

# Cloning of a rumen fungal xylanase gene and purification of the recombinant enzyme via artificial oil bodies

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**Abstract** A gene encoding a xylanase, named *xynS20*, was cloned from the ruminal fungus *Neocallimastix patriciarum*. The DNA sequence of *xynS20* revealed that the gene was 1,008 bp in size and encoded amino acid sequences with a predicted molecular weight of 36 kDa. The amino acid sequence alignment showed that the highest sequence identity (28.4%) is with insect gut xylanase XYL6805. According to the sequence-based classification, a putative conserved domain of glycosyl hydrolase family 11 was detected at the N-terminus of XynS20 and a putative conserved domain of family 1 carbohydrate-binding module (CBM) was observed at the C-terminus of XynS20. An Asn-rich linker sequence was found between the N-terminal

catalytic domain and the C-terminal CBM of XynS20. To examine the activity of the gene product, *xynS20* gene was cloned as an oleosin-fused protein, expressed in *Escherichia coli*, affinity-purified by formation of artificial oil bodies, released from oleosin by intein-mediated peptide cleavage, and finally harvested by concentration of the supernatant. The specific activity of purified XynS20 toward oat spelt xylan was 1,982.8 U mg<sup>-1</sup>. The recombinant XynS20 was stable in the mild acid pH range from 5.0 to 6.0, and the optimum pH was 6.0. The optimal reaction temperature of XynS20 was 45°C; at temperatures below 30 and above 55°C, enzyme activity was less than 50% of that at the optimal temperature.

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## Introduction

Xylan constitutes the major component of hemicellulose and is the second most abundant renewable resource with a high potential for degradation to useful end products (Collins et al. 2005). Xylan is a heteropolysaccharide containing substituent groups of acetyl, 4-*O*-methyl-D-glucuronosyl and  $\alpha$ -arabinofuranosyl residues linked to the backbone of  $\beta$ -1,4-xylopyranosyl units and has binding properties mediated by covalent and noncovalent interactions with cellulose, lignin, and other polymers (Subramaniyan and Prema 2002). Xylan varies in structure between different plant species, and the homopolymeric backbone chain of  $\beta$ -1,4-xylopyranosyl units can be substituted to varying degrees with other side-chain groups (Collins et al. 2005). Due to its heterogeneity and complexity, the complete hydrolysis of xylan requires a large variety of cooperatively

acting enzymes such as xylanase, xylosidase, arabinofuranosidase, glucuronidase, acetylxylan esterase, ferulic acid esterase, and *p*-coumaric acid esterase (Kulkarni et al. 1999; Subramaniyan and Prema 2002; Collins et al. 2005). All of these enzymes act cooperatively to convert xylan into its constituent sugars. Of them, xylanase is of particular significance. It can catalyze the random endohydrolysis of  $\beta$ -1,4-xylosidic linkages in xylan to produce xylooligosaccharide and xylose (Wu et al. 2006).

Xylanases are fast becoming a major group of industrial enzymes, finding significant application in the paper and pulp industry because the hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the amount of chlorine used as the bleaching agent. The importance of xylanases, however, is not exclusive to the paper and pulp industry. Potential applications of xylanases also include the bioconversion of lignocellulosic material and agro-wastes to fermentative products, clarification of juices, improvement in consistency of beer, and the digestibility of animal feed stock (Subramaniyan and Prema 2002).

Xylanases can be found in marine algae, protozoans, crustaceans, insects, snails, and seeds of land plants; however, most xylanases are thought to be produced mainly by microorganisms (Subramaniyan and Prema 2002). Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase levels are higher than those produced by yeasts and bacteria (Polizeli et al. 2005). Rumen fungi are able to degrade the most resistant plant cell-wall polymers; thus, the rumen fungal population represents a rich and underutilized source of xylanases with tremendous potential for industrial and agricultural applications. Many xylanase genes have been cloned from rumen specific strains such as *Orpinomyces* PC-2, *Neocallimastix patriciarum*, and *N. frontalis* SK (Huang et al. 2005; Li et al. 1997; Liu et al. 1999).

Recent advances in recombinant DNA technology have made overproduction of target proteins in cells easily achievable. To facilitate protein purification, a variety of affinity tags have been explored and used to isolate target proteins. Artificial oil bodies (AOBs) reconstituted with the three essential components, triacylglycerol, phospholipid, and oleosin, have been used to develop an expression/purification system for the production of recombinant proteins (Chiang et al. 2005; Peng et al. 2004). In this method, the target protein is expressed in *Escherichia coli* as an insoluble recombinant protein fused to oleosin, a unique structural protein of seed oil bodies, by an intein fragment. Then, stable artificial oil bodies are constituted with triacylglycerol, phospholipid, and the insoluble oleosin fusion protein. Target protein is subsequently released through self-splicing of intein induced by 1,4-dithiothreitol, with the remaining oleosin–intein residing in oil bodies. Finally, the target protein is harvested by concentrating the

supernatant (Chiang et al. 2005). This novel technique offers a powerful and competitive alternative to the affinity chromatography conventionally used for protein purification.

