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Cloning, High-Level Expression, Purification, and Properties of a Novel Endo-β-1,4-Mannanase from *Bacillus subtilis* G1 in *Pichia pastoris*

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A novel gene coding for an endo-β-1,4-mannanase (*manA*) from *Bacillus subtilis* strain G1 was cloned and overexpressed in *P. pastoris* GS115, and the enzyme was purified and characterized. The *manA* gene consisted of an open reading frame of 1,092 nucleotides, encoding a 364-aa protein, with a predicted molecular mass of 41 kDa. The β-mannanase showed an identity of 90.2–92.9% (≤95%) with the corresponding amino acid sequences from *B. subtilis* strains deposited in GenBank. The purified β-mannanase was a monomeric protein on SDS-PAGE with a specific activity of 2,718 U/mg and identified by MALDI-TOF mass spectrometry. The recombinant β-mannanase had an optimum temperature of 45°C and optimum pH of 6.5. The enzyme was stable at temperatures up to 50°C (for 8 h) and in the pH range of 5–9. EDTA and most tested metal ions showed a slightly to an obviously inhibitory effect on enzyme activity, whereas metal ions (Hg^{2+}, Pb^{2+}, and Co^{2+}) substantially inhibited the recombinant β-mannanase. The chemical additives including detergents (Triton X-100, Tween 20, and SDS) and organic solvents (methanol, ethanol, *n*-butanol, and acetone) decreased the enzyme activity, and especially no enzyme activity was observed by addition of SDS at the concentrations of 0.25–1.0% (w/v) or *n*-butanol at the concentrations of 20–30% (v/v). These results suggested that the β-mannanase expressed in *P. pastoris* could potentially be used as an additive in the feed for monogastric animals.

Keywords: *Bacillus subtilis* G1, endo-β-1,4-mannanase gene, cloning, overexpression, *Pichia pastoris*, properties

Hemicelluloses, the second most abundant polysaccharides in nature, in plant cell walls, consists of mannans classified into four subfamilies: linear mannan, glucomannan, galactoglucomannan, and galactomannan.

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β-D-Mannanase (endo-1,4-β-D-mannan mannohydrolase, E.C. 3.2.1.78) hydrolyzes 1,4-β-D-mannopyranosyl linkages within the main chain of mannans, glucomannans, galactomannans, and galactoglucomannans. Mannans and heteromannans are a part of the hemicellulose fraction in plant cell walls. Thus, β-mannanases have been used widely in the food, animal feed, detergent, and paper and pulp industries [1, 3].

For further applications, several genes encoding β-mannanases from *Bacillus* species have been cloned and expressed in heterologous expression systems, and properties of the recombinant β-mannanases have been characterized. β-Mannanases from *Bacillus* species including *B. subtilis* WL-7 [10], *B. subtilis* WL-3 [24], *B. licheniformis* [20], *B. subtilis* B36 [13], *B. circulans* CGMCC 1416 [12], and *B. stearothermophilus* [4] were expressed in *E. coli* cells with a high specific activity of up to 10,080 U/mg. Heterologous expression of *Bacillus* β-mannanase by a yeast expression system provides an alternative approach. However, β-mannanases from *Bacillus* sp. including *B. subtilis* MA139 [17] and alkaliphilic *Bacillus.* sp. N16-5 [6] were expressed in the eukaryote yeast *P. pastoris* with a low specific activity of up to 85 U/mg protein. Thus, our study focused on the high-level expression of a novel mannanase from *B. subtilis* G1 in this host strain, and the purified enzyme showed a highest specific activity of 2,718 U/mg protein (32 times as high) among *Bacillus* mannanases ever expressed in *P. pastoris*.

Materials and Methods

Chemicals and Reagents

Locust bean gum (LBG) was from Sigma-Aldrich Co. (St. Louis, USA); peptone and yeast extract were purchased from Bio Basic Inc. (New York, USA); and 3,5-dinitrosalicylic acid (DNS) was from Fluka, a Sigma-Aldrich Company (St. Louis, USA). Restriction enzymes, *Taq* DNA polymerase, and T4 ligase were supplied from Fermentas, part of Thermo Fisher Scientific Inc. (Waltham, USA).

The kit ProBond™ Nickel-Chaleting Resin was obtained from Invitrogen Corp. (Carlsbad, USA).

Vectors, Strains, and Culture Conditions

The bacterial strain *Bacillus subtilis* G1 from the Laboratory of Molecular Microbiology, Institute of Biotechnology, Hanoi, Vietnam was used as the source of the β-mannanase gene. *Escherichia coli* DH5α and pJET1/blunt (Fermentas, Thermo Fisher Scientific Inc., Waltham, USA) were used for DNA manipulations and amplification. *Pichia pastoris* host strain GS115 and pPICZαA (Invitrogen Corp., Carlsbad, USA) were used for expression of the β-mannanase. Luria–Bertani medium (LB) containing 1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl, at a pH of 7–7.5, was used for the cultivation of *E. coli* and *B. subtilis*. LB agar contained additionally 2% (w/v) agar and 100 μg ampicillin/ml; low salt LB [1% (w/v) bacto tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] agar contained 25 μg zeocin/ml.

