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# Identification of New GH 10 and GH 11 Xylanase Genes from *Aspergillus versicolor* MKU3 by Genome-Walking PCR

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**Abstract** Xylanases randomly clear the backbone of xylans, which are hemicelluloses representing a considerable source of fixed carbon in nature. Consequently, these enzymes have important industrial applications. To characterize the genes responsible for producing these enzymes, we cloned xylanase genes belonging to the GH11 and GH10 families from *Aspergillus versicolor* MKU3 using a 2-step polymerase chain reaction (PCR) protocol involving degenerate PCR and genome-walking PCR (GWPCR). We amplified a family 10 xylanase consensus fragment using degenerate PCR primers exhibiting specificity for conserved motifs within fungal family 10 xylanase genes. We identified a single family 10 xylanase gene (*xynv10*) and determined its entire gene sequence during the second step of GWPCR, which was used to amplify genomic DNA fragments upstream and downstream of *xynv10*. The *xynv10* sequence contains a 1,378-bp open reading frame separated by 8 introns with an average size of 49 bp. We also amplified a partial GH11 xylanase gene sequence (*xynv11*) using degenerate PCR and genome-walking methods. Amplification of the C-terminal region of *xynv11* using a degenerate primer designed from sequences revealed strong homology with the partial GH11 xylanase gene of *A. versicolor* MKU3. The structural region in *xynv11* was approximately 680 bp and has one intron that is approximately 64 bp in length. Further expression and characterization of these genes will give better understanding of the role of these genes in xylan degradation by *A. versicolor*. © KSBB

*Keywords:* Aspergillus versicolor *MKU3, glycosyl hydrolase, xylanases, genome-walking PCR, cloning*

## INTRODUCTION

Xylans are the major hemicelluloses in differentiated hardwood; they are also abundant in the secondary cell walls of gymnosperms and the primary cell walls of grasses. Hence, xylans represent a considerable reservoir of fixed carbon in nature [1]. Structurally, the xylans are complex and highly variable polysaccharides with a β-1,4-linked backbone of xylopyranosyl residues containing 4-*O*-methyl-glucuronosyl, 4-*O*-arabinosyl, and acetic acid side groups. Endo-1,4-β-D-xylanases (EC 3.2.1.8) are responsible for random cleavage of the xylan backbone and, hence, are industrially significant. Amino acid sequence comparisons of several endo-1,4-β-xylanases indicate that most of them are from families 10 and 11 of the glycosyl hydrolases [2].

The genome-walking method is used to identify unknown regions flanking a known DNA sequence. Unknown flanking regions can be isolated by screening a genomic library using a known DNA sequence as the probe. However, it takes a considerable amount of time to prepare libraries and to screen them for the desired DNA fragment. The use of polymerase chain reaction (PCR) to isolate unknown flanking regions of known DNA sequence is becoming increasingly popular, because it is efficient, fast, and does not require construct and screen libraries. Several genome-walking methods have been developed for cloning the flanking regions of a gene without known restriction sites [3-5].

Xylanases are inducible enzymes; thus, searching for new xylanase genes is not easy, because they are produced in multiple forms in culture. Many xylanases have been identified and characterized; however, new and novel xylanase genes are

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**Table 1.** Primers used in this study

| Primer | Sequence (5′-3′) | Length (bp) |
|---|---|---|
| Xyn11F2 | GARTACTACATYGTYGASDMNTA | 23 |
| Xyn11R | CCANRMNTYRAARTGRTT | 18 |
| Xynf10F | GAGAAYAGCATGAARTGGGAYG | 22 |
| Xynf10R | TKRTARTTGCYRTCRAANAGCA | 22 |
| V10GWF | GGGCAATGATTAACAGAAAGTAGGACTCTTGGC | 33 |
| V10GWR | GCATCCATCAGCCGTGCTCAATCAATACAAT | 31 |
| H3P1 | AGCTGCGCAGGAAACAGCTATGACCGGTTGCA | 32 |
| E1SA1 | AATTGCGCAGGAAACAGCTATGACCGGTAGCT | 32 |
| X1K1 | CTAGGCGCAGGAAACAGCTATGACCGGTGTAC | 32 |
| B1SP1 | GATCGCGCAGGAAACAGCTATGACCGGTCATG | 32 |
| NCOB | CATGGCGCAGGAAACAGCTATGACCGGT | 28 |
| V11GWF | CTCAATCACGGGAACAAGTACGTTCACGCAG | 31 |
| V11GWR | AGTGTCGGTGCAGACTTGGTACGTGCTTCC | 30 |
| V10ORFF | ATGGTCTATATCAAAACCCTGGC | 23 |
| V10ORFR | TTACAGAGCGTTCATGACGGCA | 22 |
| V11ORFF | ATGAAAGTCACTGCCGCTT | 19 |
| V11ORFR | TYAAGARGAKATCGTGACACTGGC | 24 |