In the present study, a xylanase gene from the ruminal fungus *N. patriciarum* S20 was cloned, expressed as an oleosin-fused protein in *E. coli*, affinity-purified by formation of AOBs, released from oleosin by intein-mediated peptide cleavage, and finally harvested by concentration of the supernatant. The specific activity and optimal temperature/pH of the recombinant xylanase were also analyzed.

## Materials and methods

### Isolation of xylanase-producing fungal strains

The xylanase-producing fungal strains were isolated from the rumen fluid of Taiwanese water buffalo (*Bubalus bubalis*) at the Taiwan Livestock Research Institute, Hualien Animal Propagation Station (Hualien, Taiwan) as described by Ho and Bauchop (1991). Among them, the isolate S20 showed the highest xylanase production and was identified as *N. patriciarum* based on the morphology and ribosomal ITS1 and 18S rRNA sequences (Brookman et al. 2000). *N. patriciarum* S20 strain was incubated in rumen fluid-containing maintenance medium (Ho and Bauchop 1991) using rice straw as the sole carbon source under anaerobic conditions at 39°C and transferred every 2–4 days. All of the anaerobic methods followed the Hungate technique (Bryant 1972).

### Cloning of xylanase gene

For total RNA isolation, *N. patriciarum* S20 was grown in 500-ml serum bottles containing 250 ml maintenance medium supplemented with 1% glucose as the sole carbon source under anaerobic conditions. After 3–5 days incubation at 39°C without shaking, the cells were collected by centrifugation at 4°C, 5,000  $\times$ g for 30 min. Total RNA was isolated by TRizol reagent (Invitrogen, Carlsbad, CA, USA), and mRNAs were purified using the PolyAtract mRNA Isolation System Kit (Promega, Madison, WI, USA). The *N. patriciarum* S20 cDNA library was constructed using the SMART cDNA Library Construction Kit (BD Bioscience, Palo Alto, CA, USA) and packaged with the Gigapack III Gold Packaging Extract kit (Stratagene, San Diego, CA, USA) following the manufacturer's instructions. Recombinant phages were used to transfect *E. coli* XL1-Blue cells (BD Bioscience) and screened for xylanase activity by blending in 0.7% (w/v) soft agarose overlays containing 0.3% (w/v) oat spelt xylan (Sigma Chemical Co., St. Louis, MO, USA) and 2.5 mM isopropyl-D-thiogalactopyranoside (IPTG; Sigma). Xylan hydrolysis

was detected by the Congo red staining procedure (Teather and Wood 1982). The presence of a yellow halo indicated a level of xylanase activity of the phages, which were then converted to plasmids by infecting *E. coli* BM25.8 cells according to manufacturer's instructions (BD Bioscience). The resultant plasmids (pTriplEx2-S20) were purified and the sequence of the xylanase gene (*xynS20*) insertions was determined by automatic sequencing (Mission Biotech Inc., Taipei, Taiwan). The nucleotide sequence of *xynS20* was deposited in GenBank (accession no. EU030626). SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP-3.0/>) was used to identify signal sequence cleavage sites (Bendtsen et al. 2004). Sequences were aligned in the BioEdit Sequence Alignment Editor program (Hall 1999) and a phylogenetic tree was constructed by the neighbor-joining method in ClustalW (Thompson et al. 1994) and displayed with the TreeView program (Page 1996).

### Subcloning of xylanase gene

The DNA sequences encoding *xynS20* were amplified by polymerase chain reaction (PCR) from pTriplEx2-S20 using the oligonucleotide forward primer, s20F (5' GGTGGTCATATGCAAAATGCCTGTCAACAACTC 3'), and the reverse primer, s20R (5' GGTGGTTGCTCTTCTGCATTGACATTGAGAATAC 3'). These two primers were designed to place an *NdeI* site at the 5' end and a *SapI* site at the 3' end of the PCR product, respectively. The PCR fragments encoding *xynS20* were digested with *NdeI* and *SapI* and ligated with *NdeI*–*SapI* digested pOSP2 (Chiang et al. 2005) to generate pOSP2-*xynS20*, which was sequenced to ensure that no errors were introduced by PCR. The resultant plasmids were used to transform *E. coli* BL21 (DE3) (Novagene, Madison, WI, USA) by standard techniques (Sambrook and Russell 2001). Transformants were selected on Luria–Bertani (LB) agar plates containing ampicillin (100  $\mu\text{g ml}^{-1}$ ; Sigma).

### Expression and purification of recombinant proteins

*E. coli* BL21 transformant cells were cultured in LB broth and cell growth was measured turbidimetrically at 600 nm (OD600). To produce the recombinant protein, overnight culture was prepared and subsequently seeded into fresh media. The cell cultures were maintained at 37°C and induced with 100  $\mu\text{M}$  IPTG for protein productions upon reaching an OD600 of 0.5. After 4 h of induction, the cells were harvested by centrifugation at 5,000  $\times g$  for 20 min at 4°C.

