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Purification and characterization of a thermophilic 1,3-1,4-β-glucanase from *Bacillus methylotrophicus* S2 isolated from booklice

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An extracellular 1,3-1,4-β-glucanase-producing strain S2 was isolated from booklice and identified as *Bacillus methylotrophicus*. Furthermore, a homogeneous extracellular 1,3-1,4-β-glucanase GCS2 was purified by ammonium sulfate precipitation and cation-exchange chromatography. The gene for the 1,3-1,4-β-glucanase was cloned, and the nucleotide sequence was determined. Characterization of the purified enzyme revealed the molecular mass of 26 kDa and the optimum activity at pH 7.5, 55°C. The purified enzyme can highly hydrolyze carboxymethylcellulose including oat gum, barley β-glucan, CMC and lichenan, while low activity on avicel, cellobiose, filter paper, *p*-nitrophenyl β-D-cellobioside, and *p*-nitrophenyl β-D-glucoside, but no activity against microcrystalline cellulose or salicin. The enzyme was stable at wide range of pHs 5–10 and still maintained above 60% activity at 70°C. The enzyme activity was stimulated by Trixon X-100. The property of the enzyme GCS2 makes this enzyme a broad prospect in brewing and commercial detergent industry. To our knowledge, this is the first report of a 1,3-1,4-β-glucanase from microbes associated with booklice.

[**Key words:** Booklice; *Bacillus methylotrophicus* S2; 1,3-1,4-β-Glucanase; pH stable; Thermophilic]

1,3-1,4-β-glucanases (EC 3.2.1.73, lichenase), hydrolyzing β-glucans at 1,3- and 1,4-linkage into oligomeric fractions, are one of members of glycoside hydrolase (GH) family 16 and of biotechnological interest because of their considerably potential use in industry, such as food, brewing and biomass ethanol production. For instance, 1,3-1,4-β-glucanases can be used to reduce mash viscosity and improve wort separation efficiency in Chinese breweries (1,2), to reduce the antinutrition effect and improve the digestibility of barley-grain-based diets in feed industries (3–5), to promote human health depending on the oligosaccharides digesting from β-glucan in food industries (6,7), to reduce the viscosity and increase the mixing of yeast and nutrients in the bioethanol production from barley and lichenan (5,8–10). The enzyme 1,3-1,4-β-glucanases are widely found from a variety of organisms including bacteria (1,11–13), fungi (14), and plants (15,16). However, the plant-origin endogenous 1,3-1,4-β-glucanase cannot resist high temperature, and the endogenous 1,3-1,4-β-glucanase from fungi were mostly acidic that are fit for the usage in low pH feed industry. It has been known that bacteria frequently have a higher growth rate, are easier for genetic purpose and bacterial 1,3-1,4-β-glucanases are more heat stable than plant-origin enzymes. Due to high price of the enzyme and not adequate 1,3-1,4-β-glucanases that meet the specific requirements in industrial application, searching novel thermophilic 1,3-1,4-β-glucanases from particular environments becomes an attractive alternative.

Booklice, also called psocids, were frequently found in grain or other stored food products with high temperature and humidity environments. Booklice may be also found under wallpaper, in furniture, along the sides of windows or on window sills around potted plants. The major components of endosperm cell wall polysaccharides of cereals including barley, rice, and wheat are β-glucans. Since booklice can attack directly starchy paste of cereals and books, some glucan-degrading enzymes would be contained in booklice. Therefore, we think booklice and its associated microbes maybe an ideal and valuable resource for obtaining effective glucanase. The aim of our study is to explore the undeveloped microbial and novel enzyme sources that can be potentially applied to industrial biomass conversion in booklice.

In this work, we report a new 1,3-1,4-β-glucanase-producing strain *Bacillus methylotrophicus* S2 isolated from booklice (*Psococerastis albimaculata*) collected at grain storage facilities in Henan province, China. The characteristics of the enzyme were identified and the gene *gcs2* was cloned and sequenced. Our results suggested that the enzyme had potential application in detergents. To our knowledge, this is the first report on cellulase gene isolated from booklice and associated microbes.

MATERIALS AND METHODS

Isolation, screening, and identification of strains Approximately 5 kg moldy cereals containing booklice at grain storage facilities were collected using sterile homogeneous bag. The cereal samples were transported to the lab and booklices were isolated immediately (or stored at −20°C in a freezer for later isolation) using a flotation method. About 200 booklice specimens collected were ground using 1 ml microdismembrator (Wheaton), diluted, spread onto the selective medium contains 0.3% (w/v) barley glucan, 0.1% (w/v) Congo red, and 1% (w/v) NaCl and cultured at 37°C for 20 h. Colonies showing clear hydrolysis zones were estimated as positive 1,3-1,4-β-glucanase-producing strains using 0.1% Congo red solution for 30 min followed by washing with 5 M NaCl.

