Research Article

Purification and Characterization of a Thermostable \( \beta \)-Mannanase from Bacillus subtilis BE-91: Potential Application in Inflammatory Diseases

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\( \beta \)-mannanase has shown compelling biological functions because of its regulatory roles in metabolism, inflammation, and oxidation. This study separated and purified the \( \beta \)-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 \( \beta \)-mannanase (about 28.2 kDa) showed high specific activity (79,859.2 IU/mg). The optimum temperature and pH were 65 \( ^\circ \)C and 6.0, respectively. Moreover, the enzyme was highly stable at temperatures up to 70 \( ^\circ \)C and pH 4.5–7.0. The \( \beta \)-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 \( \mu \)mol/min/mL versus 1.749 mg/mL and 33.45 \( \mu \)mol/min/mL for Konjac glucomannan, respectively. Therefore, \( \beta \)-mannanase purified by this work shows stability at high temperatures and in weakly acidic or neutral environments. Based on such data, the \( \beta \)-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 \( \beta \)-1,4-linked mannose residues. Konjac glucomannan is a randomly arranged polymer of \( \beta \)-1,4-linked glucose and mannose residues at ratio of 1.0:1.6. Both the backbones of mannan and Konjac are modified by \( \alpha \)-1,6-linked galactosyl residues to form galactomannan and galactoglucomannan, respectively [2].

\( \beta \)-mannanase (EC 3.2.1.78) is a hemicellulose that attacks the internal glycosidic bonds of mannan backbone to release the condensed \( \beta \)-1,4-manno-oligosaccharides [3]. \( \beta \)-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 \( \beta \)-1,4 mannoypyranoside linkage in mannan, galactomannan, glucomannan, and galactoglucomannan.

\( \beta \)-mannanase is widely produced by bacteria [9, 10], actinomycetes [11], fungi [12], plants, and animals [13]. Among them, \( \beta \)-mannanase from bacteria is wildly 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 \( \beta \)-mannanase-producing bacteria have been reported [15, 16], they are far from the diverse industry needs. Currently, acidic and alkaline \( \beta \)-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 \( \beta \)-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 \( \beta \)-mannanase with high activity in short fermentation time confers lower costs during the production procedures. Therefore, the exploitation of strains producing high \( \beta \)-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 \( \beta \)-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′-TACGGY-TACCTTGTAGACTT-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. \( \beta \)-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\(_2\)HPO\(_4\) 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 sugar in the reaction were quantified based on a standard DNS method. The bacterial \( \beta \)-mannanase activity was measured as the amount of protein producing 1 \( \mu \)mol/L of reducing sugar per minute (e.g., mannose) under standard conditions [20].

2.4. Purification of \( \beta \)-Mannanase. The bacterial \( \beta \)-mannanase was purified using a two-step process involving ultrafiltration (Sartorius, Germany) and gel filtration. The fermentation liquid was fractionated order by order 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 x 100 cm, Pharmacia). The eluate was obtained at a rate of 0.5 mL/min and collected in 5 mL fractions. \( \beta \)-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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-Mannanase. The activity of \( \beta \)-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\(_2\)HPO\(_4\) 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 \( \beta \)-mannanase activity was considered to be 100% when preincubated at 4°C.

2.7. The Effect of pH on the Activity and Stability of \( \beta \)-Mannanase. \( \beta \)-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\(_2\)HPO\(_4\) 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\(_2\)HPO\(_4\) buffers at pH 3.0–8.5 at 4°C. The residual \( \beta \)-mannanase activity was immediately measured after treatment by the DNS procedure.

