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Cloning and expression of a *Paecilomyces thermophila* xylanase gene in *E. coli* and characterization of the recombinant xylanase

Min Zhang, Zhengqiang Jiang *, Shaoqing Yang, Chengwei Hua, Lite Li *

Department of Biotechnology, College of Food Science and Nutritional Engineering, China Agricultural University, Beijing 100083, China

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ABSTRACT

A cDNA library of *Paecilomyces thermophila* was constructed, and the gene encoding xylanase (designated Pt *xynA*) was isolated from the library. Pt *xynA* consisted of 681 bp, and the translated protein encoded 226 amino acids. This is the first functional gene cloned from *P. thermophila*. The gene was successfully expressed in *Escherichia coli* BL21 and the recombinant xylanase (XynA) was purified to homogeneity by Ni–NTA and Sephadex G50. XynA showed an optimum activity at 75 °C and pH 7.0. Its residual activity was more than 60% after being treated at 85 °C for 30 min. K_m values of XynA for birchwood xylan, beechwood xylan and oat-spelt xylan were 4.4, 3.6 and 9.7 mg ml^{-1}, respectively. The enzyme has an endohydrolytic mode of action and can hydrolyse xylotriose to xylobiose through transglycosylation. These results indicate the XynA is a thermostable enzyme and has great potential in various industries.

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1. Introduction

Xylanases (β-1,4-Endoxylanases, EC 3.2.1.8) are enzymes which randomly hydrolyse the β-1,4-glycosidic bonds in the xylan backbone (Wong et al., 1988). They have generated considerable interest mainly because of their promising industrial applications such as in the process of cellulose biobleaching in the pulp and paper industry to reduce the usage of chlorine, in the food industry, and as feed additives in broiler and animal diets (Kulkarni et al., 1999; Polizeli et al., 2005).

A number of xylanases have been described, purified and characterized from different microorganisms including bacteria, yeast, actinomycetes and filamentous fungi (Carmona et al., 1998; Polizeli et al., 2005; Lu et al., 2008; Chen et al., 2009). Filamentous fungi are an important source of xylanases because of their high productivity (Polizeli et al., 2005). Thermophilic fungi such as *Thermomyces lanuginosus, Thermoasus auranticus*, *Talaromyces emersonii*, *Talaromyces thermophilus*, *Paecilomyces varioti*, and *Scytalidium thermophilum* produce xylanases with valuable properties, such as thermostability as well as optimum activity at elevated temperatures (Maheshwari et al., 2000; Singh et al., 2003; Polizeli et al., 2005; Maalej et al., 2009). During the past decades, substantial efforts have been put into cloning and expression of xylanase genes as well as their protein engineering by mutagenesis (Kulkarni et al., 1999; Al Balaa et al., 2006; Zhang et al., 2007; Zhou et al., 2008; Cheng et al., 2008; Ghaffar et al., 2009). To date, xylanase genes have been cloned and sequenced from many fungi, the majority of which are mesophilic fungal sources (Kimura et al., 2000; Degefu et al., 2001; Al Balaa et al., 2006; Zhang et al., 2007; Zhou et al., 2008; Ahmed et al., 2009). Only a few reports have been published regarding the xylanase genes from thermophilic fungi (Schlacher et al., 1996; Maheshwari et al., 2000; Ghaffar et al., 2009). The cloning and expression of xylanase genes from *T. lanuginosus* was achieved in *Pichia pastoris* (Schlacher et al., 1996; Damaso et al., 2003). The thermophilic fungus *Paecilomyces thermophila* J18, as a new species of *Paecilomyces* was proved to be a good producer (18,580 U g^{-1}) of xylanase in solid-state fermentation (Yang et al., 2006). The 25.8 kDa xylanase from this strain has been purified and has an optimal temperature range of 75–80 °C (Li et al., 2006).

