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Research Article

Purification and Characterization of a Thermostable β-Mannanase from *Bacillus subtilis* BE-91: Potential Application in Inflammatory Diseases

Lifeng Cheng, Shengwen Duan, Xiangyuan Feng, Ke Zheng, Qi Yang, and Zhengchu Liu

Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences, Changsha, Hunan 410205, China

Correspondence should be addressed to Zhengchu Liu; liuzhengchu@caas.cn

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β-mannanase has shown compelling biological functions because of its regulatory roles in metabolism, inflammation, and oxidation. This study separated and purified the β-mannanase from *Bacillus subtilis* BE-91, which is a powerful hemicellulose-degrading bacterium using a "two-step" method comprising ultrafiltration and gel chromatography. The purified β-mannanase (about 28.2 kDa) showed high specific activity (79, 859.2 IU/mg). The optimum temperature and pH were 65°C and 6.0, respectively. Moreover, the enzyme was highly stable at temperatures up to 70°C and pH 4.5–7.0. The β-mannanase activity was significantly enhanced in the presence of Mn^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, and Al^{3+} and strongly inhibited by Ba^{2+} and Pb^{2+}. K_m and $V_{\max}$ values for locust bean gum were 7.14 mg/mL and 107.5 μmol/min/mL versus 1.749 mg/mL and 33.45 μmol/min/mL for Konjac glucomannan, respectively. Therefore, β-mannanase purified by this work shows stability at high temperatures and in weakly acidic or neutral environments. Based on such data, the β-mannanase will have potential applications as a dietary supplement in treatment of inflammatory processes.

1. Introduction

Mannan consists of a series of complex polysaccharides, which are found in the cell wall of marine algae [1]. The backbone is comprised of β-1,4-linked mannose residues. Konjac glucomannan is a randomly arranged polymer of β-1,4-linked glucose and mannose residues at ratio of 1.0 : 1.6. Both the backbones of mannan and Konjac are modified by α-1,6-linked galactosyl residues to form galactomannan and galactoglucomannan, respectively [2].

β-mannanase (EC 3.2.1.78) is a hemicellulase that attacks the internal glycosidic bonds of mannan backbone to release the condensed β-1,4-manno-oligosaccharides [3]. β-mannanases are widely applied in pulp and paper processing [4], feed [5], food [6], pharmaceutical [7], oil, and textile industries [8] to randomly hydrolyze the β-1,4 mannopyranoside linkage in mannan, galactomannan, glucomannan, and galactoglucomannan.

β-mannanase is widely produced by bacteria [9, 10], actinomycetes [11], fungi [12], plants, and animals [13]. Among them, β-mannanase from bacteria is wildely used because of numerous advantages, including extracellular secretion, economic production and purification, and novel characteristics, such as tolerance to heat and alkaline conditions [14].

Although multiple β-mannanase-producing bacteria have been reported [15, 16], they are far from the diverse industry needs. Currently, acidic and alkaline β-mannanase has been proposed to meet the industrial demands [17]. However, the requirements of high energy in production and the environmental impact limit their development. Neutral and weakly acidic β-mannanase with lower energy for production has attracted considerable interest over the past few years; however, it has rarely been characterized. It is clarified that β-mannanase with high activity in short fermentation time confers lower costs during the production procedures. Therefore, the exploitation of strains producing high β-mannanases activity is valuable and profitable. In current study, we isolated and preserved a powerful hemicellulose-degrading bacterium (BE-91). Then we explored

the efficient purification process and characterized the enzymatic properties of its β-mannanase.

