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Screening, Cloning and Characteristics of the Common Xylanase Gene in Anaerobic Fungi

Research Article

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ABSTRACT

The aim of this study was to screen, clone and characterize the xylanase genes and to determine the common xylanase gene in anaerobic fungi. For this purpose, genomic DNA of 45 anaerobic fungi were used to amplify xylanase genes using 9 different primer pairs. The PCR yield rates of the primers in fungal isolates ranged from 6.6% to 100%. The *xynA* gene encoding xylanase was amplified from all anaerobic fungal DNAs with OrpXA primers (100%). The *xynA* was cloned into *E. coli* and 17 recombinant *E. coli* strains were obtained. The nucleotide sequences of the cloned genes were determined and characterized. The molecular weight of open reading frames (ORF) regions of the cloned genes varied between 24.7-30.2 kDa and the catalytic domains are members of glycoside hydrolase family 11. The specific activity of xylanase enzymes varied between 4.99-37.6 U/mg. Xylanase enzymes showed remaining activities ranging between 71.52-100% after incubation at 50 ˚C for 1 hour. High correlation was found between specific activity and thermal stability. This study showed that the *xynA* gene is common in anaerobic fungi, but this finding needs to be validated with further studies including species from the genera not included in this study.

KEY WORDS anaerobic fungi, cloning, *in silico* analysis, ruminant, xylanase.

INTRODUCTION

Hemicellulose is the second most abundant polysaccharide found in plants after cellulose, and xylan is the main constituent in hemicellulose (Kulkarni *et al.* 1999). The backbone of xylan consists of β-1,4-linked xylosyl residues with various groups (arabinosyl, acetyl and glucuronosyl) in their side chains (Thomson, 1993). Xylanases catalyze the hydrolysis of 1,4-β-d-xylosidic bonds in xylan, and the hydrolysis products consist of D-xylose monomers and different sizes of xylo-oligosaccharides (Collins *et al.* 2005). Based on the structural criteria, while enzymes with xylanolytic activity are present in different glycoside hydrolase families, most xylanases are classified into two glycoside hydrolase (GH) families, 10 and 11 (Lombard *et al.* 2013). Xylan plays a crucial role in maintaining cell wall integrity by forming covalent and non-covalent bonds with cellulosic fibers and lignins (Verma *et al.* 2013), therefore, xylanases play an important role in microorganisms that thrive in plant sources. Anaerobic fungi have a wide variety of enzymes that degrade plant cell wall polysaccharides, and xylanase is the most active enzyme among all the endo-polysaccharide hydrolase enzymes of anaerobic fungi (Borneman *et al.* 1989). Enzymes required to degrade xylan are produced at high levels in both monocentric and polycentric rumen fungi (Comlekcioglu *et al.* 2011). Xylanase production have been reported for species belong to the genera of *Neocallimastix* (Mountfort and Asher, 1989), *Piromyces* (Paul *et al.* 2010; Dagar *et al.* 2018), *Orpinomyces* (Ljungdahl, 2008; Dagar *et al.* 2018), *Anaeromyces*

(Novotná *et al.* 2010; Dagar *et al.* 2018), *Caecomyces* (Matsui and Ban-Tokuda, 2008), *Cyllamyces* (Comlekcioglu *et al.* 2011), *Liebetanzomyces polymorphus* (Joshi *et al.* 2018), and *Pecoramyces* (Hanafy *et al.* 2017). The detailed characterization of the enzyme systems of anaerobic fungi has been achieved by cloning the enzyme genes. Rumen fungi have xylanase catalytic domains both from GH10 and GH11 (Gruninger *et al.* 2018). Additionally, it has been observed that rumen fungi carry various catalytic domains with the same or different catalytic properties on a single polypeptide (Gilbert *et al.* 1992; Xue *et al.* 1992). In addition to catalytic domains, dockerin domains, which play an important role in the formation of the cellulosome complex, have been found in rumen fungi (Garcia-Vallvé *et al.* 2000).