In this report, we describe the isolation and cloning of one new *P. thermophila* cDNA that shares 91% identity to *xynA* gene (Accession No. U35436) from *T. lanuginosus* (Schlacher et al., 1996). The xylanase gene (Pt *xynA*) was expressed in *E. coli*. Further, the recombinant xylanase (XynA) was purified and characterized.

Abbreviations: BSA, bovine serum albumin; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CMC, carboxymethylcellulose; IPTG, isopropyl β-D-thiogalactopyranoside; LB, Luria–Bertani broth; X1, xylose; X2, xylobiose; X3, xylotriose; X4, xylotetraose; X5, xylopentaose; MOPS, 3-(N-morpholino)-propane sulphonic acid; ORF, open reading frame; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography.

* Corresponding authors. Address: China Agricultural University, College of Food Science and Nutritional Engineering, Department of Biotechnology, P.O. Box 294, No.17 Qinghua Donglu, Haidian District, Beijing 100083, China. Tel.: +86 10 62737689; fax: +86 10 82388508 (Z. Jiang).

E-mail addresses: zhqjiang@cau.edu.cn (Z. Jiang), llt@cau.edu.cn (L. Li).

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2. Methods

2.1. Materials

Birchwood xylan, beechwood xylan and carboxymethylcellulose (CMC, low viscosity) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Restriction endonucleases, pMD 18-T vector, and T4 DNA ligase were from TaKaRa (Tokyo, Japan) and were used according to the manufacturers recommendation. pET-28a(+) vector was obtained from Novagen (Madison, WI, USA). Trizol Reagent was purchased from Invitrogen. Creator™ SMART™ cDNA Library Kit and Advantage 2 PCR Kit were purchased from Clontech. Ni–NTA agarose beads and QIAquick Gel Purification Kit were purchased from QIAGEN (Qiagen Co., Hilden, Germany). All other chemicals used were analytical grade reagents unless otherwise stated.

2.2. Strains and plasmids

P. thermophila J18 deposited under the number AS3.6885 at the Center for Culture Collection of Microorganisms of China was used in this investigation. *E. coli* DH5α and BL21 strains were used for propagation of plasmids and as host for expression of the xylanase gene, respectively.

2.3. Genomic DNA and total RNA isolation

For isolation of genomic DNA, *P. thermophila* J18 was grown in medium containing xylooligosaccharides (2%, *w/v*) for 3 days. Fungal mycelia were collected by centrifugation (5000×g, 10 min) and washed twice with water at 4 °C. Protoplast formation, disruption of cells, and recovery of chromosomal DNA were done by the standard method (Sambrook and Russell, 2001). For isolation of RNA as a template for cDNA library construction, cells were grown and collected as described above. The mycelia were frozen and ground under liquid nitrogen with a mortar and pestle. The total RNA was isolated using the Trizol kit (Invitrogen). The quality and integrity of RNA was determined by gel electrophoresis in 1.0% agarose containing 3.5% formaldehyde.

2.4. Construction and screening of a P. thermophila cDNA library

A *P. thermophila* cDNA library was constructed with the aid of Creator™ SMART™ cDNA Library Kit. The size of the library was 2.16×10^6 separate clones, and the average length of the cDNA inserts was 1.2 kb. First and second strand synthesis of cDNA was carried out using standard protocols as outlined by the supplier (Clontech). First-strand synthesis is initiated with a oligo(dT) primer (CDSIII/3′ PCR Primer), and the SMART IV Oligo served as a short, extended template at the 5′ end of the mRNA. After second strand synthesis, the cDNA was digested with proteinase K and *Sfi* I to provide cDNA with unique ends. A cDNA library was prepared by ligation into the pET-SMART-M expression vector and transformed into *E. coli* strain BL21. The resultant colonies were overlaid with 0.5% xylan, 1.0% lactose and 1.7% (*w/v*) agar and incubated overnight. The overlay was stained with Congo red, and then destained with 1 M NaCl. Positive colonies were detected by the presence of clear halos. The plasmid was purified and subjected to DNA sequencing.