2. Materials and Methods

2.1. Microorganism, Media, and Fermentation Conditions. *B. subtilis* BE-91, a strain used for herbaceous fiber extraction, was identified and preserved by the Institute of Bast Fiber Crops, Chinese Academy of Agricultural Science (Changsha, Hunan, China). *B. subtilis* BE-91 was cultured in Petri dish containing 0.5% yeast extract, 1% NaCl, 0.5% Konjac glucomannan, 1% bacto tryptone, 0.05% trypan blue, and 1.5% agar. The seed medium was mainly composed of 0.1% glucose, 0.4% Konjac glucomannan, 0.3% beef extract, 0.2% yeast extract, 0.5% peptone, and 0.5% NaCl. The fermentation medium primarily consisted of 0.2% yeast extract, 0.7% Konjac glucomannan, 0.5% peptone, 0.3% beef extract, and 0.5% NaCl. *B. subtilis* BE-91 was first activated in the seed medium at 35 ± 1°C for 5.5 h. Subsequently, the suspension was serially diluted, spread onto Petri dishes, and incubated at 35 ± 1°C for 18 h. The single colony exhibiting the largest hydrolytic halo was transferred into an Erlenmeyer flask with the seed medium and cultured at 35 ± 1°C for 6 h at 180 rpm. Consequently, 2% culture was inoculated in the fermentation medium and cultured for 6 h at 35 ± 1°C at 180 rpm [18].

2.2. Classification of Strain BE-91. The 16S rDNA of strain BE-91 was PCR amplified from genomic DNA using the Bacterial Identification PCR Kit (TaKaRa, Japan). The obtained 16S rDNA was sequenced by the ABI 3730XL 96-capillary DNA analyzer. The primers were as follows: P1 5′-AGAGTTTGATCMTGGCTCAG-3′ and P2 5′-TACGGYTACCTTGTTACGACTT-3′. The resulting sequence aligned closely with the related standard sequences of other bacteria retrieved from GenBank. Neighbor-joining clusters were constructed by Mega 6.0 [19].

2.3. Enzymatic Assays. β-mannanase activity was estimated by initiating the reaction at 65°C for 10 min in 0.05 mol/L citric acid/0.1 mol/L Na_2HPO_4 buffer (pH 6.0) with 0.2% (w/v) Konjac glucomannan as substrate. The amounts of reducing sugar in the reaction were quantified based on a standard curve generated with mannose using the 3,5-dinitrosalicylic acid (DNS) method. One unit (IU) of β-mannanase activity was defined as the amount of protein producing 1 μmol/L of reducing sugar per minute (e.g., mannose) under standard conditions [20].

2.4. Purification of β-Mannanase. The bacterial β-mannanase was purified using a two-step process involving ultrafiltration (Sartorius, Germany) and gel filtration. The fermentation liquid was fractionated orderly by 100 kDa, 50 kDa, and 5 kDa membrane thresholds. The solution filtered with 5 kDa < MW < 50 kDa was further purified on a Sephadex G-100 gel column (Φ1.6 cm × 100 cm, Pharmacia). The eluate was obtained at a rate of 0.5 mL/min and collected in 5 mL fractions. β-mannanase activity was determined by the DNS method, whereas the protein was quantified by the Coomassie brilliant blue staining against bovine serum albumin (BSA) standard [21].

2.5. The Determination of Apparent Molecular Weight. The molecular mass of the β-mannanase was determined by SDS-PAGE (Bio-Rad, USA), with 3% stacking gel and 12% separating gel [22]. The protein bands were stained with 0.01% Coomassie brilliant blue R-250 and destained with a water-methanol-acetic acid (9 : 9 : 2) solvent. Zymogram analysis was performed by the method of Chanhan [17]. The molecular weight of β-mannanase was derived from the relative mobility of molecular weight markers resolved simultaneously.

2.6. The Effect of Temperature on the Activity and Stability of β-Mannanase. The activity of β-mannanase was assayed at a range of temperatures between 50 and 70°C in 0.05 mol/L citric acid-0.1 mol/L Na_2HPO_4 buffer at pH 6.0. The thermostability was assessed by preincubating the enzyme, without a substrate, at different temperatures varying over 20–80°C for 30 min. The residual activity was promptly measured by the DNS method. The β-mannanase activity was considered to be 100% when preincubated at 4°C.

