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Characterization of a novel bifunctional enzyme from buffalo rumen metagenome and its effect on in vitro ruminal fermentation and microbial community composition

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ABSTRACT

To efficiently use lignocellulosic materials in ruminants, it is crucial to explore effective enzymes, especially bifunctional enzymes. In this study, a novel stable bifunctional cellulase-xylanase protein from buffalo rumen metagenome was expressed and characterized, CelXyn2. The enzyme displayed optimal activity at pH 6.0 and 45 °C. The residual endoglucanase and xylanase activities were 90.6% and 86.4% after a 60-min pre-incubation at 55 °C. Hydrolysis of rice straw, wheat straw, sheepgrass and sugar beet pulp by CelXyn2 showed its ability to degrade both cellulose and hemicellulose polymers. Treatment with CelXyn2 improved the hydrolysis of agricultural residues with an evident increase in production of total gas, lactate and volatile fatty acids. The results of 16S rRNA and real-time PCR showed that the effect on in vitro ruminal microbial community depended on fermentation substrates. This study demonstrated that CelXyn2 could strengthen lignocellulose hydrolysis and in vitro ruminal fermentation. These characteristics of CelXyn2 distinguish it as a promising candidate for agricultural application.

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1. Introduction

Agricultural residues such as rice straw, wheat straw and corn straw are generated in abundance every year in China. These agricultural straws consist of cellulose (40%), hemicellulose (20% to 30%) and lignin (20% to 30%) (Khandeparker and Numan, 2008) and are a

considerable feed source for ruminants. Cellulose is a major constituent of the plant cell wall with a β -1,4-glycosidic bond in its backbone. Cellulases can hydrolyze the β -1,4-glycosidic linkages of cellulose into soluble oligosaccharides, cellobioses and glucose (Van Dyk and Pletschke, 2012). However, the cross-linking of xylan and cellulosic regions restrict the attachment of cellulase. Xylanases break down β -1,4-xylosidic linkages in the cellulose backbone, which can enhance the accessibility of cellulose to hydrolytic cellulases (Van Dyk and Pletschke, 2012). Hence the combination of cellulase and xylanase could efficiently hydrolyze lignocellulosic materials.

Cellulases and xylanases have been used together in industrial applications like wood bio-pulping, paper, textile and agricultural waste treatment, biofuels and juice processing (Ariaenejad et al., 2020; Jemli et al., 2016). However, this combination is barely used in ruminants as a feed additive. To improve the utilization of agricultural straws in ruminants, some researchers have tried to

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use cellulases or xylanases separately to increase nutrient digestibility and growth performance (Holtshausen et al., 2011; Klingerman et al., 2009). Addition of exogenous cellulase had a marked effect on improving nutrient utilization in lambs and growth of dairy calves (Zhao et al., 2019), increased total microbial population (Beauchemin et al., 2000) and microbial protein (MCP) synthesis (Rode et al., 1999). Interestingly, adding tannins and cellulase together could significantly enhance feedstuff digestibility, intestinal development and liver protein synthesis, and hence improve growth performance (Zhao et al., 2019). With some in vitro studies, the addition of exogenous xylanases could also improve gas production, fiber degradation and ruminal fermentation (Elghandour et al., 2016; Salem et al., 2015; Togtokhbayar et al., 2015; Vallejo et al., 2016). However, some published studies reported that adding exogenous enzymes did not have an effect on ruminal fermentation (Wallace et al., 2001), feed intake and digestion (Dean et al., 2013), and animal performance (Reddish and Kung, 2007). It should be mentioned that most of these exogenous enzymes were commercial enzymes; the effect depends on the enzyme activity and stability, diet fed to the animals and the time and amount of enzyme added.

Using enzyme cocktails including cellulase, hemi-cellulase and various accessory enzymes is often an obstacle for their application because of the high cost of cellulolytic enzymes. Hence, the application of bifunctional enzymes in the hydrolysis of plant biomass can reduce the cost of applying multiple enzymes (Murashima et al., 2003; Pohlschröder et al., 1994). Generally, the forage sources of herbivores are mainly substances of plant origin, primarily comprising lignocellulose that is resistant to biological degradation. However, ruminants depend on rumen microbiota that can digest feed with various lignocellulolytic enzymes (Wang et al., 2016). Many novel enzymes involved in cellulose degradation have been identified from single rumen microorganisms through pure culture techniques (Cai et al., 2010; Hua et al., 2018; Papageorgiou et al., 2017; Zhou et al., 2017). However, most ruminal microbes cannot be isolated or cultured in vitro (Cao et al., 2021). In fact, many novel enzymes with unique properties have been directly identified from rumen metagenome in yak (Chang et al., 2011), goat (Lee et al., 2018), buffalo (Dadheech et al., 2018) and cow (Ariaeenejad et al., 2021). Briefly, a bifunctional xylanase-endoglucanase enzyme from yak rumen metagenome exhibited efficient hydrolysis in pretreated rice straw to produce xylo-oligosaccharides and cello-oligosaccharides (Chang et al., 2011). A novel KG51 bifunctional enzyme from black goat rumen metagenome exhibited high enzyme activity against konjac glucomannan (Lee et al., 2018). An acidic cellulase from buffalo rumen was characterized, and its applicability in saccharification of lignocellulosic biomass was tested (Dadheech et al., 2018). A novel bifunctional enzyme from cow rumen metagenome improved the degradation of various biomass substrates (Ariaeenejad et al., 2021).

