**Gene cloning and expression of fungal lignocellulolytic enzymes from the rumen of gayal (*Bos frontalis*)**

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A total of 6,219 positive clones were obtained by constructing a BAC library of uncultured ruminal fungi of gayal, and two clones (*xynF1* and *eglF2*) with lignocellulolytic enzyme activity were selected. The sequencing results showed that *xynF1* and *eglF2* had 903-bp, and 1,995-bp, open reading frames likely to encode β-xylanase (*XynF1*) and β-glucosidase (*EglF2*), respectively. The amino acid sequence of *XynF1* had 99% coverage and 95% homology to the endo-β-1,4-xylanase encoded by the cellulase gene of *Orpinomyces* sp. LT-3 (GenBank accession No. AEO51791.1). The amino acid sequence of *EglF2* had 99% coverage and 93% homology to the β-glucosidase encoded by the cellulase gene of *Piromyces* sp. E2 (GenBank accession No. CAC34952.1). Analysis using the SMART software showed that *XynF1* contains a glycoside hydrolase family 11 functional module and a carbohydrate-binding module, while *EglF2* contains a glycoside hydrolase family 1 functional module. *XynF1* showed the highest relative enzymatic activity, up to 95%, at 45°C and pH 4.2, while *EglF2* showed the highest relative enzymatic activity, up to 95%, at 55°C and pH 6.2. In this study, we achieved efficient expression of the *xynF1* and *eglF2* genes in *Pichia pastoris*, which laid a foundation for the practical application of the lignocellulolytic enzymes.

**Key Words:** β-glucosidase; β-xylanase; cloning; BAC library; expression; lignocellulolytic enzyme

**Introduction**

Cellulose is the most abundant carbohydrate on Earth, with up to $1.55 \times 10^9$ t of cellulose produced globally each year via photosynthesis, of which 89% has not been utilized by humans (Lynd et al., 2002). Lignocellulose is formed from cellulose, hemicellulose, and lignin via various chemical bonds. It has a complex molecular structure, which is hard to break down by any single hydrolytic enzyme, and often a synergistic effect of multiple enzymes is required to complete the degradation of lignocellulose (Jeffries et al., 2007). Cellulase is widely applied in animal feeds, foods, textiles, papermaking, environmental protection, medicine, and other fields (Cha et al., 2007; Christner et al., 1992; Li et al., 2009). Screening for highly efficient, acidophilic, alkali-resistant, and thermophilic lignocellulolytic enzymes has been an important subject of recent studies.

