

# Characterization of a Novel Xylanase Gene from Rumen Content of Hu Sheep

Qian Wang<sup>1,2</sup>  $\cdot$  Yang Luo<sup>1</sup>  $\cdot$  Bo He<sup>1</sup>  $\cdot$  Lin-Shu Jiang<sup>3</sup>  $\cdot$ Jian-Xin Liu<sup>1</sup> • Jia-Kun Wang<sup>1</sup>

Received: 22 June 2015 /Accepted: 18 August 2015 / Published online: 10 September 2015 © Springer Science+Business Media New York 2015

Abstract A novel xylanase gene, - , was cloned from a metagenomic fosmid library, which was previously constructed from the rumen contents of Hu sheep and was functionally characterized in  $E c$  er  $c$  a  $c$ . The open reading frame was composed of 1923 bp and encoded for 640 amino acids, including a catalytic domain of glycosyl hydrolase family 10 and carbohydrate-binding module 9. The gene showed 97 % identity with uncultured bacterium Contig1552 but low similarity with xylanases from known cellulolytic-degrading microorganisms in the rumen. The recombinant XYN-LXY showed a specific activity of 664.7 U mg<sup>-1</sup>. The optimal temperature and pH of the enzyme were 50 °C and 6.0, respectively. Specifically, XYN-LXY was exclusively activated by  $Mn^{2+}$  among all of the cations and reducing agents tested in this study. An enzymatic hydrolysis assay revealed that XYN-LXY degraded birchwood xylan into xylooligosaccharide with a low degree of polymerization. After incubation for 4 h, the concentration of the dominant product, xylobiose, was  $2.297\pm$ 0.175 mg ml<sup>-1</sup> (74.07 % of total product) followed by xylose with a concentration of 0.656  $\pm 0.010$  mg ml<sup>-1</sup> (21.14 % of total product). The XYN-LXY exhibited deep degradation effects

purchased from Qiagen (Shanghai, China). The E c er c a c BL21 (DE3) competent cells, T4 DNA ligase, and pGEM-T Easy vector were purchased from Promega (Madison, Wisconsin, USA). The restriction enzymes were purchased from Takara (Dalian, China). The pET30a(+) plasmid was obtained from Novagen (Madison, Wisconsin, USA). The birchwood xylan and standard XOs were purchased from Sigma (Saint Louis, MO, USA) and Megazyme (Wicklow, Ireland), respectively. All other chemicals were purchased from Sangon (Shanghai, China).

## Screening and Sequence Analysis of Novel Xylanase Gene

The fosmid library constructed previously [[26](#page-11-0)] was employed to screen for novel xylanase genes. Lysogeny broth (LB) agar plates (0.5 % yeast extract, 1 % tryptone, 1 % NaCl, and 2 % agar) containing 1 % birchwood xylan were used for Congo red staining [\[28\]](#page-11-0). Then, xylanolytic positive clones were picked and subjected to further comparison of xylanase activity by estimating the diameter of the halo generated in the plate. The clone L3 showing the highest catalytic activity was sequenced by Sangon (Shanghai, China). The open reading frame (ORF) of the potential gene and its function were predicted using the online software Softberry ([http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=](http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb) [programs&subgroup=gfindb](http://linux1.softberry.com/berry.phtml?topic=fgenesb&group=programs&subgroup=gfindb)) and PFAM (<http://pfam.xfam.org/search>), respectively. Sequence alignment was performed using a BLAST program ([http://blast.ncbi.nlm.nih.gov/](http://blast.ncbi.nlm.nih.gov/Blast.cgi) [Blast.cgi\)](http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## Cloning of a Novel Xylanase Gene xyn-lxy

A novel xylanase gene - was obtained according to the sequencing result of the L3 clone. To clone  $\qquad$ , approximately 50 ng plasmid DNA was used as the template for amplification of the  $\qquad$ - gene by using a pair of primers Xyn-lxy-F: 5' CCGGAATTCATGAAGAAGAAACTGACGAG3'(Ec RI) and Xyn-lxy-R: 5'  $CCGCTCGAGTTATACCAGCTTGGCGTTACCAA3'$ ( $X$  I). PCR reaction was performed for 35 cycles consisting of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min. The band of  $\sim$ 2 kb was purified, ligated with pGEM-T, transformed into DH5 $\alpha$  competent cells, and streaked onto a 100  $\mu$ g ml<sup>-1</sup> ampicillin LB agar plate supplemented with 100 mM isopropyl-thio-β-D-galactopyranoside (IPTG) and 20 mg  $ml<sup>1</sup>$  5-bromo-4-chloro-indolyl-D-galactoside (X-gal). Then, the positive clones were picked and sequenced by Sangon (Shanghai, China). The resulting plasmid was designated as  $p$ GEM-T/ $\alpha$ 