AOBs were prepared according to the method described by Chiang et al. (2005). The cell pellet was resuspended in 1 ml of 0.1 M sodium phosphate buffer (pH 7.4), sonicated for 10 min with an ultrasonicator (Model XL, Misonix, Farmingdale, NY, USA), and fractioned into supernatant

and pellet parts by subsequent centrifugation. AOBs were reconstituted in 1 ml of 0.1 M sodium phosphate buffer, pH 7.4, with 15 mg of olive oil (Sigma), 150  $\mu\text{g}$  of phospholipid (Sigma), and the pellet fraction of *E. coli* cell lysate containing 550  $\mu\text{g}$  of oleosin-fused recombinant proteins. The mixture was subjected to sonication. Subsequently, AOBs were collected after centrifugation and washed with the sodium phosphate buffer (0.1 M, pH 7.4) solution. To retrieve the target protein, AOBs thus prepared were treated with 40  $\mu\text{M}$  dithiothreitol (DTT) for 16 h. Finally, a centrifugation was performed to segregate the oil and aqueous phases, and the protein in each phase was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the xylanase activities were determined.

### Gel electrophoresis and zymogram analysis

SDS-PAGE analysis was done by the method of Laemmli (1970). The zymogram technique was done according to the method of Beguin (1983). Briefly, the recombinant proteins produced in each fraction were electrophoretically separated by 15% SDS-PAGE containing 0.1% xylan. After electrophoresis, the gels were washed three times for 30 min at 4°C in 100 mM acetate buffer (pH 5.0) containing 25% isopropanol for the first two washes to remove SDS, and then incubated in the same buffer for 40 min at 40°C. The gels were stained in 0.1% Congo red solution for 15 min at room temperature and destained with 1 M NaCl for another 10 min. Light yellow bands indicating degradation of xylan were visible on a deep red background.

### Xylanase activity assay

Xylanase activity was determined in 100 mM phosphate buffer (pH 6.0) containing 0.5% (*w/v*) oat spelt xylan (Sigma) at 40°C for 10 min. Reducing sugar released from the substrate was estimated by the dinitrosalicylic (DNS) acid reagent (Konig et al. 2002). One unit of enzyme activity was defined as that releasing 1  $\mu\text{mol}$  of reducing sugar equivalents per minute from the respective substrate under the assay conditions. Specific activity was expressed as U  $\text{mg}^{-1}$  protein. Protein concentration was determined according to the Lowry assay (Lowry et al. 1951) against a standard curve of bovine serum albumin, fraction V (Sigma).

### Optimum pH and temperature of xylanase

The effect of pH on xylanase activity was evaluated at 45°C for 10 min over a pH range of 2.0–11.0 using the following different buffers (100 mM) under xylanase activity assay conditions: glycine–HCl buffer (pH 2.0 and 3.0), acetate buffer (pH 4.0 and 5.0), phosphate buffer (pH 6.0 to 8.0),

Tris–HCl buffer (pH 9.0), and glycine–NaOH buffer (pH 10.0 and 11.0). The optimum temperature was measured by performing the xylanase activity assay for 10 min at temperatures ranging from 20 to 70°C at pH 6.0.

#### Kinetic parameters of xylanase

Kinetic studies of xylanase were carried out at constant enzyme concentration with different concentrations of oat spelt xylan (1 to 6 mg ml<sup>-1</sup>). Reactions were conducted at 45°C in phosphate buffer (100 mM, pH 6.0) for 10 min. A typical Lineweaver–Burk plot was obtained when 1 [v]<sup>-1</sup> was plotted against 1 [S]<sup>-1</sup> (Lineweaver and Burk 1934). Kinetic parameters ( $K_m$  and  $V_{max}$ ) were estimated by linear regression from Lineweaver–Burk plot.

## Results

### Cloning of *N. patriciarum* S20 xylanase-encoding gene

From approximately 25,000 recombinant plaques from the cDNA library of *N. patriciarum* S20, two xylanase-producing phages were isolated by Congo red plate assay. The restriction map indicated that these xylanase-positive recombinants contained cDNA sequences deriving from the same mRNAs. This cDNA sequence with the respective size of 1.4 kb was designated *xynS20* (Fig. 1).

### Characterization of XynS20

Translation of the nucleotide sequence revealed an open reading frame of 1,008 bp encoding a protein of 336 amino acids with a predicted molecular weight of 36 kDa. Database searches of the deduced amino acid sequence were performed using the National Center for Biotechnology Information's Basic Local Alignment Search Tool (Altschul et al. 1997). The deduced amino acid sequence matched several xylanases. According to the sequence-based glycosyl hydrolase (GH) classification, a putative conserved domain of GH family 11 was detected at the N-terminus of XynS20 (position 49–240). The amino acid sequence alignment of XynS20 and other GH family 11 xylanases available from the GenBank showed that the highest identity is with xylanases from insect gut uncultured microorganisms (28.4% and 27.0%, accession nos. AAS85784 and AAS85782, respectively), followed by xylanases from *Lactococcus lactis* subsp. *cremoris* SK11 (26.9%, accession no. YP\_809518), *Clostridium cellulolyticum* H10 (22.8%, accession no. ZP\_01576648), and *Piromyces communis* (20.0%, accession no. AAG18439).