Gayal (*Bos frontalis*) is a semi-wild cattle breed that lives in mountainous areas at altitudes of 1,500–3,800 m. The animals graze throughout the year and feed on bamboos, reeds, and weeds. Long-term living in the harsh environment has equipped gayals with extremely high resistance characteristics, including resistance to coarse feeds and alpine environments. Studies have found that gayal has a significantly greater digestive ability than the local cattle (*Bos taurus*) for dry matters, organic matters, neutral detergent fiber, acidic detergent fiber, and acidic lignin of bamboo (Deng et al., 2007). Besides, gayal has a significantly higher stress resistance to harsh environments and a higher growth rate than the local cattle (Xi et al., 2007), which may be associated with its special germplasm resource of ruminal microorganisms. The study by Leng et...
al. (2011) has revealed that the rumen of gayal is characterized by a higher diversity of bacterial species than that of the Yunnan yellow cattle, and 85% of the bacteria of total sequences could not be enriched by in vitro culture. The microbial metagenomic library technique enables a highly efficient detection of novel functional genes from ruminal microorganisms involved in the degradation of crude fibers in feeds through a large amount of genetic information and high-throughput screening. Ferrer et al. (2005) have successfully constructed an expression library of dairy cows with a mean insert size of 5.5 kb and selected several cellulase genes via functional screening of the library. Duan et al. (2009) have constructed a ruminal metagenomic library of water buffalo (Bubalus bubalus) and have found 14 cellulase genes in the library. Li et al. (2016) have constructed a fosmid library of ruminal microorganisms from gayal and found a novel cellulase gene (Umcel-1) among the 38,400 clones obtained. Meta-omics studies of ruminal microorganisms in Holstein cows showed that ruminal microorganisms of ruminants have abundant resources of lignocellulolytic enzymes (Hess et al., 2011; Huggett et al., 2013; Pitta et al., 2016). Abundant resources of lignocellulolytic enzymes (Hess et al., 2011; Huggett et al., 2013; Pitta et al., 2016) have shown that ruminal microorganisms of ruminants have a higher diversity of bacterial species than that of the Yunnan yellow cattle, and 85% of the bacteria of total sequences could not be enriched by in vitro culture. The microbial metagenomic library technique enables a highly efficient detection of novel functional genes from ruminal microorganisms involved in the degradation of crude fibers in feeds through a large amount of genetic information and high-throughput screening. Ferrer et al. (2005) have successfully constructed an expression library of dairy cows with a mean insert size of 5.5 kb and selected several cellulase genes via functional screening of the library. Duan et al. (2009) have constructed a ruminal metagenomic library of water buffalo (Bubalus bubalus) and have found 14 cellulase genes in the library. Li et al. (2016) have constructed a fosmid library of ruminal microorganisms from gayal and found a novel cellulase gene (Umcel-1) among the 38,400 clones obtained. Meta-omics studies of ruminal microorganisms in Holstein cows showed that ruminal microorganisms of ruminants have abundant resources of lignocellulolytic enzymes (Hess et al., 2011; Huggett et al., 2013; Pitta et al., 2016).

Gayal has a much greater utilization rate for crude fibers in feeds than other ruminant livestock. Hence, its ruminal microorganisms are the best options to screen for cellulase genes. In the process of cellulose degradation by ruminal microorganisms, fungi are the first microorganisms that attach to fibrous matters (Bauchop, 1979; Grenet et al., 1989), thus playing a leading role in cellulose degradation. Compared with bacteria, the number of ruminal anaerobic fungi is significantly lower, accounting for 20% of the total ruminal microorganisms (Sirohi et al., 2012), but they can produce highly active cellulases and hemicellulases (Dagar et al., 2011; Eckart et al., 2010; Lee et al., 2000). To date, there has been no report on the functional genes of lignocellulolytic enzymes from ruminal fungi of gayal. Therefore, we constructed a bacterial artificial chromosome (BAC) library of ruminal fungi from gayal, containing large fragments, and we screened for genes of highly active lignocellulolytic enzymes. We also studied their enzymatic properties by expressing the enzymes in Pichia pastoris, which laid a theoretical basis for the development of lignocellulolytic enzymes to use in feeds.

Materials and Methods

Source of samples. In January 2014, ruminal contents were sampled, using a gastric catheter, from four gayals in Gongshan County of Nujiang, Yunnan Province, China (altitude: 2,260 m, latitude: 27°46′15″, and longitude: 98°39′99″) and stored these at −80°C.

Construction of a BAC library and screening for lignocellulolytic enzymatic activity. A total of 10 g of the ruminal contents from the four gayals was weighed and mixed, followed by the extraction of large-size genomic DNA from ruminal fungi, according to the method described by Chaudhary et al. (2008). The DNA was purified using the Nucleo-Bond® BAC 100 column chromatography kit (Macherey-Nagel, Inc.) according to the instructions in the user manual. A total of 30 μL of purified DNA was added to 5 μL of buffer and 2 μL of the HindIII enzyme, and the reaction volume was adjusted to 50 μL with sterile water. After restriction digestion at 37°C for 10 min, the digested DNA was separated by 0.8% agarose gel electrophoresis. The gel block containing DNA > 80 kb was recovered using the kit. A total of 100 ng of the HindIII-digested large-size DNA was mixed with 1 μL of the plasmid vector (CopyControl pCC1BAC), followed by overnight ligation at 16°C, and the ligation product was used for electrotransformation (1.6 kV, 200 Ω, 25 mF) using a GenePulser II (Bio-Rad, USA). Immediately after the electroporation, 1 mL of Luria-Bertani (LB) culture medium was added, and the mixture was transferred into a 1.5-mL centrifuge tube, followed by incubation at 37°C, 220–230 rpm for 2 h. The culture was then spread onto an LB agar plate containing chloramphenicol (12.5 μg/mL), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; 40 μg/mL), and isopropyl β-D-1-thiogalactopyranoside (IPTG; 0.4 mol/L). The library was screened for enzymatic activity by the Congo red staining method described by Teather and Wood (1982), and the enzyme production was preliminarily assessed based on the formation of a hydrolysis zone around the colony.

