably thermostable at 60 °C and the specific activity of MANB36 is 927.84 U/mg. Metal cations (except $Hg^{2+}$ and $Ag^{+}$), EDTA and 2-mercaptoethanol (2-ME) have no effects on enzyme activity. This enzyme exhibits high specificity with the substituted galactomannan locust bean gum (LBG). The gene encoding for MANB36, *manB36*, was cloned by PCR and sequenced. *manB36* contains a single open reading frame (ORF) consisting of 1104 bp that encodes a protein of 367 amino acids. The predicted molecular weight of 38.13 kDa, calculated by the deduced protein of the gene *manB36* without signal peptide, coincides with the apparent molecular weight of 38.0 kDa of the purified MANB36 estimated by SDS-PAGE. The mature protein of MANB36 has been expressed in *Escherichia coli* BL21 and the expressed mannanase has normal bioactivity.

*Key words:* β-Mannanase, *Bacillus subtilis*

## Introduction

Mannans are polysaccharides with a backbone chain of β-1,4-linked mannose units. They are the main component of hemicelluloses in hardwoods, softwoods and gums extracted from the endosperm of primarily leguminous seed (Kuhad *et al*., 1997). Endo-1,4-β-D-mannanase (EC. 3.2.1.78) catalyzes the random hydrolysis of β-1,4-mannosidic linkages in the main chain of mannan, galactomannan, glucomannan and galactoglucomannan. The released short- and long-chain oligomannosides can be further hydrolyzed to mannose by β-mannosidases. Because of the presence of side chain sugars, α-galactosidase, β-glucosidase and acetylmannan esterase are required to completely hydrolyze mannans (Puls, 1997). β-Mannanases are produced by bacteria (Araujo and Ward, 1990; Ma *et al*., 2004), fungi (Johnson, 1990; Franco *et al*., 2004), plants (Still and Bradford, 1997) and higher organisms (Xu *et al*., 2002).

Over the last few years, the use of mannanase for the biobleaching of wood pulps has attracted considerable interest. Replacement of the highly polluting chemical process used presently in the paper industry by enzyme technology would have a major impact on the environment (Buchert *et al*., 1993). β-Mannanase can be used both for the reduction the viscosity of instant coffee and for the clarification of fruit juices and wines (Wong and Saddler, 1993). Mannanase has wide commercial applications in industry processes such as paper and pulp (Suurnakki *et al*., 1997), foodstuff, feed (Sachslehner *et al*., 2000; Lee *et al*., 2003), pharmaceutical and energy (McCleary, 1991), oil and gas industry (Cutchen *et al*., 1996; Duffaud *et al*., 1997).

The production of β-mannanases by microorganisms is more promising due to its low cost, high production rate, and readily controlled conditions. *B. subtilis* is an useful bacterium producing mannanase which plays a significant role in industrial application. *B. subtilis* B36 conserved by our laboratory can produce β-mannanase named MANB36. In the present work we describe the purification, characterization and *N*-terminal amino acid sequencing of MANB36 as well as the gene cloning and the expression of a mature peptide of the enzyme in *E. coli*.

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## Materials and Methods

### *Materials*

*B. subtilis* B36 was from our laboratory. $T_4$DNA ligase was purchased from Invitrogen Co. (Carlsbad, USA). Plasmid pGEM-T easy was from Promega Co. (Madison, USA). *E. coli* JM109, BL21, plasmid pUC19 and pET-22b(+) were purchased from Novogen Co. (Stamford, USA). Taq polymerase and restriction enzymes were from TakaRa Shuzo Co. (Kyoto, Japan). Locust bean gum (LBG), guar gum (GG) and bovine serum albumin (BSA) were products of Sigma Chemical Co. (St. Louis, USA). Other chemicals used were of analytical grade and available commercially.

