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Characterization and gene cloning of a novel β-mannanase from alkaliphilic *Bacillus* sp. N16-5

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Abstract An alkaline β-mannanase was purified to homogeneity from a culture broth of alkaliphilic *Bacillus* sp. N16-5. The enzyme had optimum activity at pH 9.5 and 70°C. It was composed of a single polypeptide chain with a molecular weight of 55 kDa deduced from SDS-PAGE, and its isoelectric point was around pH 4.3. The enzyme efficiently hydrolyzed galactomannan and glucomannan, producing a series of oligosaccharides and monosaccharides. The β-mannanase gene (*manA*) contained an open reading frame (ORF) of 1,479 bp, encoding a 32-amino acids signal peptide, and a mature protein of 461 amino acids, with a calculated molecular mass of 50,743 Da. Strain N16-5 ManA, deduced from the *manA* ORF, exhibited relatively high amino acid similarity to the members of the glycosyl hydrolase family 5. The eight conserved active-site amino acids in family 5 glycosyl hydrolase were found in the deduced amino acid sequence of strain N16-5 ManA.

Keywords Alkaline · Alkaliphile · Mannanase

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Introduction

β-Mannanase (EC 3.2.1.78) catalyzes the random hydrolysis of the β-1,4-mannosidic linkages in the backbones of β-mannan, glucomannan, and galactomannan. Potential uses of β-mannanase have been shown to include pulp bleaching ability in the manufacture of kraft pulp (Buchert et al. 1993; Khanongnuch et al. 1999); the production of oligosaccharides from hemicellulose as one of the growth factors of *Bifidobacterium* sp. (Kobayashi et al. 1984); the hydrolysis of galactomannans, following hydraulic fracturing of oil well (McCutchen et al. 1996); and excellent cleaning performance on food and cosmetic stains in the detergent industry (Bettiol et al. 2000). β-Mannanases have been isolated from various bacteria, fungi, and plants (Braithwaite et al. 1995; Duffaud et al. 1997; Ethier et al. 1998; Reese and Shibata 1965; Shimahara et al. 1975). Several β-mannanase have been purified and well characterized (Emi et al. 1972; Akino et al. 1988) and nucleotide sequences coding for mannanase have also been reported (Arcand et al. 1993; Gibbs et al. 1996).

Alkaline β-mannanases provide obvious advantages for the applications in the manufacture of kraft pulp and in the detergent industry, where high pH processes are common. Alkaliphilic *Bacillus* strains often produce various alkaline enzymes, such as alkaline CMCase, protease, and amylase (Horikoshi 1996; Ito et al. 1998). There are also reports on alkaline β-mannanases from alkaliphilic *Bacillus* sp. AM001, *B. agaradhaerens* and *Bacillus* sp. I633 (Akino et al. 1987, 1989; Kauppinen et al. 2003). However, alkaline β-mannanase has not been intensively investigated. To date, only the mannanases of *Bacillus* sp. AM001 have been extensively characterized among alkaline mannanases. Previously, we isolated a strictly alkaliphilic *Bacillus* sp. N16-5, which had an optimum pH 10 for growth and produced significant amounts of extracellular β-mannanases (Ma et al. 1991). Here, we describe the characterization and gene cloning of an alkaline β-mannanase from alkaliphilic *Bacillus* sp. N16-5.

Materials and methods

Bacterial strains, plasmids, and media

Bacillus sp. N16-5, a producer of extracellular β-mannanases, was isolated previously from sediment of Wudunur Soda Lake in Inner Mongolia, China (Ma et al. 1991). *Bacillus* sp. N16-5 was grown in modified alkaline Horikoshi-II medium (Horikoshi 1971) containing 1% konjac mannan and 8% NaCl. Microscopy of cells, G+C content of DNA, pH and salt (NaCl) range for growth, PCR amplification, and sequencing of 16S rRNA genes were carried out as previously described (Zhang et al. 2002). *Escherichia coli* DH5α was used as the host for gene cloning and was grown in LB medium. Plasmid pUC19 was used as the cloning vector.

Purification of β-mannanase

Enzyme purification was done at a temperature below 4°C. The centrifugal supernatant of the culture broth was used as the enzyme source. Ammonium sulfate was added to the culture supernatant to achieve 80% saturation, and the resultant precipitate was collected by centrifugation and dissolved in a small volume of 50 mM sodium phosphate buffer, pH 8 (buffer A). After desalting by dialysis against buffer A, the sample was put on a DEAE-Sephadex A-25 (Pharmacia) column (2.2×30 cm) equilibrated with buffer A. Washing the column with a linear gradient of buffer A containing 0–0.6 M NaCl resulted in the elution of proteins with β-mannanase activity in a single peak. The active fractions were collected, desalted by dialysis, and applied again to a DEAE-Sephadex A-25 column. The active fraction was concentrated by ultrafiltration and then applied to a hydroxyapatite column (1.0×15 cm) equilibrated with a 10-mM sodium phosphate buffer (pH 8). Active enzyme was eluted by gradient elution (10–100 mM) with sodium phosphate buffer (pH 8).