DNA Manipulations

Genomic and plasmid DNA isolation was carried out by methods that have been previously described [18]. DNA fragments and PCR products were excised from a 0.8% agarose gel and purified by a gel extraction kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. DNA sequencing was performed on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). *E. coli* DH5α was transformed using the heat shock method as previously described [18].

DNA Amplification and Plasmid Construction

The putative endo-β-1,4-mannanase (ManA)-coding DNA fragment was amplified from *B. subtilis* G1 genomic DNA by PCR with *Taq* DNA polymerase. Based on the DNA sequence of the putative *manA* gene from the complete genome of *Bacillus subtilis* subsp. *subtilis* str. 168 (GenBank: NC_000964), two oligonucleotides, ManF (5'-GCG CGG CCG CGG GGA GTT GCA TTT-3') and ManR (5'-GCT CTA GAG GCT CAA CGA TTG GCGT-3'), were designed as primers to amplify the gene *manA* from *B. subtilis* G1 with introduction of the (underlined) *Not*I and *Xba*I restriction sites at 5' of the forward and reverse primers, respectively.

The PCR mixture contained 2.5 μl of 10× PCR buffer, 2 μl of 2.5 mM dNTP, 2 μl of 25 mM $MgCl_2$, 1 μl of genomic DNA (50 ng), 0.5 μl of 5 unit *Taq* polymerase, and 1 μl of each primer (10 pmol), supplemented with 15 μl of distillated water to a final volume of 25 μl. The thermocycler conditions were as follows: 94°C/3 min; 35 cycles of 94°C/1 min, 54°C/1 min, 72°C/1 min; and 72°C/10 min. The PCR product was inserted into the pJET1/blunt vector, resulting in pJMan, and sequenced. The obtained DNA sequence was aligned with coding sequences from GenBank using the DNAStar program. The *manA* obtained from pJMan digested by *Not*I and *Xba*I was inserted into pPICZαA, resulting in the recombinant plasmid pPMan under the control of the AOX1 promoter induced by methanol and possessing the zeocin marker. The mannanase Manhis encoded by the plasmid pPMan contains the alpha factor from *S. cerevisiae* as leader, the mature mannanase, and the 6× histidine tag.

Yeast Transformation and Screening

The plasmid pPMan linearized with *Sac*I was then transformed into *P. pastoris* GS115 according to the manufacturer's instructions for the EasySelect *Pichia* Expression Kit (Invitrogen Corp., Carlsbad, USA). Transformants were screened on YPDS [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 1 M sorbitol, and 2% (w/v) agar] plates containing zeocin at a final concentration of 1,000 μg/ml. The presence of the mannanase gene in the transformants was confirmed by PCR using yeast genomic DNA as a template and *manA*-specific primers.

Gene Expression

The transformant *P. pastoris* GS115/pPMan exhibiting mannanase actvity was grown in 20 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone, 4×10^{-5} (w/v) biotin] added 1% (w/v) glycerol containing an appropriate concentration of zeocin at 30°C, with agitation at 220 rpm until an $OD_{600\ nm}$ of 5–6 was reached. The cell pellet was harvested by centrifugation at 6,000 rpm for 5 min. For *AOX1* promoter-controlled expression of β-mannanase, the cell pellet was resuspended in 25 ml of YP medium [1% (w/v) yeast extract, 2% (w/v) peptone, 4×10^{-5}% (w/v) biotin] added 1% (v/v) methanol and methanol was added to a final concentration of 1% (v/v) every 24 h to maintain induction. Cultivation was performed at 30°C and 220 rpm. The culture supernatant was collected periodically to detect for the expression of the β-mannanase.

Purification of Recombinant β-Mannanase

The culture supernatant containing the β-mannanase was applied to a ProBond Ni^{2+}-charged affinity chromatography column (Invitrogen Corp., Carlsbad, USA). The purification of the recombinant His-tagged β-mannanase was carried out according to the manufacturer's instructions.

Protein Electrophoresis and Quantification

The homogeneity and molecular mass of the β-mannanase were determined by 12.5% SDS polyacrylamide gel electrophoresis with Biometra equipment (Göttingen, Germany) [11]. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Protein concentrations were measured by Bradford assay with bovine serum albumin as the standard [2].

MALDI-TOF Mass Spectrometry

The β-mannanase was identified by MALDI-TOF mass spectrometry as previously described [21]. The predicted protein band on SDS-PAGE was cut out and the target protein was digested by trypsin treatment into small peptide fragments. The mixture of peptides was analyzed by nano-LC liquid chromatography and ionized by ESI (electrospray ionization). The mass spectra were obtained on the QSTAR, XL mass spectrometer (Applied Biosystems, MDS SCIEX, Canada) with a nano-ESI ion source. Protein fragments were identified by the Mascot v.1.8 Search Software from the database (NCBInr, SwissProt). Peptide fragments showing ion scores above 42 were identified uniquely or high-similarly with $p<0.05$.