2.7. The Effect of pH on the Activity and Stability of β-Mannanase. β-mannanase activity was evaluated by incubating the purified enzyme at different pH conditions ranging from 4.0 to 8.0 in 0.05 mol/L citric acid-0.1 mol/L Na_2HPO_4 buffer at 4°C. The stability at a particular pH was tested by preincubating the purified enzyme, without a substrate, for 30 min in various 0.05 mol/L citric acid-0.1 mol/L Na_2HPO_4 buffers at pH 3.0–8.5 at 4°C. The residual β-mannanase activity was immediately measured after treatment by the DNS procedure.

2.8. The Effect of Metal Ions on the Activity of β-Mannanase. In order to examine the effects of metal ions on the activity of β-mannanase, the enzyme was incubated for 30 min at 4°C in the presence of various 1.0 mmol/L metal ions, $CaCl_2 \cdot 2H_2O$, $ZnCl_2$, $FeCl_3$, $PbCl_2 \cdot 2H_2O$, $MnCl_2 \cdot 4H_2O$, $MgCl_2 \cdot 6H_2O$, KCl, $CuCl_2 \cdot 2H_2O$, $AlCl_3$, $BaCl_2$, and NH_4Cl. The residual β-mannanase activity was measured at a specific condition and that of the treatment in the absence of additives as a control.

2.9. Substrate Specificity and Kinetic Parameters. Various glycans, such as Konjac glucomannan [23], locust bean gum from *Ceatonia siliqua* seeds (Sigma, G0753), carob galactomannan (Megazyme, P-GALML), guar galactomannan (Megazyme, P-GGMMV), ivory nut mannan (Megazyme, P-MANIV), 1,4-beta-D-mannan (Megazyme, P-MANCB), wheat arabinoxylan (Megazyme, P-120601a), beechwood xylan (Megazyme, P-141101a), and carboxymethyl cellulose (Megazyme, P-CMC4M) were examined. In brief, 0.2% (w/v) glycans were incubated with β-mannanase at 65°C for 10 min in 0.05 mol/L citric acid-0.1 mol/L Na_2HPO_4 buffer at pH 6.0, and the reducing sugars were measured by DNS. The Michaelis-Menten kinetic parameters, V_{max} and K_m, were calculated for β-mannanase. The assays of the purified enzyme were carried out by the standard DNS procedure, as described

Table 1: β-mannanase activities of five bacteria.

Bacterium number	Activity (IU/mL)[a]	Specific activity (IU/mg)[a]
BE-23	0	0
BE-78	191.5 ± 4.5	879.8 ± 13.2
BE-46	83.2 ± 2.1	311.6 ± 9.4
BE-83	70.7 ± 1.6	119.7 ± 25.5
BE-91	273.7 ± 6.5	2,319.2 ± 26.3

[a]Data are mean ± SD, $n = 3$.

above, using 1–5 mg/mL locust bean gum and 0.5–2.5 mg/mL Konjac glucomannan as substrates. The kinetic constants were determined from the Michaelis-Menten equation by directly inputting the initial rates from Lineweaver-Burk plots or the nonlinear regression [24].

2.10. Statistical Analysis. Each β-mannanase activity experiment was performed in triplicate and expressed as mean ± SD (standard deviation). The statistical analyses were performed with SPSS 15.0 (SPSS Inc., Chicago IL, USA). One-way or two-way analysis of variance (ANOVA) was used to compare various treatment groups.

3. Results and Discussion

3.1. Screening of the High β-Mannanase Activity Producing Strain. Four bacteria were stochastically selected for the β-mannanase activity assay. Figure 1 exhibited the halos produced on the screening plate. Table 1 summarized the β-mannanase activity of the four bacteria (strain BE-23 without β-mannanase activity was used as a negative control). Strain BE-91 fermented for 9 h exhibited the highest activity, up to 273.7 IU/mL. Wild-type *B. subtilis* MA139 yielded a maximum β-mannanase activity of 170 IU/mL after 3 days of fermentation, and the maximum enzyme activity of *B. subtilis* TJ-102 was 205.3 IU/mL at 38 h [25, 26]. Notably, BE-91 secreted β-mannanase with higher activity in shorter time.