β-Mannanase assay and protein estimation

With locust bean gum as the substrate, β-mannanase activity was assayed at 70°C and pH 9.5 by measuring the reducing sugars liberated during the hydrolysis of mannan, as described previously (Akino et al. 1987). One unit of activity was defined as the amount of enzyme catalyzing the production of 1 μmol of the reducing sugar per minute, using mannose as the standard. The protein concentration was measured by the method of Bradford (1976), using a protein assay kit (Bio-Rad, USA), with bovine serum albumin as a standard.

Electrophoretic analysis

SDS-PAGE was performed as described by Laemmli (1970) with 10% (w/v) polyacrylamide gels. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. Activity detection of mannanase in slab gels was done by overlaying an agar sheet containing 1% konjac mannan and 1.2% agar (pH 9.5) on the gel for 10 min at 70°C after SDS-PAGE. The band corresponding to mannanase appeared as clear zone on a red agar sheet after staining with Congo Red solution (Teathor and Wood 1982).

Molecular masses were estimated by SDS-PAGE [10% (w/v) acrylamide gel] with low-range molecular mass standards (Shanghai) and BlueRange Prestained Protein Molecular Weight Marker Mix (Pierce). Isoelectric points (pI) of the purified enzyme were determined by isoeletric focusing with a Multiphore II electrofocusing system with a PAG-Plate gel and a broad pI calibration kit (Pharmacia Fine Chemica, Uppsala, Sweden).

N-terminal amino acid sequencing

After SDS-PAGE of the β-mannanase purified from strain N16-5, the protein was transferred to a PVDF membrane (Millipore, USA), using Trans-Blot SD (Bio-Rad) and analyzed for the N-terminal amino acid sequence, using an ABI automated protein sequencer (model 491A, Applied Biosystems, USA).

Product analysis of mannanase

The hydrolysis products of the β-mannanase were analyzed by paper chromatography. After development of the products in a solvent system of butanol:pyridine:water [6:4:3 (v/v)], the spots were visualized by the silver nitrate dip method (Welker and Campbell 1963).

General DNA manipulation

Standard DNA recombination techniques were used for DNA manipulation (Sambrook et al. 1989). Genomic DNA from alkaliphilic *Bacillus* sp. N16-5 was partially digested with *Eco*RI. The DNA fragments between 2 and 10 kb in size were recovered and then ligated into the dephosphorylated *Eco*RI site of pUC19 and transformed into the competent *E. coli* DH5α. Transformed cells were grown on LB agar plates supplemented with ampicillin, Xgal, and IPTG at 37°C for 16 h. All white colonies were transferred to an LB plate containing 1% konjak mannan and ampicillin. The clones expressing β-mannanase activity were identified by the formation of clear halos around colonies, using Congo Red stain (Teathor and Wood 1982). The plasmid (pMAN1) expressing mannanase activity was digested

Table 1 Purification of β-mannanase from culture broth of *Bacillus* sp. N16-5

Step	Total activity ($\times 10^3$ U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification (*n*-fold)	Recovery (%)
Culture broth	443	1,380	321	1	100
80% $(NH_4)_2SO_4$	318	342	930	2.9	72
First DEAE-Sephadex A25	168	127	1,323	4.1	38
Second DEAE-Sephadex A25	106	48	2,208	6.9	24
Hydroxyapatite	39	7.7	5,065	15.8	8.8

with single- and multiple-restriction enzymes and subcloned. Southern hybridization was done to identify that the cloned gene originated from *Bacillus* sp. N16-5. An 8-kb probe was prepared from the *Eco*RI fragment in pMAN1, and labeled with DIG-dUTP, using the DIG DNA labeling kit (Boehringer Mannheim, Germany). Hybridization, washing, and development procedures were followed according to the manufacturer's manual. The nucleotides of both strands of the DNA insert were determined by the Sanger dideoxy chain termination methods, using an ABI 377S DNA sequencer (Applied Biosystems). Homology search in the GenBank and UniProt databases were carried out by using the BLAST program (Altschul et al. 1990).