Enzyme Activity Estimation

The β-mannanase activity of the culture medium from methanol-induced expression strains was assayed by the 3,5-dinitrosalicylic acid (DNS) method for reducing sugar analysis [15], using 0.5% (w/v) locust bean gum as the substrate in 20 mM potassium phosphate buffer (pH 7). The reactions were carried out at 40°C for 5 min. One enzyme unit was defined as that liberating 1 μmol of reducing sugar (glucose) from the substrate solution per minute under standard assay conditions.

Temperature and pH Optima

The temperature and pH optima of the β-mannanase were determined by measuring the activity, as described above, using 20 mM potassium phosphate buffer (pH 6.5) in the temperature range of 30–70°C; and 20 mM acetate buffer (pH 4–5), phosphate buffer (pH 6–8), and 20 mM Tris-HCl buffer (pH 8–9) at 45°C, respectively.

Temperature and pH Stability

For the determination of temperature and pH stability, the purified enzyme, 0.17 μg for each reaction, was preincubated at the temperature range of 30–60°C and at pH 6.5 for 1–8 h, and under various pH conditions (with 100 mM acetate at pH 3–5, 100 mM phosphate at pH 6–8, and 100 mM Tris buffer at pH 9) and at 30°C for 4 h, respectively. The residual activity was then determined.

Effects of Metal Ions, Organic Solvents, and Detergents

The purified enzyme, 0.17 μg for each reaction, was preincubated in the presence of 1–5 mM of various metal ions (Ca^{2+}, Cu^{2+}, Fe^{3+}, Fe^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, K^{+}, Ag^{+}) and EDTA or 0.5–1.5 mM of Co^{2+}, Hg^{2+}, and Pb^{2+}, in the presence of 10–30% (v/v) of various solvents (methanol, ethanol, isopropanol, *n*-butanol, and acetone), and in the presence of 0.25–1% (w/v) of various detergents (SDS, Triton X-100, and Tween 20) at 37°C for 24 h. The residual activity was then determined.

All measurements were carried out in triplicate, with the resulting values being the mean of the cumulative data obtained.

RESULTS AND DISCUSSION

Gene Cloning and Analysis

The gene encoding an endo-β-1,4-mannanase was amplified by PCR with specific primers and sequenced. The 1,092 bp insert revealed one complete ORF, predicted to encode an endo-β-1,4-mannanase (364 aa, 41.2 kDa, with a pI of 6.33). Using the SignalP predictions (http://www.cbs.dtu.dk/services/SignalP-2.0/), it was revealed that the putative β-mannanase had a signal peptide of 28 amino acids [16]. The gene and its putative β-mannanase were aligned with sequences from the GenBank using DNAstar. The sequence of the gene *manA* from *B. subtilis* G1 showed an identity of 93.7–97.1% with corresponding sequences from *B. subtilis* strains [DQ351940 (97.1%), AF324506 (96.7%), AY601725 (96.4%), AY827489 (96.2%), AX002665 (96.1%), AL009126 and D88802 (95.9%), AB016163 (93.7%)]. The putative amino acid sequence of ManA showed an identity of 90.2–92.9% (lower than 93%) with the corresponding amino acid sequences from *B. subtilis* strains [AY601725 (92.9%), AY827489 (92.4%), AL009126 and D88802 (92.1%), AB016163 (91.8%), DQ351940 (90.7%), AX002665 (90.7%), AF324506 (90.2%)]. The sequence was deposited in GenBank with an accession number of DQ309335. This might be a novel endo-β-1,4-mannanase because of a low identity of 90.2–92.9% (≤93%) with the corresponding amino acid sequences with *B. subtilis* strains deposited in GenBank.

The β-mannanase gene from *B. subtilis* strains showed almost the same length: (gene/mature protein/signal peptide): 1,080 bp/336 aa/24 aa [14]; 1,086 bp/336 aa/26 aa [9, 10]; 1,014 bp/338 aa/- [17]; 1,080 bp/336 aa/24 aa [23]; 1,104 bp/367 aa/- [13]. The β-mannanase gene *manA* from *B. subtilis* strain G1 in this study was also similar with 1,092 bp/34 aa/28 aa.

The nucleotide sequence of the β-mannanase gene from other *Bacillus* species had a longer length. The nucleotide sequence of the mannanase gene *manF* from *B. stearothermophilus* corresponded to an open reading frame of 2,085 bp that codes for a 32-amino-acid signal peptide and a mature protein with a molecular mass of 76 kDa [4]. The sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus* sp. strain JAMB-750 had an open reading frame of 2,994 bp, encoding a protein of 997 amino acids [5].

