Journal of Bioscience and Bioengineering
VOL. 108 No. 1, 24 – 29, 2009

www.elsevier.com/locate/jbiosc

Cloning and expression of GH11 xylanase gene from *Aspergillus fumigatus* MKU1 in *Pichia pastoris*

Marimuthu Jeya,[1,2,a] Sairam Thiagarajan,[1,a] Jung-Kul Lee,[2] and Paramasamy Gunasekaran[1,*]

Department of Genetics, Centre for Excellence in Genomic Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai – 625021, Tamil Nadu, India[1] *and Department of Chemical Engineering, Konkuk University, 1 Hwayang-Dong, Seoul 143701, Republic of Korea*[2]

Received 15 October 2008; accepted 2 February 2009

A xylanase gene, *xynf11a* of *Aspergillus fumigatus* MKU1 was cloned and expressed in *Pichia pastoris* X33. Two exons of the *xynf11a* gene were amplified separately and fused by overlap extension PCR. The fused product was cloned in yeast expression vector pPICZB and expressed in *P. pastoris* under the control of the AOX1 promoter. *P. pastoris* transformants expressing recombinant xylanases were selected on xylan agar plate and their ability to produce the xylanase was evaluated in flask cultures. *P. pastoris* X33 (pZBxynf11aFP) efficiently secreted the recombinant xylanase into the medium and produced the high level of xylanase activity (14 U/ml) after 96 h of growth. The recombinant xylanase produced by *P. pastoris* showed maximum activity at pH 6.0 and temperature 60 °C. The recombinant xylanase did not exhibit any cellulase activity and hence it could be potentially used for pretreatment of paper pulp before bleaching.

[**Key words:** Xylanase; *Aspergillus fumigatus*; Overlap extension PCR; Expression; *Pichia pastoris*]

β-1,4-Xylans, the major components of hemicellulose, are heterogeneous polysaccharides consisting of a homopolymeric backbone of β-1,4-linked D-xylopyranose units and short side chains, including *O*-acetyl, α-L-arabinofuranosyl, and α-D-glucuronyl residues. β-1,4-Xylanases (EC 3.2.1.8) are the key enzymes that hydrolyze the backbone structure of β-1,4-xylans to initiate degradation of the complex polysaccharides by microorganisms. Several microorganisms produce multiple xylanases, implying a strategy for effective hydrolysis of β-1,4-xylan. Each enzyme may have a specialized function in the degradation of the complex polysaccharides, and specialized functions of individual xylanases may be useful for applications in the food, feed, and paper industries (1, 2).

The genome sequence of *Aspergillus fumigatus* Af293, a clinical isolate, has now been completed. The size of the genome is 29.4 Mb and, it contains 9922 predicted genes and several of them exhibit extensive similarity with genes in *S. cerevisiae* (3). The functions of many open reading frames (ORFs) identified in genome-sequencing projects are unknown. Genes encoding for putatively interesting proteins have to be identified, the corresponding proteins have to be characterized. Extensive works have been made on only two of the xylanases belonging to glycoside hydrolase families, namely GH10 and GH11, yet several xylanases belonging to families GH5, GH7, GH8 and GH43 have also been identified and studied to a lesser extent. Hence, functional characterization of putative xylanase genes of *A. fumigatus* becomes important and this is the first report on the expression and characterization of GH11 xylanase from *A. fumigatus*.

In recent years, several industrial yeasts have been developed as recombinant host systems for commercial production of heterologous proteins. These organisms combine ease of genetic manipulation with the ability to perform many eukaryotic posttranslational modifications (4). One of the most commonly used systems is the methylotrophic yeast *Pichia pastoris*, in which expression is driven by one of the strongest known regulated promoters, the alcohol oxidase I (*AOX1*) promoter, which is induced by methanol and repressed by other carbon sources such as glucose, glycerol, and ethanol (5). Another important feature of this system is its ability to achieve extremely high cell densities, enabling efficient protein production and secretion (6). The present study was aimed to functionally characterize the putative GH11 (*xynf11a*) xylanase of *A. fumigatus* MKU1.

MATERIALS AND METHODS

Strain The strain *A. fumigatus* MKU1 used in this study was previously isolated in our laboratory (7). *Escherichia coli* TOP10F′ and *P. pastoris* X33 (mut$^+$) were from obtained from Invitrogen, California, USA.

PCR PCRs were performed in a PTC-200 programmable thermal cycler (MJ Research, Massachusetts, USA) with one cycle of 94 °C for 5 min followed by 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 50–60 °C) and extension (60 s at 72 °C), with a final extension of 72 °C for 10 min. For analysis, 10 μl of reaction product was electrophoresed on a 1% agarose gel and stained with ethidium bromide (5 μg/ml). Primers used in this study are listed in Table 1.

Cloning of GH11 xylanase Genomic DNA of *A. fumigatus* MKU1 was isolated from mycelia by the method of Murray and Thompson (8). DNA from agarose gel was also extracted using Prefect prep Gel extraction column (Eppendorf, Germany) according to the manufacturer's instructions.

* Corresponding author. Fax: +91 452 2459873.
E-mail address: pguna@eth.net (P. Gunasekaran).
[a] Authors contributed equally.