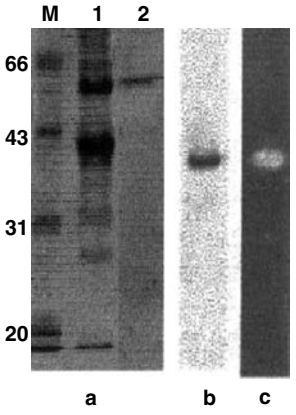

Fig. 1a–c SDS-PAGE analysis of the purified mannanase from *Bacillus* sp. N16-5. **a** SDS-PAGE with Coomassie Brilliant Blue staining. **b** SDS-PAGE of purified enzyme without preboiling treatment, staining with Coomassie Brilliant Blue. **c** SDS-PAGE of purified enzyme without preboiling treatment, activity staining with Congo Red. *Lane 1* Proteins from culture, *lane 2* purified enzyme, *lane M* molecular mass markers

Results and discussion

Taxonomic characterization of strain N16-5

The isolate, designated N16-5, was a facultative anaerobic, spore-forming, motile, Gram-positive, rod-shaped (0.6–0.8 by 2.5–5.0 μm) bacterium with peritrichous flagella. It was able to grow well over the pH range from 8.5–11.5, with an optimum at pH 10. The range of NaCl concentration for growth was over 0–15%, with optimal NaCl concentration of 8%. The G + C content of DNA was 37 mol%. The 16S rDNA sequence of strain N16-5 showed the highest similarity to *Bacillus agaradhaerens* (98% sequence similarity).

Purification and properties of β-mannanase

One mannanase was purified from cultures of strain N16-5 by precipitation with ammonium sulfate and the three-step column chromatographic procedure, with a yield of 8.8% (Table 1). An approximately 15.8-fold purification to a specific activity of 5,065 U mg of protein^{-1} was obtained for the β-mannanase activity when measured at 70°C and in 50 mM glycine–NaOH buffer (pH 9.5). The protein was homogeneous as judged by SDS-PAGE (Fig. 1). When the mannanase was boiled up to 5 min in the presence of 1% SDS prior to electrophoresis, SDS-PAGE revealed that the mannanase consisted of a single band at 55 kDa. Without preboiling the sample, SDS-PAGE revealed the mannanase to be a single band, corresponding to a lower molecular mass (40 kDa). Activity staining showed that only the non-boiled sample band was active, suggesting that SDS treatment without boiling was insufficient to denature the enzyme. The purified enzyme was also eluted as a single peak from Bio-gel P150 and estimated to have a molecular weight of about 50 kDa. Thus, the enzyme was composed of a single polypeptide with a molecular weight of 50–55 kDa. The pI was estimated to be around pH 4.3. The N-terminal amino acid sequence was SSGFYVDGNT.

Effects of pH, temperature, and metal ions on activity and stability

The effect of pH on the mannanase activity was determined in 50-mM volumes of various buffers at 70°C for 10 min, with locust bean gum as the substrate. As shown in Fig. 2a, the maximum activity was observed at pH 9.5, and more than 70% of the maximum activity was detectable between pH 7 and 11. To examine the pH stability of the mannanase, the enzyme was incubated in various buffers (50 mM each) at 50°C for 60 min, and then, residual activity was assayed by the standard method. The mannanase was stable at pH9 and retained almost 90% of its initial activity between pH 8.5 and 10.

The effect of temperature on the mannanase activity was measured at various temperatures in 50 mM glycine–NaOH buffer (pH9.5). The enzyme showed maximum activity at 70°C, and more than 70% of the

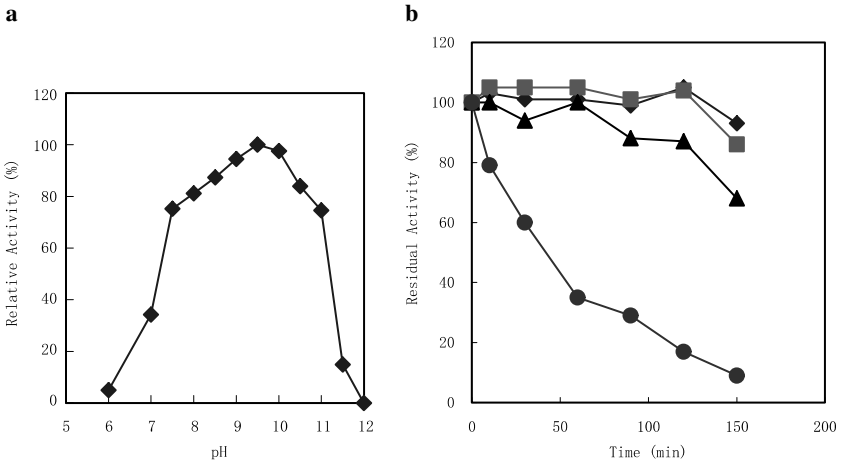

Fig. 2a, b Effects of pH and temperature on the activity and stability of β-mannanase of *Bacillus* sp. N16-5. **a** pH activity. **b** Temperature stability after preincubation at 40°C (*filled diamonds*), 50°C (*filled squares*), 60°C (*filled triangles*), or 70°C (*filled circles*). The buffers used (50 mM each) were phosphate buffer (pH 6.0–8.0), glycine–NaOH buffer (pH 8.5–10.5), and KCl–NaOH buffer (pH 11.0–12.0)

maximum activity was detectable between 60 and 75°C. The thermal stability of the mannanase was tested by preincubating the mannanase in 50 mM glycine–NaOH buffer (pH 9) at various temperatures, and then, residual mannanase activity was determined periodically under the standard assay conditions. The mannanase was stable up to 60°C during a 2-h incubation period and unstable at temperatures above 60°C (Fig. 2b).

