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Characterization of a recombinant alkaline thermostable β-mannanase and its application in eco-friendly ramie degumming

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ABSTRACT

A codon optimized synthetic alkaline thermostable *Thermobifida fusca* β-mannanase ManB (KJ806638) was expressed in *Pichia pastoris* and used in ramie degumming. To improve the expression level, a multi-copy secretion expression vector pAOhr was constructed to introduce the *ManB* gene into *Pichia pastoris* GS115. The highest secretion yield was obtained from a transformant strain containing six copies of *ManB* gene. The size of ManB protein was 34 kDa in SDS-PAGE and the secreted protein was the main protein in the culture broth. The optimal activity region of ManB was at pH 7–9 and the enzyme was quite stable at pH 6–10. At pH 9, the specific activity of ManB was 493.8 IU/mg and the optimum temperature was 70–75 °C. ManB appeared to be inhibited by Tris buffer. Molecular docking showed that Tris molecule can bind to the enzyme active site. ManB exhibited high activity for locust bean gum, whereas it showed in practice no activity for CMC-Na. Ramie degumming was performed with combined treatment by ManB and *Bacillus* sp. HG-28 expressing pectinase and xylanase. The obtained results demonstrated that the combination treatment with additional mannanase enzyme was more efficient in removing the gums than the treatment merely by the bacterial strain.

1. Introduction

Hemicelluloses forming a link between lignin and cellulose plant cell wall are polysaccharides with β-1,4-linked carbohydrate backbones. They often contain branches formed of carbohydrates and acidic molecules. Mannans as a major hemicellulose fraction are classified into linear mannan, glucomannan, galactomannan and galacto-glucomannan groups, and typically carry as side groups other carbohydrates or acid substitutions [1,2]. Due to the complicated structure of these polysaccharides, a combination of glycoside hydrolases, such as β-1,4-mannanase, β-mannosidase and α-galactosidase, is required for their complete degradation [3,4]. β-1,4-mannanase (E.C 3.2.1.78) can catalyze a random hydrolysis of the main-chain β-1,4-mannosidic linkages of mannans to mannooligosaccharides [5,6]. Alkaline β-mannanase has high enzymatic activity in alkaline conditions, and has applications in hemicellulose degradation and the textile industry [7–9]. The alkaline thermostable β-mannanase from *Thermobifida fusca* KW3 has the reported optimum temperature at 79 °C and optimum pH at 6–8, and is classified into glycoside hydrolase family GH5 [10,11]. This enzyme has potential for applications in alkaline conditions and even at high temperature.

A number of strategies for enhancing the protein production in *Pichia pastoris* expression system has been developed such as introduction of a high-efficiency transcriptional promoter and the use of an efficient signal peptide [12]. It has also been shown that the change from the native codon usage of the gene sequence to the codon usage of the expression host (codon optimization) significantly improves the expression level of recombinant proteins [13]. Furthermore, gene copy number is a critical parameter of the *P. pastoris* expression system, and it has been shown that by increasing the number of insertion sequences can boost the heterologous protein expression [14]. Therefore, it is preferable to test the transformants for progressively increasing copy numbers to identify the optimal copy number for maximal protein production.

Ramie (*Boehmeria nivea*), also named as China grass, produces one of the longest and strongest natural fibers, and is widely utilized for upscale clothing fabrics, industrial packaging, twines, cordages, canvas, car upholsteries and fiber reinforced composites [15]. The ramie bast

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fibers contain gummy substance (about 30%, w/w) consisting mainly of pectin and hemicellulose that are not easily removed during the course of natural fermentation. The traditional degumming methods for ramie fibers are mostly water retting and degumming with hot alkaline solution followed by hydrogen peroxide treatment for bleaching, resulting in problems such as environmental pollution and inferior fiber quality [16]. In recent years, cleaner and environmentally friendly pretreatment processes for ramie degumming have been studied actively, such as the application of various enzymes and microorganisms to replace the chemical treatment [17]. Recently, *Bacillus* sp. HG-28 expressing high activities of pectinase and xylanase was reported for its excellent ramie degumming ability with low cellulose damage [18].

The structure of alkaline thermostable β-mannanase from thermophilic actinomycetes *Thermobifida fusca* has been determined (PDB code 1BQC) [10,11]. The gene cloning and heterologous expression of this mannanase were not reported. In this study, the gene for ManB was synthesized, and the protein was expressed by a constructed multi-copy vector pAOhr in *P. pastoris*, and ManB was then biochemically characterized. Especially, ManB was found to significantly improve ramie degumming in combination with *Bacillus* sp. HG-28.

2. Materials and methods

2.1. Strains, vectors, and materials

The multi-copy secretive expression vector pAOhr was constructed by overlap extension PCR amplifying the secretion signal region from vector pPIC9 (Invitrogen, China) and cloning this sequence into the vector pAO815 (Invitrogen, China). *Escherichia coli* DH5α (Transgen, China) was used for gene cloning. The DNA purification kit, restriction endonucleases and LA Taq DNA polymerase were purchased from TaKaRa (Japan). Primers and the ManB gene were synthesized by Tsingke Biotech (Beijing, China). *Pichia pastoris* GS115 (Invitrogen, Beijing, China) was used as the host for heterologous protein expression. The *Bacillus* sp. HG-28 strain was preserved at CCTCC under an accession number of M2013308. Locust bean gum (LBG) and carboxymethyl cellulose-sodium (CMC-Na) were obtained from Sigma-Aldrich (USA). Ramie bast fibers (RBFs) were obtained from Hubei Jinghua Textile Group Co., Ltd. All fiber samples were vacuum-dried at 60 °C for 24 h before application. All other chemicals were of analytical grade.

