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Cloning, expression, and characterization of a xylanase 10 from *Aspergillus terreus* (BCC129) in *Pichia pastoris*

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Abstract

A full-length xylanase gene, encoding 326 amino acids belonging to the fungal glycosyl hydrolase family 10, from *Aspergillus terreus* BCC129 was cloned and sequenced. Sequence analysis suggested that the first 25 amino acids of this enzyme is the signal peptide. Therefore, only the mature xylanase gene of 906 bp was cloned into a yeast expression vector, pPICZαA, for heterologous expression in *Pichia pastoris*. A band of approximately, 33 kDa was observed on the SDS–PAGE gel after one day of methanol induction. The expressed enzyme was purified by gel filtration chromatography. The purified recombinant xylanase demonstrated optimal activity at 60 °C, pH 5.0 and a K_m of 4.8 ± 0.07 mg/ml and a V_{max} of 757 ± 14.54 μmol/min mg, using birchwood xylan as a substrate. Additionally, the purified enzyme demonstrated broad pH stability from 4 to 10 when incubated at 40 °C for 4 h. It also showed a moderate thermal stability since it retained 90% of its activity when incubated at 50 °C, 30 min, making this enzyme a potential use in the animal feed and paper and pulp industries.

Keywords: *Aspergillus terreus*; Xylanase; Glycosyl hydrolase 10; *Pichia pastoris* expression; Xylan

Xylanases (endo-β-1,4-xylanase; EC 3.2.1.8) randomly hydrolyse the β-(1,4) glycosidic linkages of xylan, the heterogeneous polysaccharide in plant cell wall, into short oligosaccharide. The complete hydrolysis of xylan requires a combination of enzymes, dependent on substrate [1,2]. In hardwoods, xylan exists as *O*-acetyl-4-*O*-methylglucuronoxylan. Arabino-4-*O*-methylglucuronoxylan is found in softwood, while arabinoxylan is typically present in grasses and annual plants. The variety of xylans resulted in a diversity of xylanases, which are classified into families 10 and 11 of the glycosyl hydrolases, based on catalytic domain, primary structure and amino acid sequence similarity [3–6]. The majority of family 10 members are endo-β-1,4-xylanases, which have a high molecular weight (>30 kDa) and a low p*I*. Family 11 consists mainly of xylanases with a high p*I* and a low molecular weight (<30 kDa) [1,4,5].

Xylanases are found in plants, algae, insects, protozoans, and microorganisms [7]. Among microbial sources, the filamentous fungi are well known as secretor of high level of xylanase enzymes into the culture medium. This property makes fungi economically effective producers of xylanases, which are widely used in various industrial applications. For instance, in the pulp and paper industry, xylanases are employed in the prebleaching process to reduce the use of the toxic chlorine chemicals [8]. In the animal feed industry, xylanases are used to increase the body weigh gains of the animals [9,10]. In the bread and bakery industry, xylanases are used to decrease the dough viscosity while increasing the bread volume and shelf life [11].

As Thailand lies near the equator, she experiences hot and humid climes, resulting in high fungal diversity within her border. Here at BIOTEC, we have accumulated thousands of

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locally found fungi within our collection (http://mycology.biotec.or.th/current_research/diversity/maindiversity.htm). Preliminary screening of approximately 766 fungi showed that one of these, *Aspergillus terreus* (BCC129), possessed high xylanolytic activity at a wide pH range (pH 3–10). This strain may therefore be a potential source of industrial xylanase. However, *A. terreus* was reported to produce mycotoxins, which are harmful to animals and humans [12,13]. Thus, in this paper, heterologous expression of BCC129 xylanase was performed in *Pichia pastoris* to eliminate the unwanted effects of the mycotoxins. Specifically, the gene encoding this broad range pH xylanase was isolated and expressed using *P. pastoris* expression system. The secreted heterologous protein is produced as a major component in the culture supernatant, facilitating downstream enzyme purification. Moreover, biochemical characterization of the purified recombinant BCC129 xylanase was also performed. Furthermore, of the numerous fungal xylanases found in the database, this is the first report from the *A. terreus* species.