The amino acid sequence of catalytic domain of Xyn20S was further compared to the reported GH family 11

xylanases by phylogenetic analysis. The phylogenetic tree shows that XynS20 branches with xylanases from insect gut and *L. lactis* subsp. *cremoris* SK11 and is more closely related to them than it is to other xylanases of eukaryotic origin from rumen (Fig. 2).

According to the sequence-based carbohydrate-binding module (CBM) classification, a putative conserved domain of family 1 CBM was found at the C-terminus of XynS20 (position 301–335; Fig. 1). The amino acid sequence alignment of CBM showed that the highest sequence identity of XynS20 was 66% with that of the XynC from *N. patriciarum* (accession no. AAF14365), followed by 61% with that of XynB from *N. patriciarum* (accession no. AAB30669), 56% with that of CelA from *N. patriciarum* (accession no. AAC49315), and 50% with that of CelF from *Orpinomyces* sp. PC-2 (accession no. AAC09067).

### Expression and purification of XynS20

PCRs were set up to subclone the DNA fragments of *xynS20* genes into the AOB expression/purification system vector pOSP2. XynS20 was expressed in *E. coli* as a recombinant protein fused to the N terminus of oleosin by a linker polypeptide, intein *M*. The expressed recombinant protein, XynS20-intein *M*-oleosin was predominately found in the insoluble fraction of cell lysate after centrifugation (Fig. 3a, lanes 3 and 4). AOBs were reconstituted with the insoluble pellet of cell lysate consisting mainly of XynS20-intein *M*-oleosin. The XynS20-intein *M*-oleosin as well as other insoluble bacterial proteins was almost entirely present in the oil-body fraction after centrifugation (Fig. 3a, lanes 5 and 6). Release of XynS20 from AOBs was achieved via self-splicing of the intein linker induced by the inclusion of DTT. After centrifugation, XynS20 was found predominately in the supernatant, whereas intein *M*-oleosin remained in AOBs (Fig. 3a, lanes 7 and 8). The yield of the purified enzyme was 0.62±0.15 mg, starting from 1.5 g (wet weight) of *E. coli* cells.

The enzymatic activities of XynS20 from each step of protein purification were further confirmed by zymographic analysis of xylan-SDS-PAGE. When presented in the insoluble fraction of cell lysate or embedding in AOBs, XynS20-intein *M*-oleosin did not show any enzymatic activity. However, after release from AOBs by inclusion of DTT to induce intein linker self-splicing, XynS20 revealed a xylanase activity band of about 36 kDa (Fig. 3b).

The enzymatic activities of XynS20 from each step of protein purification were also confirmed by DNS reagent method. The expressed recombinant protein presented in the insoluble fraction of cell lysate showed no detectable enzyme activity but showed activity after embedding in AOBs or release from AOBs by inclusion of DTT (data not shown). The specific activity of purified XynS20 toward

