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A multi-tolerant low molecular weight mannanase from *Bacillus* sp. CSB39 and its compatibility as an industrial biocatalyst

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ABSTRACT

Bacillus sp. CSB39, isolated from popular traditional Korean food (Kimchi), produced a low molecular weight, thermostable mannanase (MnCSB39); 571.14 U/mL using locust bean gum galactomannan as a major substrate. It was purified to homogeneity using a simple and effective two-step purification strategy, Sepharose CL-6B and DEAE Sepharose Fast Flow, which resulted in 25.47% yield and 19.32-fold purity. The surfactant-, NaCl-, urea-, and protease-tolerant monomeric protein had a mass of ~30 kDa as analyzed by SDS-PAGE and galactomannan zymography. MnCSB39 was found to have optimal activity at pH 7.5 and temperature of 70 °C. The enzyme showed □55% activity at 5.0–15% (w/v) NaCl, and □93% of the initial activity after incubation at 37 °C for 60 min. Trypsin and proteinase K had no effect on MnCBS39. The enzyme showed □80% activity in up to 3 M urea. The N-terminal amino acid sequence, ALKGDGX, did not show identity with reported mannanases, which suggests the novelty of our enzyme. Activation energy for galactomannan hydrolysis was 26.85 kJmol^{-1} with a K_{cat} of 142.58 × 10^4 s^{-1}. MnCSB39 had K_m and V_{max} values of 0.082 mg/mL and 1099 ± 1.0 Umg^{-1}, respectively. Thermodynamic parameters such as ΔH, ΔG, ΔS, Q_{10}, $\Delta G_{E\text{-}S}$, and $\Delta G_{E\text{-}T}$ supported the spontaneous formation of products and the high hydrolytic efficiency and feasibility of the enzymatic reaction, which strengthen its novelty. MnCSB39 activity was affected by metal ions, modulators, chelators, and detergents. Mannobiose was the principal end-product of hydrolysis. *Bacillus subtilis* CSB39 produced a maximum of 1524.44 U mannanase from solid state fermentation of 1 g wheat bran. MnCSB39 was simple to purify, was active at a wide pH and temperature range, multi-stress tolerant and catalyzes a thermodynamically possible reaction, characteristics that suggests its suitability for application as an industrial biocatalyst.

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1. Introduction

Hemicelluloses are the second most abundant heteropolymer in nature. They are classified as mannans, xylans, arabinogalactans, or arabinans depending on their sugar backbone composition. Mannans are constructed from the simple sugar mannose as plant polysaccharides. These include mannan, glucomannan, galactomannan, and galacto- glucomannan, which consist of a β-1,4-linked linear backbone of mannose residues that carry other carbohydrates or acid substitutions. There are three enzymes that participate in the complete decomposition and conversion of mannan, namely *endo*-1,4-β-mannanase, *exo*-1,4-β-mannanase, and β-mannosidase [1,2]. Nowadays, mannan and its degradation products (mannooligosaccharides) have been attracting attention of researchers in the food and pharmaceuticals industries because these oligosaccharides and poly-oligosaccharides exhibit various beneficial effects on human health [2].

Mannan and heteromannans are the major part of the hemicellulose fractions of plant cell walls. Mannan *endo*-1,4-β-mannosidase or 1,4-β-D-mannan mannanohydrolase (EC 3.2.1.78) catalyzes the random hydrolysis of the β-1,4 mannosidic linkages of mannans, glucomannan, galactomannan, and galactoglucomannan to yield mannooligosaccharides [3]. The synergistic action of *endo*-1,4-β-mannanases, β-mannosidases, β-glucosidases, α-galactosidases, and acetyl mannanesterases are required for the complete hydrolysis of softwood mannans. These enzymes belong to glycosyl hydrolase (GH) families 5 and 26 according to the carbohydrate active enzymes database (http://www.cazy.org) [4]. There are various applications of mannanases in pharmaceuticals and

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industrial processes, such as bio-bleaching of soft-wood pulps in the paper and pulp industries, improving the quality of food and feed, reducing the viscosity of coffee extracts, oil drilling, and as hydrolytic agents in detergents, slime control agents, and fish feed additives, etc. [5,6].

The production of β-mannanases by microorganisms is more feasible due to its low cost, high production rate, and easily controlled conditions. β-1,4-mannanase has been isolated from a wide range of microorganisms, including *Bacillus subtilis* B36 [7], *Bacillus subtilis* NM-39 [8], *Bacillus amyloliquefaciens* CS47 [9], *Bacillus* sp. N16-5 [10], *Bacillus* sp. MG-33 [11], *Paenibacillus illinoisensis* ZY-08 [12], *Bispora* sp. MEY-1 [13], *Aspergillus niger* [14], *Vibrio* sp. Strain MA-138 [15], *Sclerotium rolfsii* [16], *Cellulosimicrobium* sp. strain HY-13 [17], *Streptomyces lividans* 66 [18], *Streptomyces thermolilacinus* [19], and *Streptomyces* sp. S27 [20]. Microbial mannanases are mainly extracellular and inducible in nature [5].

Different parameters influence the production of mannanase. The nutritional and physicochemical factors such as incubation time, temperature, pH, carbon and nitrogen sources, inorganic salts, agitation and dissolved oxygen concentration, affect the production of mannanase [2]. To overcome the problem of low yield of mannanase and high production costs for industrial application, we screened the predominant microorganism from fermented food (kimchi) in order to find a new strain that would produce mannanase with high activity. In our study, we selected the strain that can produce the extracellular enzyme to degrade the galactomannan (locust bean gum). This work describes the isolation of *Bacillus* sp. CSB39 from kimchi and purification of novel mannanase. Further, the biochemical and thermodynamic characterizations of the enzyme were performed to determine the potential of the enzyme for bio-industrial applications.