Over 40 years since the discovery of anaerobic fungi, culture-based studies on rumen fungi have resulted in the discovery of new species, and 20 genera have been reported for rumen fungi (Stabel *et al.* 2021). However, studies on regulation, function, production, structures, and functional mechanisms of enzymes of anaerobic fungi are still limited. Several different xylanase genes have been reported from anaerobic fungi, however, which xylanase is more common in anaerobic fungi remains unclear.

The purpose of this study is to determine the prevalence of xylanase genes in rumen fungi that have been isolated in previous studies. In this context, xylanase genes of 45 anaerobic fungal isolates were screened using 9 different primer pairs. The *xynA* gene was found to be present in all anaerobic fungal isolates. Then, *xynA* was cloned to *E. coli* and 17 recombinant *E. coli* strains were obtained. Xylanase activities of recombinant *E. coli* strains were determined, and high correlation was found between specific activity and thermal stability. The DNA sequences of *xynA* were revealed, and *in silico* analysis were performed to demonstrate several physicochemical properties of XynA enzymes.

MATERIALS AND METHODS

Screening and cloning of xylanase genes

Genomic DNA of 45 anaerobic fungal isolates were used to screen and isolate xylanase encoding genes by PCR. Genomic DNA was isolated using DNA Extraction Kit (Vivantis Technologies, Malaysia) according to the manufacturer's protocol, and then stored at -20 ˚C for further analysis. Primer pairs used in this study are given in Table 1. The vector pGEMT-Easy (Promega, USA) was used to clone PCR products. Recombinant plasmids were transferred to *E. coli* EC1000 by the $CaCl_2$ method (Mandel and Higa, 1970). Transformant strains expressing the xylanase gene were selected according to the method of Teather and Wood (1982).

Microorganisms and culture conditions

In this study, 45 anaerobic fungi (*Neocallimastix* sp.=27, *Orpinomyces* sp.=13, *Caecomyces* sp.=4, *Cyllamyces* sp.=1) were obtained from the culture collection of Kahramanmaras Sutcu Imam University, Faculty of Agriculture, Department of Animal Science. The anaerobic medium was prepared according to Orpin (Orpin, 1976). As energy sources, wheat straw and glucose (0.5%) were used for subculture and DNA isolation, respectively. *Escherichia coli* EC1000 was used in cloning studies. *E. coli* was grown in Lysogeny broth (LB) at 37 ˚C in a shaking incubator (150 rpm).

Determination of physiochemical properties

The open reading frames (ORF) were searched using ORF Finder and the deduced amino acid sequences of ORFs were analysed for putative conserved domain susing CDD-BLAST (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Analysis of isoelectric point (pI), molecular weight (MW), instability index (II, stability of proteins), aliphatic index (AI, relative volume of protein occupied by aliphatic side chains), and Grand Average of Hydropathicities (GRAVY, sum of all hydropathicity values of all amino acids divided by number of residues in a sequence) were done by using ExPASy-ProtParam tool (https://web.expasy.org/protparam/) (Gasteiger *et al.* 2005). The predicted protein solubility (Sol) is calculated by using Protein-Sol tool (https://protein-sol.manchester.ac.uk/).

Enzyme assays

Endo-β-(1,4)-xylanase activity was determined using dinitrosalicylic acid (Miller, 1959). One unit of β-(1,4)-endoxylanase activity was defined as 1 µmol of reducing sugar released from beech wood xylan (Sigma) per minute under the standard assay conditions (50 ˚C, pH 6.0). The effect of pH was determined for different pH values ranging from 3.0 to 10.0 by using the substrate in 50 mM sodium acetate buffer (for pH 3.5–5.5), sodium phosphate buffer (for pH 6.0–7.5) and Tris-HCl buffer (for pH 8.0–9.0) solutions at 50 ˚C. Optimum temperature was determined by assaying the enzyme activity at different temperatures ranging from 30 to 90 ˚C with 10 ˚C increments at pH 6.0. The pH stability was determined by incubating xylanases with different buffers ranging from 3.0 to 10.0 at 50 ˚C for 30 min. Thermal stability was determined by incubating xylanases at 40, 50, 60, 70, 80 and 90 ˚C for 1 h at pH 6.0.