3.2. Classification of B. subtilis BE-91. The 1,508 bp sequence of 16S rDNA of strain BE-91 was analyzed by a phylogenic tree (Figure 2). The homology between BE-91 16S rDNA (gi 260159552) and *B. subtilis* 16S rDNA (gi 530330588 and gi 341831474) was 99%. It was confirmed that the similarity of *B. subtilis* type strains about 16S rRNA gene sequence is higher than 98% [27, 28]. We also obtained ≥98% similarity to 16S rRNA gene sequences of *B. subtilis* isolates.

3.3. Isolation and Purification of β-Mannanase. 2,000 mL of fermentation liquor was purified by ultrafiltration and chromatography. Specific activity, recovery, and multiple purifications at each step were summarized in Table 2. The recovery of β-mannanase in *B. subtilis* BE-91 exceeded 66.0%; multiple purifications achieved 32.9-fold pure β-mannanase activity, and the specific activity of the purified enzyme reached 79,859.2 IU/mg. The purified β-mannanase was shown to be homogeneous judged by SDS-PAGE analysis (Figure 3). Compared with the previous separation and purification

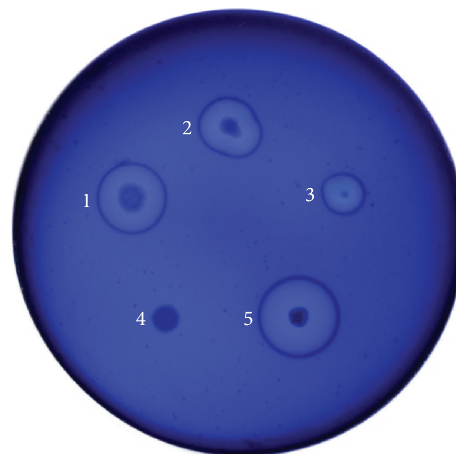

Figure 1: Clear halos produced by control and active colonies with β-mannanase activity 1, BE-78; 2, BE-46; 3, BE-83; 4, BE-23 (negative control); 5, BE-91.

methods [29, 30], the two-step method has the advantages of high efficiency, high yield, and easy operation.

3.4. Apparent Molecular Weight of β-Mannanase. The apparent molecular weight of β-mannanase was 28.2 kDa (Figure 3), lower than those of the most known β-mannanases from *Bacillus* spp. (*Bacillus licheniformis* THCM 3.1, 40 kDa; *B. subtilis* WY34, 39.6 kDa; *B. subtilis* Z-2, 38 kDa; *Bacillus circulans* CGMCC1554, 32 kDa) [28, 31–34]. Similarly, the molecular weights of β-mannanases from *Penicillium occitanis* Po16 and *Bacillus halodurans* PPKS-2 were 22 and 18 kDa, respectively [30, 31]. Due to low molecular weights, these enzymes may rapidly penetrate the lignocellulose systems and depolymerize the mannans more efficiently [35].

3.5. Optimal Temperature and Thermostability of β-Mannanase. The purified β-mannanase was maximally active at 65°C (Figure 4) and remained more than 80% active at 70°C (Figure 5). Compared with the optimal temperatures obtained for other β-mannanases (40°C for *Penicillium occitanis* Pol6; 50°C for both *Bacillus circulans* TN-31 and *B. subtilis* B36; 60°C for *Paenibacillus* sp. DZ3) [29, 31, 36], β-mannanase of BE-91 showed a pronounced activity at higher temperatures. As compared to the thermostability of the β-mannanase from wild-type *B. subtilis* BCC41051 (60°C for 30 min) [37], this β-mannanase retains 80% residual activity after incubation at 20–70°C for 30 min, indicating enhanced thermostability.

3.6. Optimal pH and Stability of β-Mannanase. The optimal pH and the stability of BE-91 β-mannanase were measured at various pHs. The optimum enzyme activity was obtained at pH 6.0 (Figure 6), and more than 80% maximal activity was retained at pH 4.5–7.0 (Figure 7). Interestingly, the optimal pH of BE-91 β-mannanase was the same as that of *B. subtilis* MA139 (pH 6.0), an enzyme that can potentially be used

Table 2: Purification of β-mannanase by ultrafiltration and gel chromatography.