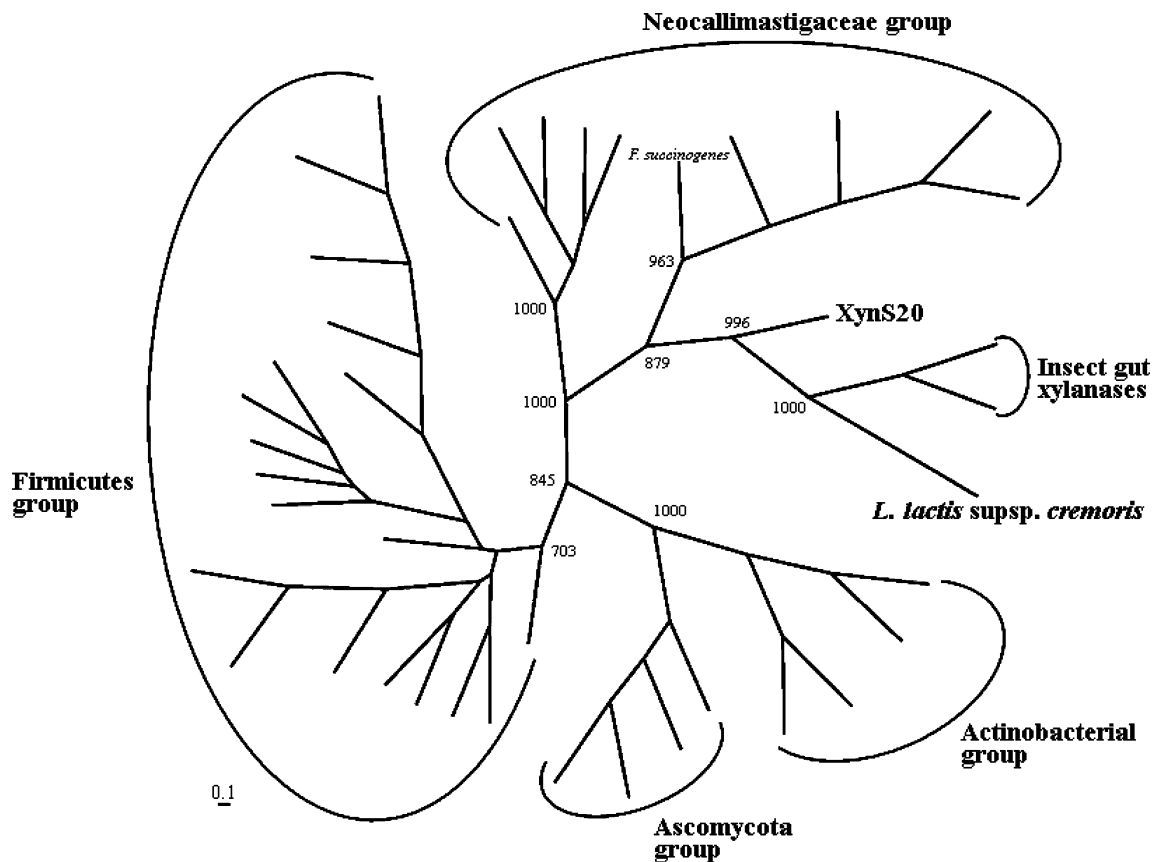
**Fig. 1** Nucleotide sequence of xylanase gene *xynS20* and its flanking regions and deduced amino acid sequence. The deduced amino acid sequence of *xynS20* gene product is indicated in *boldface*. The translational stop codon is indicated by *asterisks*. The signal peptide sequence is *underlined*. The putative region of glycosyl hydrolase family 11 is *shaded*. The carbohydrate-binding module is *boxed*

1	TTAGGAATAAAAAATATTTTGTATTTTAACGAATAATAAAAAATTTTAAATTTTCTTATTA	GA	2
3	TATATATAAACTTTGTATTTAGATAAAAAATTTTATAAAAAATATATTTTGGAAATATAT		62
63	TAATTATAAAAAATAGTATTTGTTTTTAAATTTTCATAAAAAATTTTTTTGTAAAGAA		122
123	ATGTTACGCAAATTAGTCACTGGTGCTTTAGCTGCCGCTTTATTACTTTCTGGTCAAAGT		183
183	<b><u>M L R K L V T G A L A A A L L L S G Q S</u></b>		242
1			
243	AATGCTCAAAATGCCTGTCAACAACTCAACAATTAAGTGGTGAAGAAGTATTAACAAC		302
21	<b><u>N A Q N A C Q Q T Q Q L S G G R T I N N</u></b>		
303	AAAAATGAACTGGTAATGGTAATGGAACTACAAGTATGAAATCTGGAGAGATGGTAAT		382
41	<b><u>K N E T G N G N G N Y K Y E I W R D G N</u></b>		
363	GGTGGTTCTTAACTCTTTATCCAAAGGATGCCGCTTTCAAGGCTTCATGGAACAATCTC		422
61	<b><u>G G S L T L Y P K D A A F K A S W N N S</u></b>		
423	GGTGATTTCTCGTCTGTGGTTAACTTTCAATAAGCCAGCTGCTACTAATCTTGGT		482
81	<b><u>G D F L G R V G L T F N K P A A T N L G</u></b>		
483	GGTGATCTTATTGCTAACTACAACACTACAAGAAGAGTGGTAGTGATGGAGGTACATACTCT		542
101	<b><u>G D L I A N Y N Y K K S G S D G G T Y S</u></b>		
543	TACATTGGTATTTACGGTTGGATGGATAATCCACAAATTGAATACTATGTTGTTGATGAC		602
121	<b><u>Y I G I Y G W M D N P Q I E Y Y V V D D</u></b>		
603	TGGATGCATAACAGAGGAGCTCCAGGTGGTTCCTACATGGGATCTCAAAGGGTACTATT		662
141	<b><u>W M H N R G A P G G S Y M G S Q K G T I</u></b>		
663	ACTGTTGATGGAGGTACATACAAGTCTGGTCTGGACAACGTACTGGTGCTTCCAAGTGG		722
161	<b><u>T V D G G T Y K V W S G Q R T G A S K W</u></b>		
723	GGTACTTCTACCTTCACTCAAATCTTCAGTATTCGTAAGTCCACGTCAATGTGGTTCT		782
181	<b><u>G T S T F T Q I F S I R T S P R Q C G S</u></b>		
783	ATCAATGTTTCTGAACATTTAGACAATGGCAAAAATTAGGACTTCGTTTAGGTGGTCTT		842
201	<b><u>I N V S E H F R Q W Q K L G L R L G G L</u></b>		
843	ATGGAAGCTCAACTTTTAGCTGAATCTGGTGGTGGTCTGGTTACGTTGATTTCACTTAT		902
221	<b><u>M E A Q L L A E S G G G S G Y V D F T Y</u></b>		
903	GCTACTATTACAATGGTGGATCTTCTAGTAATGCTAGTGCCCAAGCAACAACAACAAT		962
241	<b><u>A T I T I G G S S S N A S A P S N N N N</u></b>		
963	AACAACAACAATAACAACGACAACAATGGCAACTGGAACAAGTGAACAACAATAACAAC		1022
261	<b><u>N N N N N N D N N G N W N N W N N N N N</u></b>		
1023	AATAACAACAATAACAACAATAACAACAACAACAACAACAAGGTGGTGGTAAAT		1082
281	<b><u>N N N N N N N N N N N N N N N Q G G G N</u></b>		
1083	TGTGCTGCTATCTGGGGACAATGTGGAGGTAGTGATACAATGGACCAAAATGTTGTAAA		1142
301	<b><u>C A A I W G Q C G G S G Y N G P K C C K</u></b>		
1143	CAAGGTTTTGTAAACAATTAACCAATGGTATTTTCAATGTCAATAAGAAATATAAAA		1202
321	<b><u>Q G S C K Q I N Q W Y S Q C Q</u></b> *		
1203	TATAAATCGTTACTTTCAATATGAAAATTTTAAATTTTATTTAAAATAAAAAGTTGAAT		1262
1263	ATTTTAAATTTTFTTTTTCATTTATGTTAATAAAAAATTTAAAACAAAAACATGTAT		1322
1323	AAATAAAGACTTATATATAAATAGCGTTTAACTACAATAAATAAATAAATAAATAAAT		1382
1383	TTTACCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA		

