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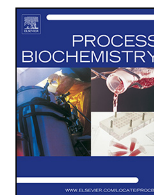

Cloning, expression, and characterization of a thermostable β-xylosidase from thermoacidophilic *Alicyclobacillus* sp. A4

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ABSTRACT

A β-xylosidase gene (*xylA4*) was identified in the genome sequence of thermoacidophilic *Alicyclobacillus* sp. A4. The deduced amino acid sequence was highly homologous with the β-xylosidases of family 52 of the glycoside hydrolases (GH). The full-length gene consisted of 2097 bp and encoded 698 amino acids without a signal peptide. The gene product was successfully expressed in *Escherichia coli* with an activity of 564.9 U/mL. Recombinant XylA4 was purified by Ni^{2+}-NTA affinity chromatography with a molecular mass of 78.5 kDa. The enzyme showed optimal activity at pH 6.0 and 65 °C, and remained stable over the pH range of 5.0–9.0. The thermostability of XylA4 is noteworthy, retaining almost all of the activity after 1 h incubation at 65 °C. Using *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX) as the substrate, XylA4 had the highest specific activity (261.1 U/mg) and catalytic efficiency (601.5/mM/s) known so far for GH52 xylosidases. The enzyme displayed high tolerance to xylose, with a K_i value of approximately 88.7 mM. It also had synergy with xylanase XynBE18 from *Paenibacillus* sp. E18 in xylan degradation, releasing more xylose (up to 1.43 folds) than XynBE18 alone. Therefore, this thermostable xylose-tolerant β-xylosidase may have a great application potential in many industrial fields.

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1. Introduction

Xylan, the major component of hemicellulose in plant cell walls, is composed of β-1,4-linked D-xylopyranose units [1]. Due to its complexity and heterogeneity, complete degradation of xylan requires the synergistic action of several enzymes [2]. They are β-xylanase (EC 3.2.1.8) that hydrolyzes the glycosidic bond of the xylan backbone into xylooligosaccharides, β-xylosidase (EC 3.2.1.37) that cleaves xylooligosaccharides into xylose monomers, and accessory enzymes responsible for the cleavage of side chains including α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase, and feruloyl or coumaroyl esterase [3].

β-Xylosidase is a rate-limiting enzyme in xylan degradation and critical for alleviating end product inhibition of xylanases [4]. It has a significant potential application value in medicine, food, energy, paper and pulp, and biofuel industries [5]. β-Xylosidases have been identified in various microorganisms, including bacteria, archaea and fungi. Except the β-xylosidases from *Fusarium proliferatum* [6], *Paecilomyces thermophila* [7], *Geobacillus thermodenitrificans* TSAA1 [8] and *Bacillus stearothermophilus* 21 [9] that are extracellular, most of them are cell-associated. Based on the amino acid sequence similarities, β-xylosidases have been classified into glycoside hydrolase (GH) families 1, 3, 30, 39, 43, 51, 52, 54, 116 and 120 (http://www.cazy.org/glycoside-hydrolases.html) [10]. To date, all known β-xylosidases of family 52 are from bacteria, and five of them, three from *Geobacillus stearothermophilus* (formally *B. stearothermophilus*) [11–13], and two from each *Aeromonas caviae* [14] and *Paenibacillus* sp. [15] have been functionally characterized.

The thermoacidophilic bacterial strain *Alicyclobacillus* sp. A4 has been reported to be an excellent producer of glycosyl hydrolases [16–18]. The objective of this study was to obtain the β-xylosidase from *Alicyclobacillus* sp. A4. As a result, we identified XylA4 of family 52 in *Alicyclobacillus* sp. A4. The coding gene was expressed in

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Escherichia coli, the purified recombinant enzyme was characterized, and its synergistic effects with the xylanase XynBE18 from *Paenibacillus* sp. E18 [19] on the degradation of various xylans were also determined. XynBE18 is a bifunctional enzyme that has both xylanase and glucanase activities. Its synergy with an α-L-arabinofuranosidase of the same microbial source had been deeply studied [20], and thus represents a good candidate for enzyme combination.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

The donor strain *Alicyclobacillus* sp. A4 [16] was obtained from our laboratory stocks. *E. coli* JM109 and BL21 (DE3) cells were grown aerobically in Luria-Bertani (LB) broth or agar plates supplemented with 50 μg/mL kanamycin for recombinant plasmid amplification and gene expression, respectively. Vectors pEASY-T3 (Tiangen, Beijing, China) and pET-30a(+) (Novagen, Darmstadt, Germany) were used for gene cloning and recombinant protein expression, respectively. *p*-Nitrophenyl-β-D-xylopyranoside (*p*NPX), beechwood xylan, birchwood xylan, xylotriose, xylobiose, xylose, and arabinose were purchased from Sigma (St. Louis, MO). Soluble wheat arabinoxylan was supplied by Megazyme (Wicklow, Ireland). DNA purification kits, restriction endonucleases, and T4 DNA ligase were purchased from TaKaRa (Otsu, Japan). All other chemicals were of analytical grade and commercially available.