Purification step	Total activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Recovery (%)	Purification multiple (fold)
Fermentation liquor	429,650.8	176.7	2,431.4	100	1
Ultrafiltration	328,317.4	8.6	38,070.2	76.4	15.6
Gel chromatography	283,500.2	3.6	79,859.2	66.0	32.9

Bacillus circulans DSM 11 AY724690
Bacillus deserti KCTC 13246 GQ465041
Bacillus eiseniae KCCM 90092 HM035089
Bacillus horneckiae MTCC 9535 EU861362
Bacillus crescens KCTC 33627 LN625239
Bacillus acidicola DSM 14745 AF547209
Bacillus filamentosus DSM 27955 KF265351
Bacillus cibi DSM 16189 AY550276
Bacillus idriensis KCCM 90024 AY904033
Bacillus aerophilus MTCC 7304 KM055067
Bacillus galliciensis DSM 21539 FM162181
Bacillus subtilis DSM 10 AJ276351
Strain BE-91
Bacillus abyssalis DSM 25875 JX232168
Bacillus fengqiuensis DSM 26745 KC291653
Bacillus berkeleyi KCTC 12718 JN187498
Bacillus hemicellulosilyticus DSM 16731 AB043846
Bacillus daliensis CGMCC 1.10369 GU583651
Bacillus gibsonii DSM 8722 X76446
Bacillus cecembensis MTCC9127 AM773821
Bacillus cereus DSM 31 AE016877
Bacillus thuringiensis DSM 2046 D16281
Bacillus beijingensis DSM 19037 EF371374
Bacillus endoradicis CCBAU05776 GU434676
Bacillus kokeshiiformis KCTC 33163 JX848633
Paenibacillus amylolyticus DSM 11730 D85396
Paenibacillus alvei DSM 29 AJ320491
Paenibacillus kobensis DSM 10249 AB073363

Figure 2: Phylogenetic tree based on 16S rDNA sequences of strain BE-91 and other bacteria by Mega 6.0 using neighbor-joining analysis with 1000 bootstrap replicates.

as a feed additive for monogastric animals [25]. At pH < 4.0, the β-mannanase activity was negligible, retaining $<80\%$ of its maximal value obtained after incubation at pH > 7.5, 4°C for 30 min. A relatively broad zone of optimum activity was observed. Therefore, BE-91 β-mannanase can be considered a weakly acidic and neutral enzyme, thereby rendering suitability for animal feed industry [38].

3.7. The Effect of Metal Ions on β-Mannanase Stability. The effect of a variety of metal ions on β-mannanase activity was measured (Table 3). The highest induction was achieved with Mn^{2+}, which showed 168% baseline activity, followed by Al^{3+}, Ca^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, and NH_4^+, respectively. K^+ and Fe^{3+} had no obvious effects on β-mannanase activity in these conditions. Ba^{2+} and Pb^{2+} greatly inhibited the enzyme activity to a final rate of 83% and 74%, respectively. This suggests that BE-91 β-mannanase should not be contaminated by Ba^{2+} and Pb^{2+}.

Table 3: Effects of different metal ions (1 mmol/L) on β-mannanase activity.

Metal ions	Relative activity (%)[a]
Blank	100
K^+	99 ± 3.2
NH_4^+	103 ± 2.7
Ca^{2+}	117 ± 3.6
Zn^{2+}	115 ± 2.9
Mn^{2+}	168 ± 4.5
Cu^{2+}	116 ± 2.1
Mg^{2+}	107 ± 2.8
Ba^{2+}	83 ± 3.1
Pb^{2+}	74 ± 2.9
Fe^{3+}	99 ± 3.6
Al^{3+}	121 ± 4.3

[a]Data are mean ± SD, $n = 3$.

