



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

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Abstract A novel xylanase gene, *XYN-LXY*, was cloned from a metagenomic fosmid library, which was previously constructed from the rumen contents of Hu sheep and was functionally characterized in *Escherichia coli*. 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⁻¹. The optimal temperature and pH of the enzyme were 50 °C and 6.0, respectively. Specifically, XYN-LXY was exclusively activated by Mn²⁺ 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 ± 0.175 mg ml⁻¹ (74.07 % of total product) followed by xylose with a concentration of 0.656 ± 0.010 mg ml⁻¹ (21.14 % of total product). The XYN-LXY exhibited deep degradation effects

purchased from Qiagen (Shanghai, China). The *E. coli* 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] 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]. 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=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/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, approximately 50 ng plasmid DNA was used as the template for amplification of the gene by using a pair of primers Xyn-lxy-F: 5' CCGGAATTCATGAAGAAGAACTGACGAG3' (*Ec* RI) and Xyn-lxy-R: 5' CCGCTCGAGTTATACCAGCTTGCGTTACCAA3' (*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 ~2 kb was purified, ligated with pGEM-T, transformed into DH5 α competent cells, and streaked onto a 100 μ g ml⁻¹ ampicillin LB agar plate supplemented with 100 mM isopropyl-thio- β -D-galactopyranoside (IPTG) and 20 mg ml⁻¹ 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 pGEM-T/.

The pGEM-T/ 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.com/vector/pET-30a.pdf>), which was previously digested with the same enzymes. Then, the ligation product was transformed into *E. coli* BL21 (DE3) and streaked onto an LB agar plate containing 100 μ g ml⁻¹ 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 μ g ml⁻¹ 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_2HPO_4 , 2 mM KH_2PO_4)

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 °C for 10 min. Then, the xylanase activity was assayed under the optimum conditions (pH 6.0, 50 °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×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⁻¹. Finally, the released sugars from the birchwood xylan were quantified by comparing the relative areas to standard xylose, xylobiose (X2), xylotriose (X3), xylotetraose (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]. The clone L3, showing the highest xylanolytic 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 *XYN-LXY*, 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>) 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/), respectively, with a signal peptide of 22 AA locating at the N-terminus (<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 *Lactobacillus* bacterium (WP_035658042, 61 %), *Escherichia* (BAA09971, 59 %), *Ruminococcus* (WP_022112477, 55 %), and *Bacteroides* (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]. 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) and AA alignment (Fig. 2).

