A xylanase-producing thermophilic strain, \textit{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, \textit{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 D-glucopyranose [17]. The combined actions of several hydrolytic enzymes, such as endo-β-1,4-xylanase (E.C. 3.2.1.8), exoxylanase (β-D-xylan xylohydrolase), and β-D-xyllosidase (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 \textit{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 spelts (Sigma), 0.05% (NH₄)₂SO₄, 0.03% MgSO₄·7H₂O, 0.02% CaCl₂·2H₂O, 0.01% K₂SO₄, 0.02% NaCl, 0.1% KH₂PO₄, 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'-AGAGTTTGTATCTGGCAG-3') and 1492R (5'-GCGTACCTTGTTACGACTT-3') [11]. The PCR product was cloned into the pMD-18T vector (Takara, China) and sequenced by TianGen (Beijing, China). Homology searches to other sequences were analyzed using the GenBank database (entry numbers: P40943, P36917, P23557, P51584, Q12603, Q60037, and Q60042), the following degenerate primers were synthesized to amplify the xylanase-encoding DNA fragment from the TC-W7 strain chromosomal DNA: sense primer Xyn [5'-CAT(C)ACA(G)C(T)TGTTTGCCA-3'] and antisense primer Xyn [5'-AT(T)CCCAG(A)AAC(T)GTG(A)AC-3']. The PCR was as follows: an initial denaturation step at 95°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s; and 72°C for 10 min. Homology to other sequences was analyzed using the GenBank BLAST program.

**Cloning of the Gene Encoding Xylanase**

According to the different xylanase sequences available in the SwissProt database (entry numbers: P40943, P36917, P23557, P51584, Q12603, Q60037, and Q60042), the following degenerate primers were synthesized to amplify the xylanase-encoding DNA fragment from the TC-W7 strain chromosomal DNA: sense primer Xyn [5'-CAT(C)ACA(G)C(T)TGTTTGCCA-3'] and antisense primer Xyn [5'-AT(T)CCCAG(A)AAC(T)GTG(A)AC-3']. The PCR was as follows: an initial denaturation step at 95°C for 4 min; 30 cycles at 95°C for 1 min, 1 min at 55°C, and 1 min at 72°C; followed by 10 min at 72°C. The amplified PCR product (492 bp) was purified by agarose gel electrophoresis and directly cloned to the pMD18-T Vector and sequenced by TianGen Biotech (Beijing) Co., Ltd. The PCR conditions were as follows: 95°C for 5 min; 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s; and 72°C for 10 min. Homology to other sequences was analyzed using the GenBank BLAST program.

**Expression and Purification of Xylanase**

The plasmid pMD18-T-Xylanase was double-digested with BamHI and EcoRI. The digested xylanase fragment was ligated into the pGEA-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 OD₆₅₀ 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 25% saturation. The supernatant was then isolated and subjected to 70% ammonium sulfate saturation. The precipitate was then collected by centrifugation (6,000 × g, 20 min) and then dissolved in 0.1 M citric acid-Na₂HPO₄, (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, (pH 7.2). The thermostability of xylanase was determined as the residual enzyme activity after 1 h of incubation at 60°C.