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Full Length Research Paper

Characterization of 6 *Bacillus subtilis* β-mannanases and their genes

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Six *Bacillus subtilis* strains (CD-3, CD-6, CD-9, CD-10, CD-23 and CD-25) that produce β-mannanase were separated from the Konjacflour plant, Tin, in the Yunnan province of China. The optimal temperatures of the 6 β-mannanases ranged from 45 to 65°C and their optimal pH ranged from 5.0 to 6.5; showing significant differences. The genes of the β-mannanase from 6 *B. subtilis* were amplified and sequenced. Except for CD-6, the genes shared the great homology (above 98%) with the *B. subtilis* β-mannanase gene deposited in GenBank. The β-mannanase gene sequence of CD-6 showed the highest homology with the sequence of β-mannanase of *Bacillus* sp. 5H, but the homology was only 70%. Alignment of the amino acid sequences of the 6 genes indicated that the 6 amino acid sequences shared near total homology with that of the *B. subtilis* β-mannanase sequence in GenBank. The homology between the amino acid sequence of CD-6 and that of β-mannanase of *Bacillus* sp. 5H was 72%. The homologies of the other five strains were all above 99%. The 6 amino acid sequences showed a high degree of conservation and the 2 most similar were CD-3 and CD-10, with a similarity of 99.5%.

Key words: β-mannanase, enzyme characterization, gene cloning.

INTRODUCTION

β-mannanase, also known as β-1,4-D-mannan-mannano-hydrolase (EC.3.2.1.78) belongs to the hemicellulases (Qi et al., 2002). It is an endonuclease that can hydrolyze mannan-oligosaccharides (MOS) and mannan polysaccharide with β-1,4-D-mannose glycosidic bonds. β-mannanase can hydrolyze the β-1,4 glycosidic bonds in the main chain of mannan from plant gum into mannose and mannan oligosaccharides composed of 2-10 mono-saccharides, which have physiological functions. Its products can significantly promote proliferation of beneficial bacterial flora, e.g., bifidobacterium, in the intestines of humans and animals to enhance the function of the digestive system, reduce the virulence of pathogens, regulate the immune system and improve the immunity of animal (Mao, 2000). β-mannanases have been extensively researched for their uses in the fields of food, medicine, fodder, paper making, printing and dyeing, textiles, oil extraction and biotechnology research.

β-mannanases exist widely in nature, being discovered in plants, such as sesame (Carvalho et al., 2001), the seeds of *Lactuca sativa* (Nascimento et al., 2004), corms germinated from konjacflour of araceae plants (Du and Li, 2000), in the digestive tract of ocean mollusca such as *perna viridis* (Xu et al., 2002), in bacteria of the human intestinal and in the rumen of herbivores. The main source of β-mannanase is microbial and it has been reported that β-mannanase are present in bacteria such as *Bacillus subtilis* (Tian et al., 1993), *Bacillus licheniformis* (Zhang et al., 2001), in thermophilic *Bacilli* (Ma et al., 2006) and in fungi such as *Aspergillus (*Li et al., 2002; Pi et al., 2006), *Sclerotium rolfsii* (Gubitz et al., 1996) and in *Actinomycete* (Wu and He, 2000). β-mannanases from different sources hydrolyze substrates in different ways and the degree of hydrolysis differs too. *B. subtilis* is the main source of

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Table 1. Primers were designed for cloning the β-mannanase genes.

Gene	Forward primer	Reverse primer
CD-3	5'-TGATTACGAATTCGAGCTCGGTAC-3'	5'-TGCAGGCATGCAAGCTGGCACTGC-3'
CD-6	5'-TGATTACGATTCGAGCTCGGTACC-3'	5'-CGAAATTTGGAATGGAATCGTCGA-3'
CD-9	5'-GCCAGTGCCAGCTTGCATGCCTGC-3'	5'-GTACCGAGCTCGAATTCGTAATCA-3'
CD-10	5'-GCCAGTGCCAGCTTGCATGCCTGC-3'	5'-TACCGAGCTCGAATTCGTAATTCA-3'
CD-23	5'-GGCAGTGCCAGCTTGCATGCCTGC-3'	5'-TACCGAGCTCGAATTCGTAATCAT-3'
CD-25	5'-ACGGCAGTGCCAGCTTGCATGCCT-3'	5'-GGTACCGAGCTCGAATTCGTAATC-3'

β-mannanase.

We isolated 6 β-mannanase producting *B. subtilis* strains from a Konjacflour plant, Tin, in Yunnan province, China. The enzymes' characteristics were studied and the 6 β-mannanase genes from the different strains were amplified and sequenced. BLASTN homology alignment analysis was performed on the GenBank database. Open reading frames (ORFs) were deduced from the DNA sequences using EditSeq in DNAStar software. BLASTP homology of the amino acid sequences encoded by the ORFs were analyzed, their evolutionary position was determined and differences among the 6 amino acid sequences were noted. In combination with their enzymatic properties, the relevance of the protein primary structure to their functions was discussed.

MATERIALS AND METHODS

Isolation

Six *B. subtilis* strains (CD-3, CD-6, CD-9, CD-10, CD-23 and CD-25) were isolated from a Konjacflour plant, Tin, in the Qujing district of Yunnan province.