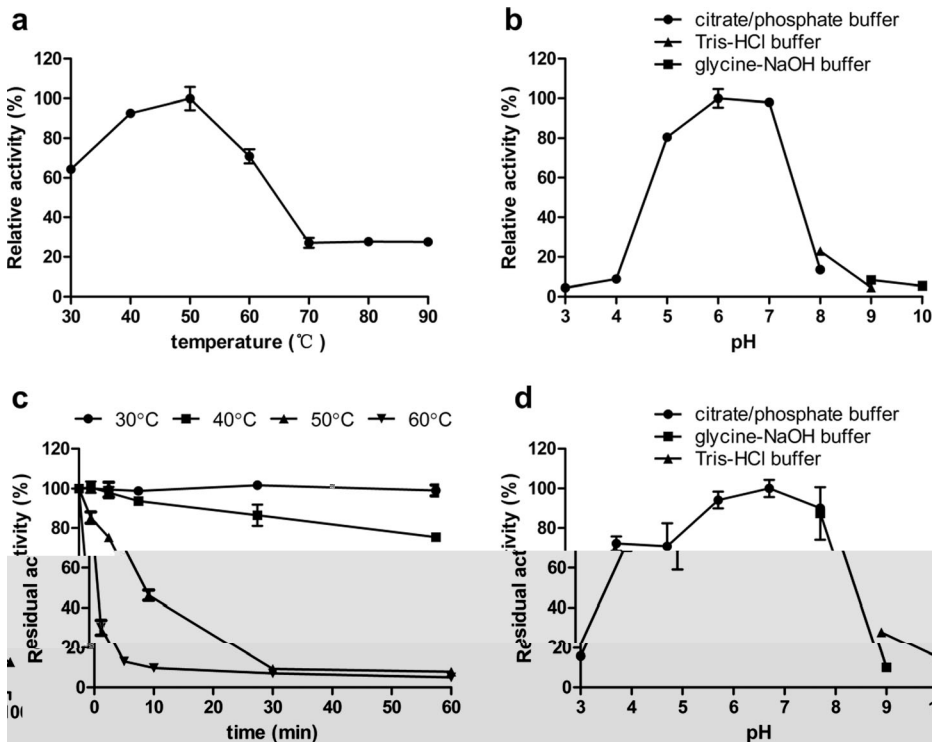


Fig. 4 Effects of temperature and pH on catalytic activity of XYN-LXY. **a** The optimal temperature of XYN-LXY. **b** The optimal pH of XYN-LXY. **c** Thermostability of XYN-LXY from 30 to 60 °C. **d** The pH stability of XYN-LXY. Assays were performed as described in the “Materials and Methods” section 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]. 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 (/) were extensively investigated in this study. As seen in Table 2, 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 Co^{2+} , Ca^{2+} , Na^{+} , and Ni^{2+} were only marginal. Interestingly, Mn^{2+} 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, 35], although most studies

Table 2 Effects of various cations on activity of XYN-LXY

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

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

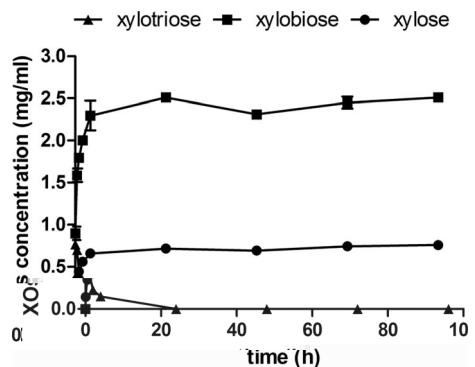
^b The concentration of SDS was 1 % (/)

revealed that Mn²⁺ showed inhibitory activity on xylanase activity [36–39]. Usually, cations such as Ca²⁺, Co²⁺, Cu²⁺, and Zn²⁺ were believed to function as activators of xylanase activity [23, 35, 37]. However, it was also reported that Ca²⁺, Co²⁺, Cu²⁺, Zn²⁺, and Fe³⁺ inhibited enzymatic activities, especially at high concentrations (5 to 20 mM) [39–42]. 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 XO₅ 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 $0.897 \pm 0.079 \text{ mg ml}^{-1}$ (49.67 % of total product, the same below) and $0.764 \pm 0.024 \text{ mg ml}^{-1}$ (42.35 %), respectively. However, xylose showed only a slight increase, with a concentration of $0.144 \pm 0.018 \text{ mg ml}^{-1}$ (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 $0.656 \pm 0.010 \text{ mg ml}^{-1}$ (21.14 %) and $2.297 \pm 0.175 \text{ mg ml}^{-1}$ (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, 22, 24] or GH 11 [17–19, 21, 23] 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] or X5 only [22] or both X4 and X5 [18] directly or indirectly (via the transglycosylation reaction). Noticeably, negligible xylose was detected in all these studies. However, Ali et al. [43] 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, 45]. 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 *Sre. ce* sp. S9 xylanase [46], 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], 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 °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.