2.2. Cloning and expression of the β-xylosidase gene

The full-length β-xylosidase gene (*xylA4*) was identified in the genome sequence of *Alicyclobacillus* sp. A4 (whole-genome sequencing on progress). The gene was amplified by PCR using a primer set (xylA4F: GGGGAATTCATGCCGAAGAATATGTATTTTAATGCAC and xylA4R: GGGGCGGCCGCTTACTCGTCGAGCCAGAGAATACTC; *Eco*RI and *Not*I sites underlined, respectively). The PCR product was connected to the pEASY-T3 vector for sequencing. The correct clone was digested by *Eco*RI and *Not*I and inserted into corresponding sites of the vector pET-30a(+) in-frame with a His-tag. The single colony of a positive transformant containing pET-*xylA4* was picked with a sterile tooth stick and grown overnight at 37 °C with agitation (220 rpm) in 50 mL LB broth containing 50 μg/mL kanamycin. The culture was then inoculated into 900 mL of fresh LB medium with 50 μg/mL kanamycin (1:100 dilutions) and grown aerobically at 37 °C to A600 of 0.6–0.8. Protein expression was induced at 30 °C for 6–12 h by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.8 mM.

2.3. Sequence analysis

Vector NTI Advance 10.0 software (Invitrogen, Carlsbad, CA) was used to evaluate sequence similarities and predict the molecular mass of protein. BLASTx and BLASTp programs (http://www.ncbi.nlm.nih.gov/BLAST/) were used to analyze the nucleotide and deduced amino acid sequences, respectively. SignalP4.0 server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide. Based on the amino acid sequences of XylA4 and 24 representative GH52 members from bacteria, a phylogenetic tree was constructed by means of neighbor-joining method with 1000 replicates and MEGA 5.05.

2.4. Purification of recombinant XylA4

The induced cells were harvested by centrifugation (6000 rpm, 4 °C, 10 min), and resuspended in sonication buffer (20 mM Tris–HCl, pH 7.6) at a ratio of 1 g cell wet weight to 5 mL buffer. The cell suspension was sonicated on ice in an Ultrasonic Cell Disruption System (JY92-IIN, 250 Hz; SCIENTZ, Ningbo, China) with 100 short bursts of 6 s followed by intervals of 15 s for cooling. The cell debris was removed by centrifugation, and the concentrated supernatant (crude enzyme, 15 mL) was applied to a 1-mL Ni^{2+}-NTA chelating column (Qiagen, Hilden, Germany) that had been pre-equilibrated with 10 mL buffer A (20 mM Tris–HCl, 500 mM NaCl, 10% glycerol, pH 7.6). The bound protein was eluted at a flow rate of 3 mL/min by applying a step gradient of imidazole (0, 20, 40, 60, 80, 100, 200, and 300 mM each in 10 mL of buffer A). The eluted fractions with enzyme activities were collected and evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by the Bradford method using bovine serum albumin as the standard [21].

2.5. Enzyme activity assays

The β-xylosidase activity was assayed as described previously [22]. The reaction mixture contained 100 μL of properly diluted His-tagged enzyme, 150 μL of 100 mM McIlvaine buffer (200 mM Na_2HPO_4, 100 mM citric acid, pH 6.0) and 250 μL of 5 mM *p*NPX (the final concentration to 2.5 mM). After incubation at 65 °C for 10 min, the reaction was stopped by addition of 1.5 mL of 1 M Na_2CO_3. The absorbance at 405 nm was measured. One unit (U) of β-xylosidase activity was defined as the amount of enzyme that released 1 μmol of *p*NP per minute under the conditions described above. Xylanase activity was determined by measuring the release of reducing sugars from xylan as previously described [16].

2.6. Enzymatic characterization

The enzyme properties of His-tagged XylA4 were determined with 2.5 mM *p*NPX as the substrate. The experiments were repeated three times independently, each time in triplicate. To verify the interaction of pH and temperature, the optimal pH for enzymatic activity was determined in the pH range from 3.0 to 8.0, 55 °C or 65 °C for 10 min. To assay pH stability, the enzyme was pre-incubated without substrate in the pH range from pH 2.0 to 12.0, 37 °C for 1 h, 6 h, 12 h or 24 h, and the residual activities were determined under standard conditions (pH 6.0, 65 °C, 10 min). The buffers used were 100 mM glycine–HCl for pH 2.0–3.0, 100 mM McIlvaine buffer for pH 3.0–8.0, 100 mM Tris–HCl for pH 8.0–9.0, and 100 mM glycine–NaOH for pH 9.0–12.0.

The optimal temperature for XylA4 activity was determined in the temperature range from 40 °C to 80 °C, pH 6.0 for 10 min. The thermostability of XylA4 was monitored by pre-incubating the enzyme without substrate in 100 mM McIlvaine buffer (pH 6.0) at 65 °C, 70 °C and 80 °C for 5 min, 10 min, 20 min, 30 min, 60 min, 2 h, 3 h, 6 h, 12 h, and 24 h, respectively. The residual enzyme activity in each case was determined under standard assay conditions (pH 6.0, 65 °C, 10 min).

The effects of different metal ions and chemical reagents on the recombinant enzyme activity was assessed in McIlvaine buffer (pH 6.0) containing 1 mM or 5 mM of NaCl, LiCl, KCl, $AgNO_3$, $MgCl_2$, $MnCl_2$, $Pb(CH_3COO)_2$, $NiCl_2$, $ZnCl_2$, $CoCl_2$, $CuCl_2$, $CaCl_2$, $CrCl_3$, $FeCl_3$, SDS, EDTA, or β-mercaptoethanol. Reactions without additive were used as the control.

2.7. Determination of kinetic parameters

To determine the kinetic values, *p*NPX of 0.25–3.5 mM was used as the substrate. First-order reactions were carried out at pH 6.0 and 65 °C for a shorter time (5 min) to ensure the linear relationship between the rate of reaction and the concentration

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