It is generally known that the forage sources of buffalo are grasses, leaves and bark of trees. Hence, exploring some novel bifunctional enzymes from buffalo rumen is possible. In the present study, we discovered a novel bifunctional enzyme from buffalo ruminal microbiota big data and subsequently expressed in *Escherichia coli* (*E. coli*) BL21 (DE3). The enzymatic properties of CelXyn2 towards various substrates were determined. To evaluate its agricultural application potential, it was used to hydrolyze rice straw, wheat straw, sheepgrass and sugar beet pulp to produce fermentable sugar. We further investigated its effects on in vitro ruminal fermentation and microbial community. To our best of our knowledge, CelXyn2 was the first bifunctional cellulase-xylanase enzyme from buffalo rumen metagenome, and its feasibility was evaluated as a feed additive in ruminants.

2. Material and methods

2.1. Animal ethics statement

All animal procedures of this experiment were conducted in accordance with Institutional Animal Care and Use Committee of Nanjing Agricultural University (GB14925, NJAU-CAST-2011-093).

2.2. Materials

The *E. coli* DH5 α and BL21 (DE3) competent cells were obtained from Vazyme (Nanjing, China). pET28a (+) vector from Novagen (Darmstadt, Germany) was used for protein expression. Prime STAR Max DNA polymerase, DNA and protein ladders were purchased from Takara (Osaka, Japan). The T5 Exonuclease and DpnI from NEB (Ipswich, MA, USA) were used for In-Fusion clone. The Ni²⁺ affinity resin (Qiagen, Germany) was utilized for protein purification. Carboxymethylcellulose (CMC), locust bean gum, Avicel and sugarcane xylan were obtained from Macklin (Shanghai, China). α and β -*p*-Nitrophenyl- β -D-glucopyranoside (α -pNPG and β -pNPG) were from Sigma-Aldrich (St. Louis, MO, USA). Beechwood xylan, oat spelt xylan, wheat arabinoxylan, β -glucan, lichenan, laminarin and xyloglucan were obtained from Megazyme (Bray, Ireland). Whatman filter paper was obtained from GE (Boston, USA).

2.3. Bioinformatic analyses of CelXyn2

The online analytical tool ProtParam (https://web.expasy.org/compute_pi/) was used to predict molecular mass and isoelectric point. The signal peptide sequence of CelXyn2 at N-terminal was discovered by using the SignalP-5.0 online tool (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>). The BLAST tool was used to find the conserved domain (<https://blast.ncbi.nlm.nih.gov>) and homologous sequences. The online NetNGlyc 1.0 Server (<https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0>) and NetOGlyc 4.0 Server (<https://services.healthtech.dtu.dk/service.php?NetOGlyc-4.0>) were used for N-linked and O-linked glycosylation site analysis, respectively. The homology modelling structures of CelXyn2 were obtained by Swiss-Model web tool (<https://swissmodel.expasy.org>).

2.4. Expression and purification of recombinant proteins

The CelXyn2 gene sequence (GenBank: MBR6042486.1) from buffalo metagenome (NCBI SRA database BioProject: PRJNA815894) was synthesized in TsingKe (Beijing, China). PCR fragments were obtained from PCR amplification via the primers CelXyn2-F (5-ctgggtgacagcaaatgggtATGAGAAAAAATTTAGGTTTCAGCCTTGTTG-3) and CelXyn2-R (5-tgttagcagccgatctcaTTATTTCCGCTAAGAATA GCAGGCAAT-3). The linearized pET28a (+) vector was obtained by inverse PCR via the primers pET-F (5-tgagatccggctgctaacaag-3) and pET-R (5-gctttgttagcagccgat-3). The linearized vector was then incubated with DpnI to remove circular original plasmids. The purified CelXyn2 PCR fragments and linearized pET28a (+) vector were treated with T5 Exonuclease and then ligated. The *E. coli* DH5 α was transformed with the ligation mixture and spread onto Luria-Bertani (LB) medium supplemented with 50 μ g/mL of kanamycin. The selected positive transformants were verified by colony PCR via the primers Clony-F (5-tgtggacagcaaatgggt-3) and Clony-R (5-gctttgttagcagccgat-3), and further sequenced before their introduction into expression host *E. coli* BL21 (DE3).

The single transformant was cultivated in 6 mL fresh LB medium supplemented with 50 μ g/mL kanamycin at 37 °C with shaking (200 rpm) overnight. The seed-culture was then transferred into 600 mL LB medium with kanamycin (50 μ g/mL) at 37 °C with

shaking (200 rpm) until the absorbance at 600 nm reached 0.5 to 0.6. Subsequently, isopropyl β -D-thiogalactopyranoside (IPTG) was added to LB medium at a final concentration of 0.5 mM for 24 h at 16 °C. The cells were finally harvested by centrifugation at $12,000 \times g$ for 30 min, resuspended in lysis buffer (50 mM NaH_2PO_4 , 300 mM NaCl, 10 mM imidazole, pH 8.0), and then sonicated (250 W, 25 min) on ice. The cell lysate was collected by centrifugation at $12,000 \times g$ for 30 min. Protein purification was conducted by using a Qiagen Ni-NTA Fast Start Kit (Hilden, Germany) under manufacturer's instructions. The purified enzyme fractions were checked by 12% SDS-PAGE. The protein concentration was measured using the Bradford method (Bradford, 1976).