Fermentation of bacteria and preparation of enzymes

A small amount of bacteria were inoculated onto LB solid medium by the streak-plate technique. After incubation at 37°C for 24 h, the bacteria were re-plated twice. A single colony was then picked into 20 ml of seed liquid in a 100 ml flask. The flask was incubated at 37°C overnight with shaking at 180 rpm. The seed culture was transferred into 30 mL basic fermentation medium in a 250 mL flask using a 2% inoculum and incubated at 37°C for 24 h at 180 rpm. The fermented broth was centrifuged for 10 min at 10,000 rpm and the supernatant retained as the crude enzyme liquid.

Purification of β-mannanase

The crude β-mannanases were isolated using a combination of ammonium sulfate, DEAE-ion exchange (a gradient from 1 to 500 mM NaCl in Tris-HCL, pH 6.5 was used to elute the proteins), SephacrylTMS-200 gel filtration chromatography (200 mM Tris-HCL, pH 6.5 was used to elute the proteins) and Mono S HR 5/5 chromatography (A gradient from 1 to 500 mM NaCl in Tris-HCL, pH 6.5 was used to elute the proteins) by AKTA purifier-900. The homology of the β-mannanases were determined by SDS-Polyacrylamide gel electroration.

Determination the activity of β-mannanase

Determination was performed by the DNS method (Akino et al., 1987).

Amplification of β-mannanase genes by PCR

Primers were designed according to the β-mannanase genes reported by GenBank as Table 1.The PCR conditions were: 94°C for 5 min, 30 cycles of 94°C for 1 min, 55°C for 1 min 30s and 72°C for 2 min 30 s and a final extension at 72°C for 20 min. The PCR products were purified by a silver beads DNA gel recovery kit (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd). The PCR products were sent to Dalian TaKaRa company for sequencing. Homology alignment analysis was made by BLASTN and BLASTP in GenBank.

RESULTS

The β-mannanass produced by 6 different strains were purified to homology by gel filters, which were one band in SDS-polyacrylamide gel electroration (data were not showed). Enzymatic characteristics of β-mannanases produced by 6 different strains were determined.

Optimal temperature of enzyme reaction and tolerance of temperature

Substrate was prepared with Na_2HPO_4-citric acid buffer, pH 6.0. After adding the enzyme solution at appropriate protein content, the reaction was incubated in different thermostatic water baths (35-75°C) for 10 min to determine the enzyme activity. Figure 1 shows that the optimal reaction temperatures of the β-mannanases produced by the 6 strains are different. The optimal reaction temperatures of β-mannanase produced by CD-3, CD-6 and CD-25 are 65°C, while those of CD-9, CD-10 and CD-23 are 45, 55 and 60°C, respectively.

Reaction mixtures containing appropriate dilutions of the enzymes were incubated in different thermostatic water baths for 30 min to determine their residual enzyme activities. Figure 2 shows that the temperature tolerances of the β-mannanases are also different. The β-mannanases produced by CD-3, CD-23 and CD-25 showed

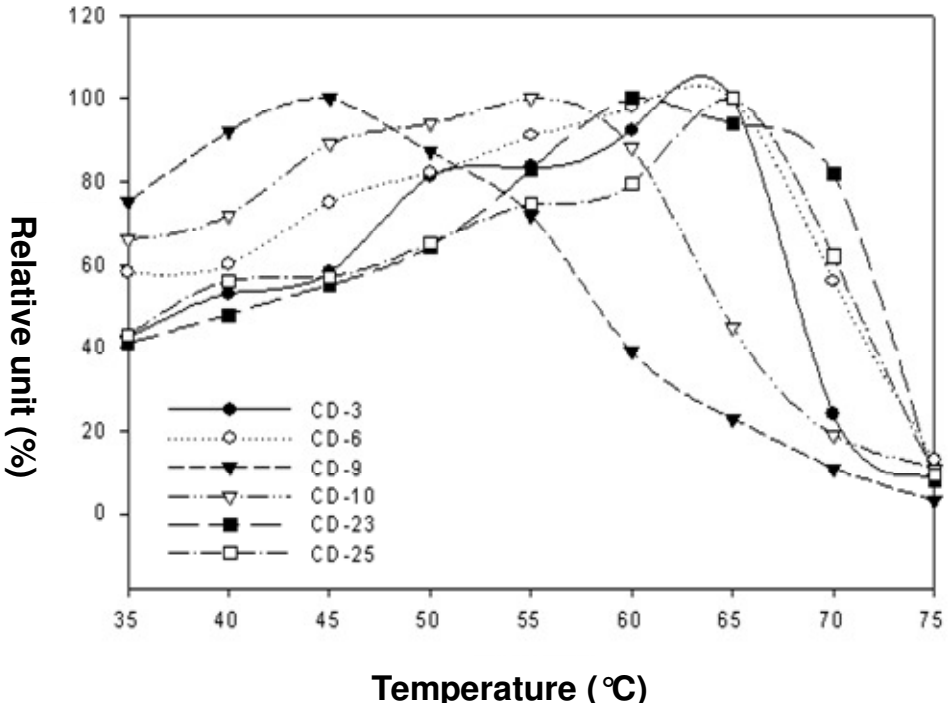

Figure 1. Effects of temperature on activity of β-mannanase.