Identification of cellulase genes. Two positive clones showing high cellulase activity were sequenced. The DNAStar software was used to find the open reading frames (ORFs); BlastP of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) was used to search the GenBank database; and glycoside hydrolase (GH) databases (http://www.cazy.org/Glycoside-Hydrolases.html) was used for the active enzymes analysis.

Expression and purification of recombinant XynF1 and EglF2 proteins. Based on the sequencing results and predicted ORFs from the gene sequences, primers were designed using Vector NTI, and restriction sites (underlined) were inserted as follows: *xynF1*-F: 5′-ACAGAATTCATGACTCTAGGTATTGGTGCA-ACC-3′; *xynF1*-R: 5′-CATCTCAGGTAAAAACCACAACACCACATT-TGC-3′; *eglF2*-F: 5′-CCTGAAATTCATGAGACTCTTTTCTATTTTTCAC-TAC-3′; *eglF2*-R: 5′-CCTCAGGATTTGTTTGTCAACATTTTCGAG-3′.

The *xynF1* and *eglF2* genes were amplified by a polymerase chain reaction (PCR) using plasmid DNA as the template. The PCR products and plasmid pPICZ-a-A were double digested with EcoRI and XhoI. The digested products were recovered using a gel recovery kit (Tiangen Biotech Co., Ltd.), ligated, and transformed into competent cells of *P. pastoris* X33. The cells were spread on an LB agar plate and incubated overnight. Then, a single colony was picked and inoculated into a 5-mL tube containing 2 mL of LB broth, followed by incubation at 30°C, 300 rpm for 12 h. After confirming the transformation by
Na₂CO₃ solution was added to terminate the reaction, and was measured using the method reported by Lachke (1988), the xylanase activity substrate (pNPX), 185 pNPX conversion into 1 was defined as the amount of enzyme required to catalyze (pNPX) as the substrate, wherein one enzyme unit (IU).

Measurement of enzymatic activity. According to the method reported by Lachke (1988), the xylanase activity was measured using p-nitrophenyl-β-D-xylopyranoside (pNPX) as the substrate, wherein one enzyme unit (IU) was defined as the amount of enzyme required to catalyze pNPX conversion into 1 μmol of pNP per minute. The total assay volume was 800 μL, and the reaction volume was 200 μL containing 10 μL of 20 mmol/L substrate (pNPX), 185 μL of 100 mmol/L potassium hydrogen phthalate-imidazole buffer (pH 6.0), and 5 μL of a properly diluted crude enzyme solution. The mixture was reacted at 40°C for 5 min, then 600 μL of a 1 mol/L Na₂CO₃ solution was added to terminate the reaction, and the colorimetric intensity was measured at 405 nm. The activity of β-glucosidase was measured using p-nitrophenol-β-D-glucopyranoside (pNPG) as the substrate, wherein 1 IU was defined as the amount of enzyme required to catalyze pNPG conversion into 1 μmol of pNP per minute. A total of 0.3 mL of the crude enzyme was added to 0.9 mL of sodium diphosphate-citrate buffer (pH 6.0) and preheated at 40°C for 5 min, followed by the addition of 0.6 mL of a 1.6 mmol/L pNPG solution (preheated for 10 min). After a precise 30-min reaction, 2.5 mL of a 0.5 mol/L Na₂CO₃ solution was added immediately to terminate the reaction, and the mixture was cooled to room temperature prior to the measurement of OD₄₁₀.