### *Medium and culture conditions*

The composition of the medium for enzyme production was as follows: 2% LBG, 2% yeast extract, 0.3% $NH_4Cl$, 0.03% $KH_2PO_4$, 0.3% $CaCl_2$, 0.06% $MgCl_2 \cdot 6H_2O$, 0.35% $Na_2CO_3$, pH 7.0. Incubation was done on an orbital shaker at 37 °C for 48 h. The producing enzyme medium was inoculated with 1% (v/v) inoculum of a cell suspension of shaking cultivation with the medium consisting of 1% tryptone, 0.3% beef extract, 0.5% NaCl, pH 7.0, at 37 °C for 16 h. *E. coli* JM109 used for cloning experiments and *E. coli* BL21 used for expressing experiments were grown at 37 °C in Luria-Bertani (LB) medium containing ampicillin (0.1 mg/mL).

### *Purification and property assay of mannanase MANB36*

*B. subtilis* B36 enzyme production culture medium was centrifuged at $12{,}000 \times g$ for 30 min at 4 °C and the supernatant was precipitated with ammonium sulfate (30%–80% saturation) in an ice bath. The precipitate was dissolved in 100 mL citric acid/NaOH buffer (pH 7.0), then concentrated to 8 ml by a ultra filtration system (5,000-molecular weight cutoff) and the buffer was exchanged by 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.5). The enzyme was put onto a HiTrap_Q_Sepharose_XL column (Pharmacia) equilibrated with the same buffer. Elution was done by NaCl solution with a linear concentration gradient (0–0.6 mol/L) at a flow rate of 3 mL/min. Mannanase fractions were pooled and concentrated by a ultra filtration system, then put on a Superdrex_S200 column (Pharmacia) and eluted with citric acid/NaOH buffer (pH 7.0) at a flow rate of 1 mL/min. Active fractions were pooled as the purified enzyme. The purified enzyme was named MANB36.

The properties of MANB36, including optimum pH and temperature, thermostability, enzyme ability under different pH values and effects of various chemicals on the activity and substrate specific activity, were measured according to the methods of Zhang *et al.* (2002).

### *N-Terminal sequence analysis*

After SDS-PAGE, the purified protein on the polyacrylamide gel was electrophoretically transferred to the PVDF membrane (BBI product) and the fragments of the PVDF membrane blotted protein were used for *N*-terminal amino acid sequencing according to the Edman method by ShangHai GenCore BioTecnologies Company.

### *Enzyme activity assays*

A modified method of Somogyi-Nelson measurement (Somogyi, 1952) was used as a standard method to estimate the mannanase activity under various conditions in this study. The standard assay reaction mixture contained 0.4 mL 0.3% (w/v) LBG dissolved in citric acid/NaOH buffer (pH 6.5) and 0.1 mL diluted enzyme, and the final volume was 0.5 mL. The reaction mixture was incubated at 50 °C for 10 min. One mannanase unit was defined as the amount of enzyme required to liberate 1 $\mu$mol of mannose per min at the assay temperature.

### *Cloning of the manB36 gene*

Genomic DNA of *B. subtilis* B36 using as template was prepared by the method described by Wang *et al.* (2001). The full length gene sequence

Table I. Primers used in this study.

| Primer | Sequence (5′-3′)[a] |
|---|---|
| P1 | CCACCAR[1]AACCAY[2]TCD[3]CC |
| P2 | AATGCV[4]M[5]AS[6]CAR[1]ACR[1]ACAAA |
| P3[b] | GCGAATTCTCTCATCCTTAATGAATGG |
| P4[c] | CAAAGCTTCCAAGCCGAAATGGC |
| P5[d] | CGCCATGGCGCATACTGTGTCGC |
| P6[c] | CGAAGCTTTCACTCAACGATTGGCG |

[a] $R^1$ stands for A or G; $Y^2$ for C or T; $D^3$ for A, G or T; $V^4$ for A, C or G; $M^5$ for A or C; $S^6$ for C or G.
[b] The *Eco*R I restriction site is underlined.
[c] The *Hind* III restriction site is underlined.
[d] The *Nco* I restriction site is underlined.

encoding MANB36 was produced by PCR with the degenerated primers P1 and P2 and the primers P3 and P4 (Table I). The resulting gene was sequenced (ABI3730 Co., SanBo, China) and named *manB36*.