The purified enzyme was incubated with various cations (1 mM) or chemical reagents at 40°C for 30 min, and then, residual activities were assayed by the standard method. As shown in Table 2, the mannanase activity was enhanced by Mg^{2+} and not affected by Ca^{2+}, Ni^{2+}, or Pb^{2+}. The mannanase activity was inhibited slightly by Zn^{2+}, Fe^{2+}, Sn^{2+}, and EDTA and strongly by Ag^{+} and Hg^{2+}. The mannanase was resistant to various surfactants, such as 0.1% SDS, 0.05% linear-alkylbenzene sulfonate, sodium α-sulfonated fatty acid ester, and polyxyethylene alkyl ether.

Table 2 Effects of metal ions and reagents on the activity of β-mannanase from *Bacillus* sp. N16-5

	Relative activity (%)
Metal ion (1.0 mmol l^{-1})	
None	100
Ca^{2+}	103
Mg^{2+}	118
Cu^{2+}	105
Pb^{2+}	101
Ni^{2+}	100
Sn^{2+}	88
Zn^{2+}	80
Fe^{2+}	78
Co^{2+}	60
Cd^{2+}	54
Mn^{2+}	36
Hg^{2+}	27
Ag^{+}	18
Reagent	
None	100
EDTA (10 mM)	88
SDS (0.1%)	115
Linear alkylbenzone sulfonate (0.05%)	101
Sodium sulfonated fatty acid ester (0.05%)	100
Polyoxyethylene alkyl ether (0.05%)	102

Substrate specificity and product analysis of mannanase

The purified mannanase was examined for its ability to hydrolyze various substrates under the standard conditions of the assay. As shown in Table 3, it showed the highest specific activity on locust bean gum, with 42 and 37% of locust bean gum activity on konjac glucomannan and sesbania gum, respectively. No activity was detected on yeast mannan, CMC, chitin, xylan, glucan, or starch. Thus the enzyme was specific toward β-1,4-mannosidic linkages of mannopolysaccharides.

The products were analyzed by paper chromatography, using locust bean gum and konjak mannan as substrates. The enzyme exhibits a random cleavage pattern, and a mixture of mannose, mannobiose, mannotriose, and mannotetraose was produced (Fig. 3). This indicated that the enzyme was an endo type of β-mannanase.

Table 3 Substrate specificities of mannanase of *Bacillus* sp. N16-5

Polysaccharides	Relative activity (%)
Locust bean gum	100
Konjak glucomannan	42
Sesbania gum	38
Guar gum	13
Yeast mannan	N[a]
Xylan	N
Pectin	N
Glucan	N
CMC	N
Chitosan	N
Soluble starch	N
Pullulan	N

[a] *N* No activity

Fig. 3 Paper chromatography of the products from mannans by β-mannanase of *Bacillus* sp. N16-5. *G* Glucose, *C* cellulobiose, *M* mannose, *K* konjac glucomannan degradation products with the enzyme, *L* locust bean gum degradation products with the enzyme

Cloning and nucleotide sequence analysis of the mannanase gene

Through the shotgun cloning method, one positive clone that showed clear halos in the selection medium was selected. The recombinant plasmid (pMAN1) possessed by the clone had a DNA insert of 8 kb. Restriction analysis of the insert of the recombinant plasmid and Southern hybridization using the 8-kb insert as a probe showed that the plasmid contained a genomic DNA fragment of strain N16-5. Construction of subclones showed that a 2.0-kb *Acc*I–*Hin*cII fragment (pMANA) showed mannanase activity.

A total of 2,058-bp nucleotides were sequenced (Fig. 4). In addition, one open reading frame (ORF) corresponding to an initiation ATG codon beginning at 403 of the determined sequence and ending with a TAG codon at 1,882 was found. The ORF (*manA*) corresponding to mannanase (ManA) consists of 1,479 bp and codes for a protein of 493 amino acid residues, with an estimated molecular mass of 54,215 Da. At the seven-base upstream region from the start codon (ATG), a presumed ribosome-binding site (GGAGGAG) was identified. No transcription terminator sequence was found downstream from the stop codon (TAG).

Primary structure of ManA

Computer analysis of the deduced amino acid sequence of ManA with sequences in the GenBank and UniProt databases showed that ManA consists of a single catalytic domain. The N-terminal region of ManA appears to have the features of a bacterial signal peptide: a relatively short basic region, followed by a hydrophobic region and a signal peptidase-recognition site (S-E-A) at amino acid residue 32 (Perlman and Halvorson 1983). Residues 33–42 of the deduced amino acid sequence, which is SSGFYVDGNT, coincide precisely with the N-terminal amino acid sequence determined from purified mannanase. The calculated molecular mass of the mature protein was 50,743 Da, and this agrees with the molecular mass of 50–55 kDa for the purified native mannanase.

