Characterization of a Recombinant Thermostable Xylanase from Hot Spring Thermophilic Geobacillus sp. TC-W7

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A xylanase-producing thermophilic strain, Geobacillus sp. TC-W7, was isolated from a hot spring in Yongtai (Fuzhou, China). Subsequently, the xylanase gene that encoded 407 amino acids was cloned and expressed. The recombinant xylanase was purified by GST affinity chromatography and exhibited maximum activity at 75°C and a pH of 8.2. The enzyme was active up to 95°C and showed activity over a wide pH range of 5.2 to 10.2. Additionally, the recombinant xylanase showed high thermostability and pH stability. More than 85% of the enzyme’s activity was retained after incubation at 70°C for 90 min at a pH of 8.2. The activity of the recombinant xylanase was enhanced by treatment with 10 mM enzyme inhibitors (DDT, Tween-20, 2-Me, or TritonX-100) and was inhibited by EDTA or PMSF. Its functionality was stable in the presence of Li⁺, Na⁺, and K⁺, but inhibited by Hg²⁺, Ni²⁺, Co²⁺, Cu²⁺, Zn²⁺, Pb²⁺, Fe³⁺, and Al³⁺. The functionality of the crude xylanase had similar properties to the recombinant xylanase except for when it was treated with Al³⁺ or Fe³⁺. The enzyme might be a promising candidate for various industrial applications such as the biofuel, food, and pulp industries.

Keywords: Thermostable xylanase, recombinant expression, characterization, stable pH, Geobacillus sp. TC-W7

Hemicellulose is the second most abundant renewable polysaccharide in nature after cellulose. Xylan is the main component of hemicellulose and is composed of a backbone of β-1,4-linked xylopyranose residues [18], usually with branches composed of β-1,3-linked l-arabinose and α-1,2-linked α-glucopyranose [17]. The combined actions of several hydrolytic enzymes, such as endo-β-1,4-xylanase (E.C. 3.2.1.8), exo-xylanase (β-D-xylan xylohydrolase), and β-D-xylosidase (E.C. 3.2.1.37), are required for the complete breakdown of xylan [6]. These enzymes cooperatively hydrolyze the β-1,4-glycosidic bonds of xylan to produce several xylooligomers.

In recent years, xylanases have been the subject of worldwide research interests because of their potential biotechnological application in various industrial processes [3, 4, 7]. Commercial suggested uses for xylanases involve the conversion of xylan, which is present in wastes from agriculture and the food industry, into xylose [10]. Xylanase can also be used for the clarification of juices, improvement in the consistency of beer, and improving the digestibility of chemical feedstock. Currently, the most promising application of xylanase is the pre-bleaching of pulps [1, 4, 13], which could improve pulp fibrillation and water retention, reduce the beating times for virgin pulps, improve the restoration of bonding and increase freeness in recycled fibers, and improve the selective removal of xylans from dissolving pulps [4].

Many xylanases have been isolated and characterized from various microorganisms, and some xylanase genes have been cloned and expressed in Escherichia coli [5, 8, 15, 21]. However, most of these enzymes are active at a neutral or acidic pH, and they usually have lower optimal activity temperatures [20]. The commercial applications of xylanases generally require a higher optimal pH or temperature, a better thermostability, and a broadly active pH range [1, 4, 16]. Therefore, it is necessary to find a robust xylanase for industrial applications.

In this study, more than 70 thermophilic bacteria were isolated from a hot spring in Yongtai (Fuzhou, China). Many of these isolates displayed a high xylanase activity.
Among them, a bacterium assigned to *Geobacillus* sp. TC-W7 based on its 16S rRNA gene sequence showed the highest xylanase activity. Therefore, the xylanase gene from this bacterium was cloned and expressed, and the recombinant xylanase and the crude xylanase were investigated further.