oat spelt xylan was 1,982.8 U mg<sup>-1</sup> with *K<sub>m</sub>* of 4.9 mg ml<sup>-1</sup> and *V<sub>max</sub>* of 3,886 μmol min<sup>-1</sup> mg<sup>-1</sup>. No activity was observed against carboxy methyl cellulose, β-glucan, lichenan, laminarin, agarose, starch, and κ-carageenan.

Temperature and pH profiles

The xylanase activity assay at various pH values revealed that truncated XynS20 was stable in the mild acid pH range



**Fig. 2** Phylogenetic tree of GH family 11 catalytic domain sequences generated by the neighbor-joining method. At major nodes, bootstrap percentages for 1,000 resamplings are shown. The Neocallimastigaceae group contains xylanases from rumen fungal *Neocallimastix* and *Piromyces* (GenBank accession nos. CAA57820, Q12667, AAG18439, AAF14365, AAF14365, CAA57717, AAB23454, AAT99015, AAN07082, and AAY18781) and a sequence from *Fibrobacter succinogenes* (P35811). The xylanase discovered in this study (XynS20) branches with xylanases from insect gut (accession nos. AAS85782 and AAS85784) and *Lactococcus lactis* subsp. *cremoris*

SK11 (accession no. YP\_809518). The fungal xylanases from phyla Ascomycota form a group (GenBank accession nos. AAQ73530, BAB20794, AAZ03776, and AAB50278). The actinobacterial xylanases also form a group (GenBank accession nos. CAD48747, AAV64879, NP\_626540, and 1HIXA). The Firmicutes group contains organisms of the orders Bacillales and Clostridiales (GenBank accession nos. 1909152A, P33558, JQ1935, Q8GJ44, CAD48307, BAA33542, AAC04578, BAA33543, AAC04579, YP\_001039362, ZP\_01576648, 1H4H, 1QH6, CAA41784, P83513, AAA85198, P17137, 1007159A, and AAN32825).

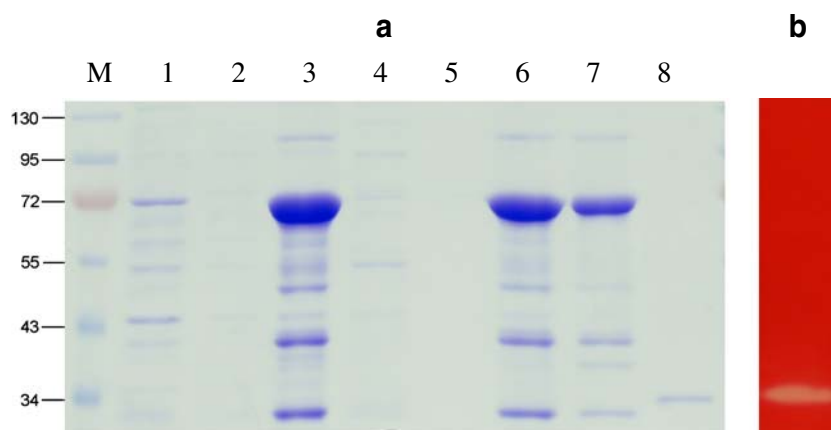
from 5.0 to 6.0, and the optimum pH was 6.0. When pH was below 4.0 or above 7.0, less than 60% of the optimal activity was retained and the enzyme showed no activity at pH 9.0 (Fig. 4a). The activities of XynS20 were also measured at different temperatures. The optimal reaction temperature of XynS20 was 45°C; at temperatures below 30 or above 55°C, enzyme activity was less than 50% of that at the optimal temperature (Fig. 4b).