The amino acid sequence of ManA showed high homology to family 5 mannanases: 96.8% sequence similarity to ManA of *B. agaradhaerens* (GenBank accession number: AAN27517), 58.2% to ManG of *B. circulans* (BAA25878), 52.3% to ManA of *Bacillus* sp. I633 (AAQ31835), 39.4% to ManA of *Thermobifida fusca* (CAA06924), 34.4% to ManA of *Vibrio* sp. MA-138 (BAA25188), and 33.1% to ManA of *Streptomyces lividans* 66 (S30386). No significant similarity was found to the alkaline β-mannanases from *Bacillus* sp. AM001, which belong to the glycosyl hydrolase family 26.

The eight conserved residues of all family 5 enzymes were demonstrated to be responsible for the catalytic activity of the enzyme (Sakon et al. 1996). The multiple sequence alignment showed that these eight essential active site residues were also conserved in strain N16-5 ManA as Arg-83, His-119, Asn-157, Glu-158, His-224, Tyr-226, Glu-254, and Trp-283 (Fig. 5).

On the basis of sequence comparisons by homology and hydrophobic cluster analysis (Henrissat 1991), the catalytic domain of glycosyl hydrolases have been classified into 91 families (http://afmb.cnrs-mrs.fr/CAZY/). β-Mannanases that have been sequenced so far belong to only two families, 5 and 26. As the amino acid sequence of members of family 5 were divergent, members of family 5 have been classified in eight subfamilies, subfamilies A1–A8 (Hilge et al. 1998). Accordingly, based on amino acid sequence comparisons, the mannanase domain of *Bacillus* sp. N16-5 belongs to a member of glycosyl hydrolase family 5, subfamily A8.

The β-mannanase reported here was active and stable in alkaline conditions. It is different from the other mannanases previously described to show maximum activity at alkaline pH. The mannanase of *Bacillus* sp. N16-5 had maximum activity at pH 9.5 and 70°C, whereas the mannanase of *B. agaradhaerens* had optimum activity at pH 8–10 and 60°C (Bettiol and Showell 2002); the mannanase of *Bacillus* sp. I633 had optimum activity at 50°C (Kauppinen et al. 2003); the three enzymes from *Bacillus* sp. AM001 had maximum activity at pH 8.5–9.0 and 60–65°C (Akino et al. 1988). *Bacillus* sp. N16-5 mannanase shows higher optimal and stable temperature values than that of other alkaline mannanases. In terms of molecular weight measured by SDS-PAGE, *Bacillus* sp. N16-5 mannanase was 55 kDa, *B. agaradhaerens* mannanase was 38 kDa, *Bacillus* sp. I633 mannanase was 34 kDa, and the three enzymes from *Bacillus* sp. AM001 were 58, 59, and 42 kDa. The molecular weight of *Bacillus* sp. N16-5 mannanase is more similar to that of the mannanases from *Bacillus* sp. AM001. The activity of the mannanases from *Bacillus* sp. AM001 were not inhibited by Mn^{2+}, Hg^{2+}, or Co^{2+},