**MATERIALS AND METHODS**

**Isolation and Identification of Thermophilic Strain *Geobacillus* sp. TC-W7**

Strain TC-W7 was isolated from a hot spring in Yongtai, Fuzhou, China. The strain was cultivated in basal agar medium containing the following: 0.3% xylan oat spelt grains (Sigma), 0.05% (NH₄)₂SO₄, 0.03% MgSO₄·7H₂O, 0.02% CaCl₂·2H₂O, 0.01% K₂SO₄, 0.02% NaCl, 0.1% KPO₄, 0.01% yeast extract, 0.05% tryptone, and 1.6% agar at a pH of 7.2. Xylanolytic colonies were visualized by flooding the agar plates in a Congo red plate assay [19]. Colonies harboring a higher ratio of clear zone to colony size were purified three times.

The 16S rRNA gene was PCR amplified from the genomic DNA of strain TC-W7 using primers 27F (5'-AGAGTTTGATCTTATGATGACTGCTCACG-3') and 1492R (5'-AAGGAGGTGATCCCTGGCTCAG-3') [11]. The PCR product was cloned into the pMD18-T vector (Takara, China) and sequenced by TianGen (Beijing, China). Homology searches to the available sequences in the GenBank database using the BLAST program indicated that the 492 bp DNA sequence was a partial xylanase gene (EU599644), the xylanase gene was PCR amplified from the genomic DNA of TC-W7 using the following specific primers: up-stream primer (5'-TGCCATCGCCTGAAAGATCCGAAAAGACG-3') and down-stream primer (5'-CCGGTCTGAGTCACTTATGATGATGAAATGCACCCATT-3') engineered with the BamHI and EcoRI restriction sites, respectively. The amplified fragment was then cloned into the pMD18-T vector and the presence was confirmed by sequencing. Homology searches were performed against the available sequences in the GenBank database using the BLAST program. Alignment of the indicated amino acid sequence was generated with the DNAMAN program.

**Expression and Purification of Xylanase**

The plasmid pMD18-T-Xylanase was double-digested with *Bam*HI and *Eco*RI. The digested xylanase fragment was withdrawn and ligated into the pGEX-4T-2 expression vector that was pre-digested with the same restriction enzymes. The recombinant plasmid containing the xylanase gene was expressed in *Escherichia coli* BL21 as a fusion protein with glutathione S-transferase (GST) and confirmed by sequencing.

Five milliliters of Luria–Bertani medium containing 50 µg/ml of ampicillin was inoculated with freshly transformed *E. coli* harboring the appropriate recombinant plasmid and incubated at 37°C overnight. Subsequently, the culture was transferred into 500 ml of fresh medium and incubated at 37°C on a rotary shaker at 250 rpm. When the OD₆₀₀ reached 0.6, heterologous expression of the xylanase gene was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM and incubated 5 h with vigorous agitation at 30°C. The culture was harvested by centrifugation at 6,000 × g for 20 min at 4°C and washed with ice-cold phosphate-buffered saline (PBS, pH 7.2). The cell pellet was resuspended in the same buffer and lysed by sonication on ice. The dialyzed supernatant was mixed with glutathione-agarose beads and incubated at 4°C for 1 h. The sample was then packed onto a Sepharose-4B column that was then washed with three volumes of PBS to elute the unbound proteins. The bound xylanase was pooled with a reducing buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purified xylanase was then digested with thrombin (Sigma) and analyzed by SDS-PAGE as described previously [9].

**Preparation of the Crude Xylanase**

The crude xylanase was obtained by ammonium sulfate precipitation [14]. The crude extracellular xylanase was harvested by centrifugation of the culture broth at 6,000 × g for 20 min at 4°C. The crude supernatant was gently added to the solid ammonium sulfate to a maximum of 70% saturation. The precipitate was then collected by centrifugation (6,000 × g, 20 min) and then dissolved in 0.1 M citric acid-Na₂HPO₄ buffer (pH 7.2) and dialyzed in the same buffer. The final preparation was used for further experiments.

**Enzyme Activity Assays**

The assay to measure xylanase activity was performed according to the method of Bailey *et al.* [2]. The reaction mixture was prepared and contained 2 ml of 1% xylan in 0.1 M citric acid-Na₂HPO₄ buffer (pH 7.2) and the appropriate diluted enzyme sample. The mixture was incubated at 70°C for 30 min and the reaction was stopped with 2 ml of 3,5-dinitrosalicylic acid reagent (DNS). The reaction was subsequently boiled for 5 min and rapidly cooled to room temperature. Xylanase activity was determined by measuring the release of reducing sugar from oat spelt xylan using DNS as described by Miller [12]. One unit of xylanase activity was defined as the amount of enzyme that produced 1 µM of xylose equivalent per minute.