Results

Construction of a BAC library and screening for lignocellulosic enzyme activity

The mixed DNA macromolecules of ruminal microorganisms, prepared using the above-mentioned DNA isolation method, had a large proportion of highly pure DNA fragments > 2.2 Mb. Restriction digestion resulted in 80 kb to 200 kb DNA fragments, and 0.1–0.4 μg/mL of gel-recovered DNA fragments was used for library construction. Our experiment resulted in a total of 9,216 clones. Plasmids from 55 randomly selected clones were digested with HindIII, and the results revealed that there was only one empty plasmid. One clone had an insert size less than 80 kb, nine clones had insert sizes of 80–110 kb, 14 clones had insert sizes of 110–140 kb, 19 clones had insert sizes

| Products | No. | Description | Max score | Total score | Query cover | E value | Identity | Accession number |
|----------|-----|-------------|-----------|-------------|-------------|---------|----------|-----------------|
| XynF1    | 1   | Endo-1,4-beta-xylanase [Orpinomyces sp. LT-3] | 561       | 561         | 99%         | 0.0     | 95%      | AEO5179.1       |
|          | 2   | Xylanase [Orpinomyces sp. PC-2] | 543       | 543         | 99%         | 0.0     | 93%      | AAD04194.1      |
|          | 3   | RecName: Full = Bifunctional endo-1,4-beta-xylanase A; Short = XYL; Flags: Precursor >emb|CAA46498.1| xylanase A [Neocallimastis patriciarum] | 527 | 895 | 99% | 0.0 | 86% | AAB23454.1 |
|          | 4   | Xylanase A, XYL[Neocallimastis patriciarum, Peptide, 607 aa] | 527       | 895         | 99%         | 0.0     | 86%      | AAB23454.1      |
| EglF2    | 1   | Beta-glucosidase [Pyromyces sp. E2] | 1300      | 1300        | 99%         | 0.0     | 93%      | CAC34952.1      |
|          | 2   | Beta-glucosidase Cel1C [Pyromyces sp. E2] | 1087      | 1087        | 99%         | 0.0     | 77%      | AAP30745.1      |
|          | 3   | Beta-glucosidase [Orpinomyces sp. PC-2] | 1077      | 1077        | 99%         | 0.0     | 78%      | AAD45834.1      |
|          | 4   | Putative cellulase [Neocallimastis patriciarum] | 981       | 981         | 99%         | 0.0     | 72%      | AEX92705.1      |

Table 1. Homology analysis of XynF1 and EglF2 gene products.

Fig. 1. Screening of clones with lignocellulosic enzyme activity.

Fig. 2. SDS-PAGE analysis of XynF1 and EglF2 recombinant protein expressed in Pichia pastoris. M, premixed protein marker; 1, pPICZ-a-A-xynF1; 6, pPICZ-a-A-eglF2; 2 and 5, blank control; 3 and 4, purified XynF1 and EglF2, respectively.
of 140–170 kb, 10 clones had insert sizes of 170–200 kb, and one clone had an insert size greater than 200 kb. Analysis showed that the constructed library was stable. The mean insert size was 162.2 kb per clone, the library size was 1,494.835 Mb, and the empty vector was carried by 2% of the clones. A total of 3,300 clones were selected from the constructed BAC library, and two positive clones with lignocellulolytic enzyme activity were identified by screening on Congo red agar (CRA). The bacterial cultures containing the positive clones were enriched for 12 h, and the enriched cultures were further confirmed on CRA. The results showed that both clones could produce an obvious hydrolysis zone. In Fig. 1, sample 1 and sample 2 are the monoclonal bacterial strains with plasmids harboring the \textit{xynF1} and \textit{eglF2} genes, respectively.