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   1  TTAGTTCGGATAAAGGTAAGTCAATTGTCGGCACGTTAATCCACTCCTCTTTATTTATATTCAAATTACCTTAAGGATAACTACTTAATA   90
  91  GTTTACCTGAAAAATGAATAACCTCAATCCCTGTGCGACTTTTTATTCAATTTAATAAAACATTATATAACACACTATATTAAATTTTGT  180
 181  TATTAAGTAAATGGTATTAAAATAAACCTCTTTATTGACTTTATCGATTACCTAATTTACCTTTAAGGAAACGCTTTCTTTTTTGCCATT  270
 271  TTGAAAGATAGATTAAAAGGCTTGGTCACTTGAGCCTACTTCCGCTTCTTGATTATCTGAAGAACGGCCCGAAAGGAGGAGTGTGACTTG  360
                                                                                          -35
 361  CCACTCAGATAATATAAATAACCCACAAGGAGGAGTAATAACATGAAAAAAAAGTTATCACAGATTTATCATTTAATTATTTGCACACTT  450
   1                -10             SD          M  K  K  K  L  S  Q  I  Y  H  L  I  I  C  T  L   16
 451  ATAATAAGTGTGGGAATAATGGGGATTACAACGTCCCCATCAGAAGCAAGTTCAGGCTTTTATGTTGATGGCAATACGTTATATGACGCA  540
  17   I  I  S  V  G  I  M  G  I  T  T  S  P  S  E  A  S  S  G  F  Y  V  D  G  N  T  L  Y  D  A   46
 541  AACGGGCAACCATTTGTCATGAAAGGCATTAACCATGGACATGCTTGGTATAAAGACACCGCTTCAACAGCTATTCCTGCCATTGCAGAG  630
  47   N  G  Q  P  F  V  M  K  G  I  N  H  G  H  A  W  Y  K  D  T  A  S  T  A  I  P  A  I  A  E   76
 631  CAAGGCGCGAACACGATACGTATTGTTTTATCAGATGGCGGTCAATGGGAAAAAGACGACATTGACACCGTTCGTGAAGTTATTGAGCTT  720
  77   Q  G  A  N  T  I  R  I  V  L  S  D  G  G  Q  W  E  K  D  D  I  D  T  V  R  E  V  I  E  L  106
 721  GCGGAGCAAAATAAAATGGTGGCTGTCGTTGAAGTTCATGATGCCACGGGCCGTGATTCACGCAGTGATTTAGATCGGGCAGTCGATTAT  810
 107   A  E  Q  N  K  M  V  A  V  V  E  V  H  D  A  T  G  R  D  S  R  S  D  L  D  R  A  V  D  Y  136
 811  TGGATAGAGATGAAAGATGCACTTATCGGCAAAGAGGATACTGTCATTATTAACATTGCAAACGAATGGTATGGCAGTTGGGATGGCGCC  900
 137   W  I  E  M  K  D  A  L  I  G  K  E  D  T  V  I  I  N  I  A  N  E  W  Y  G  S  W  D  G  A  166
 901  GCTTGGGCTGATGGCTACATTGATGTCATTCCGAAGCTTCGCGATGCCGGCTTAACACACACCTTAATGGTTGATGCAGCAGGATGGGGG  990
 167   A  W  A  D  G  Y  I  D  V  I  P  K  L  R  D  A  G  L  T  H  T  L  M  V  D  A  A  G  W  G  196
 991  CAATATCCGCAATCTATTCATGATTACGGACAAGATGTGTTTAATGCAGATCCGTTAAAAAATACGATATTCTCCATCCATATGTATGAG 1080
 197   Q  Y  P  Q  S  I  H  D  Y  G  Q  D  V  F  N  A  D  P  L  K  N  T  I  F  S  I  H  M  Y  E  226
1081  TATGCTGGTGGTGATGCTAACACTGTTAGATCAAATATTGATAGAGTCATAGATCAAGACCTTGCTCTCGTAATAGGTGAGTTCGGTCAT 1170
 227   Y  A  G  G  D  A  N  T  V  R  S  N  I  D  R  V  I  D  Q  D  L  A  L  V  I  G  E  F  G  H  256
1171  AGACACACTGATGGCGATGTTGATGAAGATACAATCCTTAGTTATTCTGAAGAAACTGGCACAGGATGGCTCGCTTGGTCTTGGAAAGGC 1260
 257   R  H  T  D  G  D  V  D  E  D  T  I  L  S  Y  S  E  E  T  G  T  G  W  L  A  W  S  W  K  G  286
1261  AACAGTGCCGAATGGGATTATTTAGACCTTTCAGAAGATTGGGCTGGTAACCATTTAACTGATTGGGGAAATAGGATTGTCCACGGGGCA 1350
 287   N  S  A  E  W  D  Y  L  D  L  S  E  D  W  A  G  N  H  L  T  D  W  G  N  R  I  V  H  G  A  316
1351  AATGGCTTGCAGGAAACCTCCAAACCATCCACCGTATTTACAGATGATAACGGTGGTGCCCCTGAACCGCCAACTACTACTACCTTGTAT 1440
 317   N  G  L  Q  E  T  S  K  P  S  T  V  F  T  D  D  N  G  G  A  P  E  P  P  T  T  T  T  L  Y  346
1441  GACTTTGAAGGAAGCACACAAGGGTGGCATGGAAGCAACGTGATGGGTGGCCCTTGGTCCGTAACAGAATGGGGTGCGTCAGGCAACTAC 1530
 347   D  F  E  G  S  T  Q  G  W  H  G  S  N  V  M  G  G  P  W  S  V  T  E  W  G  A  S  G  N  Y  376
1531  TCTTTAAAGGGCGATGTCAATTTAAGCTCAAATTCTTCACATGAACTGTATAGTGAACAAAGTCGTAATCTACACGGATACTCTCAGCTA 1620
 377   S  L  K  G  D  V  N  L  S  S  N  S  S  H  E  L  Y  S  E  Q  S  R  N  L  H  G  Y  S  Q  L  406
1621  AATGCAACCGTTCGCCATGCCAATTGGGGAAATCCCGGTAATGGCATGAATGCAAGACTTTACGTGAAAACGGGCTCTGATTATACATGG 1710
 407   N  A  T  V  R  H  A  N  W  G  N  P  G  N  G  M  N  A  R  L  Y  V  K  T  G  S  D  Y  T  W  436
1711  TATAGCGGTCCTTTTACACGTATCAATAGCTCCAACTCAGGTACAACGTTATCTTTTGATTTAAACAACATCGAAAATAGTCATCATGTT 1800
 437   Y  S  G  P  F  T  R  I  N  S  S  N  S  G  T  T  L  S  F  D  L  N  N  I  E  N  S  H  H  V  466
1801  AGGGAAATAGGTGTGCAATTTTCAGCTGCAGATAATAGCAGCGGTCAAACTGCTCTATACGTTGATAATGTTACTTTAAGATAGAATTAG 1890
 467   R  E  I  G  V  Q  F  S  A  A  D  N  S  S  G  Q  T  A  L  Y  V  D  N  V  T  L  R  *        493
1891  CACGATCACATAAGGTGTGCTTCATTGTTAAAGGGGCACACCTTACTTTTAAACGGATAGTGAACTCGCTTCAGCAAGCTAAAATATCGA 1980
1981  GAAATCAGGTGGCTTCTCTTAAAAGTTTCCATCTACACGATGCATTGAGGTGGGGGCTATCTAACCCAAAGGTTAACG.  2058.
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Fig. 4 Nucleotide sequence and deduced amino acid sequence of the *manA* gene from *Bacillus* sp. N16-5. The nucleotide sequence of the 2,058-bp fragment containing the *manA* gene appears in the GenBank database under accession number AY534912. The locations of the −35 and −10 sites and ribosome-binding sites are *underlined*. The sequence of the signal peptide is shown in *boldface* and *underlined*. The nucleotides and amino acid residues that are different from ones in the mannanase gene of *B. agaradhaerens* are indicated on *grey backgrounds*