essential for several industrial applications. Xylanase production and purification methods have been reported [6], but no information is available concerning the xylanase-encoding genes in *Aspergillus versicolor*. In this article, we describe a method for cloning xylanase genes from *A. versicolor* MKU3 isolated from a paper mill effluent sample. This organism had higher xylanase activity than other fungal strains [7]. We detected GH11 and GH10 family xylanase-encoding genes within the *A. versicolor* MKU3 genome by PCR and used a 2-step PCR approach (degenerate PCR and genome-walking PCR [GWPCR]) to identify and clone *xynv10* and *xynv11*. This is the first report in the literature of xylanase-encoding genes in *A. versicolor* MKU3 being cloned.

## MATERIALS AND METHODS

### Strain and Genomic DNA Isolation

The *A. versicolor* MKU3 strain used in this study was isolated during our previous study [7]. It was grown for 24 h in a glucose medium at 30°C on a rotary shaker (200 rpm). Fungal mycelia were collected by centrifugation. Genomic DNA was isolated from the mycelia using the method described by Murray and Thompson [8].

### PCR Cloning and DNA Sequencing of the Xylanases

By comparing the amino acid sequences of more than 10 xylanases in family 10 and family 11 from fungi, we were able to design 4 degenerate primers (Table 1). PCR was carried out in 1X buffer, 4 μL of 10 mM dNTPs, 10 pmol/μL of each primer, and 5 units of *Taq* DNA polymerase in 100 μL. Amplification was performed in an MJ Research PTC-200 programmable thermal controller (Global Medical Instrumentation, Inc., Ramsey, MN, USA) with one cycle at 94°C for 5 min followed by denaturation (35 cycles of 60 s each at 94°C), annealing (60 s at 45~55°C), and extension (180 s at 72°C), followed by a final extension of 72°C for 10 min. The analysis was carried out by electrophoresis of 10 μL of the reaction mixture on a 1% agarose gel and staining with an ethidium bromide solution (5 μg/mL). The purified PCR products were ligated to pTZR/T and sequenced with M13 primers from both strands by Macrogen (Seoul, Korea). DNA was extracted from an agarose gel using a Prefect prep Gel extraction column produced by Eppendorf (Hamburg, Germany) or Qiagen (Hilden, Germany) according to manufacturer's instruction.

### Genome-Walking PCRs

The GWPCR procedure consisted of (1) digesting genomic DNA with a restriction enzyme, (2) ligating double-stranded oligonucleotide linkers (adaptors) to the genomic fragments to form a restriction fragment linker library, and (3) carrying out a PCR from the linker library with an internal walking primer and a linker primer. Five linkers were designed according a method described by Morris *et al.* [9], each being compatible with 2 restriction fragment ends. Linkers were assembled by annealing 100 pmol each of the upper and lower linker oligonucleotides in 100 μL of TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA) at 50°C for 30 min. Approximately 100 ng of genomic DNA was digested by 25 U of restriction enzyme overnight in a 30-μL reaction mixture. Linker libraries were prepared by ligating 2 μL of the appropriate linker to 5 μL of digested genomic DNA in a 10-μL reaction mixture. The reaction mixture was cleaned, and the library volume was then increased to 50 μL with TE buffer.

### GWPCR Conditions

GWPCR was performed in a standard 50-μL PCR mixture using 1 μL of linker library as template DNA, 100 ng of an *A. versicolor* xylanase-specific walking primer, and 100 ng of the appropriate linker primer. Reaction mixtures were cycled with the following profile: step 1, 94°C for 1 min; step 2, 35 cycles at 94°C for 30 sec, and then at 64°C for 45 sec then 72°C for 1 min; step 3, 72°C for 10 min; step 4, 4°C for 5 min. Purified PCR products were cloned into pTZR/T and sequenced with M13 primers.

### Sequence Analysis

Nucleotide and deduced amino acid sequences were analyzed using sequence analysis tools. The signal peptide was analyzed using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP). Related sequences were obtained by searching the database using BLAST software [10]. Phylogenetic analyses were performed in MEGA 2.1 using the minimum evolution approach. The GENSCAN online tool (www.genes.mit.edu/GENSCAN.html) was used to identify gene features (*e.g.*, exons and splice sites) in genomic DNA.