2.8. The Effect of Metal Ions on the Activity of \( \beta \)-Mannanase. In order to examine the effects of metal ions on the activity of \( \beta \)-mannanase, the enzyme was incubated for 30 min at 4°C in the presence of various 1.0 mmol/L metal ions, CaCl\(_2\)-2H\(_2\)O, ZnCl\(_2\), FeCl\(_3\), PbCl\(_2\)-2H\(_2\)O, MnCl\(_2\)-4H\(_2\)O, MgCl\(_2\)-6H\(_2\)O, KCl, CuCl\(_2\)-2H\(_2\)O, AlCl\(_3\), BaCl\(_2\), and NH\(_4\)Cl. The residual \( \beta \)-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 glucans, 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 mannannan (Megazyme, P-MANIV), and P-MANCB, wheat arabinoxylan (Megazyme, P-120601a), beechnut xylan (Megazyme, P-14101a), and carboxymethyl cellulose (Megazyme, P-CMC4M) were examined. In brief, 0.2% (w/v) glucans were incubated with \( \beta \)-mannanase at 65°C for 10 min in 0.05 mol/L citric acid-0.1 mol/L Na\(_2\)HPO\(_4\) buffer at pH 6.0, and the reducing sugars were measured by DNS. The Michaelis-Menten kinetic parameters, \( V_{\text{max}} \) and \( K_m \), were calculated for \( \beta \)-mannanase. The assays of the purified enzyme were carried out by the standard DNS procedure, as described
Table 1: \(\beta\)-mannanase activities of five bacteria.

<table>
<thead>
<tr>
<th>Bacterium number</th>
<th>Activity (IU/mL) (\pm) SD</th>
<th>Specific activity (IU/mg) (\pm) SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE-23</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BE-78</td>
<td>191.5 (\pm) 4.5</td>
<td>879.8 (\pm) 13.2</td>
</tr>
<tr>
<td>BE-46</td>
<td>83.2 (\pm) 2.1</td>
<td>311.6 (\pm) 9.4</td>
</tr>
<tr>
<td>BE-83</td>
<td>70.7 (\pm) 1.6</td>
<td>119.7 (\pm) 25.5</td>
</tr>
<tr>
<td>BE-91</td>
<td>273.7 (\pm) 6.5</td>
<td>2,319.2 (\pm) 26.3</td>
</tr>
</tbody>
</table>

\(\alpha\) Data are mean \(\pm\) 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 \(\beta\)-mannanase activity experiment was performed in triplicate and expressed as mean \(\pm\) 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 \(\beta\)-Mananase Activity Producing Strain. Four bacteria were stochastically selected for the \(\beta\)-mannanase activity assay. Figure 1 exhibited the halos produced on the screening plate. Table 1 summarized the \(\beta\)-mannanase activity of the four bacteria (strain BE-23 without \(\beta\)-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 \(\beta\)-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 \(\beta\)-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 phylogenetic 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 \(\geq 98%\) similarity to \(16S\) rRNA gene sequences of \(B. subtilis\) isolates.

3.3. Isolation and Purification of \(\beta\)-Mananase. 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 \(\beta\)-mannanase in \(B. subtilis\) BE-91 exceeded 66.0%; multiple purifications achieved 32.9-fold pure \(\beta\)-mannanase activity, and the specific activity of the purified enzyme reached 79,859.2 IU/mg. The purified \(\beta\)-mannanase was shown to be homogeneous judged by SDS-PAGE analysis (Figure 3). Compared with the previous separation and purification methods [29, 30], the two-step method has the advantages of high efficiency, high yield, and easy operation.

3.4. Apparent Molecular Weight of \(\beta\)-Mananase. The apparent molecular weight of \(\beta\)-mannanase was 28.2 kDa (Figure 3), lower than those of the most known \(\beta\)-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 \(\beta\)-mannanases from \(Penicillium\ occitanis\) Pol6 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 mannan more efficiently [35].

3.5. Optimal Temperature and Thermostability of \(\beta\)-Mananase. The purified \(\beta\)-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 \(\beta\)-mannanases (40°C for \(Penicillium\ occitanis\) Pol6; 50°C for both \(Bacillus\ circulans\) TN-31 and \(B. subtilis\) B36; 60°C for \(Paeonibacillus\ sp. DZ3) [29, 31, 36], \(\beta\)-mannanase of BE-91 showed a pronounced activity at higher temperatures. As compared to the thermostability of the \(\beta\)-mannanase from wild-type \(B. subtilis\) BCC41051 (60°C for 30 min) [37], this \(\beta\)-mannanase retains 80% residual activity after incubation at 20–70°C for 30 min, indicating enhanced thermostability.

3.6. Optimal pH and Stability of \(\beta\)-Mananase. The optimal pH and the stability of BE-91 \(\beta\)-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 \(\beta\)-mannanase was the same as that of \(B. subtilis\) MA139 (pH 6.0), an enzyme that can potentially be used
Table 2: Purification of \( \beta \)-mannanase by ultrafiltration and gel chromatography.