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BspN165-ManA     35 GFYVDGNTLYDANGQPFVMKGINHGHAWYKDTASTAIPAIAEQGANTIRIVLSDGGQWEKDDIDTVREVIELAEQNKMVAVVEVHDATGR
Bag-ManA         35 GFYVDGNTLYDANGQPFVMRGINHGHAWYKDTASTAIPAIAEQGANTIRIVLSDGGQWEKDDIDTIREVIELAEQNKMVAVVEVHDATGR
Bci-ManG         37 GFYVSGTKLLDATGQPFVMRGVNHAHTWYKDQLSTAIPAIAKTGANTIRIVLANGHKWTLDDVNTVNNILTLCEQNKLIAVLEVHDATGS
VspMA138-ManA    22 GFYVSNGVLYEANGSAFKIRGINHAHTWYTDKLSVALSGIAATGANTVRVVLSNGYRWTKNDVSDVTNIINLAKANNLIAILEVHDTTGY
Sli-ManA         39 GIHVSNGRVVEGNGSAFVMRGVNHAYTWYPDRT-GSIADIAAKGANTVRVVLSSGGRWTKTSASEVSALIGQCKANKVICVLEVHDTTGY
Tmfu-ManA         1 GLHVKNGRLYEANGQEFIIRGVSHPHNWYPQHT-QAFADIKSHGANTVRVVLSNGVRWSKNGPSDVANVISLCKQNRLICMLEVHDTTGY
                    *  *         *  *   *  *   **          *   **** * **  *  *                *      **** **

BspN165-ManA    125 DSR---SDLDRAVDYWIEMKDALI-GKEDTVIINIANEWYGS--WDGAAWADGYIDVIPKLRDAGLTHTLMVDAAGWGQ-YPQSIHDYGQ
Bag-ManA         125 DSR---SDLNRAVDYWIEMKDALI-GKEDTVIINIANEWYGS--WDGSAWADGYIDVIPKLRDAGLTHTLMVDAAGWGQ-YPQSIHDYGQ
Bci-ManG         127 DSL---SDLDNAVNYWIGIKSALI-GKEDRVIINIANEWYGT--WDGVAWANGYKQAIPKLRNAGLTHTLIVDSAGWGQ-YPDSVKNYGT
VspMA138-ManA    112 GEESSAASLDSAADYWIELKNELI-GQEDYVIINLGNEPFGNN-NDAVAWVNDHVSAIQRLRSAGINHTIMVDAPNWGQDWKGFMLNNAQ
Sli-ManA         128 GKD-GATSLDQAGDYWVGVKSAAWRAQEDYVVVNIGNEPFGN--TNYAAWTDATKSAIGKLRGAGLGHALMVDAPNWGQDWSGTMRSNAA
Tmfu-ManA         90 GEQSGASTLDQAVDYWIELKSVLQ-GEEDYVLINIGNEPYGNDSATVAAGAWDTSAAIQRLRAAGFEHTLVVDAPNWGQDWTNTMRNNAD
                           *  *  **   *       ** *  *  **  *       *        *  ** **  *   **   ***