BioEdit (version 7.0.4.1) was used for sequence editing and analysis.

## RESULTS AND DISCUSSION

### Degenerate PCR

Degenerate primers were designed based on conserved catalytic amino acid regions of known GH10 and GH11 xylanases of the *Aspergillus* species. Using *A. versicolor* genomic DNA as template, PCR was performed with degenerate primers Xynf10F and Xynf10R to amplify GH10 xylanase. An expected 1.1-kb amplicon was obtained then eluted from the gel and cloned into a pTZ57R/T vector to obtain pTZAV10. Both strands of the insert in the pTZAV10 plasmid were sequenced. The sequence exhibited homology for the sequences of other fungal GH10 xylanases in the GenBank and EMBL databases. Similarly, PCR was performed with degenerate primers Xyn11F2 and Xyn11R to amplify the GH11 xylanase gene. A resultant 220-bp amplicon was eluted from the gel and then cloned into pTZ57R/T. Analysis of this sequence revealed strong homology with the sequences of other fungal GH 11 xylanases in the GenBank and EMBL databases.

### Genome-Walking PCR

We designed genomic walking primers (V10GWF, V10 GWR, V11GWF, and V11GWR) based on sequences of the *xynv10* and *xynv11* partial genes to amplify DNA fragments upstream and downstream of the xynv10 and xynv11 genes in *A. versicolor* MKU3. To facilitate ligation of the oligocassette to various DNA fragments, we generated an upper strand primer with restriction sites overhanging its 5′ and 3′ ends. The oligocassette linkers were made by annealing the upper strand primer with a universal lower strand primer (Table 1). Different linker libraries were constructed by digesting *A. versicolor* genomic DNA with various restriction enzymes and by ligating the resultant DNA segments with appropriate oligocassette linkers. GWPCR was performed with a specific primer and appropriate linker primers having a linker library (*Pst*I, *Hind*III, *Bam*HI, *Sph*I, *Eco*RI, *Sac*I, and *Nco*I) as a template. Successful amplification was achieved using the *Pst*I and *Sph*I linker libraries. The strategy for identifying the xylanase genes is shown in Fig. 1.

PCR with the V10GWR walking primer and H3P1 linker primer resulted in amplification of a 900-bp fragment from the *Pst*I linker library. Similarly, PCR with the primers V10GWF walking primer and the H3P1 linker resulted in a 500-bp fragment from the *Pst*I library. The PCR fragments were gel-eluted and then sequenced on both strands. The National Center for Biotechnology Information (NCBI) nucleotide Basic Local Alignment Search Tool (BLAST) search results confirmed that the sequence belongs to GH10 xylanases. The N- and C-terminal sequences were partly overlapped by an already sequenced internal fragment, and then the full-length region of *xynv10* was identified. Finally, PCR with V10ORFF

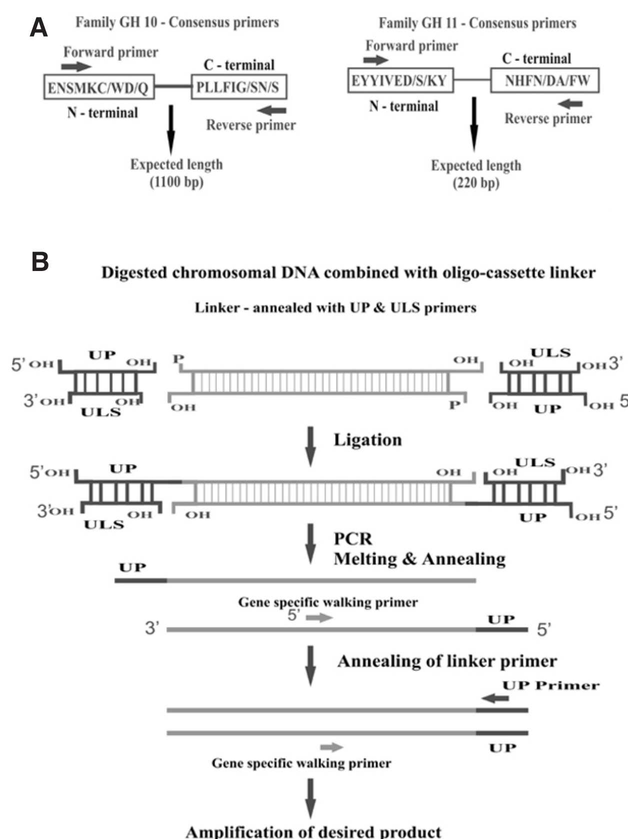


**Fig. 1.** (A) Consensus primer design for degenerate PCR. Primers were designed based on the conserved domains of GH10 and GH11 xylanase genes. Conserved domains were identified by multiple sequence alignment of the *Aspergillus* xylanase genes. (B) Schematic representation of the construction of oligocassette-mediated linker libraries. Amplification of oligocassette libraries using the cassette-specific primer and a gene specific primer.