*Expression of manB36 in E. coli*

The nucleotide fragment *manB36P*, encoding for mature protein of MANB36, was obtained with primers P5 and P6 (Table I) and was fused in the expression vector pET-22b(+) by *Nco* I and *Hind* III restriction sites. The generated plasmid pET22b-*manB36P* was transformed into *E. coli* BL21. After cultured in LB medium and induced with isopropyl-$\beta$-D-1-thiogalactopyranoside (IPTG), the cells of positive transformants were harvested by centrifugation and the cell pellet was resuspended in citric acid/NaOH buffer (pH 7.0). The cell suspension was disrupted by sonication. The cell lysate was centrifuged for 10 min at $14{,}000 \times g$ and the supernatant was analyzed by SDS-PAGE and assay of enzyme activity.

## Results and Discussion

*Purification of MANB36*

The results of each step of the purification procedure of MANB36 are presented in Table II. After purification, there was a 178.4-fold increase in enzyme specific activity which was from 5.20 to 927.84 U/mg. The yield of MANB36 was about 7% of the relative mannanase activity of the culture supernatant. The purified MANB36 appeared as a single protein band on SDS-PAGE (Fig. 1) and the molecular weight of the protein was 38.0 kDa.

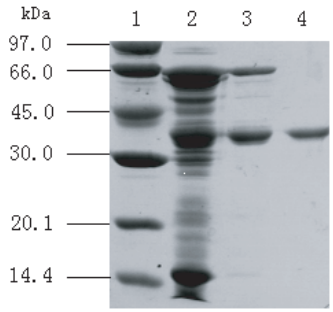


Fig. 1. SDS-PAGE analysis of MANB36. Lane 1, standard protein molecular weight; lane 2, culture supernatant after ammonium sulfate precipitation; lane 3, MANB36 after anion exchange chromatography; lane 4, MANB36 after size chromatography.

*Effects of pH value on the activity and stability of MANB36*

The mannanase activity of MANB36 at various pH values was measured. The reaction pH values were adjusted from 2.2 to 10.0 with citric acid/$Na_2HPO_4$ buffer (pH 2.2–7.5), Tris-HCl buffer (pH 7.5–9.0) and Gly/NaOH (pH 9.0–10.0). The optimum pH of MANB36 was 6.4 and more than 50% of its maximal activity was retained in the pH range from 4.6 to 9.0 (Fig. 2A). No activity was determined below pH 3.0. However, 25% activity was detected at pH 10.0. The stability of the enzyme was determined by incubating the enzyme at 52 °C for 30 min at different pH values, and the residual activity was measured by the standard assay method. The result indicated that MANB36 shows considerable stability; more than 70% of the maximum activity was retained from pH 5.0 to 8.0. Besides, the enzyme retained 44% of the maximum activity at pH 10.0 (Fig. 2B).

*Effects of temperature on the activity and stability of MANB36*

The enzyme had an optimal temperature of 50 °C (Fig. 3A). The thermal stability of MANB36 was measured by incubating the enzyme at 60 °C, 70 °C and 80 °C for various times and the residual activity was measured. MANB36 retained 90% of

Table II. Purification of MANB36 from *Bacillus subtilis* B36.

| Enzyme sample | Volume [mL] | Protein concentration [mg mL$^{-1}$] | Total activity [U] | Specific activity [U mg$^{-1}$] | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|
| Culture supernatant | 800 | 3.579 | 14872 | 5.20 | 100 | 1.0 |
| Ammonium sulfate precipitate | 110 | 4.22 | 12952 | 27.90 | 87 | 5.37 |
| Anion exchange chromatography | 44 | 0.096 | 2671.24 | 632.40 | 18 | 121.62 |
| Size chromatography | 32 | 0.037 | 1098.56 | 927.84 | 7 | 178.43 |