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Strain S2 was identified via morphological, physiological, and biochemical characteristics according to the procedures in Bergey's manual of systematic bacteriology (17). PCR amplification of the 16S rRNA gene was performed as described by Li et al. (18). 16S rRNA gene sequences were aligned manually with reference sequences retrieved from the GenBank database following BLAST searches. Phylogenetic trees were constructed using the software package MEGA version 3.1 (19) after multiple alignment of the data by CLUSTAL_X (20). Distances and clustering were based on the neighbor-joining and maximum-parsimony methods. Bootstrap analysis was used to evaluate the topology of the neighbor-joining tree by performing 1000 resamplings (21).

The strain S2 was collected as No. CICC10833 by the China Center of Industrial Culture Collection.

Purification of the glucanase GCS2 The strain S2 was inoculated a Luria Bertani (LB) medium (composition g/l: tryptone, 10; yeast extract, 5 and NaCl, 10) and cultured at 37°C for 3 days under shaking (200 rpm). A 500 ml supernatant culture of the S2 stain was treated for purification: The supernatant collected was subjected to 45–80% ammonium sulfate saturation. Then the precipitate was dissolved in a 5-ml of 50 mM sodium phosphate buffer (PBS) (pH 7.5) and designated as the crude extract for enzyme activity and chromatography. The crude extract was desalted by dialyzing against sterilized water, adjusted to pH 6.0, and applied to a Source 15Q PE4.6/100 column (Pharmacia Biotechnology) previously equilibrated with 10 mM phosphate buffer (pH 6.0). Buffers used were 10 mM PBS (pH 6.0) (A) and 10 mM PBS (pH 6.0) and 1 M NaCl (B). Bound enzyme was eluted with a linear gradient of 0–40% B for 2 min, 40–60% B for 1 min, and 60–80% B for 1 min, and 80–100% for 1 min, with a flow rate of 2 ml/min, which means that bound protein was eluted with a linear gradient of increasing NaCl (0–1.0 M, pH 6.0). Each fraction was collected, dialyzed, and assayed for enzyme activity.

The purified enzyme was checked on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and the protein content was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard during each purification step.

Cloning of the gene encoding the purified enzyme The N-terminal amino acid sequence of the first ten amino acids of the purified enzyme was blasted in the National Center for Biotechnology Information (NCBI) GenBank.

The oligonucleotide primers were designed based on the gene sequence of β-1-3,1-4-endoglucanase from *Bacillus licheniformis* (GenBank: AAN64132): 5′-GTGTTGCTGCTTCTTGTCAC-3′ and 5′-TTTTTTTGTATAGCGCACCC-3′. Cycling conditions were 4 min at 95°C for the first denaturation; 30 cycles of 40 s at 95°C, 50 s at 53°C, and 1.5 min at 72°C for amplification; then 10 min at 72°C for extension.

The PCR product was inserted into pGEM-T vector according to the manual (Promega) and sequenced. The deduced amino acid sequence was aligned with other 1,3-1,4-β-glucanases that have been reported and the phylogenetic tree of GCS2 was constructed using software MEGA5.0. The gene sequence submitted to the NCBI GenBank was assigned the accession number KR067577.

Properties of the enzyme GCS2 The 1,3-1,4-β-glucanase activity was determined using 1% azo blue barley β-glucan as substrate. The reducing sugar content was measured using the 3,5-dinitrosalicylic acid (DNS) method (22): A 1.8 ml reaction mixture containing 0.5 μg of the purified enzyme GCS2 with 1% β-glucan in 0.1 M pH 6.0 citrate phosphate buffer (appropriate diluted as needed) was incubated at 50°C for 20 min. The amount of reducing sugar released by hydrolysis was measured by 3,5-dinitrosalicylate (DNS). One unit (U) of endoglucanase activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per min from the substrate. Cellulase activity was measured by the absorbance value of the mixture at 540 nm. The mixture without adding enzymes was as negative control. All the activity assays were performed in triplicate and the standard deviations were calculated.

The optimum pH of the enzyme was determined at different pH between 2 and 12. pH stability of the purified enzyme was investigated by adding it to buffers with different pH, incubating at 4°C for 24 h, and adjusting pH to 6, followed by the cellulase activity assay as described above.

The optimal temperature assay was determined by incubating the reaction mixture at different temperatures, ranging from 30°C to 70°C. To determine the enzyme stability at different temperatures, the residual activity of the GCS2 was assayed after incubation at different temperatures ranging from 40°C to 70°C for 30 min.