Expression and Purification of Recombinant Mannanase

P. pastoris GS115/pPMan transformants were cultivated in YP medium for the β-mannanase production. After 108 h of methanol induction, the culture supernatants were collected and used for enzyme activity assay. The *P. pastoris* GS115/pPMan transformant showing the highest production of the β-mannanase (224 U/ml, Fig. 1A) was used for enzyme

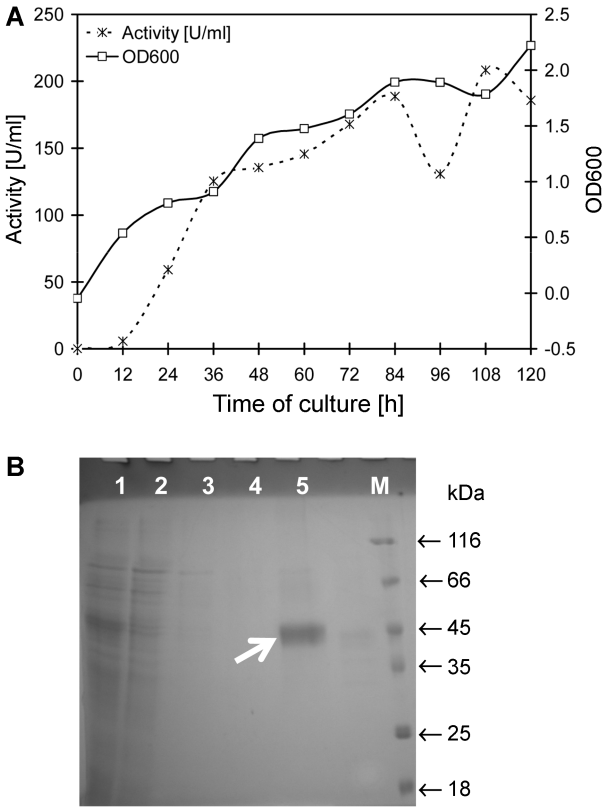

Fig. 1. Production of the β-mannanase during growth of recombinant strain M1 (**A**) and SDS-PAGE of *B. subtilis* β-mannanase expressed in *P. pastoris* (**B**).

Lane 1: Culture supernatant of M1; Lanes 2, 3, 4: Column washing solutions; Lane 5: eluate of the recombinant β-mannanase; Lane M: molecular mass standard protein.

production, purification, and characterization. The induction of 1% (v/v) of methanol showed the highest production of the recombinant β-mannanase among different amounts of methanol induction from 0.5% to 2% (data not shown). The recombinant β-mannanase was purified from the culture supernatant of *P. pastoris* GS115/pPMan by affinity chromatography Ni^{2+}-ProBond™ resin and showed only one protein band of about 45 kDa on SDS-PAGE (Fig. 1, lane 5), a little higher than the calculated value (41.2 kDa). Using the glycosylation prediction program NetOGlyc-3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/) it was revealed that no threonine or serine was the predicted *O*-glycosylation site in the ManA sequence, with a G-score of less than 0.442 (threonine 360). If the G-score is >0.5, the residue is predicted as being glycosylated; the higher the score, the more confident the prediction [7]. However, the ManA sequence was predicted to be *N*-glycosylated (Asn-Xaa-Ser/Thr sequons) in the asparagine position 54 of the Man sequence (NRT) using the glycosylation prediction program NetNGlyc-1.0 (http://www.cbs.dtu.dk/services/NetNGlyc-1.0/), with a predicted potential of 0.7513 (threshold 0.5). It demonstrated why the obtained protein showed a higher molecular mass (45 kDa) than the putative one (41.2 kDa).

The protein expression level of the recombinant β-mannanase in the culture medium was 155 mg/l. The purified β-mannanase had a specific activity of 2,718 U/mg, which was lower than that from *B. subtilis* WL-7 (10,080 U/mg) [10] and *B. subtilis* WL-3 (5,900 U/mg) [24], but higher than that from *B. subtilis* B36 (928 U/mg) [13] and *B. subtilis* MA139 (85 U/mg) [17]. However, the recombinant β-mannanase showing a higher specific activity [from *B. subtilis* WL-7 (10,080 U/mg) and *B. subtilis* WL-3 (5,900 U/mg)] were both expressed in *E. coli*. In this study, the recombinant β-mannanase from *B. subtilis* G1 expressed in the yeast *P. pastoris* showed the highest specific activity (2,718 U/mg, 32 times higher) in comparison with the recombinant β-mannanase from *B. subtilis* MA139 (85 U/mg). The *Bacillus* β-mannanase was also expressed in *B. subtilis*, but showed an insignificant activity (36.3 U/mg) [5] in comparison with those expressed in *E. coli* (10,080 U/mg) [10] or *P. pastoris* (2,718 U/mg) (this study).

The activity discussed here for the other recombinant β-mannanases was the enzyme activity toward the same substrate locust bean gum. β-Mannanases (E.C. 3.2.1.78) catalyze the same reaction: the random hydrolysis of β-1,4-mannosidic linkages in the backbones of β-mannan, glucomannan, and galactomannan, which consist of a β-1,4-linked linear backbone of mannose residues that carry other carbohydrates (glucose and galactose) or acid substitutions. Locust bean gum (LBG, also known as carob gum, carob bean gum, carobin, E410) is a galactomannan vegetable gum extracted from the seeds of the Carob tree, found mostly in the Mediterranean, and consists of mannose and galactose at a ratio of 4:1.