2.5. Nucleotide sequence accession number and sequence analysis

The GenBank accession number of Pt *xynA* cDNA sequence is FJ593504.

Nucleotide and deduced aminoacid sequences were analyzed using the ExPASy Proteomics tools (http://www.expasy.ch/tools/). Database homology searches of nucleotide sequences obtained were carried out using BLAST in GenBank at the NCBI. Signal peptide was analyzed by Signal P 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Search analysis of conserved domain and signature sequences was carried out using ScanProsite (http://www.expasy.ch/tools/ScanProsite). *N*- and *O*-Glycosylation sites were predicted using NetNGlyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc3.1 (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

2.6. Expression of the gene in E. coli

In order to efficiently express Pt *xynA* gene, the ORF region of the cDNA gene was amplified by PCR with *xynA*ORF-F (5′-CCATGGTGATCGGTATTACCTCC-3′) and *xynA*ORF-R (5′-AAGCTTGCCGACGTCAGCGACGGTGAT-3′) primers. Nco I and Hind III sites (underlined) were added to the forward and reverse primers, respectively. For the amplification of Pt *xynA* gene, PCR conditions were as follows: a hot start at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min, followed by one cycle of 72 °C for 10 min. To amplify this region from the *P. thermophila* genomic DNA, the same set of primers and PCR conditions were used. The purified PCR products were ligated with pMD18-T and sequenced with M13 primers from both strands.

The amplified PCR product of the cDNA was purified and cloned into the pMD18-T vector, and transferred into *E. coli* DH5α. Finally, the recombinant plasmid, containing Pt *xynA*, was recovered and digested with Nco I and Hind III, subcloned into pET28a (+) vector (Novagen), digested with the same restriction enzymes, and transformed into *E. coli* BL21 competent cells (Stratagene). Seed culture (10 ml) of *E. coli* BL21 harboring Pt *xynA* in pET28a vector was prepared by growing cells on a rotary shaker (200 rpm) for about 15 h at 37 °C. The prepared seed culture was used to inoculate LB medium (100 ml) containing kanamycin (50 μg ml^{-1}) which was cultured at 30 °C on a rotary shaker (200 rpm) until the optimal density at 600 nm reached 0.5–0.6. IPTG (isopropyl β-D-thiogalactopyranoside) was added to a final concentration of 1 mM, and incubation was then continued for 12 h.

2.7. Purification of the recombinant xylanase (XynA)

The cells were harvested by centrifugation, and suspended in 50 mM phosphate buffer (pH 8.0) and disrupted by sonication. The clear supernatant was collected by centrifugation and applied to Ni–NTA agarose resin column (1 × 5 cm) equilibrated with 50 mM phosphate buffer (pH 8.0) containing 20 mM imidazole and 300 mM NaCl. The enzyme was eluted with a linear gradient of 45–175 mM imidazole in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. The sample was dialyzed over night against 50 mM citrate buffer (pH 6.0) and was concentrated using an Amicon® Ultra centrifugal stirred cell with a 10,000 molecular weight cut off membrane (Millipore, Bedford, MA). The concentrated sample (0.8 ml) was applied to a Sephadex G50 column (100 × 1.0 cm) equilibrated with 50 mM citrate buffer (pH 6.0). Recombinant xylanase was eluted with the above buffer at a flow rate of 0.1 ml min^{-1}. The protein eluted as a single peak and was used as a purified xylanase for enzyme characterization. The homogeneity of the purified enzyme was monitored by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). SDS–PAGE was performed in a 12.5% (*w/v*) polyacrylamide gel according to the method of Laemmli (1970). The marker proteins were protein molecular weight standards in the range of 14.4–97 kDa (GE Healthcare). Protein bands were visualized by Coomassie brilliant blue R-250 staining.

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