## Discussion

Rumen fungi are able to degrade the most resistant plant cell-wall polymers; thus, the rumen fungal population represents a rich and underutilized source of novel enzymes with tremendous potential for industrial and agricultural applications. A thorough review of the relevant literature revealed that at least 30 different xylanase genes have been cloned from ruminal

fungi including genera of *Neocallimastix*, *Orpinomyces*, and *Piromyces*. According to the Carbohydrate-Active Enzymes (CAZY) database (<http://afmb.cnrs-mrs.fr/CAZY/>) (Coutinho and Henrissat 1999), enzymes with xylanase activity are found in families 5, 7, 8, 10, 11, and 43. Further, all of the rumen fungal xylanases are found only in families 10 and 11. Certain regions, which would appear to be conserved amongst the GH family 11 xylanases including YGW and EYY residues, were found in the N-terminus of XynS20. Therefore, XynS20 was classified as a member of this family.

Some xylanases contain distinct CBMs which have an important role in crystalline cellulose binding. Although there are domains that bind specifically to xylan (denoted xylan-binding module), many xylanases contain domains that bind specifically to cellulose rather than xylan (Raghothama et al. 2000). It has been suggested that the reason for this is that cellulose is a uniform, crystalline, and ubiquitous ligand, whereas xylan can be very variable

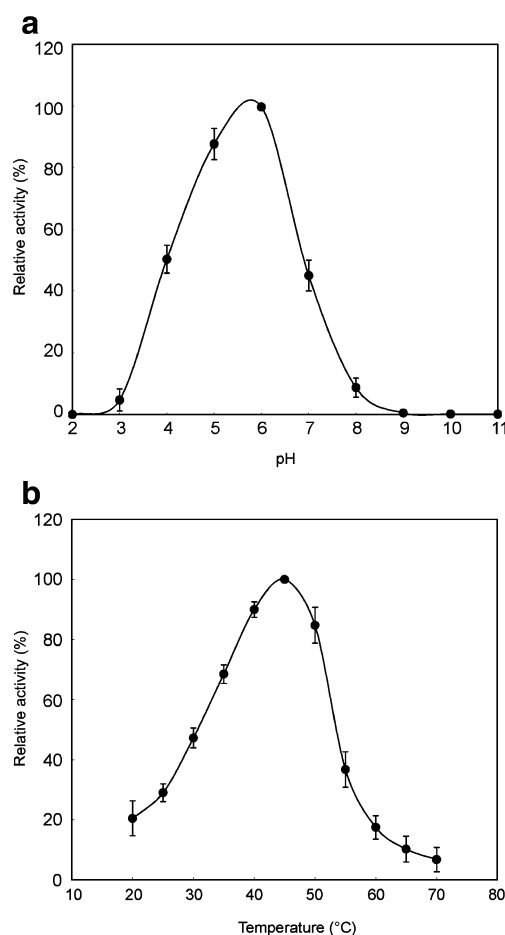


**Fig. 3** SDS-PAGE and zymographic analysis of XynS20 purified from AOBs. **a** SDS-PAGE of expressed and purified protein encoded by xylanase gene. Lane M, molecular weight marker; lane 1, pellet fractions of *E. coli* cells before IPTG induction; lane 2, supernatant fractions of *E. coli* cells before IPTG induction; lane 3, pellet fractions

of *E. coli* cells after IPTG induction; lane 4, supernatant fractions of *E. coli* cells after IPTG induction; lane 5, the remaining supernatant after AOBs recovered; lane 6, AOBs; lane 7, digested AOBs; lane 8, purified XynS20. **b** Zymogram of purified XynS20 showing xylanase activity

depending on the species or differentiation state of the plant. In the plant cell wall, xylan is always closely associated with cellulose and, therefore, domains that bind to cellulose will also locate the enzyme close to xylan (Hashimoto 2006). CBMs from different enzymes and different taxonomic origins have been classified into families with similar amino acid sequences and 3D structures (Lehtio et al. 2003). According to the sequence-based CBM classification, a putative conserved domain of family 1 CBM was observed at the C-terminus of XynS20. A thorough review of the relevant literature revealed that most of the CBMs from rumen fungal xylanases belong to family 10 and only the CBMs from *N. patriciarum* xynB (accession no. AAB30669) and xynC (accession no. AAF14365) belong to family 1, which have a relatively simple molecular architecture. It is possible that as the hyphae from fungi grow into the cell wall, the delivery of the secreted enzyme to the target substrate does not require an elaborate repertoire of CBMs (Lehtio et al. 2003).