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

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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-Mannanase Stability. The effect of a variety of metal ions on \( \beta \)-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 \( \beta \)-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 \( \beta \)-mannanase should not be contaminated by Ba\(^{2+}\) and Pb\(^{2+}\).

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

<table>
<thead>
<tr>
<th>Metal ions</th>
<th>Relative activity (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>100</td>
</tr>
<tr>
<td>K(^+)</td>
<td>99 ± 3.2</td>
</tr>
<tr>
<td>NH(_4)(^+)</td>
<td>103 ± 2.7</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>117 ± 3.6</td>
</tr>
<tr>
<td>Zn(^{2+})</td>
<td>115 ± 2.9</td>
</tr>
<tr>
<td>Mn(^{2+})</td>
<td>168 ± 4.5</td>
</tr>
<tr>
<td>Cu(^{2+})</td>
<td>116 ± 2.1</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>107 ± 2.8</td>
</tr>
<tr>
<td>Ba(^{2+})</td>
<td>83 ± 3.1</td>
</tr>
<tr>
<td>Pb(^{2+})</td>
<td>74 ± 2.9</td>
</tr>
<tr>
<td>Fe(^{3+})</td>
<td>99 ± 3.6</td>
</tr>
<tr>
<td>Al(^{3+})</td>
<td>121 ± 4.3</td>
</tr>
</tbody>
</table>

\(^a\)Data are mean ± SD, \( n = 3 \).
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 $\beta$-mannanase exhibited the highest activity with Konjac glucomannan, enriched in glucose units. This finding suggests that $\beta$-mannanase of BE-91 preferentially hydrolyzes the $\beta$-1,4-linkage of the glucosylated mannan backbone.

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

<table>
<thead>
<tr>
<th>Substrate (0.5%, w/v)</th>
<th>Relative activity (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Konjac glucomannan</td>
<td>100</td>
</tr>
<tr>
<td>Locust bean gum</td>
<td>88.15 ± 1.8</td>
</tr>
<tr>
<td>Carob galactomannan</td>
<td>91.85 ± 1.7</td>
</tr>
<tr>
<td>Guar galactomannan</td>
<td>35.70 ± 0.6</td>
</tr>
<tr>
<td>Ivory nut mannan</td>
<td>32.74 ± 0.3</td>
</tr>
<tr>
<td>1,4-Beta-D-mannan</td>
<td>46.22 ± 0.4</td>
</tr>
<tr>
<td>Wheat arabinoxylan</td>
<td>0</td>
</tr>
<tr>
<td>Beechwood xylan</td>
<td>0</td>
</tr>
<tr>
<td>Carboxymethyl cellulose</td>
<td>0</td>
</tr>
</tbody>
</table>

*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$_2$HPO$_4$ buffer.  
$^a$Data are mean ± SD, $n = 3$. 

$K_m$ and $V_{max}$ values of this $\beta$-mannanase estimated by the Lineweaver-Burk plot were 7.14 mg/mL and 107.5 $\mu$mol/min/mL, respectively, for locust bean gum, versus 1.749 mg/mL and 33.45 $\mu$mol/min/mL for Konjac glucomannan,
respectively. These results displayed higher affinity of \( \beta \)-mannanase towards natural Konjac glucomannan (\( V_{\text{max}}/K_m \), 19.1 \( \mu \text{mol/min/mg} \)) than the locust bean gum (\( V_{\text{max}}/K_m \), 15.0 \( \mu \text{mol/min/mg} \)), similar to the values obtained for \( \text{Penicillium cirriphilum} \) C1 and \( \text{Penicillium freii} \) F63, hence constituting it as an adequate candidate in food industry for the production of oligosaccharides [17, 18, 39].

4. Conclusion

\( \text{B. subtilis} \) bacteria are abundant, moderately stable, and mostly nonpathogenic microorganisms. Our results indicated that \( \text{B. subtilis} \) BE-91 could be a prominent candidate for the production of extracellular \( \beta \)-mannanase. In addition, this study developed an advanced purification approach, “two-step method,” with high efficiency, high yield, and easy operation. Furthermore, the \( \beta \)-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 \( \beta \)-mannanase, purified and characterized from \( \text{B. subtilis} \) 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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