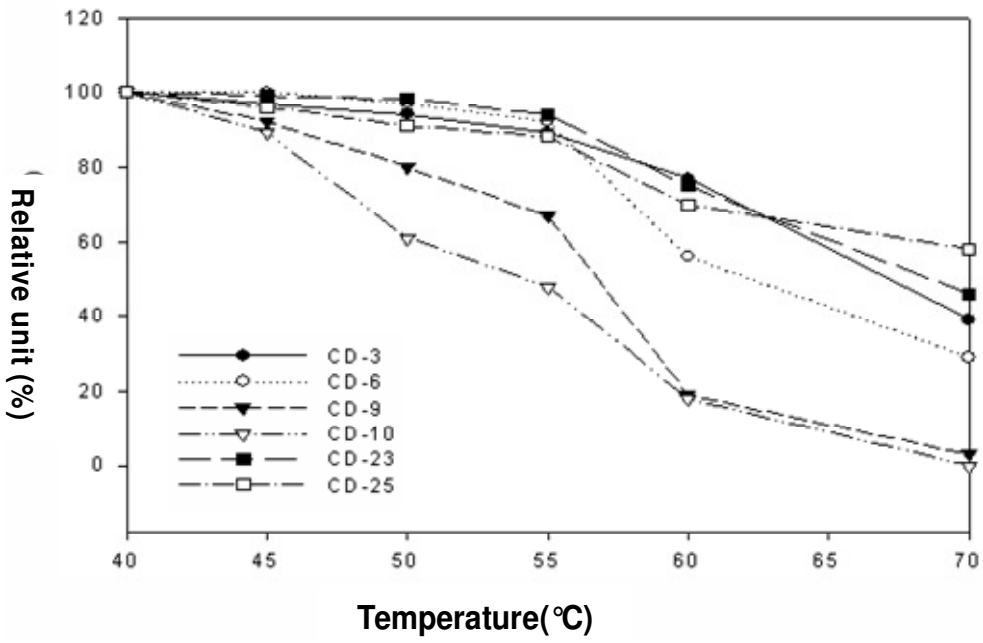

Figure 2. Effects of temperature on stability of β-mannanase.

good temperature tolerance; their residual enzyme activities remained above 70% even after incubating at 60°C for 30 min. However, the residual enzyme activities of the β-mannanases produced by CD-3 and CD-23 both dropped to below 50% after incubating in 70°C for 30 min. The temperature tolerance of CD-10 was the worst. Its enzyme activity dropped to below 50% after incubating at 55°C for 30 min.

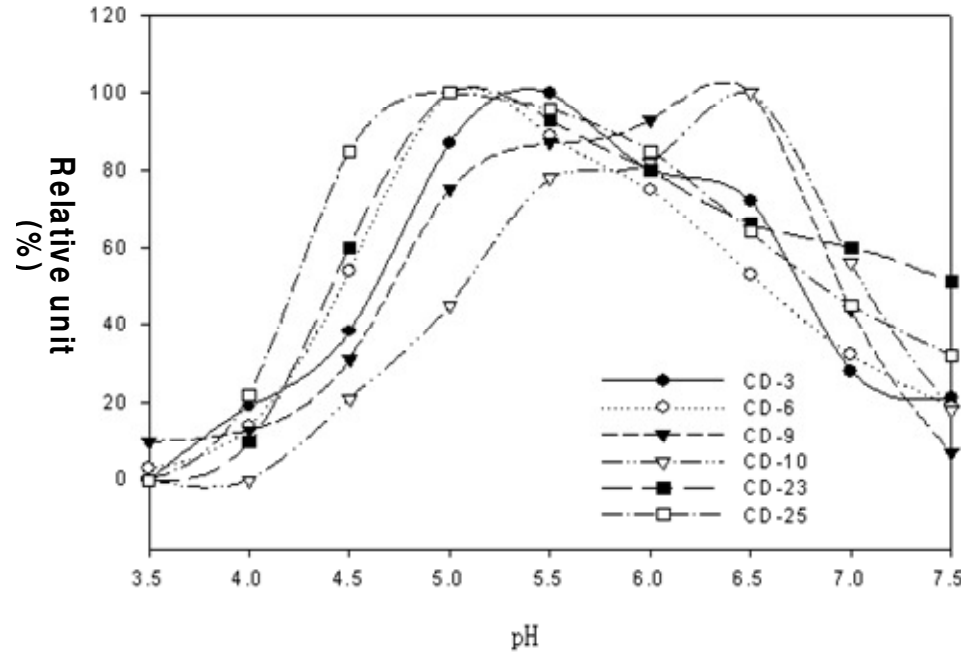

Figure 3. Effects of pH on activity of β-mannanase.