RESULTS AND DISCUSSION

In this study, genomic DNA of 45 anaerobic fungi belong to *Neocallimastix* (n=27), *Orpinomyces* (n=13), *Caecomyces* (n=4) and *Cyllamyces* (n=1) were screened for xylanase genes with different primers.

Table 1 The list of xylanase genes and primers that examined in this study

Gene (bp)	Species	Accession number	Reference*	Primer Name and Sequence (5'-3')
xynA (997)	*Orpinomyces* sp. PC-2	OSU57819	(Li *et al.* 1997)	OrpXA F:TGCCTCTGCTGGTCAAAGATTA R:ACCATTCGTTGTTTTCAACACC
xynA (572)	*Piromyces* sp.	X91858	(Fanutti *et al.* 1995)	PirXA F:GGTTATGAATTATGGGCTG R:TTTAATGTAAACCTTGGCG
xynB (609)	*Orpinomyces* sp. OUS1	AJ863170	(Nicholson *et al.* 2005)	OrpXB F:GAATTCCGTGGTCACTGTCTTA R:ATTATCACCAACACCCCAAAC
xynB(2591)	*N. patriciarum*	S71569	(Black *et al.* 1994)	NeoXB F:AGAAAAATGAAATTTAGCTCAGC R:AATTTAAAGGCATTGTGAATACC
xynS20 (1003)	*N. patriciarum*	EU030626	(Liu *et al.* 2008)	NeoXS20 F:TGTTACGCAAATTAGTCACTGG R:TTGACATTGAGAATACCATTGG
xynS20E (2019)	*N. patriciarum*	FJ529209	(Pai *et al.* 2010)	NeoXS20E F:ATGAGATTAGGTGTTGCT R:CTATTTAATTTTTAACGTAAACC
xynWF1 (995)	*P. communis*	EU314935	-	PirXW F:ACCGTCGGTAATGGTCAAAAC R:TTAAAAACCACAACCACACCAG
Xyl (763)	*Piromyces* sp. RRY-2002	AY130763	-	PirX1 F:GGTCAAAACCAACATAAGGGT R:CACTTGTAACCTTGAGCAGTAA
Xyl (569)	*P. communis*	AF297649	-	PirX2 F:ATGAATTATGGGCTGATGGT R:CGTATGGGAAATCAGCAGTAC

* Xylanases that has no references, were direct submissions to NCBI.

PCR yield rates of the primers in fungal isolates ranged between 6.6-100%. The highest rate (100%) was obtained with OrpXA primer. Approximately 900 bp size PCR product was obtained from all fungal DNAs with OrpXA primers. On the other hand, primers NeoXS20 (8.8%), NeoXB (8.8%), andNeoXS20E (6.6%) worked at a lower rate than the others (Table 2). According to this result, it can be thought that *xynS20*, *xynS20E* and *xynB* genes are not common in rumen fungi, and the *xynA* gene region obtained with OrpXA primers is a common xylanase gene for rumen fungi. Except for the *xynB* gene, all genes were detected in isolate 25 (*Neocallimastix* sp.), and only the *xynA* gene was found in isolates 28, 30 and 44 (*Neocallimastix* sp., *Caecomyces* sp. and *Cyllamyces* sp., respectively). Xylanases are produced at high levels in both monocentric and polycentric rumen fungi (Comlekcioglu *et al.* 2011). Although xylan is the most common inducer for xylanase production of rumen fungi, xylanase has also been produced in media containing different carbon sources such as cellulose, cellobiose, glucose, or xylose (Mountfort and Asher, 1989; Comlekcioglu *et al.* 2012). Therefore, it was suggested that xylanase is produced at basal level by rumen fungi (Lowe *et al.* 1987). According to the results in this study, it can be considered that the *xynA* gene isolated from all rumen fungi is responsible for basal xylanase production of rumen fungi, however, more research involving all anaerobic fungal genera is needed to confirm the presence of xynA in anaerobic fungi.