2.5. Enzymatic characterization of CelXyn2

The enzyme assays for CelXyn2 were incubated in 100 mM Citric acid- Na_2HPO_4 buffer, pH 6.0. The 200 μL reaction system contained 2.0% wt/vol CMC or beechwood xylan as substrate and 50 μL protein (0.133 mg/mL), and was incubated at 45 °C for 5 min. The reaction mixture was stopped by adding 300 μL DNS reagent and heating at 100 °C for 5 min. The cellulase or xylanase activity was calculated by the DNS method (Deshavath et al., 2020). One unit of cellulase or xylanase activity was defined as the amount of protein that produced 1 μmol of glucose or xylose per min, using glucose or xylose as a standard. For the assay of β -glucosidase activity of CelXyn2, α -pNPG and β -pNPG were used as substrates. Assay mixtures containing 150 μL 2 M α -pNPG or β -pNPG and 50 μL protein were incubated for 5 min at 45 °C. The reaction was stopped by using 300 μL Na_2CO_3 (2% wt/vol), and the released pNP was measured by using pNP as a standard. The released reducing sugar and pNP were diluted and measured at 540 nm and 405 nm by using a SPARK Multi-functional Microplate Reader (Tecan Trading, Switzerland), respectively. One unit of enzyme activity was defined as the amount of protein required to release 1 μmol of reducing sugar or pNP from the substrate per minute under the optimum assay.

Optimum pH for CelXyn2 activity was measured in 100 mM buffer solutions at room temperature for 5 min, including Citric acid- Na_2HPO_4 buffer (pH 2.0 to 7.0), Tris-HCl buffer (pH 8.0 to 9.0) and Glycine-NaOH buffer (pH 10.0 to 12.0). The optimum temperature of CelXyn2 was measured at 30 to 60 °C at the optimum pH in 100 mM Citric acid- Na_2HPO_4 buffer or 4 M NaCl solution. The pH stability analysis of CelXyn2 for cellulase or xylanase activity was conducted by incubating 0.133 mg/mL of enzyme at different pH levels, ranging from pH 2.0 to 10.0 for 16 h at 4 °C. After that, residual cellulase or xylanase activity was determined with 100 mM Citric acid- Na_2HPO_4 buffer pH 6.0 using 2% wt/vol CMC or beechwood xylan as a substrate. The thermal stability analysis of CelXyn2 was performed by incubation of the enzyme at 50 and 55 °C, for 60 min, and residual activity was measured. The residual cellulase or xylanase activity was determined as the time needed to detect protein activity losses of 50% at 50 and 55 °C (half-life, $T_{1/2}$).

Effect of different metal ions and chemicals on cellulase or xylanase activity of CelXyn2 was determined in optimal conditions in the presence of 10 mM various metal ions (K^+ , Cu^{2+} , Zn^{2+} , Fe^{2+} , Fe^{3+} , Co^{2+} and Ba^{2+}), 10 mM modulators (PMSF, EDTA and β -Mercaptoethanol), 1% wet/vol detergents (Tween 20, Tween 80, Triton X-100 and SDS) and 2 M denaturant (urea and guanidine hydrochloride). The cellulase and xylanase activities were determined using 2% wt/vol CMC and beechwood xylan as substrates, respectively. Control enzyme activity determined according to the same conditions without any additive was set as 100%. The effect of salt on enzyme activity was studied in various NaCl concentrations (1 to 5 M) and artificial sea water (NaCl, 26.29 g/L; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3.94 g/L; CaCl_2 , 0.99 g/L; KCl 0.74 g/L; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 6.09 g/L).

Effect of different lignocellulosic substrates including CMC, beechwood xylan, oat spelt xylan, sugarcane xylan, wheat arabinoxylan, β -glucan, filter paper, locust bean gum, Avicel, lichenan, laminarin, xyloglucan, α -pNPG and β -pNPG on the activity of CelXyn2 was investigated. Lignocellulosic substrates were prepared at 2% (wt/vol) in 100 mM Citric acid- Na_2HPO_4 (pH 6.0) buffer. The amount of reducing sugar was determined using the DNS method at 540 nm (Deshavath et al., 2020), and the released pNP was measured at 405 nm. Kinetic parameters were performed in 100 mM Citric acid- Na_2HPO_4 (pH 6.0) buffer at different concentrations (1 to 20 mg/mL) for 5 min. The purified enzyme was used to hydrolyze CMC and beechwood xylan, and then the kinetic parameters were calculated according to Lineweaver–Burk method.

2.6. The hydrolysis of agricultural residues and in vitro ruminal fermentation

To evaluate the capacity of CelXyn2 against natural substrates, rice straw, wheat straw, sheepgrass and sugar beet pulp were used as substrates. For this purpose, the dried rice straw, wheat straw and sheepgrass were cut to small sizes of 2 to 3 mm, and sugar beet pulp was milled and sieved through a 50-mesh screen. All these substrates were thoroughly washed with ultra-pure water to remove reducing sugar, then oven dried. The purified 50 μL of enzyme (0.5 mg/mL) was added with each substrate (2% wt/vol) to 20 mL Citric acid- Na_2HPO_4 (100 mM, pH 6.0) and incubated and shaken at 45 °C for 168 h. The reducing sugar liberated in the reaction was quantitated by the DNS method at 3, 6, 14, 24, 48, 72, 96, 120, 144 and 168 h, respectively.

The effect of CelXyn2 on ruminal fermentation and microbial community of rice straw, wheat straw and sheepgrass was studied by an in vitro experiment according to previous studies (Li et al., 2018a; Zhang et al., 2020). The in vitro study was performed in 120 mL bottles containing 0.6 g substrate and 60 mL buffered rumen fluid. The buffered rumen fluid was composed of 15 mL rumen fluid collected from three ruminally fistulated Hu sheep fed a diet consisting of 700 g/kg alfalfa and 300 g/kg concentrates and 45 mL artificial anaerobic buffer (Menke and Steingass, 1988). Adding 2 mg CelXyn2 to bottles was set as treatments, while PBS buffer was a control. All samples were incubated in triplicate, and were incubated and shaken at 39 °C for 48 h. At the end of fermentation, the ruminal total gas and pH value were measured immediately, and the samples were collected and stored at -20 °C for volatile fatty acids (VFA), lactate, $\text{NH}_3\text{-N}$ and MCP synthesis determination according to a previous study (Jin et al., 2017). Briefly, 1 mL fermentation fluid was stored by adding 0.2 mL deproteinizing solution (100 g/L metaphosphoric acid and 0.6 g/L crotonic acid) for VFA measurement by Agilent 7890B gas chromatography (California, USA), and 5 mL fermentation fluid was preserved to measure $\text{NH}_3\text{-N}$, MCP and lactate concentration via spectrophotometry using a SPARK Multi-functional Microplate Reader (Tecan Trading, Switzerland). In addition, the microbial DNA extracted from samples and the 16S rRNA high throughput sequencing were conducted by following protocols outlined in previous research (Xie et al., 2019).