Optimal pH and pH tolerance of the 6 β-mannanases

Substrate was prepared in buffers of different pH (3.5 - 7.5), Na_2HPO_4- citric acid buffer with pH from 3.5 to 6.0 and Na_2HPO_4-NaOH buffer with pH from 6.0 to 7.5. After adding enzymes at the appropriate dilution, the enzyme activities were measured. Figure 3 shows that the optimal pH of the β-mannanases are different. The optimal pH of the β-mannanase produced by CD-6, CD-23 and CD-25 was 5.0; the optimal pH of the enzyme produced by CD-3 was 5.5 and the optimal pH of those enzymes produced by CD-9 and CD-10 was 6.5.

Enzyme solution was prepared in buffers of different pH (4.0-8.0), Na_2HPO_4- citric acid buffer with pH from 4.0 to 6.0 and Na_2HPO_4-NaOH buffer with pH from 6.0 to 8.0. The enzyme solutions were left at room temperature for 1 h before having their pH adjusted to their optimal pH to determine their residual enzyme activities. Figure 4 shows that the acid tolerance of the β-mannanase produced by CD-3 is relatively good. When kept at pH 4.5 at room temperature for 1 h, its residual enzyme activity was still above 80%. However, the residual enzyme activities of the other strains dropped to below 60% at pH 4.5, indicating relatively poor acid tolerance.

Determination of specific activity

The protein content of the test solutions after appropriate protein content was determined for the 6 purified β-mannanases (purification data not shown). The result is shown in Table 2.

Determination of isoelectric point

Buffers of different pH (3.5, 3.8, 4.0, 4.2, 4.5, 4.8 and 5.0) were prepared. Test solution was diluted appropriately with these buffers and kept at room temperature for 1 h. Then supernatant was extracted by centrifugation at 12,000 rpm for 10 min and tested for their residual enzyme activities. As shown in Figure 5, the isoelectric point of β-mannanase produced by CD-3 and CD-10 is 3.8 and that of the other strains is 4.0.

PCR and sequence analysis

Primers were designed according to the β-mannanase gene sequences deposited in GenBank. 6 amplification fragments of about 1 kb were obtained by PCR using genomic DNA from the 6 strains as templates. After purification, the products were sent to Dalian TaKaRa company for sequencing. The results showed that the PCR products from CD-3, CD-6, CD-9, CD-10, CD-23 and CD-25 were 1001 bp, 1014 bp, 1000 bp, 1000 bp, 1001 bp and 1000 bp, respectively. GenBank accession numbers were assigned (Table 3). BLASTN similarity search and homology comparisons were carried out using the GenBank database. Table 3 shows that, except the amplification product of CD-6, the amplification products of the strains show homology of more than 98% with the β-mannanase genes from *Bacillus subtilis*. The amplification product from CD-6 shares the greatest homology with the β-mannanase genes from *Bacillus sp.* 5H, but the homology is only 70%.

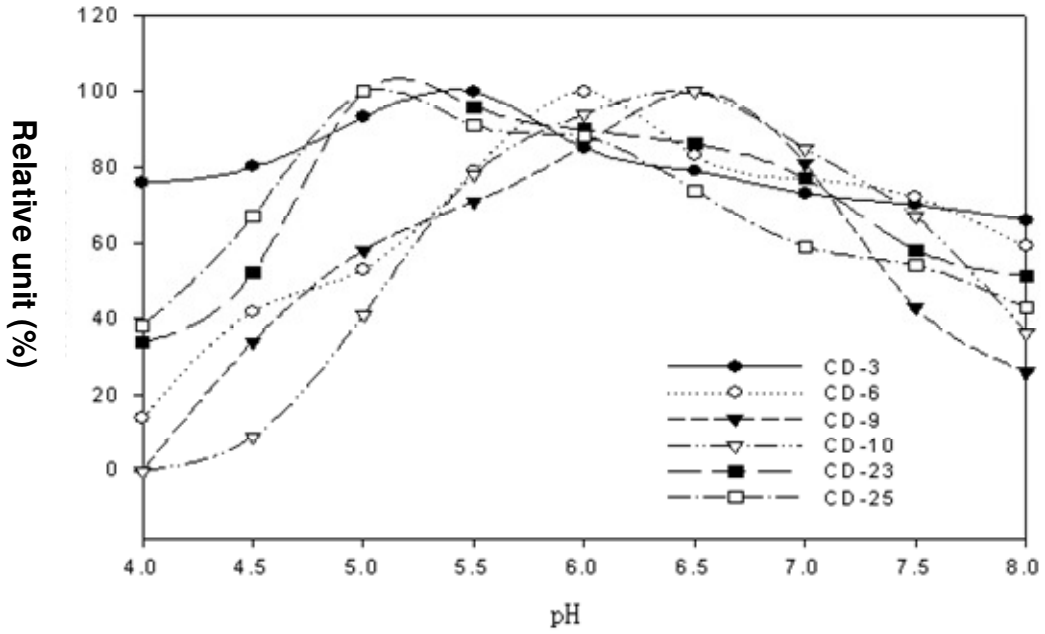

Figure 4. Effects of pH on stability of β-mannanase.

Table 2. Specific activity of β-mannanase from different strains.