and V10ORFR primers resulted in a 1,400-bp amplicon, which was cloned into a pTZ57R/T vector and designated pTZV10. Both strands of the insert were sequenced and submitted to GenBank (accession no. EF158475). A BLAST search for *A. versicolor xynv10* in the NCBI database revealed a high degree of homology with the GH 10 xylanases of fungal origin.

PCR with the V11GWR walking primer and the B1SP1 linker primer resulted in a 900-bp amplicon fragment from the *Sph*I linker library, which was eluted and then sequenced on both strands. A NCBI nucleotide BLAST search confirmed that the sequence belongs to GH 11 xylanases. The sequence was aligned with an already sequenced 220-bp amplicon to identify the N terminal of *xynv11*. Similarly, GWPCR was performed using different linker primers and a V11GWF walking primer to amplify the downstream region of *xynv11*. However, no distinct product was obtained using any linker/primer combinations. To amplify the C terminal of *xynv11*, the partial nucleotide sequence was aligned with sequences that showed a greater percentage of homologous

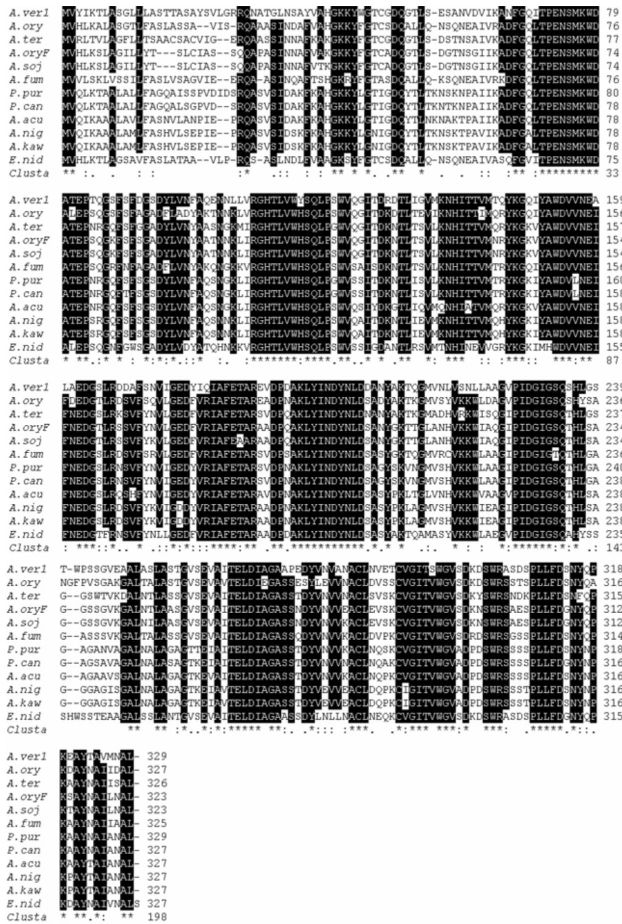

**Fig. 2.** Comparison of GH family 10 xylanases with XYNV10 of *A. versicolor* MKU3. Multiple alignment was obtained by ClustalW in a FASTA format using the neighbor-joining method. The amino acid residues are numbered, and the conserved regions are boxed. * – invariant residues, : – similar amino acids, . – less similar amino acids.
A.ver1, *A. versicolor* MKU3 (EF158475); A.ory, *A. oryzae* xylanase F1 (AB011212); A.ter, *A. terreus* (DQ087436); A.oryF, *A. oryzae* xylanaseF3 (AB066176); A.fumi, *A. fumigatus* (DQ156555); A.soj, *A. sojae* (AB040414); P.pur, *P. purpurogenum* (AF249328); P.can, *P. canescens* (AY756109); A.acu, *A. aculeatus* FIA (O59859); A.nig, *A. niger* (JT0608); A.kaw, *A. kawachii* (P33559); E.nid, *E. nidulans* (ABF50851).

regions, and then a degenerate reverse primer (V11ORFR) was designed. The forward primer (V11ORFF) was designed from the N terminal of the *xynv11* gene. The ORF of *xynv11* (685 bp) was amplified using V11ORFF and V11ORFR. The fragment was cloned and sequenced, and the gene sequence was submitted to GenBank (accession no. EF158476).