The pGEM-T/ $\blacksquare$ - plasmid was digested with Ec RI and X I and subjected to ligation with pET30a(+) (the vector map could have been acquired from [http://www.synthesisgene.](http://www.synthesisgene.com/vector/pET-30a.pdf) [com/vector/pET-30a.pdf](http://www.synthesisgene.com/vector/pET-30a.pdf)), which was previously digested with the same enzymes. Then, the ligation product was transformed into  $E. c$  BL21 (DE3) and streaked onto an LB agar plate containing 100 μg ml<sup>-1</sup> kanamycin. Plasmids of the transformants were isolated and used for screening by PCR (Supplemental Fig. S1). The resulting strain was designated as  $BL21/pET30a(+)$ 

## Expression and Purification of XYN-LXY

The BL21/pET30a(+)/ - was inoculated into 5-ml LB medium containing 100  $\mu$ g ml<sup>-1</sup> kanamycin and incubated at 37 °C overnight. On the next day, the culture was inoculated into a 250-ml flask containing 100-ml LB medium, followed by shaking at 200 rpm at 37 °C until  $OD_{600} = 0.5^{-1}$ . After the addition of IPTG to a final concentration of 1 mM, the flask was further incubated at 150 rpm at 25 °C for 8 h. The culture was chilled on ice for 30 min and then centrifuged at 12,000 rpm at 4 °C for 15 min. The cell pellets were collected and resuspended in 30 ml PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH2PO

To investigate the effects of metal ions, EDTA, and SDS on the catalytic activity of XYN-LXY, the purified enzyme was incubated with various chemicals at 50  $\degree$ C for 10 min. Then, the xylanase activity was assayed under the optimum conditions (pH 6.0, 50  $^{\circ}$ C).

The kinetic constants of XYN-LXY were determined under standard conditions (pH 6.0, 50 °C) for 10 min. The concentration of the birchwood xylan substrate ranged from 0.4 to 15 mg/ml. The data were fitted with linear regression for a Lineweaver-Burk plot using Microsoft Excel 2010.

## Analysis of the Hydrolysis Products of Birchwood Xylan

To evaluate the hydrolysis profile of XYN-LXY, approximately 80 U pure enzyme was incubated with 1 % birchwood xylan at 50 °C. The portions were collected at different time intervals (5 min, 30 min, 1 h, 2 h, 4 h, and 24 h) and immediately boiled for 10 min. Then, all samples were subjected to a Waters Alliance HPLC system (separations module e2695, Waters, Milford, MA, USA) equipped with a Sugar-Pak TM 1 column (300 mm $\times$ 6.5 mm) and refractive index detector (Waters 2414). Distilled water was applied as the mobile phase with a flow rate of 0.5 ml min<sup>-1</sup>. Finally, the released sugars from the birchwood xylan were quantified by comparing the relative areas to standard xylose, xylobiose (X2), xylotriose (X3), xylotetroase (X4), and xylopentose (X5).

## Results and Discussion

## Identification of a Novel Xylanolytic Gene from a Fosmid Library

In our previous study, 18 clones out of 12,704 that produced halo zones on LB plates supplemented with 1 % xylan were obtained from a Hu sheep rumen fluid fosmid library [[26](#page-11-0)]. The clone L3, showing the highest xylanolyic activity, was sequenced by shotgun pyrosequencing, and 44 potential ORFs were functionally predicted by the online softwares Softberry and PFAM. Among them, a xylanase-encoding ORF of 1923 bp, designated as

, was cloned. The gene coded for 640 amino acids (AA), with a GH 10 catalytic domain locating at its N-terminus (50–443 AA) and a carbohydrate-binding module (CBM) 9 locating at its C-terminus (456–640 AA) [\(http://pfam.xfam.org/search\)](http://pfam.xfam.org/search) and a linker sequence between them. The online software prediction indicated that the theoretical molecular weight and isoelectric point of XYN-LXY were 71.3 kDa and 4.95 ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)), respectively, with a signal peptide of 22 AA locating at the N-terminus [\(http://www.cbs.dtu.dk/](http://www.cbs.dtu.dk/services/SignalP/) [services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)).

The data derived from BLAST revealed that XYN-LXY showed low similarity to xylanases from known cellulolytic-degrading microorganisms in the rumen, such as *Lachnos* raceae bacterium (WP 035658042, 61 %), E bac er r a (BAA09971, 59 %), R eb r a a (WP\_022112477, 55 %), and B\_r br recac (WP\_013279709, 52 %). However, it shared 97 % identity with the uncultured bacterium Contig1552 (AHF25111), which was recently uncovered in a metagenomic study of the rumen digesta of Jersey cows [[32\]](#page-11-0). It was suggested that XYN-LXY was a novel xylanase gene of interest for use as a feed additive due to the high digestibility of the rumen toward cellulosic stuffs. The close relationship between XYN-LXY and xylanases from other rumen microbes was also confirmed via phylogenetic tree analysis (Fig. [1\)](#page-5-0) and AA alignment (Fig. [2](#page-5-0)).