Strain	Enzyme activity (U/mL)	Protein content (mg/mL)	Specific activity (U/mg)
CD-3	143.22	0.2253	635.69
CD-6	189.35	0.1996	948.65
CD-9	87.53	0.1875	466.83
CD-10	66.49	0.2954	224.25
CD-23	109.88	0.2078	528.78
CD-25	97.43	0.2309	421.96

Nucleotide sequence comparisons of six β-mannanase genes from different strains

Alignments of the 6 β-mannanase genes from the different strains were conducted by MegAlign in the DNA Star software (ClustalW Method). Percent identity and divergence of the 6 genes were analyzed using sequence distances. Figure 6 indicates that the 6 β-manna-nase genes from the different strains show differences. The β-mannanase genes from CD-9 and CD-25 share the highest similarity (99.5%). CD-23 and CD-25, CD-9 and CD-23, CD-3 and CD-10 also show very high similarities (99.4, 99.4 and 99.3%, respectively). The genes that showed the lowest similarity were from CD-3 and CD-6, with a similarity of only 70.4%.

Cluster analysis of these 6 genes was made with the β-mannanase genes that had been deposited in GenBank database and shared a high homology with the 6 genes to create a phylogenetic Tree (Figure 7). The 5 genes from CD-3, CD-9, CD-10, CD-23 and CD-25 show a close genetic relationship. However, they have a more distant relationship to the gene from CD-6. The 6 genes all show a close genetic relationship with the gene from *Bacillus* sp. 5H. Cluster analysis of these 6 genes alone was performed (Figure 8). The results indicate that the β-mannanase genes from CD-9 and CD-25 have the closest evolutionary relationship, which is consistent with the result of the similarity analysis shown in Figure 6.

β-mannanase amino acid sequence analysis

EditSeq in the DNAstar software was used to find the largest ORFs in the β-mannanases nucleotide sequences and the amino acid sequences encoded by the ORFs were deduced. The amino acid sequences were analyzed by BLASTP homology alignment against the GenBank database and the result are shown in Table 4.

The result demonstrates that the 6 deduced amino acid sequences share the greatest homology with the β-mannanases from *Bacillus* sp. 5H and also show great homology with β-mannanases from *Bacillus* strain GHF26

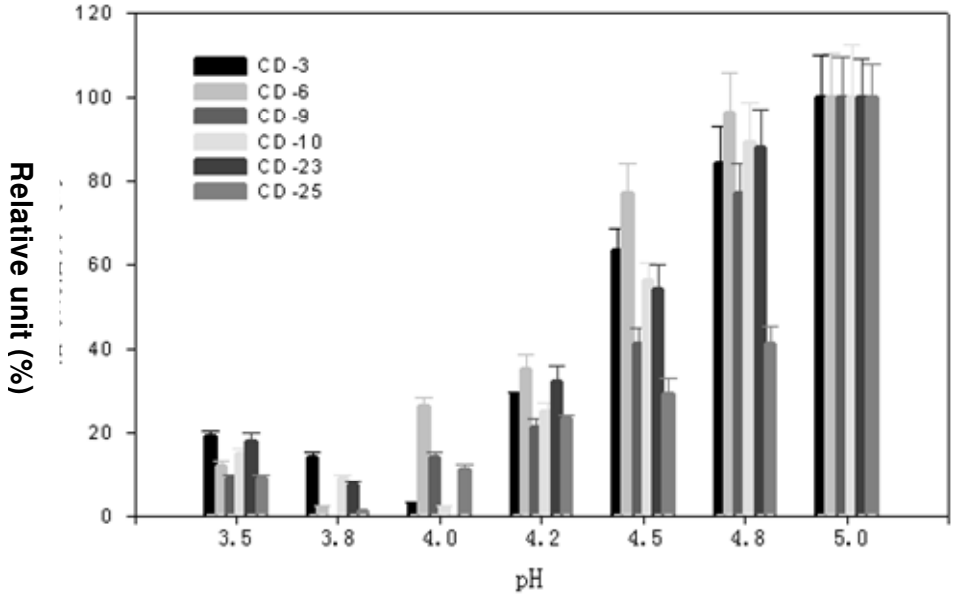

Figure 5. The determination of isoelectric point from protein.

Table 3. Alignment analysis in GenBank of PCR amplification products.

Strain	Length (bp)	GenBank accession No.	Homolog	Homology (%)
CD-3	1001	EU755322	*B. subtilis* strain A33 *beta*-mannanase (man) gene	98
CD-6	1014	EU755323	*Bacillus sp.5H beta*-mannanase (man) gene	70
CD-9	1000	EU755324	*B. subtilis* isolate WL-7 *beta*-mannanase precursor (manA) gene	98
CD-10	1000	EU755325	*Bacillus subtilis* strain A33 *beta*-mannanase (man) gene	98
CD-23	1001	EU755326	*B. subtilis* isolate WL-7 *beta*-mannanase precursor (manA) gene	98
CD-25	1000	EU755327	*B. subtilis* isolate WL-7 *beta*-mannanase precursor (manA) gene	98