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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. *Proceedings of the National Academy of Sciences*, *109*(42), 17117–17122.
2. Haki, G. D., & Rakshit, S. K. (2003). Developments in industrially important thermostable enzymes: a review. *Biotechnology*, *89*(1), 17–34.
3. Collins, T., Gerday, C., & Feller, G. (2005). Xylanase, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews*, *29*(1), 3–23.
4. Kulkarni, N., Shendye, A., & Rao, M. (1999). Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews*, *23*(4), 411–456.
5. Watanabe, S., Kodaki, T., & Makino, K. (2005). Complete reversal of coenzyme specificity of xylofuranose dehydrogenase and increase of thermostability by the introduction of structural zinc. *Journal of Biochemistry*, *280*(11), 10340–10349.
6. Bocchini, D. A., Gomes, E., & Silva, R. D. (2008). Xylanase production by *Bacillus cereus* DL using maltose as carbon source. *Applied Biochemistry and Biotechnology*, *146*(1-3), 29–37.
7. Miyazaki, K., Takenouchi, M., Kondo, H., Noro, N., Suzuku, M., & Tsuda, S. (2006). Thermal stabilization of *Bacillus* family-11 xylanase by directed evolution. *Journal of Biochemistry*, *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 Microbiology 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. *World Journal of Microbiology*, *28*, 929–935.
10. Silva, J. P. A., Mussatto, S. I., Roberto, I. C., & Teixeira, J. A. (2011). Ethanol production from xylose by *Pichia NRRLY-7124* in a stirred tank bioreactor. *Brazilian Journal of Chemical Engineering*, *28*(3), 151–156.
11. Cardona, C. A., Quintero, J. A., & Paz, I. C. (2010). Production of bioethanol from sugarcane bagasse: status and perspectives. *Biotechnology*, *101*(13), 4754–4766.
12. Matsushika, A., Watanabe, S., Kodaki, T., Makino, K., & Sawayama, S. (2008). Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, NADP⁺-dependent xylofuranose dehydrogenase, and xylofuranosyltransferase. *Journal of Biochemistry*, *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.

19. Zhang, M., Jiang, Z., Yang, S., Hua, C., & Li, L. (2010). Cloning and expression of a *Paecilomyces thermophilus* xylanase gene in *Escherichia coli* and characterization of the recombinant xylanase. *Bioresour Technol*, *101*(2), 688–695.
20. Zhang, J., Siika-aho, M., Puranen, T., Tang, M., Tenkanen, M., & Viikari, L. (2011). Thermostable recombinant xylanases from *Novospharia foveola* and *Thermotoga maritima* show distinct properties in the hydrolysis of xylans and pretreated wheat straw. *Bioresour Technol*, *102*(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 Biotechnol*, *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 *Marcobacterium* sp. *J Biotechnol*, *173*(8), 2028–2041.
23. Gao, H., Yan, P., Zhang, B., & Shan, A. (2014). Expression of *Aspergillus* IA-001 Endo- β -1, 4-xylanase in *Pichia pastoris* and analysis of the enzymic characterization. *Appl Biochem Biotechnol*, *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, *Serratia* HY-14. *J Microbiol*, *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.), *Microbiome of the rumen* (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. *Chin J Microbiol*, *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. *Appl Biochem Biotechnol*, *23*(3), 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. *Appl Environ Microbiol*, *43*(4), 777–780.
29. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*(5259), 680–685.
30. Bailey, M. J., Biely, P., & Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *J Biotechnol*, *23*(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. *Anal Biochem*, *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. *PLoS 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 *Ustilago* xylanase in *Pichia pastoris*. *Bioresour Technol*, *173*(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. *Bioresour Technol*, *102*(3), 3330–3336.
35. Knob, A., & Carmona, E. C. (2010). Purification and characterization of two extracellular xylanases from *Pectinococcus*: a novel acidophilic xylanase. *Appl Biochem Biotechnol*, *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. *World J Microbiol*, *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 *Gaerhmanniella* KMM 241. *Appl Microbiol Biotechnol*, *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- β -1, 4 xylanase from an alkaliphilic *Candida* sp. CKMX1 isolated from mushroom compost. *World J Microbiol Biotechnol*, *30*(10), 2597–2608.

39. Zhang, W., Lou, K., & Li, G. (2010). Expression and characterization of the *Dc* Rt46B.1 xylanase gene (*B*) in *Bacillus* *AedBce* *r* *a* *d* *B* *ec*, 160(5), 1484–1495.
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 *Acetivibrio* sp. Xz-8 with high catalytic efficiency and application potentials in the brewing industry. *J. R. A. Arc* *r* *a* *d* *F* *d* *C* *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, *Gaec* *a* *e* *a* KMM241. *Mar. e* *D* *r*, 11(4), 1173–1187.
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. Arc* *r* *a* *d* *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. r* *d* *a* *c* *e* *b* *c* ATCC 824. *J. R. A. I* *d* *r* *a* *M* *c* *r* *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* *a* *c* *e* *b* *c*.