PCR products obtained with OrpXA primers were cloned for comparative analysis of *xynA* genes. As a result of the cloning process, 17 xylanase genes belong to *Neocallimastix* (n=12), *Orpinomyces* (n=3), *Caecomyces* (n=1) and *Cyllamyces* (n=1) were cloned to *E. coli*. BLAST analysis of amino acid sequences showed that all XynA contained a single catalytic domain belonging to the glycosyl hydrolase 11 family in this study. It has been observed that rumen fungi carry various catalytic domains with the same or different catalytic properties on a single polypeptide (Gilbert *et al.* 1992; Xue *et al.* 1992).

The glycosyl hydrolase 11 family includes low molecular weight (<30 kDa) xylanases which show high activity towards heteroxylan with a backbone of xylose units (Haki and Rakshit, 2003).

Computational analysis determining the physico-chemical properties of proteins in the gene families is of great importance to figure out the functions of the protein encoded by genes *in vitro*. According to gene sequences, the molecular weight and % GC (guanine-cytosine content) content of the open reading frame (ORF) region of the cloned genes varied between 24.7-30.2 kDa and 42.5-47.5%, respectively.

Table 2 PCR results that obtained with the primers used in this study

Primer	Primer annealing temperature* (°C)	Approximate PCR product size** (bp)	PCR yield rate (%)
OrpXA	59	900	100
PirX1	45	800	75.5
OrpXB	50	600	71.1
PirXA	50	570	57.7
PirX2	50	550	40.0
PirXW	55	900-1000	31.1
NeoXS20	50	1000	8.8
NeoXB	50	2500	8.8
NeoXS20E	50	2000	6.6

* The temperature that yielded the highest PCR product yield.
** The approximate molecular sizes of PCR products in agarose gel.