Materials and methods

Strains, plasmids, culturing conditions, and primers

Aspergillus terreus (BCC129) was obtained from BIOTEC Culture Collection (BCC), Thailand. It was isolated from soil in the central region of Thailand at Pa Sak, Lop Buri province. It was aerobically cultured in 100 ml, 5% wheat bran at 30 °C with 200 rpm shaking. After 2 days of cultivation, the culture was harvested by filtration through a 0.5 mm diameter mesh. *Escherichia coli*, DH5α, was used as host for plasmid propagation. It was cultured in low salt LB medium. *P. pastoris* KM71 (Invitrogen, USA), used as a host for expression of xylanase, was grown in YEPD [2% (w/v) peptone, 2% (w/v) glucose (Sigma, USA), and 1% (w/v) yeast extract]. The *P. pastoris* transformants were cultured under a selective condition in YEPD containing 100 μg/ml Zeocin. pGEM-T Easy vector (Promega, USA) was used in cloning of PCR fragments. pPICZαA vector (Invitrogen) was used in *P. pastoris* expression. All synthetic oligonucleotides used in this study were obtained from the Bio Service Unit (BSU), BIOTEC, Thailand (Table 1).

Table 1
Oligonucleotides used in the studies

Primer names	Sequence 5′ → 3′
PM1	CCGGAATTCAAGCTTCTAGAGGATCCTTTTT TTTTTTTTTTT
PM2	CCGGAATTCAAGCTTCTAGAGGATCC
Xyl2-F1	ACNCCNGARAAYTCNATGAAR
Xyl2-B1	HATYTCRTTNACNACRTCCCA
Xyl2-B2	RTCRTTHATRTANAGYTTNGC
3′ RazXyl-Ast1	TCGGTCTTGAAGAACCACATC
5′ RazXyl-Ast1	TCAGCACCACCAAAGCTGAAC
5′ RazXyl-Ast2	GATTCGGCTCAGTCGCATC
AstXyl-F1	CCGCTCGAGAAAAGACAGGCGGCTTCGAGC
AstXyl-R1	CTAGTCTAGATTACAAGGCGGAGATAATTG

For degenerate primer, the following abbreviations are used (N = A, T, C, G; H = A, T, G; Y = C, T; M = A, C; S = C, G; R = A, G).

Total RNA isolation, cDNA synthesis, and semi-nested PCR

BCC129 mycelia were frozen in liquid nitrogen before grounded up in an ice-cold mortar until powdery consistency was achieved. Tri-Reagent (Molecular Research Center, USA) was used for total RNA extraction from powdered BCC129 mycelia, following the manufacturer's instruction. The quality and integrity of RNA was determined by gel electrophoresis in 1% agarose containing 3.5% formaldehyde as described by Sambrook et al. [14].

First-strand cDNA from BCC129 was synthesised using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Lithuania) according to the supplier. First-strand cDNAs were then used as template in the semi-nested PCR. The first round of PCR was performed with 1 U of DyNAzyme EXT DNA polymerase (Finnzyme, Finland) in a total volume of 50 μl containing 20 μM of Xyl2-F1 and Xyl2-B1 degenerate primers, 1× DyNAzyme buffer, 2.5 mM each of dNTPs, 2 mM $MgCl_2$, and 4 μl cDNA. The PCR condition was: one cycle of 4 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 40 °C, and 30 s at 72 °C, and finally 10 min at 72 °C. The second PCR condition was the same as in the first round amplification except here it contained 4 μl of the previous PCR products as template and Xyl2-F1 and Xyl2-B2 as primers. The PCR products were analyzed by agarose gel electrophoresis. The amplicon of the expected size was gel-purified using QIAquick gel extraction kit (Qiagen, Germany) and cloned into pGEM-T Easy vector (Promega, USA). Sequencing was performed by Macrogen (Korea).

Isolation of the full-length xylanase gene by 3′ RACE and 5′ RACE

For the 3′ RACE, first-strand cDNAs were generated from 4 μg of total RNA from BCC129 mycelia using oligo-(dT) adaptor (PM1 primer) and MMLV-H minus reverse transcriptase, under the conditions suggested by the manufacturer (Fermentas, Lithuania). PCR was performed as described above, using 3′ RazXyl-Ast1 and PM2 primers with an annealing temperature of 55 °C.

For the 5′ RACE, first-strand cDNAs were synthesized as described above, except here, random hexamer instead of gene specific primer, as was commonly employed at this step, was used in the reaction. The RNA template was eliminated by adding NaOH to the final concentration of 0.5 M and incubated at 55 °C for 30 min. The reaction was then neutralized with 72 μl of 1% (v/v) acetic acid and gel-purified. Next, poly(A) tail was added to the 3′ end of the obtained first-strand cDNAs using terminal deoxynucleotidyl transferase (Promega, USA) according to the supplier's instruction. To amplify the 5′ end of the cDNA, nested-PCR was performed using 5′ RazXyn-Ast1 and PM1, and 5′ RaceXyn-Ast2 and PM2 primers consecutively. The PCR conditions for both