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A novel thermostable GH5_7 β-mannanase from *Bacillus pumilus* GBSW19 and its application in manno-oligosaccharides (MOS) production

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ABSTRACT

A novel thermostable mannanase from a newly isolated *Bacillus pumilus* GBSW19 has been identified, expressed, purified and characterized. The enzyme shows a structure comprising a 28 amino acid signal peptide, a glycoside hydrolase family 5 (GH5) catalytic domain and no carbohydrate-binding module. The recombinant mannanase has molecular weight of 45 kDa with an optimal pH around 6.5 and is stable in the range from pH 5–11. Meanwhile, the optimal temperature is around 65 °C, and it retains 50% relative activity at 60 °C for 12 h. In addition, the purified enzyme can be activated by several ions and organic solvents and is resistant to detergents. Bpman5 can efficiently convert locus bean gum to mainly M2, M3 and M5, and hydrolyze manno-oligosaccharides with a minimum DP of 3. Further exploration of the optimum condition using HPLC to prepare oligosaccharides from locust bean gum was obtained as 10 mg/ml locust bean gum incubated with 10 U/mg enzyme at 50 °C for 24 h. By using this enzyme, locust bean gum can be utilized to generate high value-added oligosaccharides with a DP of 2–6.

1. Introduction

Hemicellulose is a heterogeneous polymer composed of mainly pentoses (D-xylose, D-arabinose), hexoses (D-mannose, D-glucose, D-galactose) and sugar acids, which account for one third of all components available in plants and are the second most abundant heteropolymers present in nature [1–3]. Mannans are hemicellulosic polysaccharides in the plant primary cell wall with two major physiological roles: as storage carbohydrates in the of softwoods; and as structural components of the hemicellulose–cellulose network with a similar function to xyloglucans. [2,4]. For instance, locust bean gum (LBG) from *Ceratonia siliqua*, one of the most commercialized mannan materials, is a popular natural polymer consisting of a β-1,4-linkage mannose backbone with galactose monomers linked to it randomly by α-1,6 bonds [1]. It is naturally abundant in the Mediterranean region since ancient times and now extends to different regions such as North Africa, South America, and Asia, which makes it easily available and relatively cheap [5–6]. Traditionally, LBG and other mannan materials are mostly used in food industry and pharmaceutical industry as additives. During the past decades, the oligomeric form of mannans- the manno-oligosaccharides (MOS) draws attention of a lot of researchers due to its excellent physiological properties acting as dietary fiber and prebiotics [1,7–9]. Furthermore, MOS was proved to be efficient to enhance antibiotics and laccases production in some certain cases [10–12].

Mannan endo-1,4-β-mannosidases are usually found in bacteria, plants and fungi. Mannan endo-1,4-β-mannosidases that belong to glycoside hydrolase family 5 (GH5) are found in bacteria, fungi and higher plants. Until now, only a few GH5 mannanendo-1,4-β-mannosidases from the genus *Bacillus* have been cloned, expressed and characterized [13–15]. Mannan endo-1,4-β-mannosidase (EC 3.2.1.78, commonly named β-mannanase) can randomly hydrolyze the β-1,4 linkages in the main chain of linear mannan polymer or its derivatives to low molecular weight, water soluble oligosacharrides [1,16]. Due to this property, the mannanase are widely used in food, feed, fiber, liquid fuel and other industrial fields [17–19]. Correspondingly, the enzyme prop-

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erties, such as thermo stability, pH stability, etc, should be taken into consideration in different industrial process. [16–17].

Bacillus pumilus is a Gram-positive, aerobic, spore-forming bacterium commonly found in soil and rhizosphere [20]. It is generally non-pathogenic and usually shows a high resistance to environmental stresses. Recently, the genome of the *B. pumilus* strain SAFR-032 was reported [21], revealing it comprised many potential genes for biotechnological applications. In our previous work, a *B. pumilus* strain GBSW19 was isolated from Tibet (China), and it could be able to efficiently decompose plant lignocelluloses. Subsequently comes the questions: does this strain encode novel polysaccharide degrading enzymes that are fairly stable and thus meet the needs of industrial production? How to take use of this enzyme in bioconversion of natural polymer such as cheap LBG into high value-added oligosaccharides? In this study, the endo-1,4-β-mannosidase encoding gene (*Bpman5*) from *B. pumilus* GBSW19 was cloned, expressed and purified. Various properties of the recombinant enzyme were also investigated. In addition, a simple method was proposed for MOS preparation using LBG as substrate. All these results reveals that our recombinant mannan endo-1,4-β-mannosidase from *B. pumilus* GBSW19 is thermo-, pH- and detergent-stable and thus can be a useful candidate in various applications such as bioconversion and fiber industries.

2. Materials and methods

2.1. Strains and vectors

B. pumilus GBSW19 was isolated from the soil of pea plants in Nyingchi County, Tibet, China (N29°39′6¢′, E94°21′37″, ASL 2993.88 m) and was stored in the China General Microbiological Culture Collection Center (CGMCC, Beijing, China) under the registration number CGMCC8140. *Escherichia coli* DH5α and *E. coli* BL21were stored in our laboratory. The pMD-18T simple (Takara, Tokyo, Japan) and pET-30a-c(+) (Novagen, Madison, WI,USA) vectors were used for cloning and protein expression, respectively. Restriction enzymes, *ExTaq* DNA polymerase and T4 ligase were purchased from Takara (Tokyo, Japan).