3. Results and discussion

3.1. Bioinformatic analyses of CelXyn2

A 1,146-bp open reading frame (ORF) from buffalo rumen metagenome encoding a protein of 381 amino acid (AA) residues was identified. The online analytical tool ProtParam (https://web.expasy.org/compute_pi/) predicted molecular mass and isoelectric point of the enzyme was 43.4 kDa and 6.11, respectively. The

absence of signal peptide indicates that CelXyn2 is an intracellular enzyme. Compared with reported cellulases, the BLAST result classified CelXyn2 as a member of the glycosyl hydrolase family 5 (GH5), and it only had a cellulase domain (69 to 266 AA, Fig. 1A). Besides, the online NetNGlyc 1.0 Server and NetOGlyc 4.0 Server were used for N-linked and O-linked glycosylation site analysis, respectively. They predicted an N-linked glycosylation site (N51) and two O-linked glycosylation sites (T256 and S290). These results indicated that CelXyn2 enzyme could be glycosylated.

The result of NCBI-BLAST revealed that CelXyn2 shared 100% identity with a cellulase gene (Genbank accession: MBR6042486.1) and a high degree of identity with other GH5 members. The CelXyn2 shared 70.87% identity with a *Bacteroidales bacterium* (GenBank: MBQ0029623.1) GH5 family cellulase, 69.37% identity with a *Paludibacteraceae bacterium* (GenBank: MBR4714200.1) GH5 family endoglucanase (Supplementary Table S1). The homology modelling structures of CelXyn2 were obtained by Swiss-Model web tool (<https://swissmodel.expasy.org>). The cellulase 3D structure determination of CelXyn2 is shown in Fig. 1B based on the endoglucanase model 1cec.1.A (PDB: 1CEC) (Dominguez et al., 1995), carrying 32.23% AA sequence identity with the model. The xylanase 3D structure determination of CelXyn2 is shown in Fig. 1C based on the xyloglucanase 2jep.1.A (PDB: 2JEP) (Gloster et al., 2007) with 20.00% AA sequence identity. From the predicted results, Glu182 is the active-site nucleophile and Glu322 acts as the acid-base catalyst, which is consistent with the catalytic mechanism leading to the net retention of configuration at the hetero-carbon in the deep substrate binding crack (McCarter and Withers, 1994). The tertiary

structure of CelXyn2 showed high 3D structural similarity with endoglucanase (1cec.1.A) and xyloglucanase (2jep.1.A). The final production of purified protein was 509 mg/L. The SDS-PAGE analysis of the purified CelXyn2 (Fig. 2A) found a single band which was consistent with the predicted molecular mass 43.4 kDa. In addition, CelXyn2 showed enzymatic activities against CMC or beechwood xylan (Fig. 2B). Hence, biochemical properties of CelXyn2 were further characterized in detail.

3.2. Effect of pH and temperature on enzyme activity

The effect of pH and temperature on enzymatic activity was determined using CMC and beechwood xylan as substrates, respectively. The pH profile of CelXyn2 was determined by testing the enzymatic activity at pH 2.0 to 10.0. As shown in Fig. 3A, the pH profile of CelXyn2 showed the same optimal pH value of 6.0 for both cellulase and xylanase activities, which was similar to some reported bifunctional enzymes such as *Bacillus halodurans* TSLV1 rBhcell-xyl (Rattu et al., 2016) and *Paenibacillus curdlanolyticus* B-6 Xyn10E (Sermathanaswadi et al., 2017). Notably, the enzymatic activity rapidly declined when the pH value was above the optimum. The cellulase activity was measured at 39.37% of its maximum activity at pH 2.0, while 35.46% of xylanase activity was measured under the same condition. Furthermore, CelXyn2 presented 21.88% cellulase activity and 17.17% xylanase activity at pH 10.0, respectively. The pH stability analysis of CelXyn2 was measured by incubating the enzyme for 16 h at 4 °C (Fig. 3B). The enzyme was stable between pH 4.0 and pH 8.0 and retained 96.24%