<span id="page-5-0"></span>

Fig. 1 Phylogenetic dendrogram of XYN-LXY and xylanases from related microorganisms

#### Expression and Purification of Recombinant XYN-LXY

The E. c transformant carrying xylanase gene  $\sim$  was induced with 1 mM IPTG for 8 h, and the cell pellets were collected. After centrifugation and sonication, the soluble fractions were subjected to  $6\times$  His-tag purification. Both the crude supernatant and the purified protein were then analyzed by SDS-PAGE, and a distinguishable band with an estimated molecular mass of 80 kDa was observed (Fig. [3\)](#page-6-0), which was in agreement with the calculated molecular mass. After purification, the purified XYN-LXY showed a specific activity of 664.7 U mg<sup>-[1](#page-6-0)</sup> (Table 1). The  $K<sub>m</sub>$  and  $V<sub>max</sub>$  of the recombinant XYN-LXY were 4.39 mg ml<sup>-1</sup> and 3.20 µmol min<sup>-1</sup>, respectively (Supplemental Fig. S2). The turnover number ( $_{cat}$ ) of the enzyme was 125.9 s<sup>-1</sup>, suggesting that it was a promising candidate for hemicellulose hydrolysis.

uncultured bacterium Contig1552	<b>MAFGGTMKNKEKK-</b>
XYN-LXY	<b>NKMITR-</b>
Eubacterium ruminantium Lachnospiraceae bacterium AC2031	MAAAMML SATACGKKKDE ATSNDTAAVTTE AATSTVAE VEI DEDNIL VNPYFL DDDVTAWQ - - - - - - AGQGSSKVGI ADESVPL PDGYRRCAVIDRI NILKNGYFSSDDVSAWSIEGGSLGIEVISDGNG-PDGFPTYGKINRDPESSSPYDCFAQDV------TDSEEGGVTYDYEFYAMLSDEYEGAPADQRVVD 93
uncultured bacterium Contig1552	<b>FISOSCICKAGT-</b> /FIAAGILIGLIAMI L PROTSASNAPDAMOTOLPDL 54 FAAGLICLTMI- - FACCGCKAGT- PGQTGASNAPDAVCTDI PDL
XYN-LXY Eubacterium ruminantium	SSPYDCFAQDITSSISKGTEYQFEFYAMLSSDYEGAPAEQRTVEDERYITANGSTTYLGSYSAEITEVSSQPLEPGKWTRFAGT-FTPQWSGNLDQAVIR 193
Lachnospiraceae bacterium AC2031	FAPYITVDGNTTYLGSYSAEITGNPSETLTPGEWKKFSGTFTPS <mark>E</mark> SGKAEKMEVIRIIEQGTDYGNGT <mark>O</mark> VK <mark>S</mark> DYYIAGVKLVP <mark>O</mark> DSGAATAIG <mark>K</mark> EEBUVDLI93
uncultured bacterium Contig1552	<u>vaskogu sedan vgjengoden soakuko i vikinfiaavtueden koetinfansnaapasden hkeenneegnio votus varadkandangs vaskid</u> 15
XYN-LXY	ckt vaskdgese dan vet cygdde) sdakl molvtkhf navt le nel kpet mf gnsnaapasdsi hkeen gebon o vptnsy aradkmnddi ve nnsknp
, Eubacterium ruminantium Lachnospiraceae bacterium AC2031	VI E QGTDYENEDCVKGDYYI AGFKLLGE ALEPVAYSVEKDI PSLASVVSSE DGLGADSI CGTAVTGS II DDEFI QELI SKHFNAVTLGNELKMD-AVFGY RSVØØSSE SUSODØDI SCØLSSVGVNBSNUKKUSKKØRSØGDUNDNAGDSI HEYNDOØDØDGSUNBE HANDENT DE ØRDUDF SØRE AT DEE DEARDE ENR 29
uncultured bacterium Contig1552 XYN-LXY	DHKI RVRGHVLWNISGTPEWFFHEGYDKSKDYVSKEEMWKRLEWYI KTWLTYYTGDQSKYKDLFYGFDVWNELI SDASATYRTDSEQGGDNLTDDTHSSK dhki rvrghvi. Maisci peverie sydkskdyvsko ennkri envi ki M. I Yvigdoskykdi. Fygfdmwel4 I Sdasatyri dieosodi tiddihissk
"Eubacterium ruminantium	SNDTVPAIEDEEADINGEKIKVPVMDHSRAKKILDKILEINEKNGANIKVRGHVLVWHSQAPEWFFHEDYDKTKPYVDKDTMNLRLEWYIRELLNYYTGP 39
sucation ospeaning bactastics in mazzulus	<u>ㅋ~ 님께서 잘못한다. 전쟁 가지 이 가지 않아 가지 않아 있다. 이 가지 않아 그 사람들은 그 사람들은 그 사람들은 그 사람들을 하고 있다. 그 사람들은 그 사람들은 그 사람들은 그 사람들은 그 사람들을 지 않아 있다.</u>
354 incultured bacterium Contig1552	ssmikmiesije i inglikega koglasije (kvidnije spakkessi i tii ndvaaaeste infinsesmiks (vests tego barenar vde vdetoi
348 XYN-LXY	SSMWKMYGSMEYI INAFRFANKYAPASLELMWDMVECDAKKRGGI I QLI NDMKAAEGTRI ITGFGMOGHYGVNSPSVT OFEEAAKEMAKHVEKVMLITEL
$A = \frac{1}{2}$ $A = \frac{1}{2}$ $A = 3$ $B = 493$ ; $A = 1$ achnospiraceae bacterium AC2031	DRKYRDLFYGWDVVILEAI SDGTGTYRTDELPGADOLSDSTHGSKSSWWKVYGSNEFIINAFKYANRYAPATLKLYYNDYNECDELKELSLIELINAVKF SSNNXMXSRIF + 100403+40+640: SUEUXXVÖXVESS: 504: SUKØU+1 DVX: 452000 +50345000 + 34405700 + 5+ 404 × 574 + 70 % + 60
同 <sub>급 452±</sub> uncultured bacterium Contig1552。  - 日 - 446 © <sub>U</sub> bacterium - 1111 - 11  - 日 - 446 © <sub>U</sub> bacterium - 1111 - 11	LERES RELESSING SILLE SERGELIS ISLASING SALIK SULTAS UNIK DEN SUSULGSALUG SASSANG PILE DEN MALINUAL DER SULTA - ERFARFFRASEFRAFREL FFFE GOYFARE YET SKNLIKKS- SVIIFIGS TILMSVI DSNSMLGSAMMYGASDGKMYVFPLLFDEKYQAKLTMVAFVDPSKI
$\overline{\{\vee\}}$ 591 Eubacterium ruminantium	DGTRIDGFEMQGHYSVNAPTVDRIKEAIQDYSQVVDEV-MITEFDVKAGLGYDGTDETKDKEYTKQMHYFRKIYEAAKALNAEGVHFSGITMWGVVD
いる 591 / D風 4932-achnospiraceae bactedium.AC2031	
551 uncultured bacterium Contig1552	evdal - kretne to csvni dse i deannkasavkle i mas vajtodski tada: kim - kok okol ndkssdenzgdsi eve i dedhkreean
$545$ <sub>C</sub> XYN-LXY	pvdal - krptnetitoosvni doei delannkvoavklei mvosvavtodaklunoke tuyvpmdxkokdlndkssdenoodsi evfi dedhkkpeay WLGSG-NNVGGSSDGTSLGCPLLFDDNYQAKPMYWAFVD-PSKLGEEPVVVRPEINIKKGSATMDGDIEEAWDAABAVSLSIKLGSDISADGKLLW
·■리즈 - 가이 EEN 689: Ubacterium ruminantium PDDG風K 693: "seconiospasc <i>hae" os</i> con AGPA21.	
R4R; uncultured bacterium Contig1552 640 XYN-LXY	Konfonden eile Debeck abdik eras i Feede varop debe er die belevische Escolarus (1804-2005 i Struktunden 1914 Bon Santan eile Debeck abdik eras i Feede varop debe er die der verden er begende i Structung van Bonien Akt
788 Eubacterium ruminantium <b><i>SKAGVE</i></b>	LYVLADVADSVLNEDSEDDYQQDSVEIFVDENNGKSGGYEADDKQYRIS-FSNKQSFNGEKCVAENITSATKKTDDGYVVEAAIKWTDITPEVG
<sup>27</sup> 78; Lachnosoiraceae bacterium (AC2031 Ve	านทหงระทั่งพระหาระพระออกจาพรพิษุสตภ <b>์โล</b> ยาก <b>ก็อู่อิน</b> ทหงระทั่วคะสงสกะกรอย อนทัญสรรี <mark>สุ</mark> รอหาราทรงสอ <mark>ช</mark> กรกว่าทหาสหลางหลยเ
646 ¿uncultured bacterium Contig1552	
640 (XYN-LXY	
Eubacterium ruminantium Lachnospiraceae bacterium AC2031	LOVNDATAE GVRCGTISWADDT GT GYMSPE VF GT VVF E E