Besides the substrate LBG used most for the β-mannanase activity assay in these studies, konjac (glucomannan) and guar gum (mannose:galactose ~2:1) was also used for substrate specificity assay. Konjac and guar gum showed a lower β-mannanase activity in comparison with LBG (97% and 11.3% [24], 70% and 13% [10], 85% and 7% [8], respectively). Only the mannan endo-1,4-β-mannosidase from *Bacillus licheniformis* DSM13 showed a higher β-mannanase activity toward konjac (219%) and 1,4-β-D-mannan (166%) in comparison with LBG (100%) [20]. The recombinant mannanase from *B. circulans* K-1 was more active toward konjac (200%) than LBG (100%) and guar gum (9.6%) [25].

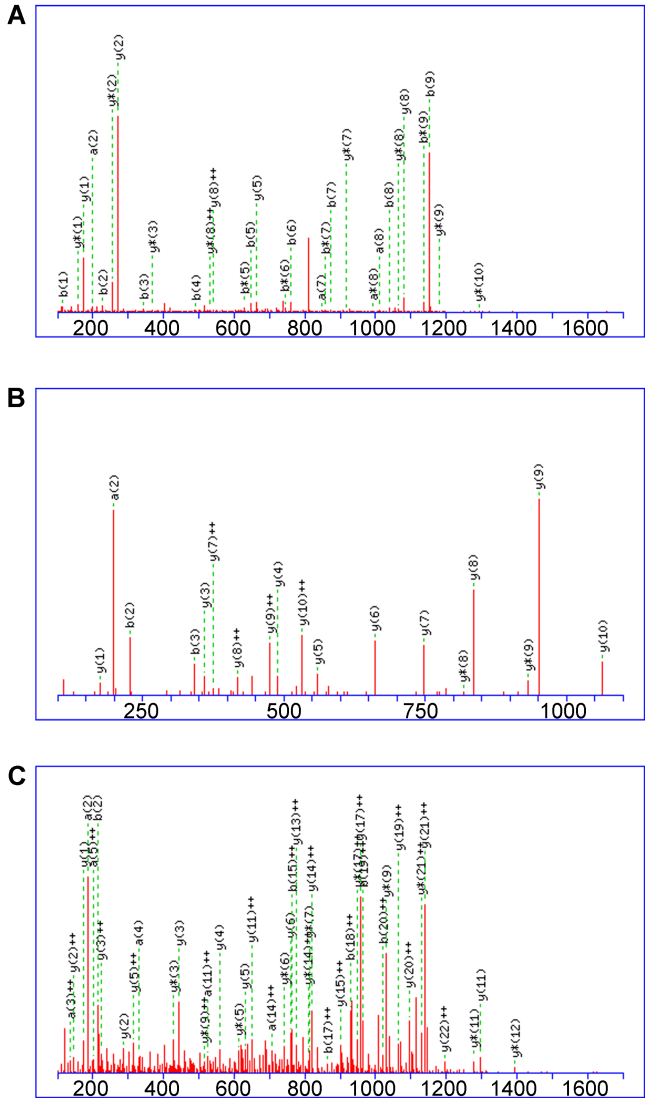

Fig. 2. Monoisotopic mass of neutral peptides ILDSSTAEGKR, position 156–166 (**A**); GLDHLIWVYSPDANR, position 235–249 (**B**); and VLSGAFGGYSHDTFSMAEADRIR, position 60–82 (**C**), found in gi|56182702, beta-1,4-mannanase (*Bacillus subtilis*), corresponding to ion scores of 65, 57, and 74, respectively.

Three peptide fragments of the purified enzyme identified by MALDI-TOF mass spectrometry agreed with those of the mannanase found in gi|56182702, beta-1,4-mannanase (*Bacillus subtilis*) ILDSSTAEGKR (position 156–166); GLDHLIWVYSPDANR (position 235–249); and VLSGAF GGYSHDTFSMAEADRIR (position 60–82) (Fig. 2), corresponding to ion scores of 65, 57, and 74, respectively. The peptide fragments showing ion scores above 42 were identified uniquely or high-similarly with $p<0.05$. These peptides showed an identity of 100% with the corresponding fragments of the putative ManA protein.

Temperature and pH Optima

The recombinant β-mannanase from *B. subtilis* G1 had an optimum temperature of 45°C (Fig. 3A) and showed high activity (≥93%) at the temperature range of 40–55°C in comparison with the maximum activity (Fig. 3A). The purified β-mannanase had an optimum pH of 6.5 (Fig. 3B), and more than 60% of the maximum activity was detectable at pH 7.5. Both ManA expressed in *P. pastoris* and expressed in *E. coli* in our previous study [19] showed a similar profile of optimum temperature and pH (45°C, pH 6.5 and 40°C, pH 7, respectively).