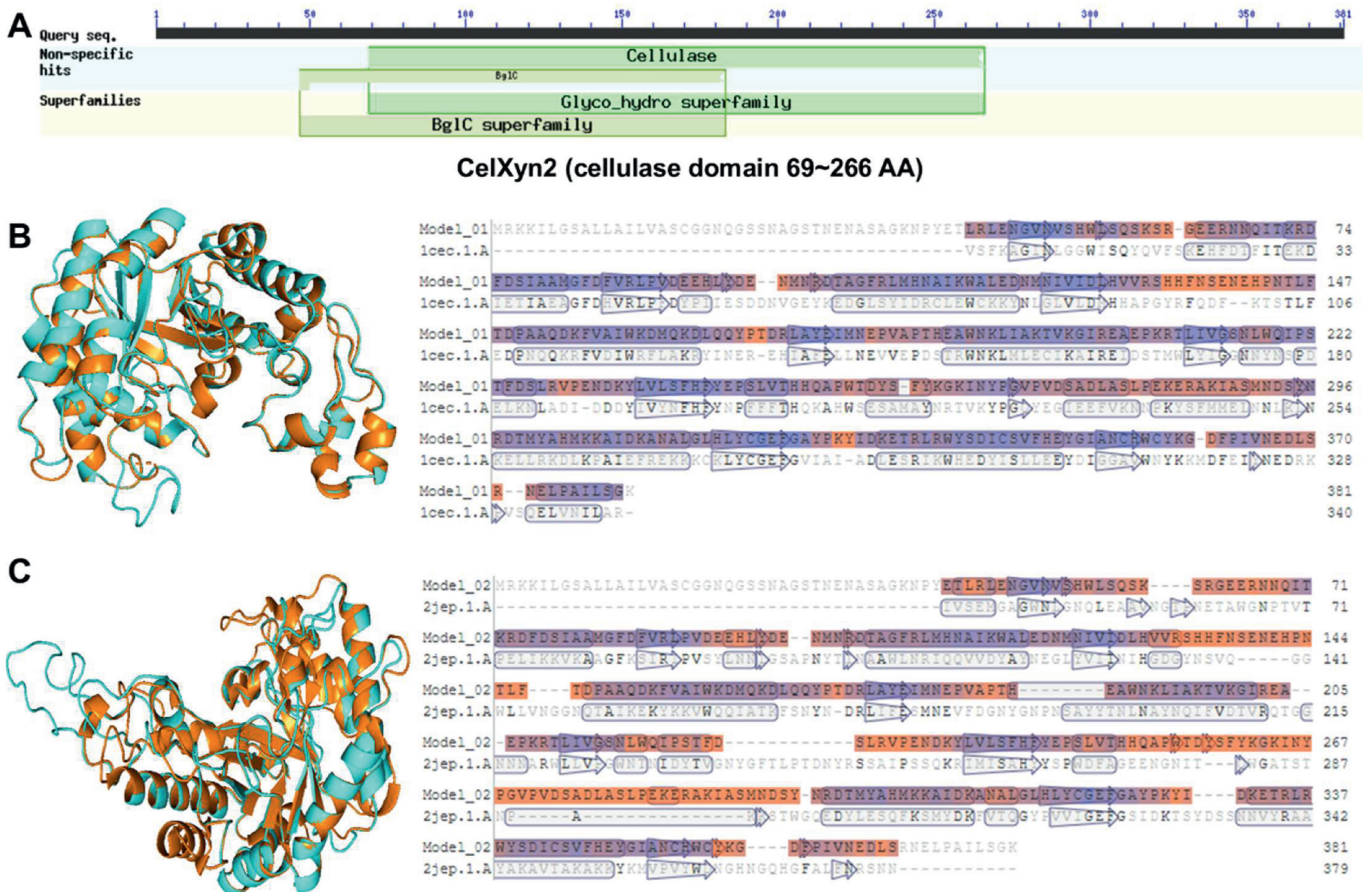


Fig. 1. Structural analysis of CelXyn2. (A) The conserved domain of CelXyn2 using NCBI Conserved Search. Structural superposition of (B) cellulase (cyan) with PDB code 1CEC (orange), and (C) xylanase (cyan) with PDB code 2JEP (orange) using PyMOL program (Bramucci et al., 2012).

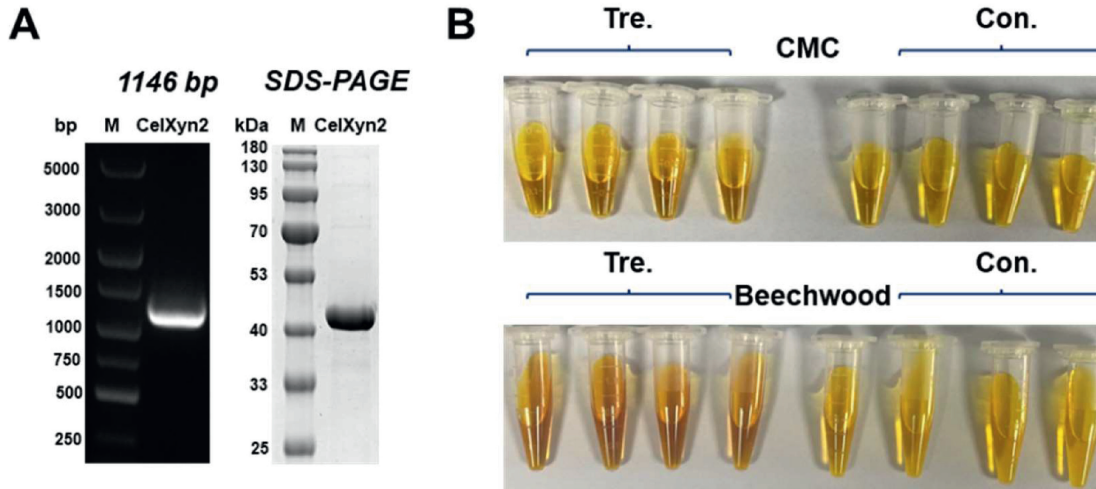


Fig. 2. Analysis of purity and functional activity of CelXyn2. (A) 1.2% agarose gel electrophoresis and 12% SDS-PAGE analysis of gene fragment and purified enzyme. Lanes: 1, DNA or protein marker; 2, The PCR product of the gene *celxyn2* or purified protein CelXyn2. (B) Enzymatic activities tested against carboxymethylcellulose (CMC) and beechwood xylan. Tre.: activated CelXyn2, Con.: inactivated CelXyn2.