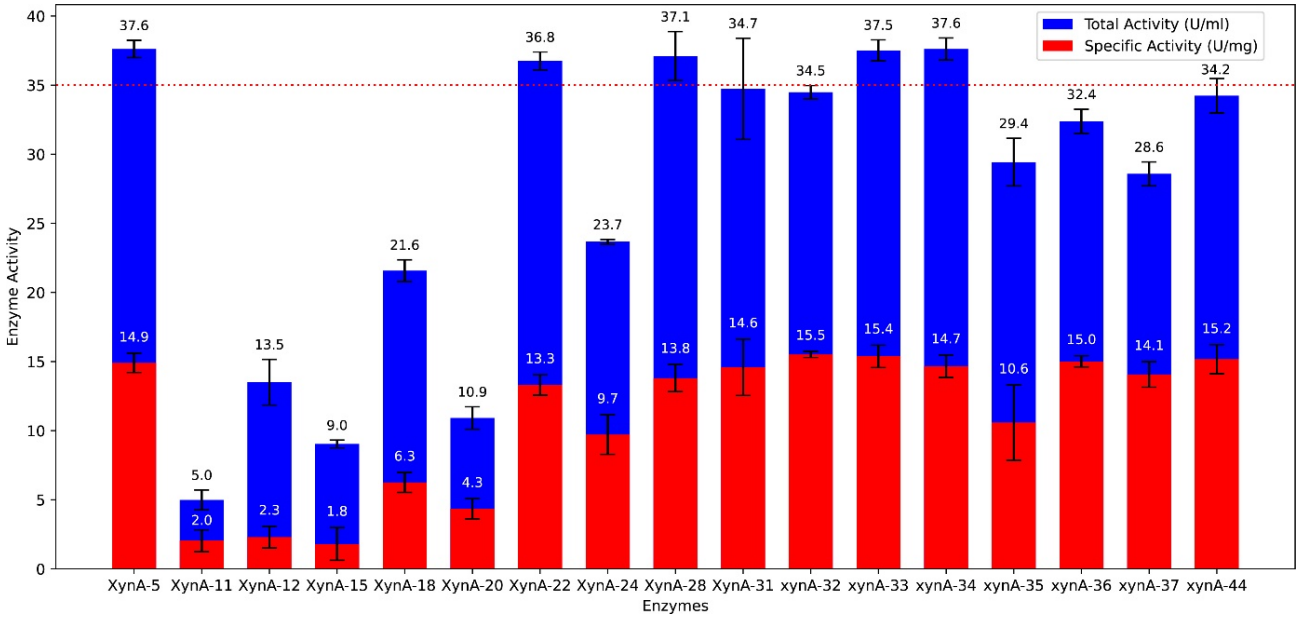

Figure 1 Bar graph showing specific and total enzyme activities of cloned xylanases. Blue color shows total activity and orange color shows specific activity. The threshold line shows the level of 35 U/mg. Enzyme activity values for each xylanases is given on bars

The secondary structure of the xylanases in this study were dominated by random coils (49.5%) followed by extended strand (34.1%), β-turn (8.6%), and α-helix (7.8%). pI values of the enzymes were found between 8.51-9.54. The highest MW value was found in the protein encoded by the xynA-31 gene. Theoretical isoelectric points are useful for understanding the protein charge stability and pI for these xylanases varied from 8.51 to 9.54. The range of GRAVY laid in between – 0.27 and – 0.60. The GRAVY value for a peptide or protein is calculated as the sum of all amino acid hydropathy values divided by the number of residues in the sequence (Chang and Yang, 2013). Lower range of GRAVY for enzymes used in this study indicates the possibility of better interaction of these enzymes with water (Sarkar *et al.* 2020). The solubility of protein is crucial in performing its functional characteristics (Yousefi and Abbasi, 2022).

Protein-Sol server was employed for solubility prediction of XynA. Based on the results, all xylanases except XynA-44 found to be above the threshold level of 0.45 which shows the solubility of proteins.

The optimum temperature and pH of the cloned xylanase enzymes were determined as 50 ˚C and 6.0-6.5, respectively. It has been determined that the enzyme activities generally decrease by 50% and 80% after 60 ˚C and 70 ˚C, respectively. Optimum pH, temperature and thermal stability of xylanases were found to be compatible with previous studies (Mountfort and Asher, 1989; Comlekcioglu *et al.* 2010).

In this study, thermal stability was calculated by measuring the remaining activity of the enzyme after 1 hour of pre-incubation at 50 ˚C, the temperature at which the enzyme works optimally. The thermal stability of enzymes was found to vary between 71.52 and 100%.