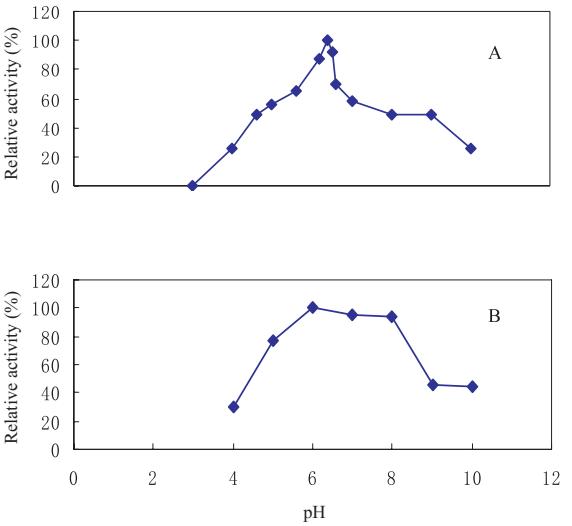


Fig. 2. The optimum pH (A) and pH stability (B) of MANB36.

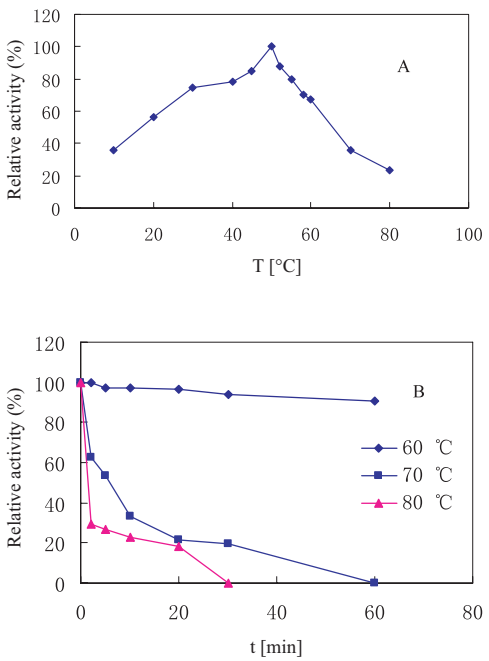


Fig. 3. The optimum temperature (A) and thermal stability (B) of MANB36.

its activity at 60 °C for 60 min and 20% at 70 °C for 30 min. At 80 °C, the enzyme showed 18% of its activity for 20 min, but no activity for 30 min (Fig. 3B).

Table III. Effects of various chemicals on MANB36.

| Chemical | Concentration [mmol/L] | Relative activity (%) |
|---|---|---|
| $FeCl_3$ | 1 | 108 |
| $CaCl_2$ | 1 | 107 |
| $AlCl_3$ | 1 | 106 |
| LiCl | 1 | 105 |
| KCl | 1 | 100 |
| $CrCl_3$ | 1 | 100 |
| $MgCl_2$ | 1 | 100 |
| $CoCl_2$ | 1 | 100 |
| $CuSO_4$ | 1 | 96 |
| $ZnSO_4$ | 1 | 95 |
| $NiSO_4$ | 1 | 95 |
| $C_4H_6O_4Pb$ | 1 | 94 |
| $MnSO_4$ | 1 | 93 |
| $AgNO_3$ | 1 | 3 |
| $HgCl_2$ | 1 | 2 |
| 2-ME[a] | 1 | 100 |
| EDTA[b] | 1 | 97 |
| PMSF[c] | 1 | 83 |
| SDS[d] | 1 | 79 |

[a] 2-ME, 2-mercaptoethanol.
[b] EDTA, ethylenediaminetetraacetic acid.
[c] PMSF, phenylmethylsulfonyl fluoride.
[d] SDS, sodium dodecyl sulfate.

### *Effects of various chemicals on the activity of MANB36 and substrate specificity*

As shown in Table III, MANB36 is strongly inhibited by $Hg^{2+}$, which is similar to the $\beta$-mannanases from *B. subtilis* SA-22 (Yu *et al.*, 2003), KU-1 (Zakaria *et al.*, 1998), BM9602 (Li *et al.*, 2000) and *B. circulans* K-1 (Yosida *et al.*, 1997), but is different from mannanase of alkalophilic *B.* sp. (Akino *et al.*, 1988). Inhibition of MANB36 by $Ag^{+}$ corresponds to the character of mannanases from *B. subtilis* KU-1, BM9602 and alkalophilic *B.* sp. (Akino *et al.*, 1988) whereas differs from that of some $\beta$-mannanases of other strains such as *B. subtilis* SA-22 and *B. circulans* K-1. Unlike the $\beta$-mannanases of *B. subtilis* SA-22 and BM9602 which are inhibited by $Al^{3+}$ and $Ca^{2+}$ and *B. subtilis* KU-1 which is inhibited by $Cr^{3+}$, MANB36 is not inhibited by $Al^{3+}$, $Ca^{2+}$ and $Cr^{3+}$ but slightly inhibited by $Cu^{2+}$ and $Mn^{2+}$.