Our recombinant β-mannanase from *B. subtilis* G1 showed a similar value of pH optimum (pH 6.5) but a little lower value of temperature optimum (45°C) in comparison with other *B. subtilis* recombinant β-mannanases (pH 5–6.5, 45–65°C). The optimal activity of the recombinant β-mannanase from *B. subtilis* strains was at pH 6 and 50°C for β-mannanase from *B. subtilis* MA139 in *P. pastoris* [17]; pH 6 and 60°C: *B. subtilis* WL-3 in *E. coli* [24]; pH 6 and 55°C: *B. subtilis* WL-7 in *E. coli* [10]; pH 6.4 and 50°C: *B. subtilis* B36 in *E. coli* [13]; and pH 5–6.5 and 45–65°C: *B. subtilis* strains CD-3, CS-6, CD-9, CD-10, CD-23, CD-25 [22].

pH and temperature optima for the mannanase from other *Bacillus* species were pH 10 and 70°C: *Bacillus* sp. N16-5 in *P. pastoris* [6]; pH 6–7 and 50–60°C: *B. licheniformis* strain DSM13 in *E. coli* [20]; pH 7.6 and 58°C: *B. circulans* strain CGMCC 1416 in *E. coli* [12]; and pH 6.5 and 70°C: *B. stearothermophilus* [4] (Table 4).

Temperature and pH Stability

The recombinant β-mannanase was stable at temperatures below 50°C (for 8 h), activated by incubation at 30–40°C within 15 h with an increase by 22–113% (Fig. 4A). This

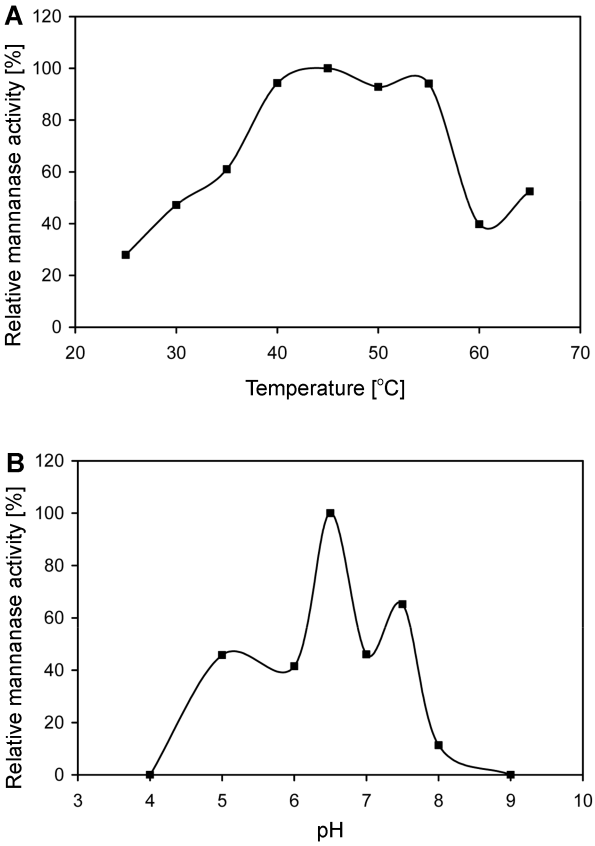

Fig. 3. Temperature (**A**) and pH (**B**) optima of the β-mannanase from *B. subtilis* G1 expressed in *P. pastoris*.

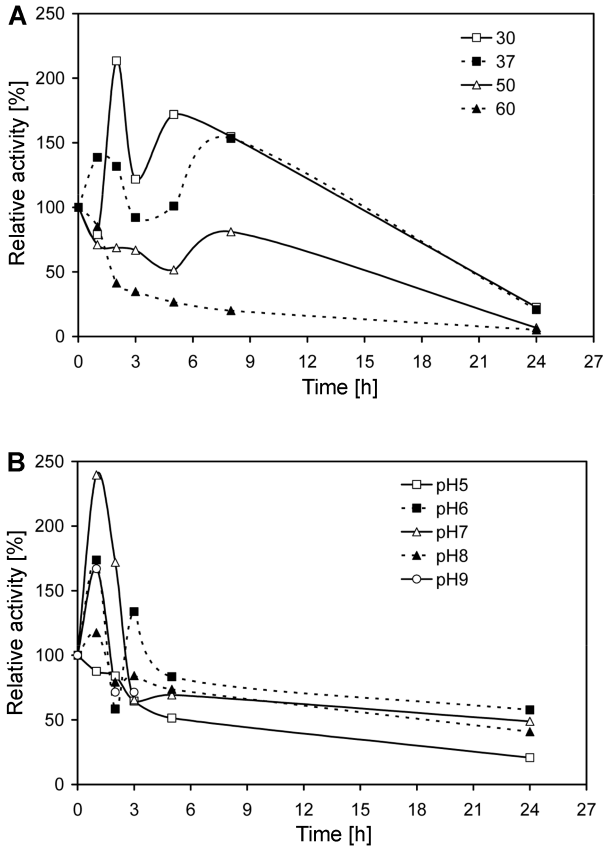

Fig. 4. Temperature (**A**) and pH (**B**) stability of the β-mannanase from *B. subtilis* G1 expressed in *P. pastoris*.