**Characterization of the Recombinant Xylanase**

To determine the optimal temperature of the xylanase, the enzyme activity was measured at temperatures ranging from 35°C to 95°C under 0.1 M citric acid-Na₂HPO₄ buffer (pH 7.2). The thermostability of xylanase was determined as the residual enzyme activity after
The isolated thermophilic bacterium TC-W7 exhibited the highest xylanase activity among the strains that were screened from the hot spring samples from Yongtai (Fuzhou, China). According to the 16S rRNA gene sequence analysis (GenBank Accession No. GQ866911), the strain had the most homology to sequences from Geobacillus sp. Based on these results, we identified the isolate as a species of the genus Geobacillus.

Gene Cloning and Sequence Analysis of the Xylanase

Using the genomic DNA from the Geobacillus sp. TC-W7, a 1,224 bp fragment that encoded a 407 amino acid protein (GenBank Accession No. GQ857066) with a calculated molecular mass of 47.4 kDa was amplified using specific primers. Using UniProt Knowledgebase searches, the amino acid sequence that was deduced showed 98%, 86%, 85%, 57%, 51%, 50%, and 42% identities with Geobacillus thermoeoenitrificans (A4IP71), Geobacillus sp. WBI (B5M201), Geobacillus sp. Y412MC61 (C9RT34), Bacillus firmus (Q6U892), Bacillus halodurans (Q17TM8), Paenibacillus sp. JDR-2 (C6D767), and Clostridium stercorarium (P40942), respectively. Amino acid homology alignment of the xylanase gene with these homologous xylanases was performed (Fig. 1). Two catalytic glutamates (Fig. 1 noted *) are located in Glu-182 and Glu-292 based on hydrophobic cluster analysis.

Expression and Purification of the Recombinant Xylanase Protein

To facilitate the purification the bacterial xylanase, the 1,224 bp fraction was expressed as a GST fusion protein in E. coli. After induction with IPTG at 30°C, the control bacterium, and induced and non-induced recombinant bacteria (containing the xylanase gene) were analyzed by SDS-PAGE. A band (approximately 73 kDa) corresponding to the GST-xylanase fusion protein was identified in the induced recombinant bacterium (Fig. 2, Lane 2). There was no protein detected at the same size in the induced and non-induced control. This indicated that the xylanase gene was expressed. After purification by affinity chromatography, SDS-PAGE analysis indicated that a purified GST-xylanase fusion protein was obtained (Fig. 2, Lane 3).

Biochemical Characterization of Xylanase

To characterize the recombinant and crude xylanases, the effects of pH, temperature, inhibitors, and metal ions on enzymatic activity were evaluated. Both the recombinant xylanase and crude xylanase exhibited optimal working temperature at 75°C (Fig. 3A and 3B), but the recombinant xylanase had almost equal enzymatic activity at temperatures between 75°C and 80°C. The crude xylanase activity decreased quickly at temperatures that were greater than 75°C (Fig. 3B),
but the recombinant xylanase remained active up to 90°C (Fig. 3A). Thermostability assays suggested that the recombinant xylanase and crude xylanase were still stable between 65°C and 70°C (pH 7.2). After incubation at 65°C and 70°C for 120 min, the recombinant xylanase retained 80% of its initial activity (Fig. 3C). Additionally, the half life of the recombinant xylanase was approximately 70 min at 75°C, and the crude xylanase had lost 60% of its activity after incubation at 75°C for 40 min (Fig. 3C and 3D).