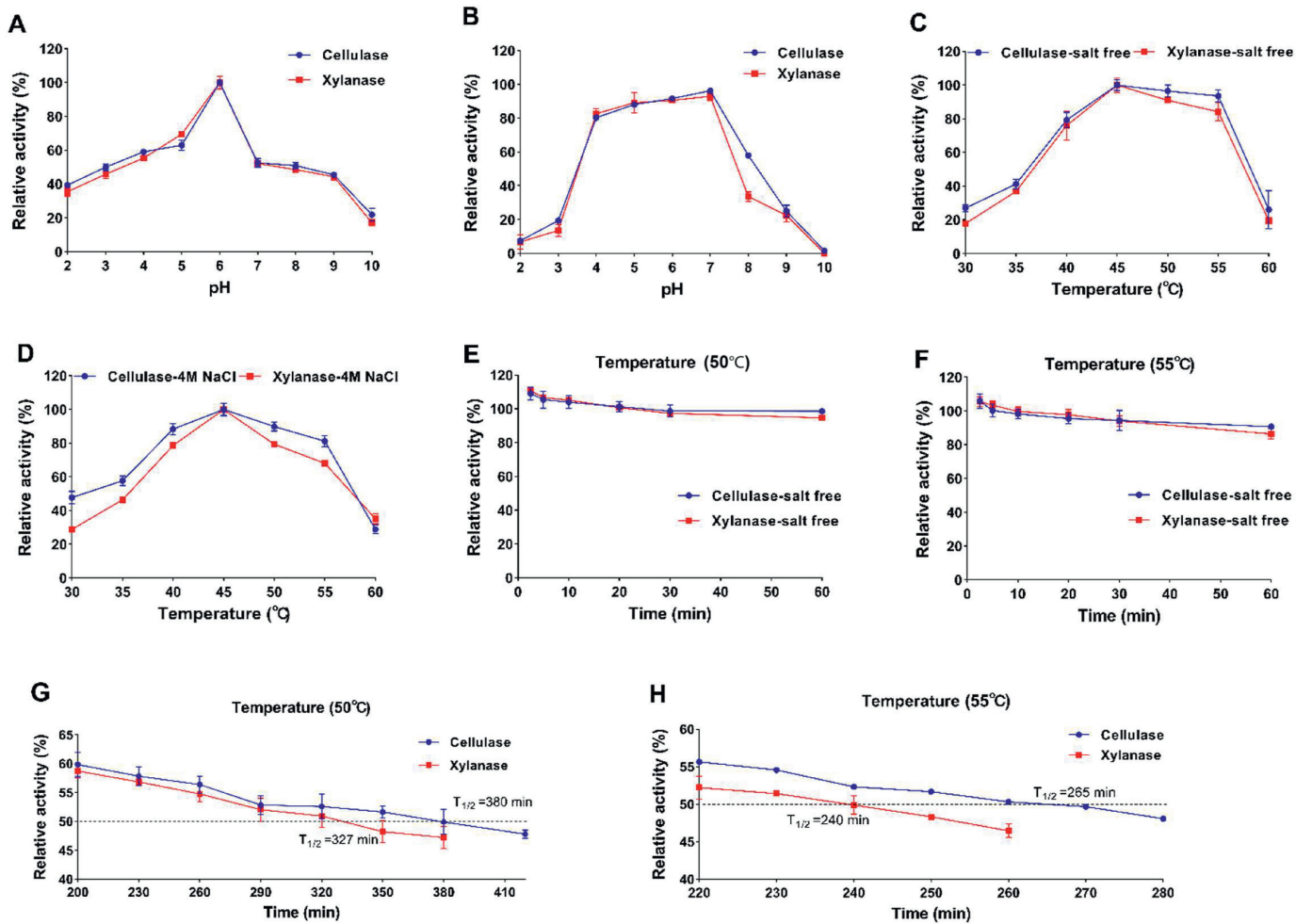


Fig. 3. Effect of pH, temperature and storage on CelXyn2 activity. (A) pH profile of CelXyn2 at pH 2.0 to 10.0. (B) pH stability. Temperature profile of CelXyn2 in (C) 100 mM Citric acid- Na_2HPO_4 buffer (pH 6.0) and (D) 4 M NaCl solution at 30 to 60 °C. Storage stability of CelXyn2 by incubating the protein for 60 min at (E) 50 and (F) 55 °C. The $T_{1/2}$ of CelXyn2 at (G) 50 and (H) 55 °C. Data is shown as mean \pm standard deviation.

cellulase activity and 92.95% xylanase activity. Surprisingly, CelXyn2 even displayed 7.50% cellulase activity and 6.71% xylanase activity at pH 2.0. However, CelXyn2 exhibited 1.58% cellulase activity and lost xylanase activity at pH 10.0. Similar results were discovered where bifunctional enzymes from *Clostridium thermocellum* (Xue et al., 2015) and camel rumen metagenome (Khalili et al., 2017) were stable in acid and neutral conditions.