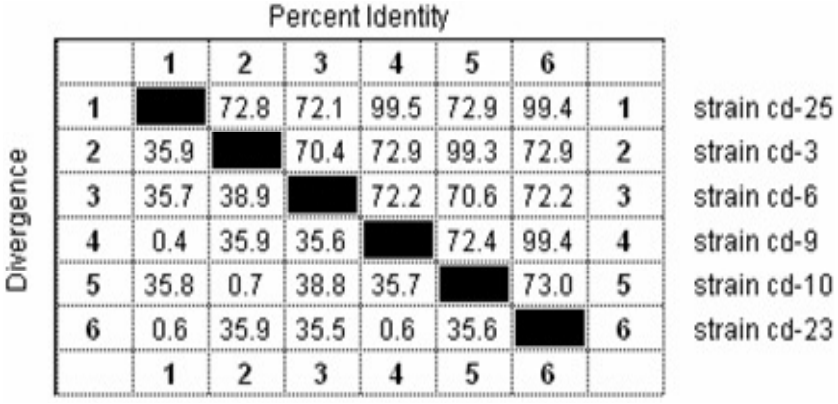

Figure 6. Percent identity and divergence of 6 sequences (by ClustalW method).

(more than 60%). Accordingly, we speculate that they might belong to *Bacillus* strain GHF26. Apart from the amino acid sequence of CD-6, which shares 72% with that of *Bacillus* sp. 5H, the other 5 sequences show a homology of more than 99% with that of proteins from *Bacillus subtilis*, which is consistent with the analysis of the alignments of the nucleotide sequences. Accordingly, we can deduce that the amino acid sequences are from

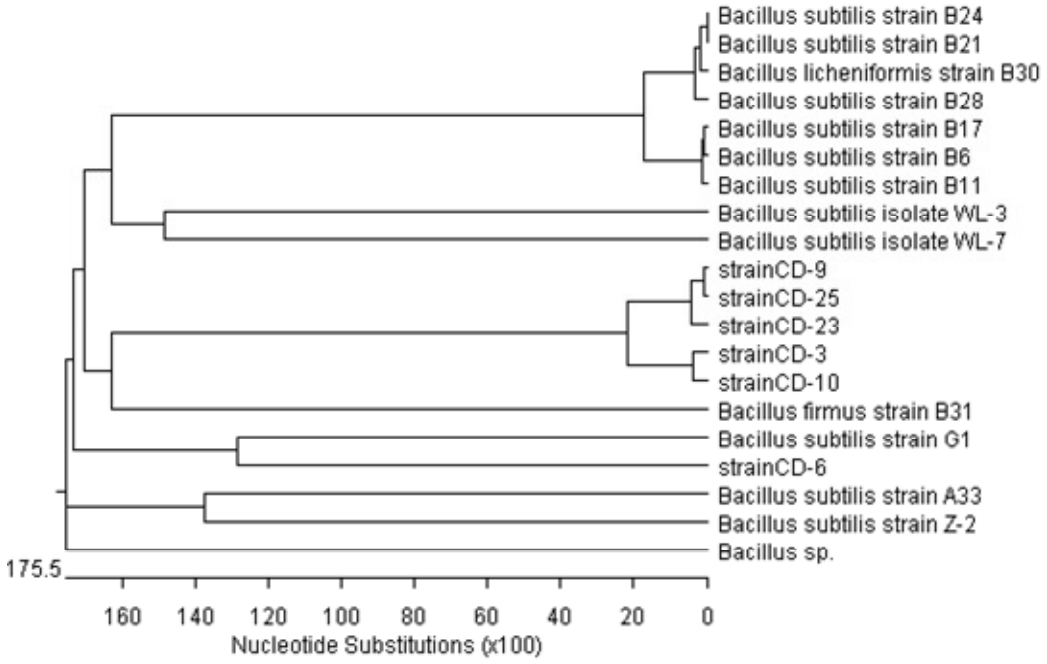

Figure 7. Cluster analysis of β-mannanases genes.

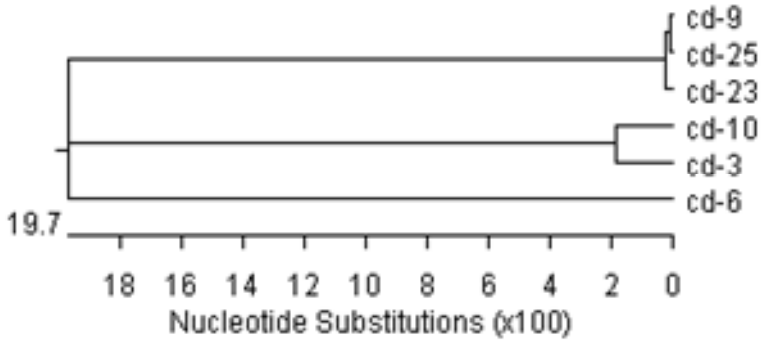

Figure 8. Cluster analysis of 6 β-mannanases genes.

Table 4. Alignment analysis in GenBank of β-mannanase deduced amino-acid sequences.