Fig. 2 Amino acid alignment of XYN-LXY and three closely related xylanases. The alignment includes xylanases from uncultured bacterium Contig1552 (AHF25111), Lac raceae bacterium (WP\_027439728), and E bac er  $\int$  a (BAA09971). Consensus amino acids were shaded in b ac. The figure was generated using DNASTAR Lasergene 10



Fig. 4 Effects of temperature and pH on catalytic activity of XYN-LXY. a The optimal temperature of XYN-LXY. b The optimal pH of XYN-LXY. c Thermostability of XYN-LXY from 30 to 60 °C. d The pH stability of XYN-LXY. Assays were performed as described in the "[Materials and Methods](#page-1-0)" section using 1 % birchwood xylan as the substrate. At the optimal pH and temperature, the highest xylanase activity was taken as 100 %. The xylanase activity under optimal conditions (50 °C, pH 6.0) was taken as 100 % in the assay to determine pH stability. For thermostability, the xylanase activity of the enzyme without a heat challenge was taken as 100 %. All of the assays were carried out in triplicate

XYN-LXY retained more than 90 % of its initial activity in the range from pH 6.0 to 8.0. However, the hydrolytic activity of the XYN-LXY dramatically declined when assays were conducted at pHs <6.0 or pHs >8.0 (Fig. 4d). A cold-active xylanase from XynGR40, also derived from rumen content, had an optimum pH at 6.5 and exhibited high activity from pH 5.5 to 7.5 [\[34](#page-11-0)]. Both XYN-LXY and XynGR40 showed high catalytic activity and good stability at neutral pHs, which was probably an adaptation to the normal environmental condition (pH 5.5–7.5) of the rumen for feed digestion.