The N and C terminals of a partial *xynv10* gene were amplified from the *Pst*I linker library, and the N terminal of a partial *xynv11* gene was amplified from the *Sph*I linker library using GWPCR. Unfortunately, the C terminal of *xynv11* could not be amplified from linker libraries constructed with different restriction enzymes, possibly because of a lack of appropriate restriction enzymes downstream of *xynv11*. GWPCR was used to isolate 2 xylanase genes (*xynB* and *xynC*) from *Thermotoga maritima* FjSS3B.1 [11], to determine the nucleotide sequence of the complete *xynA* gene (which encodes a novel multidomain xylanase [XynA]) in *Caldibacillus cellulovorans* [12], and to clone a gene encoding a new xylanase (*xyn*ZG) in *Plectosphaerella cucumerina* [13].

### Nucleotide Sequence Analysis of *xynv10* and *xynv11*

The *xynv10* open-reading frame is 1,378 bp in length. The predicted cDNA that was obtained using GENSCAN software and compared with identical fungal GH 10 xylanase sequences included 8 introns with an average length of 49 bp, each within the *xynv10* gene. The length of the intron was similar to those of other filamentous fungal introns, which vary from 45 to 53 bp. A conserved domain search (RPSBLAST) analysis confirmed the presence of a catalytic domain within the GH 10 xylanase. The 685-bp *xynv11* gene contained only one intron (64 bp). The intron-exon junction followed the GT-AG rule and resembled those of other filamentous fungal introns. The analysis confirmed the presence of a catalytic domain within the GH 11 xylanase. Interestingly, only one intron was found in the *xynv11* gene; similarly other fungal family 11 endoxylanase genes also have only 1 or 2 introns [14,15].

The intron-exon arrangement in family 10 glycosyl hydrolases is very different from that in family 11. They typically consist of several introns and exons, whereas family 11 glycosyl hydrolyses contain only one intron. The *xynv10* gene contains 8 introns; all of which have the consensus sequence GT….AG for splicing, except intron 6, which contains a splicing sequence AT……AC. This "deviation" has been described as an example of intron sliding, *i.e.*, this sequence could be an ancestral consensus that shifted to GT….AG in more recently evolved fungi, such as *A. kawachii* [16]. This "deviated" intron is found in several family 10 fungal endoxylanases genes, including those seen in *A. oryzae* [17], *P. purpurogenum* [18], *P. chrysogenum* [19], and *P. simplicissium* [20]. The significance of this difference between the genes of the 2 endoxylanases families is unknown.

### Comparison of Putative *xynv10* and *xynv11* with Other Xylanases

The deduced amino acid sequence of *xynv10* was compared with that of other characterized GH10 xylanases and was found to exhibit maximum homology with the xylanases in *A. oryzae* (xylanase F1) and *P. simplicissimum* (67%), followed by those in *A. terreus*, xylanase F3 of *A. oryzae* (66%), *A. sojae*, *P. purpurogenum*, and *P. canescens* (65%); xylanase A of *Streptomyces coelicolor* A3 (46%); and *Aureobasidium pullulans* var. *melanigenum* (40%). The sequence alignments of GH 10 xylanases are presented in Fig. 1. The protein comprises 329 residues, including the signal peptide. The putative signal peptide was predicted using the SignalP 3.0 Server, and the most likely cleavage site was

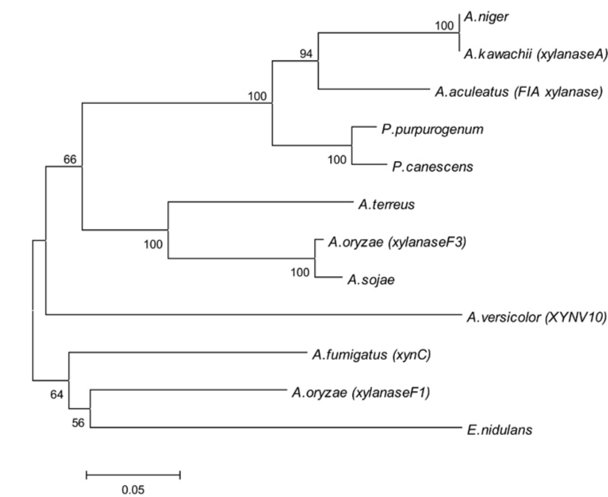


**Fig. 3.** Phylogenetic analysis of *xynv10* from *A. versicolor* MKU3. The sequences of GH10 xylanases were used to assess their phylogenetic relationships. The phylogram was generated with ClustalW in FASTA format using the neighbor-joining method and displayed in Treeview.