2.2. Synthesis and codon optimization of β-mannanase gene ManB

Based on the primary amino acid sequence of ManB (PDB structure 1BQC), a new β-mannanase *ManB* gene (GenBank accession no. KJ806638) was designed and synthesized. To enhance the production level, the synthesized gene *ManB* was optimized for higher frequencies of codon-usage in *P. pastoris*, splicing sites, GC-content, RNA secondary structure and content of restriction enzyme recognition sequences (*Eco*R I, *Not* I, *Bam*H I and *Bgl* II) by using the GeneOptimizer® expert software from Life Technologies-Invitrogen (Carlsbad, California, USA). The free energy of mRNA folding and the GC content were analyzed using RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). The designed gene *ManB* was synthesized by Tsingke Biotech (Beijing, China) with flanking *Eco*R I restriction site at the 5′ end, simultaneously terminator codon (TAA) and *Not* I restriction site at the 3′ end for expression in *P. pastoris*.

2.3. In vitro construction of the multi-copy expression vectors

The mature β-mannanase gene *ManB* was directionally cloned into vector pAOhr previously digested with *Eco*R I and *Not* I restriction enzymes and was then preserved in *E. coli* DH5α. Proper construction of expression plasmid named *ManB*-pAOhr was confirmed by colony PCR using primer 5′AOX (5′-GACTGGTTCCAATTGACAAGC-3′) and primer 3′AOX (5′-GGCAAATGGCATTCTGACAT-3′), and DNA sequencing by Tsingke Biotech (Beijing, China). A *ManB* expression cassette consisting of the AOX1 promoter, the α-mate factor, the β-mannanase gene *ManB* and the 3′-AOX-terminator was designated as one single-copy. The single-copy vector *ManB*-pAOhr was digested by *Bgl* II and *Bam*H I respectively to generate a 2.5 kb fragment containing one-copy *ManB* cassette.

The expression vector with two-copy β-mannanase gene *ManB* was generated by inserting one-copy *ManB* cassette into the unique *Bam*H I site of the original vector pAOhr to create a tandem head-to-tail repeat of two-copy *ManB* cassettes. The proper construction of expression plasmid named 2*ManB*-pAOhr was confirmed by restriction digestion. 3-copy, 4-copy, 5-copy and 6-copy vectors were generated by repeating the above steps.

2.4. Expression of β-mannanase ManB in P. pastoris GS115

The one-copy, two-copy, three-copy, four-copy, five-copy and six-copy *ManB* vectors were digested with *Stu* I and then transformed into competent *P. pastoris* GS115 cells by electroporation, respectively. Transformants were screened on minimal dextrose (MD) plates lacking histidine at 30 °C for 48 h. The positive transformants were transferred into 3 mL buffered glycerol complex medium (BMGY) and grown at 30 °C for 48 h. The cells were pelleted by centrifugation (5000*g*, 4 °C, 5 min) and resuspended in 1 mL of buffered methanol complex medium (BMMY) containing 0.5% methanol for 48 h to induce heterologous protein expression. The culture supernatant was collected by centrifugation (12,000*g*, 4 °C, 10 min) and subjected to β-mannanase activity assay. The positive transformants exhibiting the highest β-mannanase activity were selected for high-cell-density fermentation in 1-L flasks following a fed-batch mode according to the *Pichia* expression manual (Invitrogen).

Batch cultivation was carried out in 10-l bioreactor (Sartorius, Germany) to grow the optimal recombinant strain for producing the β-mannanase (ManB) protein. The cultivation parameters were as follows: agitation 200 rpm (tip speed 0.6 m/s, two Rushton type impellers), aeration 0.3 vvm (volume air per volume liquid per minute) and cultivation time for 8 days at 28 °C [19]. The cell-free culture supernatant was collected by centrifugation at 12,000*g* for 10 min at 4 °C followed by ultrafiltration with a molecular weight cutoff of 5 kDa in the membrane (Vivascience, Göttingen, Germany).

2.5. Enzyme activity and protein assays

β-Mannanase activity was determined by measuring the amount of reducing sugars released from the locust bean gum substrate (Sigma, St. Louis, MA), using the 3,5-dinitrosalicylic acid (DNS) method as reported previously [20]. All experiments were run in triplicate. For the control sample, the ManB protein was added after the DNS reagent. One unit (IU) of enzyme activity was defined as the amount of enzyme liberating 1.0 μmol of reducing sugar equivalent per min under the standard assay conditions. The enzyme activity of ManB was determined after incubation in 50 mM glycine-NaOH (pH 9.0) containing 0.5% of LBG or CMC−Na at 70 °C for 10 min. The protein concentration was measured with the TaKaRa Bradford Protein Assay Kit (Beijing, TaKaRa), utilizing bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 12.5% gel using the reported method [21]. The separated protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO) and the apparent molecular weights were estimated based on the standard marker proteins.

The optimal pH for ManB activity was determined at 65 °C for 10 min in buffers over a pH range of 4.0–10.0. The pH stability of ManB was estimated by measuring the residual enzyme activity under the conditions (pH 9.0, 70 °C, 10 min) after pre-incubation of the enzyme in buffers of pH 4.0–10.0 at 37 °C for 1 h without the substrate. The buffers used were 50 mM Na_2HPO_4-citric acid (pH 4.0–7.5), 50 mM Tris-