Between the N-terminal family 11 catalytic domain and the C-terminal family 1 CBM of XynS20, there is a 60 amino acid region (position 241–300) containing 37 Asn. This characteristic feature has previously been identified as regions of linkers separating the different domains of the proteins for several fibrolytic enzymes (Denman et al. 1996; Zhang and Flint 1992). Unlike the usual Ser/Thr/Pro-rich linker found in most other glycosyl hydrolases, the XynS20 linker sequence is extremely Asn rich. To date, the only two other identified glycosyl hydrolases that contain an Asn-rich linker sequence are a xylanase from *Ruminococcus flavefaciens* and a cellulase from *N. patriciarum* (Denman et al. 1996; Zhang and Flint 1992). Previous studies have revealed that removal of the linker sequences from xylanase did not affect the activity of the enzyme against purified arabinoxylan (Ferreira et al. 1990; Srisodsuk et al. 1993). However, the linker



**Fig. 4** Influence of pH (**a**) and temperature (**b**) on the activity of recombinant XynS20. For the pH profile, the reaction pHs were adjusted to 3.0–11.0 with various buffers at 100 mM the enzymatic activity was measured with 10  $\mu$ l purified enzyme for 10 min at 40°C. For the temperature profile, enzymatic activity was measured with 10  $\mu$ l purified enzyme in 100 mM of phosphate buffer (pH 6.0) at different temperatures for 10 min

sequences enhance the activity of enzyme against natural complex substrates consisting of cellulose and hemicellulose (Poon et al. 2007; Shen et al. 1991).

The GH family 11 phylogenetic tree shows that XynS20 branches with xylanases from insect guts (accession nos. AAS85782 and AAS85784) and *Lactococcus lactis* subsp. *cremoris* SK11 (accession no. YP\_809518). The amino acid sequence alignment of catalytic domains showed that the sequence identity of XynS20 was 40.7% with that of *L. lactis* subsp. *cremoris* SK11 xylanase. This *Lactococcus* xylanase was not listed in the CAZY database (Coutinho and Henrissat 1999) of carbohydrate active enzymes or reported in the literature to the best of our knowledge. So the details regarding this enzyme were not available. The amino acid sequences of the catalytic domain of XynS20 exhibited 41.7% and 41.2% identities to those of XYL6805 and XYL6419, two xylanases discovered from a lepidopteran intestinal tract sample and from a termite sample, respectively (Brennan et al. 2004). It is generally suggested that obligately anaerobic microorganisms inhabiting the rumen, typically not found elsewhere in nature, are unique to the particular ecosystem (Selinger et al. 1996). XynS20, like the other rumen fungal xylanases, is phylogenetically different from aerobic bacterial and fungal xylanases but phylogenetically related to XYL6805 and XYL6419. Therefore, our results suggest that the xylanases produced by the microbial symbionts inhabiting the ruminant gastrointestinal tract are closely related to those produced by the microbial symbionts inhabiting the insect gut, although the gastrointestinal tract of ruminants is very different from the insect gut.

Compared with available ligand affinity purification systems available for recombinant proteins, the AOB expression/purification system used in this study displayed equivalent purification efficiency at a lower cost by replacing the expensive ligand-coupled columns with AOB (Peng et al. 2004). Many intrinsic soluble proteins expressed in *E. coli* are inclined to form insoluble aggregates confined in inclusion bodies. Nevertheless, the AOB system used in this study provides excellent machinery to functionally recover intrinsic soluble proteins that are expressed as insoluble aggregates in *E. coli*. Our results suggest that the folding of XynS20 was first tangled with the hydrophobic fusion oleosin, and thus unable to perform its specialized function. Constitution of AOB attracts the hydrophobic oleosin portions into the TAG matrix, thus leaving XynS20 alone for self-refolding right after the physical segregation of oleosin and XynS20. As XynS20 self-folds into its correct conformation on the surface of an AOB, its functional activity may be recovered concurrently. This could be demonstrated in that XynS20 embedding in AOBs showed enzyme activity in DNS reagent method but did not show activity band in zymographic analysis since AOBs were destroyed after SDS pretreatment.

In a previous study, *N. patriciarum* xylanase gene *xynC* was fused to an oleosin gene and introduced into *Brassica napus*. Oil bodies extracted from the transgenic canola seeds exhibited xylanase activity, indicating *xynC* was immobilized on the surface of oil bodies (Liu et al. 1997). In this study, we expressed XynS20 as an oleosin-fused protein in *E. coli* and then subjected to mixing with plant oil to form AOBs. The enzyme activity assay confirmed the presence of active xylanase on the surfaces of AOBs. Further studies are needed to evaluate the application of immobilized xylanase using AOBs in the conversion of xylan in the wastes of the paper-pulp industry and the bioconversion of lignocellulosic material and agro-wastes to fermentative products.

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