The actions of the enzyme were assayed on 0.3% LBG, 0.3% guar gum, 0.3% soluble starch, 0.3% amylopectin and 0.3% methylcellulose. As shown in Table IV, MANB36 mainly hydrolyzes LBG and GG with a relative hydrolysis percentage of 100 and 21, respectively, and had no activity for methylcellulose. Both LBG and GG are galac-

Table IV. Substrate specificity of MANB36.

| Polysaccharide | Relative activity (%) | Structrue sugar of polysaccharides | Linkage style |
|---|---|---|---|
| Locust bean gum | 100 | Mannan (main chain)<br>Galactose (side chain) | $\beta$-1,4 (main chain)<br>$\alpha$-1,6 (side chain) |
| Guar gum | 21 | Mannan (main chain)<br>Galactose (side chain) | $\beta$-1,4 (main chain)<br>$\alpha$-1,6 (side chain) |
| Soluble starch | 26 | Glucose | $\alpha$-1,4 (main chain)<br>$\alpha$-1,6 (side chain) |
| Amylopectin | 2 | Glucose | |
| Methylcellulose | ND | Glucose | $\alpha$-1,4 (main chain)<br>$\alpha$-1,6 (side chain) |

ND, not detected.

tomannans with the same linkage style, but GG molecules are more galacto-substituted than LBG molecules, thus MANB36 prefers mannan with a low level of galacto substitution as the substrate. The significant activity of MANB36 on soluble starch and low activity on amylopectin, a kind of insoluble polysaccharides, were detected. The ability to hydrolyze $\alpha$-1,4-linkage of glucose in soluble starch is in disparity from other $\beta$-mannanases of *B. subtilis* reported so far, which suggests that MANB36 is a rare mannanase with activity of amylase.

*Assay for specific activity of purified MANB36*

With BSA as a standard, the protein concentration of purified MANB36 was assayed by the Coomassie Brilliant Blue G-250 method. The specific activity of purified MANB36 was confirmed to be 927.84 U/mg.

*The resistance to pepsin and trypsin of MANB36*

Purified MANB36 was treated with 80 U/mL pepsin or trypsin for 2 h at 37 °C, respectively. The residual activity was measured by the standard assay method. MANB36 treated with trypsin retained 60% of maximum activity, but had no activity after treatment by pepsin. Therefore, MANB36 has definite resistance to trypsin. This may be the first report about this specific feature.

*N-Terminal amino acid sequences of MANB36*

The sequence of eight *N*-terminal amino acids of purified MANB36 was determined as HTVSPVNP.

*Cloning and sequence analysis of the gene manB36*

For the isolation of a specific fragment of the target gene, two degenerate primers P1 and P2 were designed based on highly conserved regions in five $\beta$-mannanase gene sequences of different *B. subtilis* strains registered in Genbank. A 501 bp nucleotide fragment was generated and showed the highest identity (98.8%) with the mannanase gene from *B. subtilis* strain Z-2 (GenBank accession number AY827489). The primers P3 and P4 were designed based on the mannanase gene sequence of *B. subtilis* strain Z-2. As presumed, a fragment of about 1.3 kb was produced and named as *manB36*. The determined sequence (1283 bp in size) has been deposited in the GenBank database under accession number DQ351940.

*manB36* contains a single ORF consisting of 1,104 nucleotides encoding a protein of 367 amino acids with a predicted molecular weight of 41.603 kDa. The *N*-terminal amino acid sequence (HTVSPVNP) determined from the purified MANB36 coincides precisely with the sequence starting from His-32 to Pro-39 in the deduced amino acid sequence. Thus the signal peptide MANB36 is probably cleaved between Ala-31 and His-32. Removal of the 31 *N*-terminal residues yields a polypeptide with a calculated molecular weight of 38.13 kDa, which is in good agreement with the molecular mass of purified MANB36 (38 kDa).