## Effects of Cations and Reducing Agents on XYN-LXY

The stimulatory or inhibitory effects of XYN-LXY by 10 mM of various metal cations, 10 mM EDTA, and  $1\%$  SDS ( $\prime$ ) were extensively investigated in this study. As seen in Table [2](#page-8-0), EDTA, SDS, and most metal cations  $(Cu^{2+}, Fe^{3+}, Zn^{2+}, K^+,$  and  $Ag^+)$  tested in this study were found to dramatically inhibit XYN-LXY (P<0.05). However, the inhibitory effects of  $\text{Co}^{2+}$ ,  $Ca^{2+}$ , Na<sup>+</sup>, and Ni<sup>2+</sup> were only marginal. Interestingly, Mn<sup>2+</sup> was the only activator of the cations tested that significantly enhanced the hydrolytic activity of XYN-LXY ( $P$ <0.05). The promotive effect of  $Mn^{2+}$  was observed by two other studies [\[33,](#page-11-0) [35](#page-11-0)], although most studies

Reagents (10 mM)	Relative activity (%)	
Control	100	
$Co^{2+}$	$91.0 \pm 4.3$	
$\mathrm{Ca}^{2+}$	$90.2 \pm 3.5$	
$\mathrm{Cu}^{2+}$	$80.5 \pm 4.7^a$	
$\mathrm{Na}^+$	$87.6 \pm 14.6$	
$\mathrm{Fe}^{3+}$	$85.7 \pm 8.9^a$	
$\mathrm{Ni}^{2+}$	$89.1 \pm 9.6$	
$Zn^{2+}$	$84.1 \pm 11.0^a$	
$Mn^{2+}$	$117.2 \pm 11.5^a$	
$\rm Mg^{2+}$	$79.9 \pm 7.8^a$	
$\mbox{K}^+$	$84.9 \pm 9.4^a$	
$Ag+$	$16.0 \pm 1.8^a$	
<b>EDTA</b>	$72.1 \pm 8.5^a$	
$SDS^b$	$\overline{0}$	

<span id="page-8-0"></span>Table 2 Effects of various cations on activity of XYN-LXY

<sup>a</sup> Xylanase activity was significantly increased or decreased compared to control  $(P<0.05)$ 

 $<sup>b</sup>$  The concentration of SDS was 1 % ( / )</sup>

revealed that  $Mn^{2+}$  showed inhibitory activity on xylanase activity [\[36](#page-11-0)–[39](#page-12-0)]. Usually, cations such as  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ , and  $Zn^{2+}$  were believed to function as activators of xylanase activity [[23,](#page-11-0) [35,](#page-11-0) [37\]](#page-11-0). However, it was also reported that  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ , and  $Fe^{3+}$  inhibited enzymatic activities, especially at high concentrations (5 to 20 mM) [\[39](#page-12-0)–[42\]](#page-12-0). Taken together, the effects of cations on the catalytic activity of xylanase seem to be tricky, and further studies regarding the dosage-dependent effect and conformational change of enzymes need to be carried out to gain insight into the relationship of cations and xylanolytic activity.

## Analysis of Hydrolysis Products of Birchwood Xylan

In the current study, the hydrolysis products of birchwood xylan by endo-xylanase were analyzed by HPLC (Fig. 5). Approximately 80 U purified XYN-LXY was employed to degrade the xylan substrate. After incubation at 50 °C for 5 min, the XOs with low DP

Fig. 5 Hydrolysis profile of birchwood xylan degradation by XYN-LXY. One percent birchwood xylan substrate was incubated with approximately 80 U XYN-LXY at 50 °C. Aliquots obtained at different time intervals (5 min, 30 min, 1 h, 2 h, 4 h, 24 h, 48 h, 72 h, and 96 h) were boiled for 10 min and subjected to HPLC analysis



(xylose, X2, and X3) accumulated rapidly. The concentrations of X2 and X3 were  $0.897\pm$ 0.079 mg ml<sup>-1</sup> (49.67 % of total product, the same below) and 0.764±0.024 mg ml<sup>-1</sup> (42.35 %), respectively. However, xylose showed only a slight increase, with a concentration of 0.144 $\pm$ 0.018 mg ml<sup>-1</sup> (7.98 %). After hydrolysis for 5 min, the concentrations of xylose and X2 ascended as the reaction continued and reached a stationary phase at 4 h, with concentrations of 0.656±0.010 mg ml<sup>-1</sup> (21.14 %) and 2.297±0.175 mg ml<sup>-1</sup> (74.07 %), respectively. However, X3 started to reduce to a trace amount after hydrolysis for 4 h.