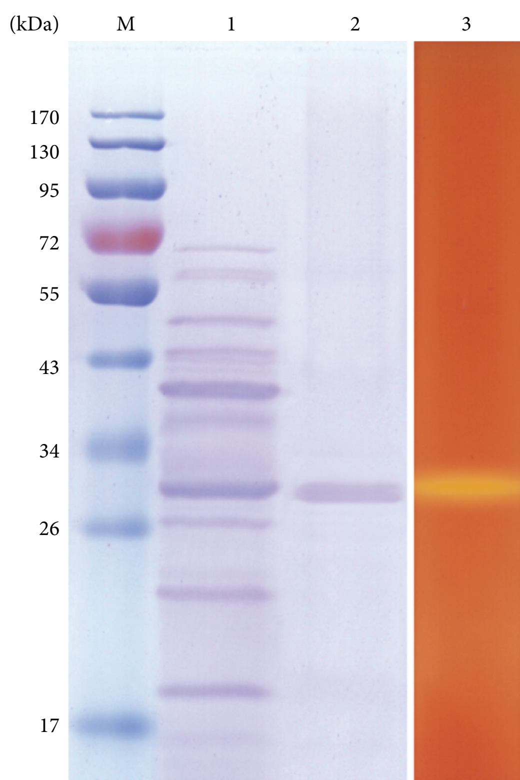

FIGURE 3: SDS-PAGE analysis of β-mannanase. Lane M: protein molecular weight standard; Lane 1: culture broth; Lane 2: purified β-mannanase; Lane 3: zymogram of purified β-mannanase.

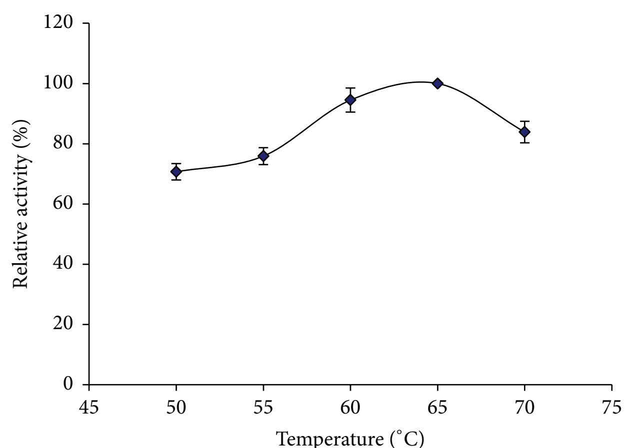

FIGURE 4: Optimum temperature curve of β-mannanase.

3.8. Kinetic Parameters. The purified enzyme hydrolyzed Konjac glucomannan but only slightly hydrolyzed ivory nut mannan, guar galactomannan, and 1,4-beta-D-mannan. Wheat arabinoxylan, beechwood xylan, and CMC were barely hydrolyzed, as shown in Table 4. This β-mannanase exhibited the highest activity with Konjac glucomannan, enriched in glucose units. This finding suggests that β-mannanase of BE-91 preferentially hydrolyzes the β-1,4-linkage of the glucosylated mannan backbone.

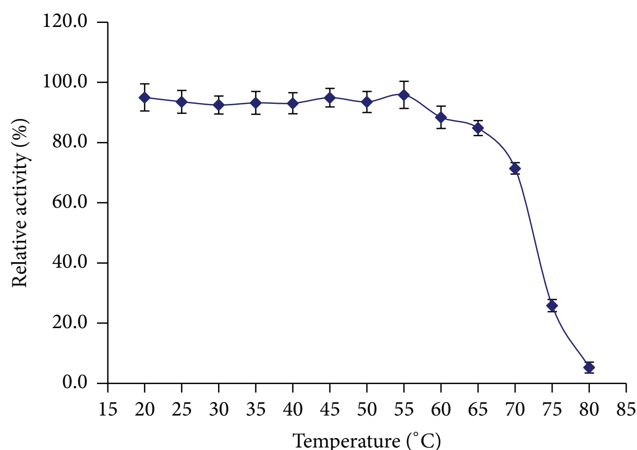

FIGURE 5: Thermal stability curve of β-mannanase.