Strain	ORF (bp)	Amino acids	Name and origin of homologous protein	Homology (%)
CD-3	935	311	beta-mannanase [*B. subtilis* A33]	99
CD-6	948	316	beta-mannanase [*Bacillus* sp.5H]	72
CD-9	909	303	beta-mannanase precursor [*B. subtilis* WL-7]	100
CD-10	723	241	beta-mannanase [*B. subtilis* A33]	99
CD-23	935	311	beta-mannanase precursor [*B. subtilis* WL-7]	100
CD-25	934	311	beta-mannanase precursor [*B. subtilis* WL-7]	100

the β-mannanases originating from *Bacillus* sp. 5H. Percent identity and divergence of the 6 amino acid sequences were analyzed and the result is shown in Figure 9. CD-3 and CD-10 share the highest similarity of 99.9% and the sequences from CD-25 and CD-23, CD-25 and CD-9, CD-23 and CD-9 also show great similarity (99.5, 99.6 and 99.3%, respectively). The highest and the lowest homology of amino acid sequence from CD-6 with that of the other 5 sequences are 71.2 and 69.2% respectively, which is consistent with the nucleotide

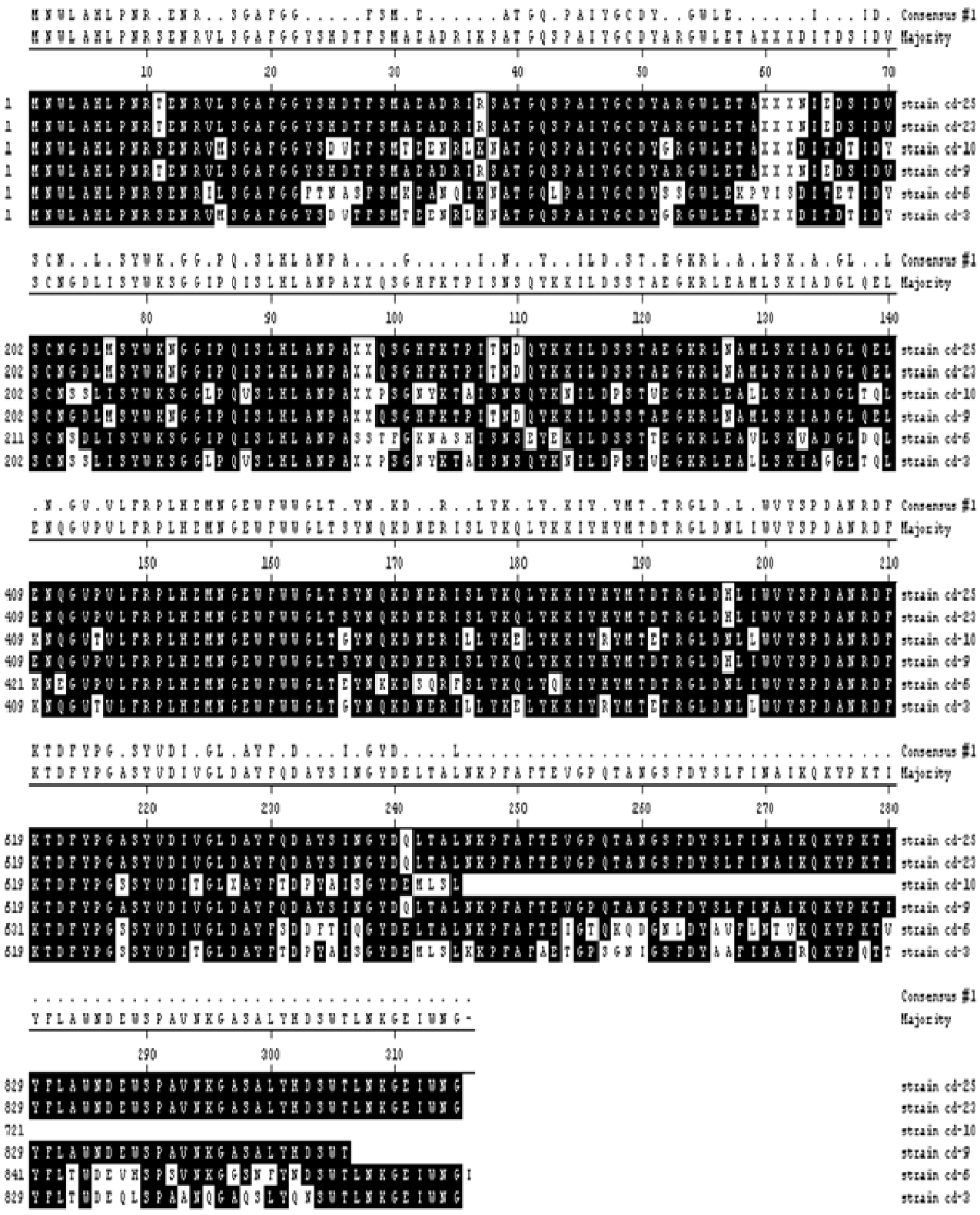

Figure 9. Alignment of deduced β-mannanase amino-acid sequences from *B. subtilis* isolates.

sequence comparisons.

The homologies of the 6 amino acid sequences were analyzed by alignment in the DNAStar software. Figure 9 shows that the homology of the 6 sequences is high, with

large sections of conserved sequences. These conserved sequences might have some relevance to the functions of β-mannanases.