It is known that two key xylanases, endo-xylanase and xylosidase, are required for the hydrolytic degradation of the main chain of xylan. Endo-xylanase is believed to catalyze xylan into XOs, and partial products can be further converted into a monosaccharide xylose by xylosidase. Consequently, XOs, mainly X2 or/and X3, and a small amount of xylose were obtained after enzymatic hydrolysis of birchwood xylan by either GH 10 [[20,](#page-11-0) [22,](#page-11-0) [24](#page-11-0)] or GH 11 [[17](#page-10-0)–[19,](#page-11-0) [21,](#page-11-0) [23\]](#page-11-0) endo-xylanase. As for XOs with higher DP, such as X4 and X5, results varied among these studies. After adequate digestion (at least 12 h), birchwood xylan was able to release X4 only [\[24](#page-11-0)] or X5 only [\[22](#page-11-0)] or both X4 and X5 [\[18](#page-10-0)] directly or indirectly (via the transglycosylation reaction). Noticeably, negligible xylose was detected in all these studies. However, Ali et al. [\[43\]](#page-12-0) reported that a Xyn10A from C r d ace b c ATCC 824 exclusively released xylose from xylan or XOs, except X2, while X2 and X3 were found to be the main products when the substrates were catalyzed by xylanases from the same strain [\[44](#page-12-0), [45](#page-12-0)]. Taken together, the various hydrolysis patterns above suggested that endo-xylanase probably functioned randomly on the main chain of xylan.

In the current study, a novel xylanase, XYN-LXY, derived from Hu sheep rumen content was employed to catalyze birchwood xylan substrate. Interestingly, X2 (74.07 %) was the prenominal product, followed by xylose (21.14 %), after hydrolysis for 4 h (Fig. 5). To our knowledge, comparable results were only found in two studies from the same group. The  $S_f$  *ce* sp. S9 xylanase [\[46\]](#page-12-0), isolated from the hottest place in China, Flaming Mountain in the Turpan basin of Xinjiang, with an air temperature of 47 °C during the daytime, and a cold-active xylanase, XynGR40 [\[34](#page-11-0)], derived from goat rumen content, were both reported to be capable of releasing high amounts of X2 and xylose from xylan in the absence of xylosidase. We speculated that the reason for the two enzymes and XYN-LXY in this study to exhibit an abnormal hydrolysis pattern was the extreme environmental conditions from which they were derived.

## **Conclusions**

In this study, a novel xylanase gene, - , was cloned from an uncultured strain in the rumen fluid. The enzyme was optimally active at 50  $^{\circ}$ C and pH 6.0. The thermostability assay revealed that XYN-LXY was a mesophilic xylanase. Interestingly, it was demonstrated that birchwood xylan could be completely degraded into monosaccharide by XYN-LXY, indicating that the enzyme might be a potential candidate for application in the feed and biofuel industries.

Acknowledgments The authors acknowledge the financial support from the Innovation Team Program of Zhejiang province (2011R50025) and the Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions (CIT&CD20130324).

#### <span id="page-10-0"></span>Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

Authors' Contribution Qian Wang drafted the manuscript. Yang Luo and Bo He carried out the studies and contributed to the drafting of the manuscript. Jia-Kun Wang, Jian-Xin Liu, and Lin-Shu Jiang participated in the project design and manuscript preparation. All authors read and approved the final manuscript.