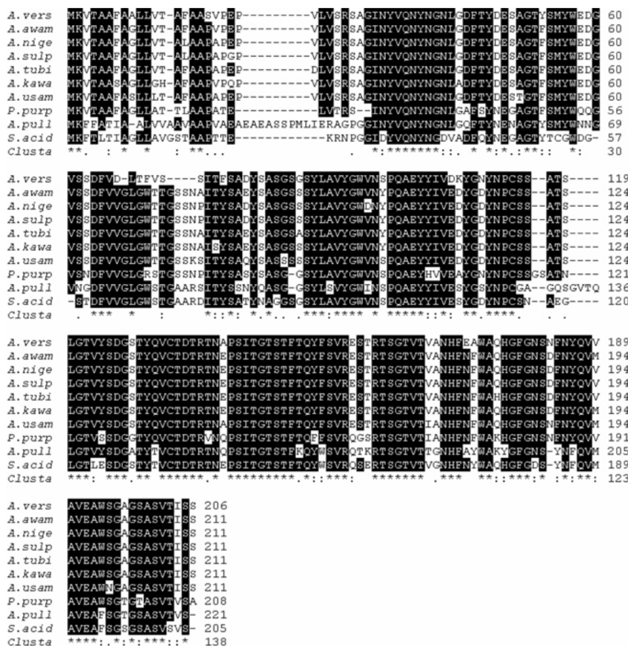

**Fig. 4.** Comparison of GH family 11 xylanases with *xynv11* of *A. versicolor* MKU3. Multiple alignment was achieved with ClustalW in FASTA format using the neighbor-joining method. The amino acid residues are numbered, and the conserved regions are boxed. * – invariant residues, : – similar amino acids, . – less similar amino acids.

A.vers, *A. versicolor* MKU1; A.awam, *A. awamori* (CAA 55005); A.usam, *A. usamii* (AAZ22340); A.tubi, *A. tubigensis* (AAB05996); A.sulp, *A. sulphureus* (ABC84852); A.kawa, *A. kawachii* (BAA03576); P.purp, *P. purpurogenum* (AAK 50762); A.pull, *Aureobasidium pullulans* (AAD51950); S.acid, *Scytalidium thermophilum* (AAQ22691).

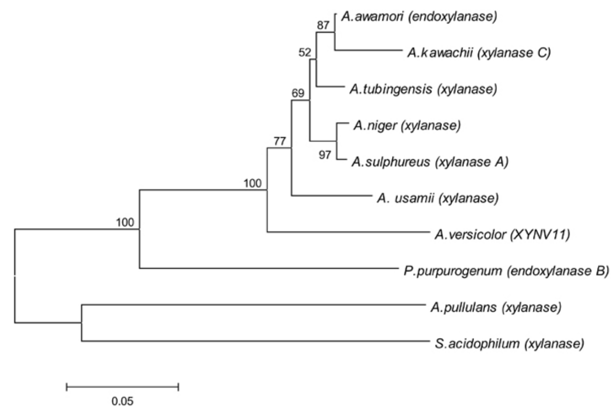


**Fig. 5.** Phylogenetic analysis of xynv11 from *A. versicolor* MKU3. The sequences of GH11 xylanases were used to assess their phylogenetic relationships. The phylogram was generated with ClustalW in FASTA format using the neighbor-joining method and displayed in Treeview.

between positions 20 and 21. The calculated molecular mass and isoelectric points (pI) of *xynv10* were 33.4 and 4.28 kDa, respectively. The protein possesses 2 cysteines at positions 283 and 289, which may form a disulfide bridge, such as those seen in *P. purpurogenum* [18] and *P. simplicissimum* xylanases [20]. The postulated catalytic residues, glu-158 and glu-265 in *xynv10*, are conserved in all the sequences. Phylogenetic analysis revealed that the *xynv10* in *A. versicolor* MKU3 forms a separate cluster (Fig. 3). A putative N-glycosylation site (N285) is present in *xynv10* in *A. aculeatus*, *P. purpurogenum*, and *P. simplicissimum*, but not in other endoxylanases.