The proteins from CD-3 and CD-10 showed the highest similarity (99.9%), which is consistent with the analysis of the alignments of the nucleotide sequences. Only one difference exists, at amino acid position 227. In CD-3 the amino acid is Asp, but in CD-10 the amino acid is missing. The similarity between CD-10 and CD-6 is the lowest (69.2%). Nevertheless, there are still large sections of conserved sequences existing among the 6 sequences. For example, in the regions of amino acid residues: 1 - 10, 12 - 14, 17 - 22, 28 - 30, 32, 39 - 42, 44 - 51, 54 - 57, 64, 68 - 69, 71 - 73, 76, 77 - 81, 83 - 84, 86 - 87, 89 - 96, 101, 107, 109, 112, 115 - 117, 119 - 120, 122 - 126, 128, 130 - 132, 134, 136 - 137, 140, 142, 144 - 145, 147 - 165, 167 - 168, 170 - 171, 174, 177 - 179, 181 - 182, 184 - 186, 188 - 190, 192 - 196, 198, 200 - 217, 128 - 223, 225 - 226, 228 - 230, 232, 236, 238 - 240 and 245. The same amino acids exist at these positions in all 6 proteins and thus they are highly conserved. We speculate that these conserved regions must be associated with the special functions of β-mannanase.

DISCUSSION

The temperature tolerance of the β-mannanase from CD-10 is worse than the others. In the CD-10 protein, the amino acid at 227 is missing, while in the others the amino acid is Asp. This one difference might be responsible for the difference in temperature tolerance.

The enzyme from CD-3 showed better pH tolerance than the other strains. In CD-3, the amino acid at position 135 is Gly, but it is Asp in the other enzymes. This change of amino acid at this position might have an influence on the pH tolerance of the CD-3 enzyme.

The isoelectric points of CD-3 and CD-10 are 3.8, while the others are 4.0. Analyzing the amino acid sequences of CD-3 and CD-10, at positions 16, 33, 36, 75, 85, 88, 114, 118, 176, 180, 187, 191, 199 and 224, the amino acids between the 2 strains are the same, however compared with the other sequences, these amino acids are different. We speculate that the amino acids at the above positions might affect the protein isoelectric point. This will need to be confirmed by further experimentation.

ACKNOWLEDGEMENTS

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REFERENCES

Akino T, Nakamura N, Horikoshi K (1987). Production of beta-mannosidase and beta-mannanase by an alkalophilic *Bacillus* sp [J]. Appl. Microbiol Biotechnol. 26: 323-327.

Carvalho P, Borghetti F, Buckeridge MS, Morhy L, Filho E (2001). Temperature-dependent germination and endo-β-mannanase activity in sesame seeds [J]. R. Bras. Fisiol. Veg. 13(2): 139-148.

Du XF, Li P (2000). Some properties of β-mananase from the tubers of *Amorphophallus albus* [J]. J. Hefei Univ. Technol. 23(5): 679-682.

Gubitz GM, Hayn M, Urbanz G, Steiner W (1996). Purification and properties of an acidic β-mannanase from *Sclerotium rolfsii* [J]. J. Biotechnol. 45: 165-172.

Li JF, Wang BL, Wu MC (2002). Fermentation process of acidic β-mannanase from *Aspergillus niger* [J]. Food Ferment. Ind. 28(9): 19-22.

Ma JS, Yao TT, Shi GY, Wang ZX (2006). Purification and characterization of β-mannanase from *Bacillus stearothermophilus* strain 2004 [J]. J. Food Sci. Biotechnol. 25(3): 25-32.

Mao S (2000). Application of β-mannanase in production of animals [J]. Cereal Feed Industry, 9: 31-33.

Nascimento WM, Cantliffe DJ, Huber DJ (2004). Ethylene evolution and endo-β-mannanase activity during lettuce seed germination at high temperature[J]. Sci. Agric. 61(2): 156-163.

Pi XE, Fei DB, Wang LY, Feng GQ, Yuan C (2006). Properties of acidic β-mannanase from *Aspergillus niger* AS6034 [J]. Feed Res. 2: 50-52.

Qi JR, Liao JS. Peng ZY (2002). Progress on the production and application of β-mannanase from microbe [J]. China Food Additives, 6: 12-15.

Tian XY, Xu Y, Ma YH, Zhou PJ (1993). Purification and p roperties of β-mannanases from alkalophilic *Bacillus* N16-5. Acta Microbiol. Sinica 33(2): 115-121.

Wu J, He BW (2000). The study on chemical modification and active site of β-D mannanase from Nocardioform Actinomycetes [J]. Chin. J. Biochem. Mol. Biol. 16(2): 227-230.

Xu BZ, Hägglund P, Stålbrand H, Janson JC (2002). Endo-beta-1, 4-mannanases from blue mussel, *Mytilus edulis*: purification, characterization, and mode of action [J]. J. Biotechnol. 92: 267-277.

Zhang J, He ZM, Hu K, Feng YY, Zhang ZG (2001). Preparation of β-mannanase from *Bacillus licheniformis* [J]. Food Ferment. Ind. 27(2): 5-7.