### References

- 1. Chiniquy, D., Sharma, V., Schultink, A., Baidoo, E. E., Rautengarten, C., Cheng, K., Carroll, A., Ulvskov, P., Harholt, J., Keasling, J. D., Pauly, M., & Ronald, P. C. (2012). XAX1 from glycosyltransferase family 61 mediates xylosyltransfer to rice xylan. Pr ceed  $e$  Na a Acade Sc e ce ,  $109(42)$ , 17117–17122.
- 2. Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *B*  $re$   $rce$   $Tec$   $\qquad$   $, 89(1), 17-34$ .
- 3. Collins, T., Gerday, C., & Feller, G. (2005). Xylanase, xylanase families and extremophilic xylanases. FEMS  $M \, \sigma \, b$  Reviews, 29(1), 3–23.
- 4. Kulkarni, N., Shendye, A., & Rao, M. (1999). Molecular and biotechnological aspects of xylanases. FEMS M cr b Re e , 23(4), 411–456.  $Re\ e\ ,\ 23(4),\ 411-456.$
- 5. Watanabe, S., Kodaki, T., & Makino, K. (2005). Complete reversal of coenzyme specificity of xylitol dehydrogenase and increase of thermostability by the introduction of structural zinc.  $J \, r \, a \, B \, ca \, C \, e \, r \, , \, 280(11), \, 10340-10349.$
- 6. Bocchini, D. A., Gomes, E., & Silva, R. D. (2008). Xylanase production by Bac crc a Dl using maltose as carbon source. A ed B c e r and B ec ,  $146(1-3)$ , 29–37.
- 7. Miyazaki, K., Takenouchi, M., Kondo, H., Noro, N., Suzuku, M., & Tsuda, S. (2006). Thermal stabilization of *Bac b* family-11 xylanase by directed evolution. *J* r a *B ca C e* r . 281(15) of Bac b family-11 xylanase by directed evolution. J r a B ca C e r,  $281(15)$ , 10236–10242.
- 8. Stephens, D. E., Singh, S., & Rumbold, K. (2009). Error-prone PCR of a fungal xylanase for improvement of its alkaline and thermal stability. FEMS  $M \, \alpha \, b$  Letters, 293(1), 42–47.
- 9. Wang, Q., Zhao, L. L., Sun, J. Y., Liu, J. X., & Weng, X. Y. (2012). Enhancing catalytic activity of a hybrid xylanase through single substitution of Leu to Pro near the active site. W r d J r a M cr b a d  $B$  ec , 28, 929–935.
- 10. Silva, J. P. A., Mussatto, S. I., Roberto, I. C., & Teixeira, J. A. (2011). Ethanol production from xylose by  $P c a$  NRRLY-7124 in a stirred tank bioreactor.  $Bra a J ra C e ca E eer$ , 28(3), Pc a NRRLY-7124 in a stirred tank bioreactor. Bra  $a \, J \, r \, a \, C \, e \, ca \, E$ 151–156.
- 11. Cardona, C. A., Quintero, J. A., & Paz, I. C. (2010). Production of bioethanol from sugarcane bagasse: status and perspectives. B  $re$   $rce$  Tec  $\frac{101(13)}{300(13)}$ , 4754–4766.
- 12. Matsushika, A., Watanabe, S., Kodaki, T., Makino, K., & Sawayama, S. (2008). Bioethanol production from xylose by recombinant Sacc ar ce cere ae expressing xylose reductase, NADP<sup>+</sup>-dependent xylitol dehydrogenase, and xylulokinase. J r a  $B$  ce ce a  $\overline{d}$  B e eer, 105(3), 296–299.
- 13. Zhang, W., & Geng, A. L. (2012). Improved ethanol production by a xylose fermenting recombinant yeast strain constructed through a modified genome shuffling method.
- <span id="page-11-0"></span>19. Zhang, M., Jiang, Z., Yang, S., Hua, C., & Li, L. (2010). Cloning and expression of a Paec ce thermophila xylanase gene in  $E. c$  and characterization of the recombinant xylanase. B  $re$  ree  $Tec$ ,  $101(2)$ , 688–695.
- 20. Zhang, J., Siika-aho, M., Puranen, T., Tang, M., Tenkanen, M., & Viikari, L. (2011). Thermostable recombinant xylanases from N raea e a and T er a c a ra ac show distinct properties raea e a and T er a c a ra ac show distinct properties in the hydrolysis of xylans and pretreated wheat straw. B  $ec$   $rB$   $e$ , 4(1), 12.
- 21. Chen, C. C., Luo, H., Han, X., Lv, P., Ko, T. P., Peng, W., Huang, C. H., Wang, K., Gao, J., Zheng, Y. Y., Yang, Y. Y., Zhang, J. Y., Yao, B., & Guo, R. T. (2014). Structural perspectives of an engineered β-1, 4 xylanase with enhanced thermostability.  $J \rightharpoonup a$   $B \neq ec$  , 189, 175–182.
- 22. Fan, G., Yang, S., Yan, Q., Guo, Y., Li, Y., & Jiang, Z. (2014). Characterization of a highly thermostable glycoside hydrolase family 10 xylanase from Ma bra c eac a ea. I er a a J r a B ca Macr ec e 70, 482–489.
- 23. Gao, H., Yan, P., Zhang, B., & Shan, A. (2014). Expression of A er  $er$  IA-001 Endo-β-1, 4xylanase in P c a a r and analysis of the enzymic characterization. A ed B c e r a d B ec .  $173(8)$ , 2028–2041.  $B, 173(8), 2028-2041.$
- 24. Kim do, Y., Shin, D. H., Jung, S., Lee, J. S., Cho, H. Y., Bae, K. S., Sung, C. K., Rhee, Y. H., Son, K. H., & Park, H. Y. (2014). Biocatalytic properties and substrate-binding ability of a modular GH10 β-1,4-xylanase from an insect-symbiotic bacterium,  $S \neq c \neq c \neq HY-14$ . J  $r \neq M \neq b$ , 52(10), 863–  $ce$  e ca HY-14. J r a M cr b  $.52(10)$ , 863– 870.