1389-1723/$ - see front matter © 2009, The Society for Biotechnology, Japan. All rights reserved.
doi:10.1016/j.jbiosc.2009.02.003

TABLE 1. Oligonucleotide primers used in this study

Primer	Sequence (5′→3′)	Length (bp)
Xyn11F2	GARTACTACATYGTYGASDMNTA	23
Xyn11R	CCANRMNTYRAARTGRTT	18
AFORF11F	ATCATGGTTTCTTTCTCCTACCTG	24
AFORF11R	CCTAGTAGACAGTGATGGAAGCAG	24
PP11NF	GCGAATTCAATAATGGTTTCTTTCTCCTACCT	32
PPF11R	CACGCGGCCGCGTAGACAGTGATGGAAGCAGAT	33
PP11E1R	GCTGCCTCCGTAGTTGATGGTTCTAGCGCTTCCAGGGTTC	40
PP11E2F	GCTGGAACCCTGGAAGCGCTAGAACCATCAACTACGGAGG	40

Exon I of *xynf11a* gene was amplified with PCR primers PP11NF and PP11E1R in the first round, the reverse primer PP11E1R has 20 base pair overlapping region from 5′ end of exon II. Similarly, Exon II of *xynf11a* gene was amplified with primers PP11E2F and PPF11R, the forward primer PP11E2F has 20 base pair overlapping region from 3′ end of exon I. Both amplified exon I and exon II were purified and mixed in 1:1 molar ratio. These fragments were joined by the second round of PCR with no primers. In the third round of PCR, the fused product was amplified with primers PP11NF and PPF11R carrying flanking restriction sites (EcoRI and NotI) to facilitate further cloning into pPICZB vector. The fusion product *xynf11aFP* with its own signal sequence was digested with EcoRI and NotI and cloned into pPICZB, the resulting plasmid pZBX11aFP which was linearized by digestion with BstXI to facilitate integration via homologous recombination at the AOX1 locus in *P. pastoris* X33 strain (Invitrogen, California, USA).

Transformation of *P. pastoris* *P. pastoris* X33 was grown overnight (30 °C at 250 rpm) in YPD broth (10 g/l yeast extract, 20 g/l peptone and 20 g/l dextrose) and transformed with the recombinant construct, according to the manufacturer's instructions (Invitrogen, California, USA).

P. pastoris X-33 (mut^+) was transformed with linearized construct (pZBX11aFP) by electroporation [BTX (ECM399), Germany]. Transformed cells were selected on YPD agar medium supplemented with 2 M sorbitol and zeocin (100 μg/ml).

Expression of xylanase in recombinant *P. pastoris* The yeast culture medium (BMG medium) consisted of (g/l) 13.4 g of YNB, 4×10^{-4} g of Biotin, 5 ml of glycerol and 100 mM phosphate buffer pH 6.0. *P. pastoris* was grown in BMG medium in an orbital shaker (250 rpm) at 30 °C to an $OD_{600\ nm}$ of 1.3 to 1.6. The cells were harvested by centrifugation 1500 ×*g* for 10 min and the pellet was resuspended in 25 ml of BMMY medium (BMG medium in which glycerol was replaced by methanol (5 ml/l) and further supplemented with yeast extract 10 g/l and peptone 20 g/l in 250 ml of Erlenmeyer flasks kept at the similar conditions. Methanol (0.5%) was fed to the culture every 24 h for induction and samples were withdrawn at intervals. The cells were removed by centrifugation (10,000 ×*g* for 10 min) and the supernatant was assayed for xylanase activity.

Characterization of xylanase To determine the specificity of the recombinant xylanase, the enzyme was assayed with different substrates (Sigma, St Louis, MO, USA) such as birchwood xylan, beechwood xylan, and oat spelt xylan, low viscosity carboxymethyl cellulose and *p*-nitrophenyl-β-D-xylanopyranoside (pNPX). Reducing sugars were measured by the 3,5-dinitrosalicylic acid method with xylose, glucose and paranitrophenol as the standards. The activities were compared with the highest one obtained for any of these substrates.

The effect of pH on the xylanase activity was determined by measuring the relative activity using sodium acetate (pH 5.0–5.5), sodium phosphate (pH 6.0–8.5) and sodium carbonate (pH 9.0–9.5) buffers. The maximum activity was considered as 100%, and used as reference in determining relative activities at different pH values. The effect of temperature on the reaction rate was determined by performing the standard reaction at different temperatures in the range of 30–90 °C.

The stability of the xylanase as a function of pH was determined by measuring the residual xylanase activity after incubation of the enzyme for 1 h at different pH at 30 °C. The relative activity was expressed considering the activity before incubation as 100%. Thermostability of xylanases was determined by incubating the enzyme extract at different temperatures (30–90 °C) for 30 min.