Substrate specificities of the enzyme were investigated using different kinds of substrates including oat gum, barley β-glucan, carboxymethyl cellulose (CMC), Lichenan, avicel, cellobiose, microcrystalline cellulose, *p*-nitrophenyl β-D-cellobioside (pNPC), *p*-nitrophenyl-β-D-glucoside (pNPG), salicin and Whatman No. 1 filter paper. The effect of several metal chloride salts ($FeCl_2$, $LiCl_2$, KCl, $CaCl_2$, $MgCl_2$, $CuCl_2$, $ZnCl_2$, $CoCl_2$, $HgCl_2$ and $MnCl_2$) and chemicals (SDS, EDTA, 1,10-phenanthroline Hydrate, urea, DTT, Tween 80, Trixon X-100, β-mercaptoethanol) was assayed by adding them into the reaction mixture and measuring the residual cellulase activity.

Homology modeling The 3D-structures were obtained using EasyModeller 2.0 using the crystal structures of the endo-β-1,3-1,4-glucanase from *Bacillus subtilis* strain 168 (PDB ID: 3O5S_A; Identity: 94%), β-glucanase from *B. licheniformis* (PDB ID: 1GBG_A; Identity: 87%), molecular and active-site structure of a *Bacillus* (1-3,1-4)-β-glucanase [synthetic construct] (PDB ID:1BYH_A; Identity: 75%) and crystal structure of the engineered 1,3-1,4-β-glucanase protein from *B. licheniformis* (PDB ID:3D6E_A; identity: 76%) as templates. PROCHECK and VERIFY_3D programs of Structural Analysis and Verification Server (http://nihserver.mbi.ucla.edu/SAVES/) were used to check the stereochemical quality of the 3D models obtained.

Ethics statement Written consent was received from public grain storage facility owners participated in this study. The location is not privately owned or protected in any way. Confidentiality of the information was maintained throughout the study.

RESULTS

Identification of 1,3-1,4-β-glucanase-producing strain S2 Nineteen bacterial strains were isolated from the booklice samples on the screening plates, while only seven bacterial isolates exhibited good 1,3-1,4-β-glucanase activity according to their zones of clearance by Congo red assay. The isolate S2, showing maximum clearance zone diameter was selected for further studies. The effective strain S2 was aerobic, motile, rod-shaped and formed endospores (Fig. S1). The major components of the fatty acid profile were $C_{15:0}$ anteiso, $C_{15:0}$ iso, $C_{16:0}$ iso and $C_{17:0}$ anteiso. The diagnostic diamino acid of the cell wall was *meso*-diaminopimelic acid. The 16S rDNA gene was sequenced and submitted to NCBI GenBank (accession no. KJ130061). The phylogenetic tree of the 16S rDNA sequence of strain S2 is shown in Fig. S2. The closest phylogenetic neighbors of strain S2 are *B. methylotrophicus* CBMB205^T (99.24%), *Bacillus tequilensis* KCTC 13622^T (98.77%), *B. subtilis* subsp. inaquosorum KCTC13429^T (98.77%) and *B. subtilis* NCIB 3610^T (98.70%). Therefore, on the basis of the polyphasic taxonomic data presented, strain S2 was identified as *B. methylotrophicus*.

Purification of the enzyme GCS2 in culture filtrates Purification of the enzyme with glucanase activity from culture supernatant of *B. methylotrophicus* strain S2 was performed by ammonium sulfate precipitation and cation-exchange chromatography. The fraction of 45–80% salting out of ammonium sulfate was detected as containing 1,3-1,4-β-glucanase activity. Following that, the bacterial extracellular 1,3-1,4-β-glucanase was purified to homogeneity by cation-exchange chromatography (Fig. 1A). Only the elution peak with 1,3-1,4-β-glucanase activity was pooled and SDS–PAGE of the aliquots yielded a single protein band with a molecular mass about 26 kDa (Fig. 1B). After each purification step, the enzyme activities were assayed, and the concentrate fold is shown in Table 1. About 3.2-fold purification was achieved after precipitated by $(NH_4)_2SO_4$. After applied to cation-exchange chromatography, the active fraction resulted in 19.2-fold purification with a specific activity of 15.9 IU/mg/min of the enzyme (Table 1).

Effect of pH and temperature on activity and stability of GCS2 The results of pH studies indicated a broad pH activity range of 2.0–12.0 and optimum pH at 7.5 (Fig. 2A). The enzyme was stable at wide range of pHs, still more than 60% residual activity was retained after incubation at pH 5–10 for 15 h, and more than 50% activity at pH 6–10 even after incubation for 24 h. Less than 50% activity was retained at pH 4.5 (Fig. 2B).

Temperature optimization studies at pH 7.5 showed that at 55°C, the enzyme had the maximum activity (Fig. 2C). As for the stability of temperature, the enzyme still possessed about 90% activity at 60°C after 24 h of incubation, and still maintained about 60% activity at 70°C after 8 h of incubation. While when the incubation time was extended to 24 h, less than 20% activity was determined. Almost no activity was detected at when the temperature was above 70°C (Fig. 2D).

Hydrolysis of different substrates Substrate specificity of the enzyme GCS2 was determined under optimal conditions with 1% polysaccharides (Table 2). The enzyme showed the highest hydrolytic activity against barley oat gum, and presented