A BLAST search for the deduced amino acid sequence of *xynv11* in *A. versicolor* MKU3 revealed a high degree of homology with the GH 11 xylanases in the following fungi: *A. awamori* (87%), *A. niger* (86%), *A. sulphureus* (86%), and *A. kawachii* (84%); the alignment of the homologous sequences appears in Fig. 4. Phylogenetic analysis (Fig. 5) revealed that the *xynv11* in *A. versicolor* MKU3 forms a separate cluster and is closely related to other *Aspergillus* species. Conserved GH 11 xylanase regions known as active site signature 1 and 2 (http://www.expasy.org/prosite) are present: [PSA] - [LQ] - x - E - [YF] - Y - [LIVM](2) - [DE] - x - [FYWHN] and [LIVMF] - x(2) - E - [AG] - [YWG] - [QRFGS] - [SG] - [STAN] - G - x - [SAF]. A Glu centered in the active site signature has been identified as the catalytic residue. This protein comprises 206 residues, including the signal peptide. A putative signal peptide was predicted using the SignalP 3.0 Server, and the most likely cleavage site was between positions 16 and 17 (AFA-AS). The calculated molecular mass and pI of *xynv11* were 21.97 and 4.24 kDa, respectively.

## Identification of Putative Regulatory Elements in *xynv10* and *xynv11* Promoter Regions

The transcription of the xylanolytic genes is controlled by gene-specific induction and carbon catabolite repression [21].

The 5′-noncoding region of *xynv10* was screened for various consensus sequences upstream. A TATA box-like sequence (TATAAA) was detected 119 bp upstream from the translation initiation site. The 5′-GGCTRA-3′–like sequence identified as the binding site for XlnR (the transcriptional activator of the xylanolytic system) was observed 252 bp downstream. The upstream sequence has 2 sequences that are similar to the CREA binding site (5′-SYGGRG-3′) at positions –125 and –313, which suggests that it is involved with regulation of the gene in response to glucose. Analysis of the 5′-noncoding region revealed several potential regulatory elements: a putative XlnR sequence (TTTAGCC) 316 bp downstream, 2 binding sites for the putative pH activator PacC located 255 and 275 bp downstream, and a typical eukaryotic promoter with the TATA box TATAAAT located 95 bp upstream from the translation initiation site. No CreA binding site was detected in the 395-bp promoter sequence we obtained, however a putative XlnR binding sequence was found in both the *xynv10* and *xynv11* genes of *A. versicolor* MKU3. Two CreA binding sequences were seen in *xynv10*, but none were seen in the sequenced 395-bp promoter region of *xynv11*. In *A. oryzae*, *xynG2* has 2 XlnR and 5 CreA binding sequences [22]. Only one typical CreA site for catabolite repression was found in a family 11 xylanase from phytopathogenic fungus *Ascochyta pisi* Lib [23].

GWPCR has been used to clone GH11 xylanase genes from bacteria [1,11] and nematophagous fungi [12]. However, this is the first study in which GH11 and GH10 xylanase genes were cloned from filamentous fungi (*A. versicolor* MKU3) using this method. We conclude that GWPCR is an easy and efficient method for cloning new xylanase genes from fungal sources.