**Sequence analyses of lignocellulolytic enzyme genes, \textit{xynF1} and \textit{eglF2}**

The plasmids from the two positive clones showing enzymatic activity were isolated and sequenced. The 903-bp \textit{xynF1} cellulase gene was found to encode a 300-amino acid sequence, whereas the 1,995-bp \textit{eglF2} cellulase gene was found to encode a 664-amino acid sequence, the GenBank accession numbers for these two sequences are KY606584 and KY606585. The theoretical isoelectric point (pI) of \textit{xynF1} is 5.97, and the molecular weight is 33.1 kDa; the theoretical pI of \textit{eglF2} is 5.18, and the molecular weight is 76.1 kDa, as predicted by the ExPASy Compute pI/Mw tool (http://www.expasy.org/tools/pi_tool.html). The SMART analysis showed that the amino acids 1–180 of \textit{xynF1} represented a glycoside hydrolase family 11 (GH11) functional module, while the amino acids 216–255 and 259–298 represented carbohydrate-binding module. The amino acids 1–16 of \textit{eglF2} represented a signal peptide, while the amino acids 77–289, 298–557, and 560–664 represented GH1 functional module. The BlastX analysis revealed that the amino acid sequence of \textit{xynF1} had 99% coverage and 95% homology to the endo-\textit{β}-1,4-xylanase encoded by the cellulase gene of \textit{Orpinomyces} sp. LT-3 (GenBank accession No. 12

Fig. 3. Relative enzymatic activity of \textit{xynF1} and \textit{eglF2} at different pH values.

Note: Relative enzyme activity is defined as the percentage relative to the highest enzyme activity under each enzymatic reaction condition.

Fig. 4. Relative enzyme activity of \textit{xynF1} and \textit{eglF2} at different temperatures.

Note: Relative enzyme activity is defined as the percentage relative to the highest enzyme activity under each enzymatic reaction condition.
AEO51791.1), 93% homology to the xylanase encoded by the cellulase gene of Orpinomyces sp. PC-2 (GenBank accession No. AAD04194.1), and less than 90% homology with other proteins encoded by hemicellulase genes from various fungi. Whereas that of EgI2F had 99% coverage and 93% homology to the β-glucosidase encoded by the cellulase gene of Piromyces sp. E2 (GenBank accession No.CAC34952.1) and less than 80% homology with enzymes encoded by cellulase genes from other various fungi. (Table 1).

Induced expression in Pichia pastoris and purification of the XynF1 and EgI2F enzymes

After the recombinant plasmids (pPICZ-a-A-xynF1/eglI2F) were transformed into P. pastoris and their expression was induced using 0.5% methanol, the yeast cells were harvested and disrupted by sonication, followed by centrifugation to collect both pellets and supernatants. SDS-PAGE (Fig. 2) indicated that the molecular weights of XynF1 and EgI2F were 33.1 kDa and 76.1 kDa, respectively. The bands could be clearly seen, and their mobility was consistent with the expected sizes of the target proteins. The expression levels were relatively high, while the expression levels in the yeast transformed with the empty plasmid hardly differed before and after the induction. Both XynF1 and EgI2F proteins were observed as a single band of the expected size after purification via Ni-NTA resin (Fig. 2).

Enzymatic properties of the recombinant XynF1 and EgI2F proteins

The supernatant (crude enzyme solution) harvested after 12 h of induction was reacted with the substrate (pNPX or pNPG) at 40°C and pH 3.0–8.0 for 30 min to measure the enzymatic activity under various pH conditions. The results indicated that the recombinant enzymes, XynF1 and EgI2F, showed the highest relative enzymatic activity, up to about 95%, at pH 4.2 and pH 6.2, respectively, demonstrating good stability (Fig. 3).

XynF1 and EgI2F were also reacted with the substrate for 30 min at temperatures of 30–70°C and at their optimal pH values of 4.2 and 6.2, respectively, in order to measure their enzymatic activities at different temperatures. The results showed that XynF1 had the highest relative enzymatic activity and thermostability at 45°C, while EgI2F had the highest relative enzymatic activity and thermostability at 55°C (Fig. 4).