BspN165-ManA     208 DVFNADPLKNTIFSIHMYEYAGGDANTVRSNIDRVIDQDLALVIGEFGHRHTDGDVDEDTILSYSEETGTGWLAWSWKGNSAEWDYLDLS
Bag-ManA         208 DVFNADPLKNTMFSIHMYEYAGGDANTVRSNIDRVIDQDLALVIGEFGHRHTDGDVDEDTILSYSEETGTGWLAWSWKGNSTEWDYLDLS
Bci-ManG         210 EVLNADPLKNTVFSIHMYEYAGGNASTVKSNIDGVLNKNLALIIGEFGGQHTNGDVDEATIMSYSQEKGVGWLAWSWKGNSSDLAYLDMT
VspMA138-ManA    200 FVFNSDPKLNTIFSVHMYEVYS-SYNSVNDYISSFTNNGLVLVIGEFASTHKGADVDEGSIMERSETLSLGYIGWSWSGNDTTTSDLDIV
Sli-ManA         215 SVFASDPDRNTVFSIHMYGVYD-TAAEVRDYLNAFVGNGLPIVVGEFGDQHSDGNPDEDAIMATAQSLGVGYLGWSWSGNGGGVEYLDMV
Tmfu-ManA        179 QVYASDPTGNTVFSIHMYGVYS-QASTITSYLEHFVNAGLPLIIGEFGHDHSDGNPDEDTIMAEAERLKLGYIGWSWSGNGGGVEYLDMV
                       *   **  ** ** ***                    *    **  *    **  *        *   *** **      **
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Fig. 5 Multiple sequence alignment for the catalytic domain of *Bacillus* sp. N16-5 ManA and five family 5 mannanases. *BspN165-ManA Bacillus* sp. N16-5 ManA, *Bag-ManA B. agaradhaerens* mannanase, *Bci-ManG B. circulans* ManG, *VspMA138-ManA Vibrio* sp. MA138 ManA, *Sli-ManA Streptomyces lividans* 66 ManA, *Tmfu-ManA Thermobifida fusca* ManA. Alignment was performed using the Clustal W program (Thompson et al. 1994). The *boldface letters* show the conserved active site residues of family 5 hydrolases. The *asterisks* indicate amino acid conservation in all enzymes compared

while the activities of the mannanase from *Bacillus* sp. N16-5 were moderately inhibited by these metal ions but not inhibited by Cu^{2+} and Ni^{2+}. The mannanases of *Bacillus* sp. AM001 had the specific activity of 312 and 470 U mg^{-1}, but the mannanase of *Bacillus* sp. N16-5 had the specific activity of 5,065 U mg^{-1}, which is the highest value compared to other mannanases reported to date. No information of specific activity and metal ion effects on activity were acquired for the alkaline mannanases from *B. agaradhaerens* and *Bacillus* sp. I633. The properties of high thermal activity and stability in alkaline condition, combined with the characteristics of insensitivity to some surfactants and high specific activity, would qualify *Bacillus* sp. N16-5 mannanase for application in the manufacture of kraft pulp and in the detergent industry.

Bacillus sp. N16-5 ManA was compared with other proteins in the GenBank database. It showed a high level of homology to the mannanase of *B. agaradhaerens* (97% sequence identity). Alignment of the nucleotide sequence of these two enzyme genes (95.5% sequence similarity) showed that no insertions and deletions were present, and 43 substitutions were found in the coding region of the two enzymes, which in turn led to 15 replacements at the amino acid level (Fig. 4). Since *Bacillus* sp. N16-5 mannanase is different to *B. agaradhaerens* in terms of optimal temperature for activity, to some extent the mutations should be responsible for the thermal activity and stability of the enzyme.

It is interesting to compare the sequences of mannanases from *Bacillus* sp. N16-5 and *Bacillus* sp. AM001, the only two mannanases extensively characterized among alkaline mannanases reported to date. The amino acid sequence of the β-mannanase from *Bacillus* sp. N16-5 revealed low identity (19%) to that of *Bacillus* sp. AM001. However, a higher level of similarity (48%) was observed for the C-terminal region (about 150 amino acid residues long) of these two enzymes. Akino et al. (1989) reported a β-mannanase gene from alkalophilic *Bacillus* sp. AM-001 producing two β-mannanases (ManA and ManB). The ManA and ManB had the molecular weights of 55 and 38 kDa and maximum activity at pH 9.0 and 8.5, respectively. The ManB lacks the C-terminal region of the ManA. Accordingly, we may consider that C-terminal region of the mannanase from *Bacillus* sp. N16-5, as well as that of *Bacillus* sp. AM001, may be responsible for the alkaliphilicity of the enzymes. The relationship between the structure and function of the mannanase is being pursued, which may be of value in mannanase engineering.

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