$\beta$-Mannanase sequences are classified as glycosyl hydrolase family 5 and 26 based on amino acid sequence similarity (Henrissat, 1991), and both families are in clan GH-A of retaining enzymes (Henrissat *et al.*, 1995). The amino acids 37 to 355

of MANB36 are 98.4% identical to amino acids 32 to 350 of mannanase of *B. subtilis* Z-2, which composes the catalytic domain of family 26 (http://www.sanger.ac.uk/software/Pfam). Glu-212 and Glu-320 in mature ManA (Bolam *et al*., 1996) are the catalytic carboxylic amino acids and are strictly conserved in the enzymes of family 26 (Bolam *et al*., 1996). The corresponding residues in MANB36 are Glu-183 and Glu-297. Accordingly, ManB36 is classified as a member of glycosyl hydrolase family 26.

*Expression of MANB36 in E. coli*

No mannanase activity was detected from the crude extracts of the control strain whereas distinct activity was confirmed in BL21::pET22-*manB36P* cell culture. The result of SDS-PAGE analysis indicated that the molecular weight of the expressed *manB36P* protein is about 38.0 kDa. The expressed MANB36 has the mannanase activity 32.9 U/mL.

To sum up, MANB36 carries some characters that are apparently different from those of $\beta$-mannanases from other *B. subtilis* strains and this implies that MANB36 could be a novel candidate enzyme for further application. Further experiments to overexpress the *manB36* gene in *Pichia pastoris* and the study of the active site of MANB36 are in progress.

Akino T., Nakamura N., and Horikoshi K. (1988), Characterization of three $\beta$-mannanases of an alkalophilic *Bacillus* sp. Agric. Biol. Chem. **52**, 773–779.

Araujo A. and Ward O. P. (1990), Hemicellulases of *Bacillus* species: preliminary comparative studies on production and properties of mannanases and galactanases. J. Appl. Bacteriol. **68**, 253–261.

Bolam D. N., Hughes N., Virden R., Lakey J. H., Hazlewood G. P., Henrissat B., Braithwaite K. L., and Gilbert H. J. (1996), Mannanase A from *Pseudomonas fluorescens* ssp. *cellulosa* is a retaining glycosyl hydrolase in which E212 and E320 are the putative catalytic residues. Biochemistry **35**, 16195–16204.

Buchert J., Salminen J., Sika-aho M., Ranua M., and Viikari L. (1993), The role of *Trichoderma reesei* xylanase and mannanase in the treatment of softwood kraft pulp prior to bleaching. Holzforschung **47**, 473–478.

Cutchen M. C., Duffaud D. G., Leduc P., Peterson H. A. R., Tayal A., Khan A. S., and Kelly M. R. (1996), Characterization of extremely thermostable enzymatic breakers ($\alpha$-1,6-galactosidase and $\beta$-1,4-mannanase) from the hyperthermophilic bacterium *Thermotoga neapolitana* 5068 for hydrolysis of guar gum. Biotechnol. Bioeng. **52**, 332–339.

Duffaud G. D., McCuthen C. M., Leduc P., Parker K. N., and Kelly R. M. (1997), Purification and characterisation of extremely thermostable $\beta$-mannanase, $\beta$-mannosidase and $\alpha$-galactosidase from the hyperthermophilic eubacterium *Thermotoga neapolitana* 5068. Appl. Environ. Microbiol. **63**, 169–177.

Franco P. F., Ferreira H. M., and Filho E. X. (2004), Production and characterization of hemicellulase activities from *Trichoderma harzianum* strain T4. J. Biol. Chem. **279**, 22953–22963.

Henrissat B. (1991), A classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem. J. **280**, 309–316.

Henrissat B., Callebaut I., Fabrega S., Lehn P., Mornon J. P., and Davies G. (1995), Conserved catalytic machinery and the prediction of a common fold for several families of glycosyl hydrolases. Proc. Natl. Acad. Sci. USA **92**, 7090–7094.