The activity assay at different pH values revealed that the optimal pH levels for the recombinant xylanase and crude xylanase were 8.2 and 7.2, respectively, and they were active over a range of pH values of 3.2–10.2 (Fig. 4A and 4B). Both the recombinant xylanase and the crude xylanase were stable in the alkaline pH between 7.2 and 8.2; more than 85% and 65% of the enzymatic activities were retained at 70°C after 90 min compared with their respective optimal pH (Fig. 4C and 4D). As we can see

Fig. 1. Multiple alignments of representative xylanases from thermophiles. Numbering begins at the N-termini of the proteins. Gaps are indicated with dashes. Amino acids that are identical in at least six of the aligned sequences are shaded. Two Glu residues marked with an asterisk indicate the putative active site in family 10 xylanase.
from Fig. 4C and 4D, the recombinant xylanase was observed to be less stable than the crude xylanase at pH 9.2. After incubation at 70°C for 90 min, the recombinant xylanase had lost 50% of its activity, and the crude xylanase still retained 80% of its original activity. Meanwhile, the crude and recombinant xylanases seemed more tolerant of an alkaline pH, each maintaining 75% and 55% of their residual activity, respectively, at pH 10.2 after 60 min of incubation (Fig. 4C and 4D).

The recombinant xylanase exhibited high enzyme activity across a broad temperature range from 55°C to 90°C with an optimal temperature at 75°C. Additionally, it showed over 50% activity within a pH range of 5.2–10.2 and was stable for more than 90 min when incubated at pH 10.2 (at 70°C). This suggests that it was tolerant of alkaline conditions.

The activity of the recombinant xylanase and crude xylanase in the presence of different inhibitors and detergents is shown in Fig. 5. Both the crude xylanase and recombinant xylanase exhibited similar activity profiles. Treatment with SDS, DTT, 2-ME, Tween 20, and Triton X-100 could enhance the enzymes’ activity to different degrees, and treatment with EDTA and PMSF restricted their activity.

The effects of metal cations on the enzymatic activity are shown in Table 1. When Hg²⁺, Ni²⁺, Co²⁺, Cu²⁺, Zn²⁺,
Pb\(^{2+}\), Al\(^{3+}\), and Fe\(^{3+}\) were added at 10 mM concentrations, the activity of the recombinant xylanase was strongly inhibited. We observed that in the presence of Mg\(^{2+}\), Ca\(^{2+}\), or Ba\(^{2+}\), Mn\(^{2+}\), and Fe\(^{2+}\), the activity of the recombinant xylanase was partially inhibited. Conversely, the xylanase activity was enhanced by monovalent cations such as Li\(^{+}\), Na\(^{+}\), and K\(^{+}\), with the relative activity compared with untreated controls being 109.6%, 106.9%, 114.8%, respectively. However, in our experiments, we found that the crude xylanase activity was reduced only by treatments with Hg\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\), Pb\(^{2+}\), and Fe\(^{3+}\) (Table 1). All other metal ions that were tested stimulated the enzymes’ activity, especially Al\(^{3+}\) and Fe\(^{2+}\) (their relative activities were 116.8% and 144.5%, respectively). This demonstrates that the crude xylanase is clearly different from the recombinant xylanase. We hypothesized that other xylanases in the crude extract may be present that stabilized the xylanase against metal ion shock.

Fig. 4. Effects of pH on xylanase activity (A, B) and stability (C, D).

The optimum pH was assayed after incubation in various pH buffers at 70°C for 30 min. For pH stability, the residual activities of recombinant xylanase (C) and crude xylanase (D) were evaluated after incubation in different pH buffers at 70°C for various lengths of time. The maximum enzymatic activity measured under the optimal condition was defined as having a relative activity of 100%.

Fig. 5. Effects of inhibitors and detergents on xylanase activity.

The xylanase solution was pre-incubated with various inhibitors or detergents at 70°C for 10 min, and the residual activity of crude xylanase (A) and recombinant xylanase (B) was assayed.
Our study revealed that the recombinant xylanase produced in thermophilic *Geobacillus* sp. TC-W7 was thermostable and pH stable. Considering that most currently characterized xylanases are active only at an acidic pH, the thermostable recombinant xylanase described herein, which shows a broad pH activity profile and high activity under alkaline conditions, and whose activity persisted with most of the chemical inhibitors, might be a promising candidate for various industrial applications. This novel thermostable xylanase could be useful for several industrial applications, such as the biofuel, food, and paper and the pulp industries. However, further structure–function studies will need to be performed by molecular engineering to further characterize the xylanase.

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**References**