enzyme was stable at a pH of 6–9, and it was activated within pH 6–9 after treatment for 1 h with an increase by 18–140% (Fig. 4B). Our β-mannanase showed less thermostability and a narrow pH stability range. The recombinant β-mannanases from other *Bacillus* species were stable at higher temperatures; up to 70°C for the β-mannanases from *Bacillus* sp. N16.5 [6] and *B. subtilis* WL-3 [10]; up to 60°C for the β-mannanases from *B. subtilis* WL-3 [24], *B. subtilis* B36 [13], *B. Licheniformis* THCM3.1 [8], and *B. Licheniformis* DSM13 [20]; and up to 50°C for the β-mannanase from *B. circulans* CGMCC 1416 [12]. The recombinant β-mannanases from *Bacillus* species were stable in different and wider pH range; pH 5–12 for *B. licheniformis* [20]; pH 6–11.5 for *Bacillus* sp. N16.5 [6]; pH 7–9 for *B. circulans* CGMCC 1416 [12]; pH 5–9 for *B. subtilis* MA139 [17]; and pH 3–9 for *B. subtilis* WL-7 [10] β-mannanases.

Effect of Metal Ions

In general, the tested metal ions and EDTA showed an inhibitory effect on enzyme activity (Table 1), where the higher the concentration of metal ions and EDTA was, the lower the mannanase activity remained. However, it was not clear why Co^{2+}, Hg^{2+}, and Pb^{2+} showed an opposite effect on enzyme activity. By the addition of Co^{2+}, Hg^{2+}, and Pb^{2+} at lower concentration (0.5 mM and 1 mM), the enzyme activity remained just 1–4% after 24 h, but the mannanase activity remained much higher (13–39%) by the addition of these metal ions at higher concentration (1.5 mM).

Many mannanases of other *Bacillus* strains, such as *B. circulans* CGMCC 1416 [12], *Bacillus* sp. N16.5 [6], and *B. licheniformis* THCM3.1 [8], were substantially inhibited by Ag^{+}, Hg^{2+}, and Cu^{2+}; Ag^{+} and Hg^{2+}; and Pb^{2+}, Cu^{2+}, and Ag^{+}, respectively, similar to the mannanase ManA. Pb^{2+} and Ni^{2+} strongly inhibited the mannnase ManA, but showed no effect on the β-mannanase from *Bacillus* sp. N16.5 [6]. In our study, no metal ions increased the mannanase ManA activity; however, Mg^{2+}; Fe^{2+} and Cu^{2+}; and Ca^{2+}, Co^{2+}, and Li^{+} increased the enzyme activity of mannanases from *Bacillus* sp. N16.5 [6]; *B. circulans* CGMCC 1416 [12]; and *B. subtilis* WL-3 [24] by up to 51%, respectively.

Table 1. Effects of metal ions and inhibitor on the β-mannanase from *B. subtilis* G1 expressed in *P. pastoris*.

Compound	Remaining activity (%) at concentration (mM) of		
	1	2.5	5
$CaCl_2$	78	71	36
$CuSO_4$	71	24	33
$FeCl_3$	52	70	63
$FeSO_4$	72	78	87
$MgCl_2$	80	79	76
$NiCl_2$	68	38	0
$ZnSO_4$	55	21	3
$AgNO_3$	74	39	22
KCl	90	82	72
EDTA	80	86	84
Heavy metal ion	Remaining activity (%) at concentration (mM) of		
	0.5	1	1.5
$Co(NO_3)_2$	3	2	13
$HgCl_2$	4	3	39
$Pb(NO_3)_2$	4	1	22

Table 2. Effect of detergents on the β-mannanase from *B. subtilis* G1 expressed in *P. pastoris*.

Detergent	Remaining activity (%) at concentration (%) of			
	0.25	0.50	0.75	1.00
SDS	0	0	0	0
Triton X-100	51	48	39	46
Tween 20	5	65	59	41

Effects of Organic Solvents and Detergents

Chemical additives including detergents (Triton X-100, Tween 20, and SDS) and organic solvents (methanol, ethanol, *n*-butanol, and acetone) showed an inhibitory effect on the β-mannanase activity (Tables 2 and 3). In particular, the addition of SDS at the concentration of 0.25–1.0% completely inhibited the enzyme (Table 3).