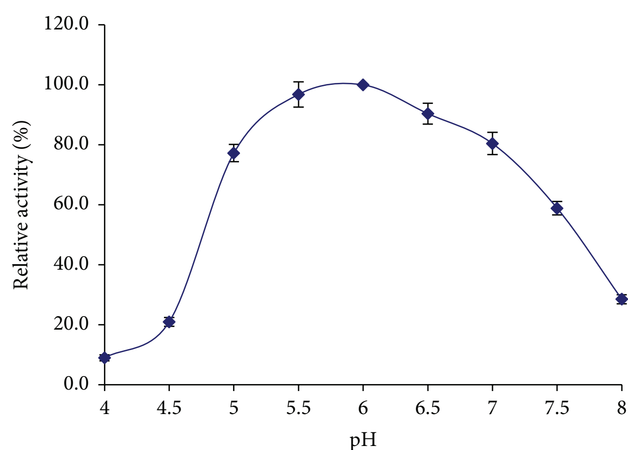

FIGURE 6: Optimum pH curve of β-mannanase.

TABLE 4: Hydrolytic activity of the purified enzyme on different polysaccharides.

Substrate (0.5%, w/v)	Relative activity (%)[a]
Konjac glucomannan	100
Locust bean gum	88.15 ± 1.8
Carob galactomannan	91.85 ± 1.7
Guar galactomannan	35.70 ± 0.6
Ivory nut mannan	32.74 ± 0.3
1,4-Beta-D-mannan	46.22 ± 0.4
Wheat arabinoxylan	0
Beechwood xylan	0
Carboxymethyl cellulose	0

Assays were carried out at 65°C at pH 6.0 for 10 min in 0.05 mol/L citric acid-0.1 mol/L Na_2HPO_4 buffer.
[a]Data are mean ± SD, $n = 3$.

K_m and V_{max} values of this β-mannanase estimated by the Lineweaver-Burk plot were 7.14 mg/mL and 107.5 μmol/min/mL, respectively, for locust bean gum, versus 1.749 mg/mL and 33.45 μmol/min/mL for Konjac glucomannan,

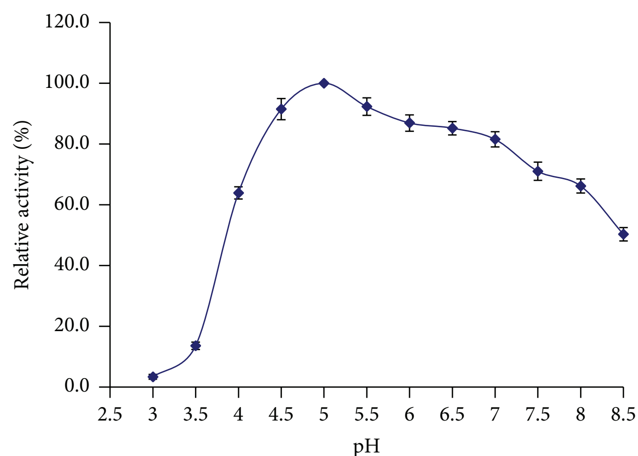

Figure 7: pH stability curve of β-mannanase.

respectively. These results displayed higher affinity of β-mannanase towards natural Konjac glucomannan (V_{max}/K_m, 19.1 μmol/min/mg) than the locust bean gum (V_{max}/K_m, 15.0 μmol/min/mg), similar to the values obtained for *Penicillium pinophilum* C1 and *Penicillium freii* F63, hence constituting it as an adequate candidate in food industry for the production of oligosaccharides [17, 18, 39].

4. Conclusion

B. subtilis bacteria are abundant, moderately stable, and mostly nonpathogenic microorganisms. Our results indicated that *B. subtilis* BE-91 could be considered a prominent candidate for the production of extracellular β-mannanase. In addition, this study developed an advanced purification approach, "two-step method," with high efficiency, high yield, and easy operation. Furthermore, the β-mannanase purified from BE-91 was extremely stable at relatively high temperatures and various weak acidic or neutral pHs. Finally, the enzyme showed a higher affinity towards natural Konjac glucomannan, a major functional food material. Therefore, this β-mannanase, purified and characterized from *B. subtilis* BE-91 for the first time, is suitable for inflammatory diseases.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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