Discussion

Ruminants are capable of efficiently utilizing cellulose, and this unique digestive function is conferred by a large amount of symbiotic microorganisms in their rumen. In this study, we directly extracted the metagenomic DNA of fungi from the rumen of gayal using a non-culture-based method to construct a BAC gene library. A total of 6,219 positive clones were obtained, of which two clones with lignocellulolytic enzymatic activity, xynF1 and eglI2F, were selected. Compared with the study carried out by Ferrer et al. (2005), the metagenomic BAC library contains more genetic resources and information, which is more favorable for the cloning of lignocellulolytic enzyme genes from ruminal fungi.

Similar to the enzyme studied by Liu et al. (2005), XynF1 has a GH11 functional module and two carbohydrate-binding module, which can specifically bind to xylan and facilitate the enzymatic reaction. It has been reported that GH11 xylanases are highly specific, with relatively narrower substrate ranges than those of GH10 xylanases, and hydrolyze xylan or xylooligosaccharides mainly into disaccharides and trisaccharides (Berrin and Juge, 2008), which can be further hydrolyzed by GH10 xylanases (Collins et al., 2005). Both GH10 and GH11 xylanases are involved in the efficient degradation of xylan. The study carried out by Li et al. (1997) has shown that the amino acid sequence of the xylanase from the monocentric ruminal fungus Neocallimastix patriciarum has a generally greater homology with other xylanases than the enzyme from the polycentric ruminal fungus Orpinomyces sp. The amino acid sequence of the xylanase of N. patriciarum has a catalytic domain (CD) and a non-catalytic repeated peptide domain (NCRPD). Studies have demonstrated that CD binds to the cellulosome through NCRPD and the synergistic catalytic effect is more efficient than that of CD alone (Ljungdahl, 2008). Meanwhile, the amino acid sequence of the xylanase of Orpinomyces sp. only has a CD, which may result in a lower xylanase enzymatic activity than that of the N. patriciarum enzyme. In the study of Tutus et al. (2014), the xylanase XynA16 had an optimal pH of 6.0 and optimal temperature of 50°C and still retained 74% of its relative enzymatic activity after incubation at this temperature for 24 h. However, in this study, the optimal pH for XynF1 was 4.2, and the enzyme reached the highest relative enzymatic activity, up to 95%, and the highest stability, after a reaction at 45°C for 30 min. XynF1 exhibits a higher enzymatic activity at pH 4.2–6.0 and temperatures of 40–55°C. Thus, XynF1 prefers an acidic pH and is able to tolerate relatively high temperatures.

The amino acid sequence of EgI2F contains a signal peptide and a GH1 functional module and exhibits only β-glucosidase enzymatic activity. Enzymes of the GH1 family are found in the intestine of many animals (Dai et al., 2015; Wang et al., 2011), and the majority of β-glucosidases belong to the GH1 and GH3 families (Henrissat et al., 1995), which are important components of the cellulolytic enzyme system. Hong et al. (2006) have found that an enzyme with both β-glucosidase and β-xylosidase activity showed a better cellulolytic efficiency. Li et al. (2014) have found in ruminal microorganisms from B. taurus a novel β-glucosidase gene, which encodes for a protein, ungluI35B12, with a molecular weight of 84.1 kDa. This protein has an optimal reaction temperature of 38°C and an optimal pH of 5.0 and shows the highest similarity with proteins from Bacteroidetes. Chen et al. (2012) have reported that a β-glucosidase, NpaBGS, from N. patriciarum W5 showed the highest enzymatic stability at 40°C and pH 5.5. When Mg2+ and Mn2+ were added to the reaction, the enzymatic stability increased with increasing temperature. In this study, EgI2F showed the highest enzymatic stability at 55°C and pH 6.2 and had a better enzymatic activity at pH 5.0–8.0 and at 50–
65°C. Our data show that EglF2 works over a wide range of pH values and is able to tolerate, to a certain extent, high temperatures. Therefore, the EglF2 protein from the GH1 family, obtained in this study, may have originated from anaerobic fungi that degrade fibers.

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