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## REFERENCES

1. Morris, D. D., M. D. Gibbs, C. W. J. Chin, M. H. Koh, K. K. Y. Wong, R. W. Allison, P. J. Nelson, and P. L. Bergquist (1998) Cloning of the *xynB* gene from *Dictyoglomus thermophilum* Rt46B.1 and action of the gene product on kraft pulp. *Appl. Environ. Microbiol.* 64: 1759-1765.
2. Henrissat, B. and A. Bairoch (1996) Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316: 695-696.
3. Kilstrup, M. and K. N. Kristiansen (2000) Rapid genome walking: a simplified oligo-cassette mediated polymerase chain reaction using a single genome-specific primer. *Nucleic Acids Res.* 28: e55.
4. Mishra, R. N., S. L. Singla-Pareek, S. Nair, S. K. Sopory, and M. K. Reddy (2002) Directional genome walking using PCR. *Biotechniques* 33: 830-834.
5. Nthangeni, M. B., F. Ramagoma, M. G. Tlou, and D. Litthauer (2005) Development of a versatile cassette for directional genome walking using cassette ligation-mediated PCR and its application in the cloning of complete lipolytic genes from *Bacillus* species. *J. Microbiol. Methods* 61: 225-234.
6. Salama, M. A., K. M. I. Ismail, H. A. Amany, A. El-Lill, and N. S. I. Geweely (2008) Biochemical studies of purified extracellular xylanases from *Aspergillus versicolor*. *Int. J. Botany* 4: 41-48.
7. Jeya, M., S. Thiagarajan, and P. Gunasekaran (2005) Improvement of xylanase production in solid-state fermentation by alkali tolerant *Aspergillus versicolor* MKU3. *Lett. Appl. Microbiol.* 41: 175-178.
8. Murray, M. G. and W. F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.
9. Morris, D. D., R. A. Reeves, M. D. Gibbs, D. J. Saul, and P. L. Bergquist (1995) Correction of the β-mannanase domain of the *celC* pseudogene from *Caldocellulosiruptor saccharolyticus* and activity of the gene product on kraft pulp. *Appl. Environ. Microbiol.* 61: 2262-2269.
10. Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman (1990) Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
11. Reeves, R. A., M. D. Gibbs, D. D. Morris, K. R. Griffiths, D. J. Saul, and P. L. Bergquist (2000) Sequencing and expression of additional xylanase genes from the hyperthermophile *Thermotoga maritima* FjSS3B.1. *Appl. Environ. Microbiol*. 66: 1532-1537.
12. Sunna, A., M. D. Gibbs, and P. L. Bergquist (2000) A novel thermostable multidomain 1,4-*β*-xylanase from *Caldibacillus cellulovorans* and effect of its xylan-binding domain on enzyme activity. *Microbiology* 146: 2947-2955.
13. Zhang, G. M., J. Huang, G. R. Huang, L. X. Ma, and X. E. Zhang (2007) Molecular cloning and heterologous expression of a new xylanase gene from *Plectosphaerella cucumerina*. *Appl. Microbiol. Biotechnol.* 74: 339-346.
14. Apel-Birkhold, P. C. and J. D. Walton (1996) Cloning, disruption, and expression of two endo-β1,4-xylanase genes, *XYL2* and *XYL3*, from *Cochliobolus carbonum*. *Appl. Environ. Microbiol.* 62: 4129-4135.
15. Kimura, T., N. Kitamoto, Y. Kito, S. Karita, K. Sakka, and K. Ohmiya (1998) Molecular cloning of xylanase gene *xynG1* from *Aspergillus oryzae* KBN 616, a shoyu koji mold, and analysis of its expression. *J. Ferment. Bioeng*. 85: 10-16.
16. Sato, Y., Y. Niimura, K. Yura, and M. Go (1999) Module-intron correlation and intron sliding in family F/10 xylanase genes. *Gene* 238: 93-101.

17. Kimura, T., H. Suzuki, H. Furuhashi, T. Aburatani, K. Morimoto, K. Sakka, and K. Ohmiya (2002) Molecular cloning, characterization, and expression analysis of the *xynF3* gene from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 66: 285-292.
18. Chavez, R., C. Almarza, K. Schachter, A. Peirano, P. Bull, and J. Eyzaguirre (2001) Structure analysis of the endoxylanases A gene from *Penicillium purpurogenum*. *Biol. Res.* 34: 217-226.
19. Haas, H., E. Friedlin, G. Stoffler, and B. Redl (1993) Cloning and structural organization of a xylanase-encoding gene from *Penicillium chrysogenum*. *Gene* 126: 237-242.
20. Schmidt, A., A. Schlacher, W. Steiner, H. Schwab, and C. Kratky (1998) Structure of the xylanase from *Penicillium simplicissimum*. *Protein Sci.* 7: 2081-2088.
21. Tsukagoshi, N., T. Kobayashi, and M. Kato (2001) Regulation of the amylolytic and (hemi-) cellulolytic genes in aspergilli. *J. Gen. Appl. Microbiol.* 47: 1-19.
22. Kimura, T., H. Suzuki, H. Furuhashi, T. Aburatani, K.Morimoto, S. Karita, K. Sakka, and K. Ohmiya (2000) Molecular cloning, overexpression, and purification of a major xylanase from *Aspergillus oryzae*. *Biosci. Biotechnol. Biochem.* 64: 2734-2738.
23. Lubeck, P. S., L. Paulin, Y. Degefu, M. Lubeck, I. Alekhina, S. A. Bulat, and D. B. Collinge (1997) PCR cloning, DNA sequencing and phylogenetic analysis of a xylanase gene from the phytopathogenic fungus *Ascochyta pisi* Lib. *Physiol. Mol. Plant Pathol.* 51: 377-389.