The temperature profile of CelXyn2 was determined by testing the enzymatic activity at 30 to 60 °C (Fig. 3C). The optimum temperature of both the cellulase and xylanase CelXyn2 was 45 °C. When the enzyme was incubating in 4 M NaCl solution, the optimum temperature of CelXyn2 was not influenced by NaCl (Fig. 3D). Notably, the relative activity was significantly stimulated at temperatures below the optimum, which was similar to a halophilic xylanase XylCMS from camel rumen metagenome. Briefly, the optimum temperature of XylCMS was not affected in 3 M NaCl (Ghadikolaei et al., 2019). The CelXyn2 maintained $\geq 20\%$ of its maximum cellulase and xylanase activities at 60 °C. Accordingly, it retained $>28\%$ of cellulase and xylanase activities under 4 M NaCl solution at 60 °C, which indicated that 4 M NaCl improved the enzymatic activity. The above results demonstrated that the enzyme could be active at high temperature. Similarly, most bifunctional enzymes also reached optimum temperatures around 40 to 70 °C (Adlakha et al., 2011; Chang et al., 2011; Ghatge et al., 2014; Xue et al., 2015). The bifunctional enzyme from *Caldicellulosiruptor bescii* CbXyn10C exhibited maximum activity at 85 °C (Xue et al., 2015) and the bifunctional enzyme cbGH5 of a *Chryseobacterium* strain isolated from straw-fed cattle even had a temperature optimum at 90 °C (Tan et al., 2018). We also mentioned that only one bifunctional enzyme Xyn-CBM-Cel from camel rumen metagenome was most efficiently hydrolyzed at 35 °C (Khalili et al., 2017). Thermostability of CelXyn2 was studied by incubating the enzyme in optimum conditions. The CelXyn2's cellulase and xylanase activities remained at over 94% after incubation at 50 °C for 60 min (Fig. 3E). Furthermore, after treatment for 60 min at 55 °C, it retained 90.60% and 86.36% of its initial activity, respectively (Fig. 3F). The $T_{1/2}$ of the enzyme at 50 and 55 °C was further determined. The $T_{1/2}$ of the cellulase activity was about 380 and 265 min at 50 and 55 °C, respectively (Fig. 3G and H). Accordingly, these values were about 327 min (at 50 °C) and 240 min (at 55 °C) for xylanase activity (Fig. 3G and H). However, most bifunctional enzymes have not had their $T_{1/2}$ determined. The thermostability of CelXyn2 is comparable with a bifunctional enzyme PersiCelXyn1 from cow rumen metagenome, which had a $T_{1/2}$ of cellulase and xylanase activities at 50 °C for more than 407 and 577 min, respectively (Ariaeenejad et al., 2021). Moreover, the

thermostability of CelXyn2 xylanase activity is higher than that of some published xylanases (Bai et al., 2012; Zhou et al., 2012), which have shorter $T_{1/2}$ when incubated at 40 or 45 °C. However, a thermostable xylanase rXynAHJ14 from a xylanolytic *Bacillus* strain displayed a longer $T_{1/2}$ at 70 °C. The CelXyn2 was active and stable under slightly higher temperatures, acidic and neutral environments and long storage. These results suggested that CelXyn2 might be a potential candidate enzyme for various biotechnological processes such as lignocellulosic biomass degradation.

3.3. Effect of metal ions and chemicals on enzyme activity

The effect of metal ions on CelXyn2 activity was assessed by calculating the relative cellulase and xylanase activities. A negative effect of K^+ , Cu^{2+} , Zn^{2+} , Fe^{3+} and Ba^{2+} on CelXyn2 activity was observed (Fig. 4), which was similar to some reported bifunctional enzymes (Chen et al., 2018; He et al., 2019; Hua et al., 2018; Kim et al., 2015; Tan et al., 2018). The xylanase activity of bifunctional enzymes was slightly inhibited by K^+ (Chen et al., 2018; Kim et al., 2015). A novel thermostable GH7 endoglucanase from *Chaetomium thermophilum* was sensitive to Cu^{2+} and Fe^{3+} (He et al., 2019). Generally, Cu^{2+} can inhibit many cellulases or xylanase (Li et al., 2018b; Verma and Satyanarayana, 2012) because of the formation of intermolecular and intramolecular disulfide bonds derived from auto-oxidation of proximal cysteine residues (Vieille and Zeikus, 2001). The activity of CelXyn2 changed slightly with the addition of Zn^{2+} (Ariaeenejad et al., 2021). The activity of cbGH5 could be slightly lowered by Ba^{2+} (Tan et al., 2018). Results showed that CelXyn2 was enhanced in the presence of Fe^{2+} and Co^{2+} , which demonstrated that Fe^{2+} and Co^{2+} might play a crucial role in stimulating activity (Ariaeenejad et al., 2021; Hua et al., 2018).

The PMSF is an enzyme inhibitor that can react with serine residues to inhibit enzymic activity. As shown in Fig. 4, CelXyn2 activity was inhibited by PMSF, which was consistent with an endoglucanase from *Fomitopsis meliae* CFA 2 (Patel and Shah, 2021) and a xylanase from *Aspergillus versicolor* (Carmona et al., 2005). The CelXyn2 activity was reduced by EDTA, suggesting that metal ions play a crucial role in catalysis of cellulase and xylanase. β -Mercaptoethanol is used to reduce the disulfide bond formed between cysteine, and it has been reported in many studies to improve the activity of glycoside hydrolase (Yang et al., 2015; Zhao et al., 2012). In the present study, the cellulase activity of CelXyn2 was inhibited by adding β -mercaptoethanol (Fig. 4A), while the xylanase activity was improved (Fig. 4B). Anionic detergent, Tween 20 and Tween 80 showed negative effects on the enzymatic activity of CelXyn2. In previous studies, the activities of bifunctional