- 25. Morrison, M., Adams, S. E., Nelson, K. E., & Attwood, G. T. (2005). Metagenomic analysis of the microbiomes in ruminants and other herbivores. In P. S. Makkar H & C. S. McSweeney (Eds.), Me d  $\alpha$  b a ec r r a (pp. 209–220). Netherlands: Springer.  $i \tau$  a (pp. 209–220). Netherlands: Springer.
- 26. Wang, J. K., An, P. P., Chen, Z. M., Ye, J. A., & Liu, J. X. (2010). Construction and analysis of fosmid library of rumen microbiota of Hu sheep. C e e J r a A a N r  $, 22, 341-345$ .
- 27. Wang, J. K., Sun, Z. Y., Zhou, Y., Wang, Q., Ye, J. A., Chen, Z. M., & Liu, J. X. (2012). Screening of a xylanase clone from a fosmid library of rumen microbiota in Hu sheep. Ann and Biotechnology, 156– 173.
- 28. Teather, R. M., & Wood, P. J. (1982). Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. A ed a d E r e a M cr b 43(4), 777–780.
- 29. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Na re, 227(5259), 680–685.
- 30. Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity.  $J \r a \r B \ncc \r 33(3), 257-270$ .
- 31. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. A a ca B c e r, 72(1), 248–254.
- 32. Wang, L., Hatem, A., Catalyurek, U. V., Morrison, M., & Yu, Z. (2013). Metagenomic insights into the carbohydrate-active enzymes carried by the microorganisms adhering to solid digesta in the rumen of cows. PL S ONE, 8(11), e78507.
- 33. Han, H., You, S., Zhu, B., Fu, X., Sun, B., Qiu, J., Yu, C., Chen, L., Peng, R., & Yao, Q. (2014). Characterization and high expression of recombinant  $U$  a a d xylanase in P c a a r. B ec Le er,  $37(3)$ ,  $697-703$ .
- 34. Wang, G., Luo, H., Wang, Y., Huang, H., Shi, P., Yang, P., Meng, K., Bai, Y., & Yao, B. (2011). A novel cold-active xylanase gene from the environmental DNA of goat rumen contents: direct cloning, expression and enzyme characterization. B  $re$   $rce$  Tec  $\qquad$ , 102(3), 3330–3336.
- 35. Knob, A., & Carmona, E. C. (2010). Purification and characterization of two extracellular xylanases from Pe c cerr : a novel acidophilic xylanase. A ed B ce r a d B ec , 162(2), 429–443.
- 36. Wang, W., Wang, Z., Cheng, B., Zhang, J., Li, C., Liu, X., & Yang, C. (2014). High secretory production of an alkaliphilic actinomycete xylanase and functional roles of some important residues. W  $r dJ - r a$  $Mcr$  b a d B ec , 30(7), 2053–2062.
- 37. Guo, B., Chen, X. L., Sun, C. Y., Zhou, B. C., & Zhang, Y. Z. (2009). Gene cloning, expression and characterization of a new cold-active and salt-tolerant endo-β-1, 4-xylanase from marine G ac ec a e a KMM 241. A ed M cr b a d B ec , 84(6), 1107–1115.
- 38. Walia, A., Mehta, P., Chauhan, A., Kulshrestha, S., & Shirkot, C. K. (2014). Purification and characterization of cellulase-free low molecular weight endo $\beta$ -1, 4 xylanase from an alkalophilic Ce cr b ce a CKMX1 isolated from mushroom compost. W r d J r a M or b a d B ec 30(10), 2597–2608.
- <span id="page-12-0"></span>39. Zhang, W., Lou, K., & Li, G. (2010). Expression and characterization of the Dictyoglomus thermophilum Rt46B. 1 xylanase gene  $(B)$  in Bac 1495.
- 40. Zhao, L., Meng, K., Bai, Y., Shi, P., Huang, H., Luo, H., Wang, Y., Yang, P., Song, W., & Yao, B. (2013). Two family 11 xylanases from  $Ac$  ae sp. Xz-8 with high catalytic efficiency and application potentials in the brewing industry. J r a A r c r a a  $dF/dC e$  r, 61(28), 6880–6889.
- 41. Guo, B., Li, P. Y., Yue, Y. S., Zhao, H. L., Dong, S., Song, X. Y., Sun, C. Y., Zhang, W. X., Chen, X. L., Zhang, X. Y., Zhou, B. C., & Zhang, Y. Z. (2013). Gene cloning, expression and characterization of a novel xylanase from the marine bacterium, G ac ec a e a KMM241. Mar e Dr ,  $II(4)$ , 1173–1187. xylanase from the marine bacterium,  $Gacec \ a \ e$
- 42. Cheng, F., Sheng, J., Dong, R., Men, Y., Gan, L., & Shen, L. (2012). Novel xylanase from a holstein cattle rumen metagenomic library and its application in xylooligosaccharide and ferulic acid production from wheat straw. J r a  $A r c$  r and  $F d C e$  r  $.60(51)$ , 12516–12524.
- 43. Ali, M. K., Rudolph, F. B., & Bennett, G. N. (2005). Characterization of thermostable Xyn10A enzyme from mesophilic  $C \cdot r d$  ace b c. ATCC 824. J r a I d r a M cr b & B ec. mesophilic C rd ace b c ATCC 824. J r a I d r a M cr b & B ec . 32(1), 12–18.
- 44. Lee, S. F., Forsberg, C. W., & Rattray, M. (1987). Purification and characterization of two endoxylanases from  $C$  r d ace b c