2. Material and methods

2.1. Materials

Locust bean gum (galactomannan) and mannose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Thin layer chromatography (TLC) silica gel plates were purchased from Merck (Darmstadt, Germany). Mannobiose and mannotriose were purchased from Megazyme (Ireland). Sepharose CL-6B was purchased from Amersham Bioscience (Uppsala, Sweden). DEAE Sepharose Fast Flow was purchased from GE Healthcare Bio-Science AB (Uppsala, Sweden). All the reagents used were of analytical grade.

2.2. Isolation and two-stage screening of bacterial strain for mannanase production

Seventy-eight samples of traditional Korean food (kimchi) collected from different provinces in Korea were selected for our study. Nature uses microorganisms to carry out the fermentation process, and for many years, humankind has used different microorganisms to make food products. The biological association of these microorganisms in humankind, their symbiotic relationship to environment, human health, made us to select food item rather than anything else for this study. For bacterial isolation, briefly, 1 g of kimchi was mixed with 0.85% NaCl and incubated for 24 h at 37 °C. Serial dilutions were performed up to 10^{-7} in Mueller-Hilton broth. From each dilution, streaking was done to find out the appropriate colony-forming unit (CFU). The appropriately diluted solution was stored as stock cultures in 20% glycerol at −70 °C. All 78 bacterial strains were subjected to the screening process. The preliminary screening procedure was carried out by streaking the stock cultures on agar plates containing 1.25% (w/v) locust bean gum galactomannan (LBG) as a carbon source, 0.5% (w/v) yeast extract and 0.5% (w/v) beef extract as nitrogen sources, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% K_2HPO_4, 0.07% KH_2PO_4, and 0.05% NaCl as metal ion sources and 1.5% agar and incubating the plates at 37 °C for 30 h. Fully grown culture plates were flooded with 0.5% Congo red for 20 min and washed with distilled water. Thereafter, the plates were flooded with 1 M NaCl for 15–20 min and washed with distilled water 2–3 times. Results were interpreted on the basis of LBG utilization. Further screening was done by culturing in a media containing 1.25% (w/v) LBG, 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.03% K_2HPO_4, 0.07% KH_2PO_4, and 0.05% NaCl. The strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL media at 37 °C with shaking at 120 rpm for 60 h. The culture broths were centrifuged at 10,000*g* for 30 min, and the supernatant was collected for enzyme activity measurements. Bacterial strain CSB39 exhibited the highest mannanase activity among the strains subjected to screening, as per agar plate assay and enzyme assay, and thus was selected for further study. Strain identification based on morphological characteristics was carried out according to Bergey's Manual of Systematic Bacteriology and further by 16S rRNA gene sequence analysis.

2.3. Solid state fermentation (SSF) of wheat bran

For SSF, wheat bran was primarily used as the solid substrate. An enriched mineral salt solution, possessing the same composition as the submerged fermentation medium excluding carbon and nitrogen sources was initially prepared, and the pH was adjusted to 7.5. Ten grams of wheat bran was thoroughly mixed with the enriched mineral salt solution at different ratios (1:1, 1:2, 1:3, and 1:4) in 250 mL Erlenmeyer flasks. Finally, overnight grown seed culture was inoculated and incubated at 37 °C for 120 h. At every 24 h, 1 g of spent solid wheat bran was withdrawn, suspended in 10 mL of Tris/HCl buffer (10 mM, pH 7.5), and vortexed thoroughly. The properly vortexed solution was centrifuged at 10,000*g* for 20 min at 5 °C to recover mannanase activity in the supernatant.

2.4. Enzyme production and purification

Bacillus sp. CSB39 was cultured in 2 L baffled flasks containing 400 mL of LBG medium at 37 °C with shaking at 120 rpm for 60 h. One percent (v/v) seed culture was inoculated into the LBG medium grown on 0.4% (w/v) beef extract, 0.4% (w/v) peptone, 0.1% (w/v) yeast extract, and 0.25% (w/v) NaCl at 37 °C for 16 h. After 60 h of cultivation, the cell-free supernatants were collected by centrifugation at 10,000*g* for 30 min. The extracellular enzyme was then further purified in series of steps. All the purification processes were carried out at 0 °C unless stated otherwise. The supernatant was subjected to ammonium sulfate precipitation at 30–80% saturation overnight. The precipitate was collected by centrifugation at 10,000*g* for 50 min, re-suspended and dialyzed in 10 mM Tris/HCl (pH 7.5) buffer overnight, and concentrated using a 50 kDa ultrafiltration centrifugal device (Millipore Corp, Darmstadt, Germany). The enzyme solution was then loaded onto a Sepharose CL-6B column (85 cm × 1.7 cm) pre-equilibrated with 10 mM Tris/HCl, pH 7.5. Proteins were eluted at 30 mL/h and 3 mL fractions were collected. The mannanase active fractions were pooled, concentrated, and loaded on to a DEAE Sepharose Fast Flow column (15 cm × 2.5 cm) pre-equilibrated with 10 mM Tris/HCl, pH 7.5. The bound proteins were eluted with 0–1.0 M KCl gradient at a flow rate of 0.9 mL/min. Active fractions were pooled, concentrated, and analyzed for purity. Further characterizations were carried out using the pure enzyme.

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