Our mannanase ManA was similar to mannanases of *B. subtilis* WL-3 [24] and *B. circulans* CGMCC 1416 [12], which were substantially inhibited by SDS, but the recombinant β-mannanase from *Bacillus* sp. N16.5 showed significant resistance to all the tested surfactants including SDS, Triton X-100, and Tween 20. In addition, an enhancement in the β-mannanase activity was observed in the presence of SDS. After the recombinant enzyme was incubated with 0.25% of SDS for 30 min at 40°C, the activity of alkaline β-mannanase was increased by 21% [6]. Hatada *et al*. [5] reported that the β-mannanase from *Bacillus* sp. strain JAMB-750 expressed in *B. subtilis* enhanced its activity to 20% by the addition of 2% of SDS at pH 7.5 in 25 mM Tris-HCl buffer and 20°C for 30 min.

The inhibition of the ManA activity by the addition of organic solvents was coincident with the *B. licheniformis* THCM3.1 β-mannanase. Its activity decreased by 11–53%

Table 3. Effects of organic solvents on the β-mannanase from *B. subtilis* G1 expressed in *P. pastoris*.

Solvent	Remaining activity (%) at concentration (%) of		
	10	20	30
Methanol	78	20	3
Ethanol	89	71	88
Isopropanol	100	106	103
n-Butanol	65	1	0
Acetone	85	88	46

Table 4. Physicochemical properties of recombinant β-mannanases from *Bacillus* species expressed in *E. coli*, *P. pastoris*, and *B. subtilis*.

Mannanase from strain	Gene bp/ protein aa/ prepeptide aa	Specific activity (U/mg)[a]	Expressed in host cells	Molecular mass (kDa)	Optimum temperature and pH	Temperature[b] and pH stability	Activation (↑) and inhibition (↓)[c]	Reference
B. subtilis MA139	1,014/337/	85	*P. pastoris*		50°C, pH 6	45°C (0.5 h), pH 5–9	↓ Cu^{2+}	[17]
B. subtilis G1	1,092/364/28	2,718	*P. pastoris*	45	45°C, pH 6.5	50°C (8 h), pH 5–9 (5 h)	↓ SDS, Pb^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Zn^{2+}, Ag^{+}	This study
B. subtilis G1	1,092/364/28	359.3 U/ml	*E. coli*	41	40°C, pH 7.0			[19]
B. subtilis WL-3	1,080/360/24	5,900	*E. coli*	38	60°C, pH 6	60°C (8 h)	↓ Fe^{2+}	[24]
B. subtilis WL-7	1,086/362/26	10,082	*E. coli*	38	55°C, pH 6	65°C (1 h), pH 3–9 (1 h)	↑ DTT	[10]
B. subtilis B36	1,164/367/	928	*E. coli*	38	50°C, pH 6.4	60°C		[13]
B. stearothermophilus	2,085/695/32	384	*E. coli*	74				[4]
Bacillus sp. N16-5	1,482/493	32.2 U/ml	*P. pastoris*	60	70°C, pH 10	70°C (2 h), pH 6.0–11.5 (1 h)	↑ SDS, Mg^{2+} ↓ Ag^{+}, Hg^{2+}, Mn^{2+}	[6]
Bacillus sp. strain JAMB-750	2,994/997/26	36.3	*B. subtilis*	107	55°C, pH 10	pH 6.0–10.5	↑ SDS ↓ Fe^{3+}, Fe^{2+}, Pb^{2+}, Hg^{2+}, Ca^{2+}	[5]
B. licheniformis DSM13		1,672	*E. coli*	45	50-60°C, pH 6–7	55°C (0.5 h), pH 5–12 (0.5 h)		[20]
B. licheniformis THCM3.1	1,080/	626	*E. coli*	40	45°C, pH 9	60°C (24 h), pH 9 (48 h)	↑ Na^{+}, K^{+}, Ca^{2+} ↓ Pb^{2+}, Fe^{3+}, Cu^{2+}, Ag^{+}, Sn^{2+}	[8]
B. circulans K-1		1,550		62	65°C, pH 6.9		↑ Al^{3+} ↓ Zn^{2+}	[25]
B. circulans CGMCC 1416	981/326/31	482	*E. coli*	31	58°C, pH 7.6	50°C (1 h), pH 7–9 (1 h)	↑ Ca^{2+}, Co^{2+}, Li^{+} ↓ Ag^{+}, Hg^{2+}, Cu^{2+}	[12]

[a]Locust bean gum as substrate.

[b]Residual activity was over 60% of the original enzyme activity for the duration of treatment.

[c]Activation: Increase in activity in comparison with the original activity. Inhibition: Decrease in activity by more than 50% in comparison with the original activity.

by the addition of organic solvents, except for *n*-hexane, which did not show an effect on the β-mannanase activity [20].

In conclusion, we have cloned a novel gene encoding an endo-β-1,4-mannanase (ManA) from *Bacillus subtilis* strain G1, which showed identity of less than 93% in amino acid sequence with the known β-mannanases from GenBank. The recombinant β-mannanase was identified by MALDI-TOF mass spectrometry and showed a specific activity of 2,718 U/mg, 32 times as high as other *Bacillus* β-mannanases ever expressed in *P. pastoris*. Its biochemical properties suggest that the β-mannanase might potentially be used in the feed industry.

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