Johnson K. G. (1990), Exocellular beta-mannanases from hemi-cellulolytic fungi. World J. Microbiol. Biotechnol. **6**, 209–217.

Kuhad R. C., Singh A., and Eriksson K. E. (1997), Microorganisms and enzymes involved in the degradation of plant fiber cell walls. Adv. Biochem. Eng. Biotechnol. **57**, 45–125.

Lee J. T., Bailey C., and Cartwright A. L. (2003), Beta-mannanase ameliorates viscosity-associated depression of growth in broiler chickens fed guar germ and hull fractions. Poult Sci. **82**, 1925–1931.

Li W., Dong Z., and Cui F. (2000), Purification and characterization of an endo-beta-1,4-mannanase from *Bacillus subtilis* BM9602. Wei Sheng Wu Xue Bao **40**, 420–424.

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

McCleary B. V. (1991), Comparison of endolytic hydrolases that depolymerize 1,4-$\beta$-D-mannan, 1,5-$\beta$-L-arabinan and 1,4-$\beta$-D-galactan. In: Enzymes in Biomass Conversion (Leatham G. F. and Himmel M. E., eds.). ACS Symp. Ser., Washington D.C., Vol. 460, pp. 437–449.

Puls J. (1997), Chemistry and biochemistry of hemicelluloses-relationship between hemicellulose structure and enzymes required for hydrolysis. Macromol. Symp. **120**, 183–196.

Sachslehner A., Foidl G., Foidl N., and Gubitz G. (2000), Hydrolysis of isolated coffee mannan and coffee extract by mannanases of *Sclerotium rolfsii*. J. Biotechnol. **80**, 127–134.

Somogyi M. (1952), Notes on sugar determination. J. Biol. Chem. **195**, 19–23.

Still D. W. and Bradford K. J. (1997), Endo-[beta]-mannanase activity from individual tomato endosperm caps and radicle tips in relation to germination rates. Plant Physiol. **113**, 21–29.

Suurnakki A., Tenkanen M., Buchert J., and Viikari L. (1997), Hemicellulases in the bleaching of chemical pulps. Adv. Biochem. Eng. Biotechnol. **57**, 261–287.

Wang S. Y., Wu S. J., Thottappilly G., Locy R., and Singh N. (2001), Molecular cloning and structural analysis of the gene encoding *Bacillus cereus* exochitinase Chi36. J. Biosci. Bioeng. **92**, 59–66.

Wong K. K. Y. and Saddler J. N. (1993), Applications of hemicellulases in the food, feed, and pulp and paper industries. In: Hemicellulose and Hemicellulases (Coughlan M. P. and Hazlewood G. P., eds.). Portland Press Ltd, London, pp. 127–143.

Xu B., Hagglund P., Stalbrand H., and Janson J. C. (2002), Endo-beta-1,4-mannanases from blue mussel, *Mytilus edulis*: purification, characterization, and mode of action. J. Biotechnol. **92**, 267–277.

Yosida S., SakoY., and Uchida A. (1997), Purification, properties, and *N*-terminal amino acid sequence of guar gum-degrading enzyme from *Bacillus circulans* K-1. Biosci. Biotech. Biochem. **61**, 251–255.

Yu H. Y., Sun Y. M., Wang W. J., Yang Y. S., and Yang Y. H. (2003), Purification and properties of *Bacillus subtilis* SA22 endo-1,4-$\beta$-D-mannanase. Chinese J. Biotechnol. **19**, 327–331.

Zakaria M. M., Yamamoto S., and Yagi T. (1998), Purification and characterization of endo-1,4-$\beta$-mannanase from *Bacillus subtilis* KU-1. FEMS Microbiol. Lett. **158**, 25–31.

Zhang H. L., Yao B., Yuan T. Z., Wang Y. R., Cao S. S., and Fan Y. L. (2002), Purification and properties of 43 kD xylanase XYNA from *Streptomyces olivaceoviridis* A1. J. Agric. Biotechnol. **10**, 10–14.