Enzyme assays Xylanase activity was assayed according to Bailey et al. (9). Suitably diluted enzyme was incubated with 1% oat spelt xylan in 50 mM sodium phosphate buffer pH 6.0 at 50 °C for 10 min. The released reducing sugars were assayed after adding 1 ml of DNS (3, 5-dinitrosalicylic acid) reagent and then boiled for 15 min. The absorbance was measured at 540 nm (10). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of xylose per min under the experimental conditions. Carboxymethylcellulase (CMCase) activity was assayed with 1% carboxymethylcellulose in 50 mM sodium citrate buffer, pH 5.0, as a substrate. One unit of cellulase activity was defined as the amount of enzyme required to release 1 μmol of glucose per minute under the given conditions.

β-Xylosidase activity was measured by determining its A400 development with *p*-nitrophenyl-β-D-xylanopyranoside (pNPX) in 50 mM phosphate buffer (pH 6.0) as substrate at 50 °C.

Protein was estimated according to the method of Lowry et al. using bovine serum albumin as the standard (11).

Sequence analysis Nucleotide and deduced amino acid sequences were analyzed with the sequence analysis tools. Signal peptide sequence was analyzed by SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Related sequences were obtained from the databases using the software BLAST. Phylogenetic analyses were performed in MEGA 2.1, using the minimum evolution (ME) approach. GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) was used for identification of gene features such as exons and splice sites in genomic DNA. BioEdit (version 7.0.4.1) was used for sequence editing and analysis.

RESULTS

Majority of xylanases belong to the two families, GH10 and GH11 glycosyl hydrolases. To clone GH11 xylanase, degenerate primers were designed (Table 1) based on conserved catalytic domain regions of known GH11 xylanases of *Aspergillus* species. Using *A. fumigatus* MKU1 genomic DNA as template, PCR was performed with degenerate primers Xyn11f2 and Xyn11R to amplify GH11 xylanase gene. The resulted amplicon of length 220 bp was cloned and sequenced. Analysis of this 220 bp sequence revealed strong homology with the sequences of fungal GH11 xylanases in the GenBank and EMBL databases. In order to design primers to amplify the full length GH11 xylanase gene, BLAST was done with partial gene sequences of *A. fumigatus* MKU1 with unannotated genome sequence of *A. fumigatus* Af293 available in TIGR database. Based on the sequences retrieved from TIGR database and primers were designed for the amplification of complete ORF of GH11 xylanase gene of *A. fumigatus* MKU1. PCR with the primers AFORF11F and AFORF11R resulted in an expected amplicon of 750 bp of GH11 xylanase gene and designated as *xynf11a*. The *xynf11a* gene was cloned and sequenced. The *xynf11a* gene sequence was submitted to GenBank (accession number EF375873).

Nucleotide sequence analysis of the *xynf11a* gene The ORF of *xynf11a* was 739 bp long and interrupted by a single intron of 52 bp. An intron/exon junction that followed the GT-AG rule was present. The size of the intron resembled those of other filamentous fungal introns. Moreover, the intron occurs at the same position as those of family 11 xylanases of filamentous fungi. Conserved domain search (RPSBLAST) analysis confirmed the presence of catalytic domain of GH 11 xylanase.

A BLAST analysis of the deduced amino acid sequence of *A. fumigatus* XYNF11a showed high degree of identity to the following GH family 11 xylanases of fungal origin in the protein sequence database: 84% with *A. terreus* exlA, 78% with *A. oryzae* XynG2, 79% with *A. nidulans* xyl, 72% with *A. kawachii* xynA, 72% with *A. usamii* xylaII, 72% with *A. niger* xylB, 72% with *A. sulphureus* xylanaseB, 70% with *Chaetomium thermophilum*, 68% with *Seophaeris turcica*, 67% with *Cochliobolus carbonum* xy12, 66% with *Cochliobolus sativus*, and 63% with *A. oryzae* XynG1. Multiple sequence alignment of amino acid sequence of XYNF11a with sequences of related fungal xylanases is shown in Fig. 1. Phylogenetic analysis revealed that XYNF11a of *A. fumigatus* formed a separate cluster with *Aspergillus* xylanases and was closely related to *Aspergillus terreus* xylanase (Fig. 2). The conserved regions of GH11 xylanases, [PSA] - [LQ] - x - E - [YF] - Y - [LIVM](2) - [DE] - x - [FYWHN], known as active site signature 1 and [LIVMF] - x(2) - E - [AG] - [YWG] - [QRFGS] - [SG] - [STAN] - G - x - [SAF] known as active site signature 2 (http://www.expasy.org/prosite) are present. Glu residue centered in the active site signature has been identified as catalytic residue. The putative signal peptide was predicted with SignalP 3.0 Server and the most likely cleavage site was between position 18th and 19th amino acids.

Expression of *xynf11a* of *A. fumigatus* MKU1 in *P. pastoris* As the *xynf11a* gene had only one intron, attempt was made to fuse the exons of *xynf11a* gene and to clone the fused fragment downstream to the AOX1 promoter and express in *P. pastoris*. Primers were designed to amplify the two exons separately.

A 287 bp exon I amplified with primers PP11NF and PP11E1R and 400 bp exon II amplified with primers PP11E2F and PPF11R were used in the overlap extension PCR. Finally, the overlap extension PCR resulted in 687 bp exon fusion product. The fusion product was sequenced to confirm the reading frame of *xynf11a*.