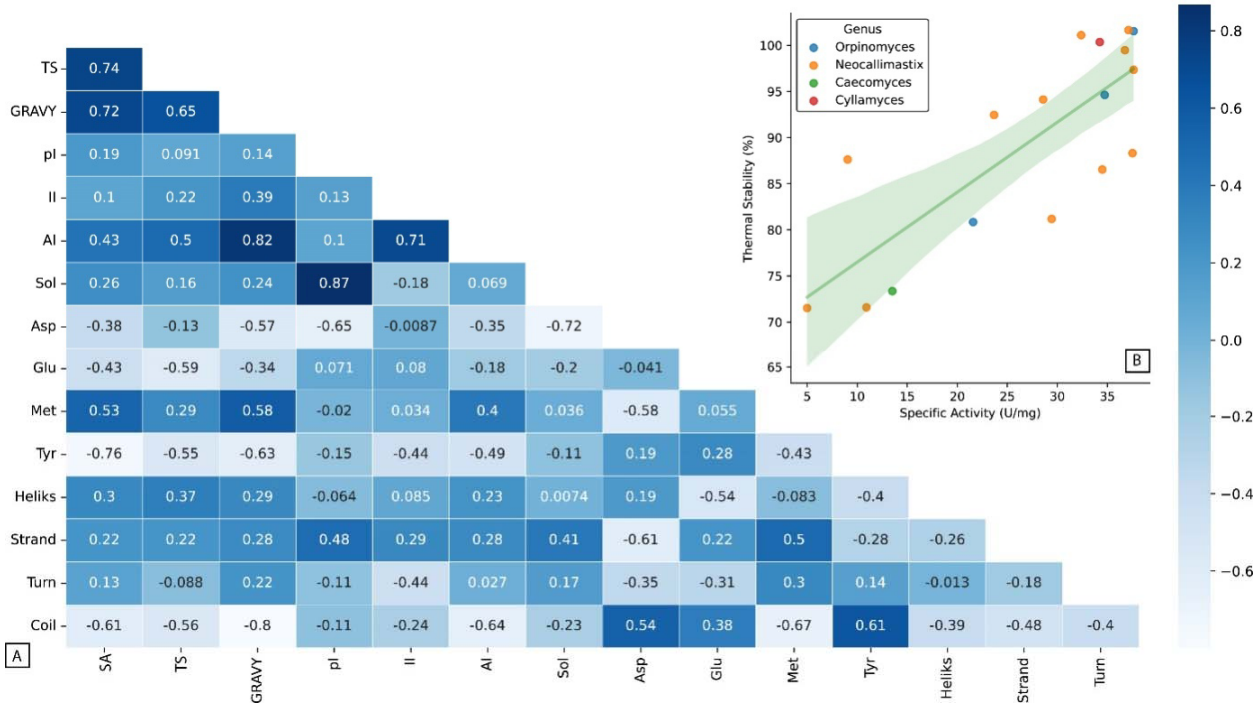

Figure 2 Spearman correlation analysis of 17 rumen fungal XynA. (A) Correlation coefficients were illustrated in heatmap. The calculated correlations are color coded white to blue. SA: specific Activity, TS: thermal stability (B) Correlation between specific activity (U/mg) and thermal stability (%). Genera were indicated as colored plots

It was observed that 29% of xylanases completely preserved their activity after 1 hour of pre-incubation at 50 ˚C. *In silico* analysis showed that the range of instability index of the xylanases found between 11.35 and 27.75. Since II was below 40, these xylanases can be predicted as stable.

However, no significant correlation was found between II and experimental thermal stability values. Since the stability of the protein may depend not only on the intrinsic nature of the protein but also on the conditions of the protein environment, II method for the estimation of protein stability under *in vitro* conditions is questionable (Gamage *et al.* 2019). Aliphatic index may be considered as a positive factor for the increase of thermostability of globular proteins (Ikai, 1980). In this study, the aliphatic index ranged between 54.51 and 81.05, and a significant correlation was found between aliphatic index and thermal stability ($P<0.05$). This study also discovered a significant correlation between GRAVY and thermal stability. At optimum temperature and pH, the specific activity of xylanases ranged from 4.99 to 37.62 U/mg, and 65% of these xylanases had specific activity higher than 35 U/mg (Figure 1). High correlation was observed between thermal stability and specific activity of xylanases ($P<0.05$). The active sites of enzymes can determine the effect of temperature on enzyme activity, which means that the evolution of the enzyme active site is likely constrained by its temperature dependence (Daniel and Danson, 2010). Additionally, both specific activity and thermal stability were highly positively correlated with GRAVY, while they were negatively correlated for random coil structure and tryptophan content ($P<0.05$). The Spearman correlation heatmap is given in Figure 2.

CONCLUSION

In the present study, previously isolated xylanase genes of anaerobic fungi were screened through genomic DNAs of 45 anaerobic fungal isolates. A xylanase gene which was named as xynA was amplified from all anaerobic fungal isolates using OrpXA primers. In this context, this study is the first report where commonness of xynA gene were revealed for anaerobic fungi. xynA of 17 different anaerobic fungal species were cloned in E. coli. The recombinant xylanases were optimally active at 50 ˚C and pH 6.0, and have good thermal stability at 50 ˚C. Positive correlations were found between thermal stability, specific activity, aliphatic index and GRAVY. However, no correlation was found between *in silico* calculated instability index values and experimentally found thermal stability values. Further studies containing all anaerobic fungal genera are needed to find out the prevalence of different xylanase genes in anaerobic fungi and investigate the relationship between the

fibrolytic capacity of anaerobic fungal isolates and the variety of xylanase genes that they own.

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