2.2. Chemicals and reagents

LBG, konjac, 1-pheny-3-methyl-5-pyrazolone(PMP) and *p*-nitrophenol were from Aladdin (Shanghai, China), β-1,4-mannans, *p*-nitrophenyl-β-D-glucopyranoside (pNPG), *p*-nitrophenyl-β-D-mannopyranoside (pNP-β-Man), *p*-nitrophenyl-α-D-mannopyranoside (pNP-α-Man), *p*-nitrophenyl-β-D-galactopyranoside (pNP-β-Gal), *p*-nitrophenyl-α-D-galactopyranoside (pNP-α-Gal), xylan (from birch wood), mannan (from *Saccharomyces cerevisiae*) carboxymethyl-cellulose (CMC) and pectin were supplied by Sigma–Aldrich (St. Louis, MO, USA). Manno-oligosaccharides (M2–M6) were from Megazyme (Bray, Ireland). 3,5-Dinitrosalicylic acid (DNS) was purchased from Sinopharm Chemical Reagent Co., Ltd (Beijing, China). In particular, LBG solution lower than 5.56 mg/ml need slowly addition with heating and stirring. For higher concentration solution, 0.9 ml solvent were added into a 2-ml tube with corresponding weight of LBG and then vortexed before use.

2.3. Gene cloning and sequencing

Based on the DNA sequence of the putative mannan endo-1,4-β-mannosidase encoding gene *ydhT* (GenBank accession: NC_009848.1) from the complete genome of *B. pumilus* SAFR-032 (GenBank: CP000813.1), two oligonucleotides, ManSigF (5′-ATGGCCGCTTTTGTGCAGAC-3′) and ManSigR (5′-TCATCGATTCTTTTCGTTCATTC-3′), were designed and synthesized to amplify the *Bpman5* gene from *B. pumilus* GBSW19. The thermocycling parameters were: 95 °C for 5 min, 30 cycles of 94 °C for 30 s, 52 °C for 60 s and 72 °C for 90 s, followed by 10 min extension at 72 °C. The PCR product was excised from a 1.0% agarose gel and purified by a gel extraction kit (Omega, Norcross, GA, USA) according to the manufacturer's instructions. The purified product was ligated to the pMD18-T simple vector and transformed into *E. coli* DH5α for sequencing.

2.4. Sequence analysis

Sequence assembly and analysis were carried out using the EditSeq software (DNASTAR, Madison, WI, USA). Signal peptide was predicted using SignalP 4.0 [22] (http://www.cbs.dtu.dk/services/SignalP/). The BLAST server was used for homology searches in Genbank (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple alignments of DNA and protein sequences were conducted using the ClustalX2 program [23] and BLAST programs (http://www.ncbi.nlm.nih.gov/BLAST/), respectively. The original protein secondary structure was predicted by Espript 3.0 [24–25] web server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) using the structure of the well-studied β-mannanase from *Trichoderma reesei* as template (PDB No.1QNO).

2.5. Vector construction and expression of recombinant protein

A pair of primers, ManF (GAAGATCTGATGGCCGCTTTTGTGCAGAC) and ManR (5′-CGAGCTCTCATCGATTCTTTTCGTTCATTC-3′), were synthesized to amplify the signal peptide sequence deleted *Bpman5* gene from *B. pumilus* GBSW19 with *Bgl*II and *Sac*I restriction sites (underlined) introduced at the 5′ end of the forward and reverse primers, respectively. The purified PCR fragment was ligated to the pMD18-T simple vector, transformed into *E. coli* DH5α and then sequenced. The target fragment was double digested using the *Bgl*II and *Sac*I restriction enzymes (Takara, Tokyo, Japan) and then ligated to the expression vector pET-30a-c(+) (Novagen, Madison, WI, USA) to generate a fusion DNA sequence encoding a hexahistidine at the N-terminal of the recombinant protein for future purification. The vector included a *tac* promoter which could be activated for high protein expression by using isopropyl β-D-1-thiogalactopyranoside (IPTG). The expression plasmid was first transformed into *E. coli* DH5α for sequencing, later extracted using a plasmid extraction kit (Omega, Norcross, GA, USA) and finally transformed into *E. coli* BL21 (DE3).

The transformed *E. coli* BL21 harboring the recombinant *Bpman5* gene was inoculated into 20 ml LB broth containing 50 μg/ml of kanamycin at 37 °C for 12 h. Thereafter, this culture was inoculated (5% v/v) into 250 ml LB broth containing 50 μg/ml of kanamycin and grown at 37 °C until the OD_{600} value reached 0.6 (~70 min). IPTG was then added to a final concentration of 0.2 mM, and the culture was shaken (200 rpm) at 28 °C for 4–6 h. The cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C and resuspended in 20 ml imidazole solution (dissolved in PBS buffer containing 20 μg/ml PMSF) after discarding the supernatant. The cells (Ultrasonic Processor; 20 amplitude, pulser 3 s, for 15 min) were sonicated on ice and then centrifuged at 8000 × *g* to collect the supernatant containing soluble proteins.

2.6. Enzyme purification, qualification and activity assay

Immobilized metal affinity chromatography (IMAC) and ultrafiltration were adopted for protein purification. Approximately 20 ml of crude protein supernatant was loaded onto a Ni-NTA column (GE Healthcare Life Science, Piscataway, NJ, USA) and with increasing