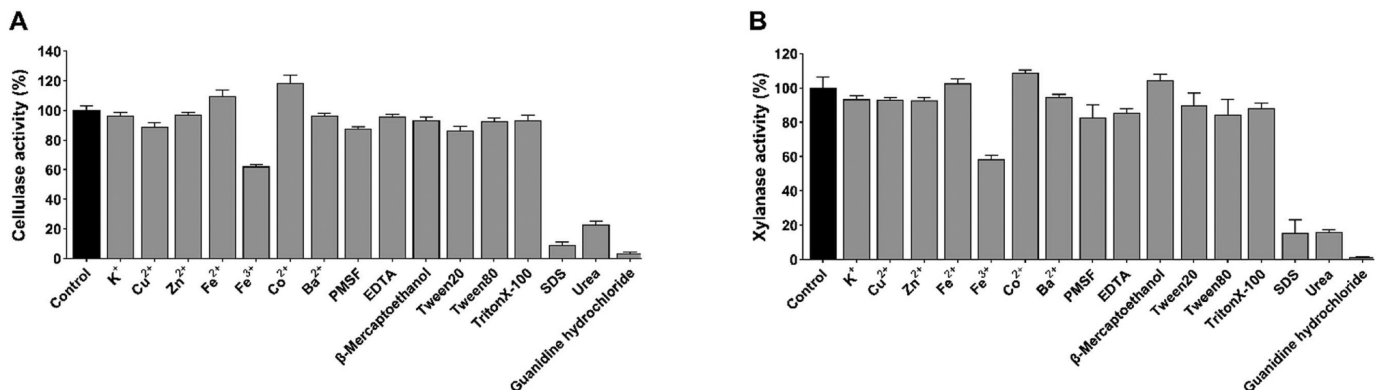


Fig. 4. Effect of metal ions and chemicals on (A) cellulase and (B) xylanase activities of CelXyn2. Data is shown as mean \pm standard deviation. PMSF = phenylmethylsulfonyl fluoride; EDTA = ethylenediaminetetraacetic acid; SDS = sodium dodecyl sulfate.

enzymes EG-M-Xyn (Chen et al., 2018) and PersiCelXyn1 (Ariaenejad et al., 2021) were decreased by adding Tween 20. In addition, Triton X-100 exhibited a decrement in both cellulase and xylanase activities of CelXyn2. The xylanase activity of EG-M-Xyn was slightly suppressed by Triton X-100 (Chen et al., 2018). An inhibitory effect was observed with SDS, which significantly suppressed CelXyn2 activity at a concentration of 1% wt/vol. This indicates that CelXyn2 has a little resistance to surfactants, which may be related to the distribution of AA on its surface. In fact, most bifunctional enzymes were inhibited by SDS (Ariaenejad et al., 2021; Chen et al., 2018; He et al., 2019; Tan et al., 2018). To our surprise, the activity of EG-M-Xyn was significantly enhanced by SDS (Chen et al., 2018). Our results showed that CelXyn2 could not withstand denaturants including urea and guanidine hydrochloride at a concentration of 2 M.

3.4. Effect of salt and artificial seawater on enzyme activity

Salt is probably the most important food additive, and salt-tolerant enzymes could be used in various food-production processes. Salt-tolerant enzymes involved in fermentation processing could reduce costs because of unnecessary sterilization. The effect of salt on the activity of CelXyn2 was performed in the presence of different concentrations of NaCl at optimum pH and temperature. NaCl concentrations in the range of 1 to 5 M were shown to stimulate cellulase and xylanase activities at 45 °C (Fig. 5A and B). The highest cellulase and xylanase activity with 15.84% and 19.65% enhancement compared with the control was obtained at 4 M NaCl, respectively. All NaCl concentrations improved the cellulase activity at 55 °C, but the xylanase activity was slightly suppressed by 5 M NaCl (Fig. 5A and B). When CelXyn2 was incubated with 4 M NaCl at 55 °C, the enzyme activity was lower than that at 45 °C (Fig. 5A and B). After 60 min incubation at 45 °C with 4 M NaCl, the CelXyn2 retained 102.49% cellulase activity and 97.37% xylanase activity (Fig. 5C). The analysis of thermostability in the presence of 4 M NaCl suggested that salt had a destabilizing effect on CelXyn2 at 55 °C

(Fig. 5D). As shown in Fig. 5D, CelXyn2 activity began to rapidly decline after 5 min incubation at 55 °C and reached $\geq 58\%$ of initial activity during 60 min. To investigate the tolerance to seawater, CelXyn2 was incubated with artificial seawater, and Fig. 5E showed that CelXyn2 retained approximately 96.4% and 71.3% of initial cellulase activity at 45 and 55 °C, respectively. Accordingly, CelXyn2 maintained approximately 89.5% and 65.9% of initial xylanase activity at 45 and 55 °C, respectively (Fig. 5F). Regardless of temperature influence, CelXyn2 activity in artificial seawater was lower than that in 4 M NaCl and salt-free control conditions, which suggested that CelXyn2 was unstable in artificial seawater. To the best of our knowledge, the only salt-tolerant bifunctional enzyme reported is Cel5 from *Hahella chejuensis* KCTC 2396 (Ghatge et al., 2014). The cellulase and xylanase activities of Cel5 were not inhibited by 5 M NaCl, and small increase in activity was detected in 0.1 to 5 M NaCl solution (Ghatge et al., 2014). Based on the above results, CelXyn2 was proven to be a halophilic enzyme with elevated cellulase and xylanase activities in high NaCl concentrations, especially 4 M, which indicated that CelXyn2 could be applied in high-salt conditions. Notably, CelXyn2 is the first halophilic bifunctional enzyme characterized from buffalo rumen microbial metagenome.

3.5. Substrate specificity and kinetic parameters

The purified recombinant CelXyn2 was also assayed to evaluate the degradation capability of various substrates under optimal reaction conditions (45 °C, pH 6.0). All the tested substrates were efficiently degraded by CelXyn2 except for α -pNPG and β -pNPG, which suggested that CelXyn2 does not have β -glucosidase activity and cannot hydrolyze the bonds of pNPG to produce glucose (Table 1). CelXyn2 presented the highest cellulase activity towards lichenan (906.26 ± 21.90 U/mg) followed by β -glucan (244.74 ± 3.59 U/mg) and CMC (205.03 ± 5.42 U/mg), and the highest xylanase activity against beechwood xylan (274.40 ± 6.39 U/mg) followed by xyloglucan (72.33 ± 2.68 U/mg) and sugarcane

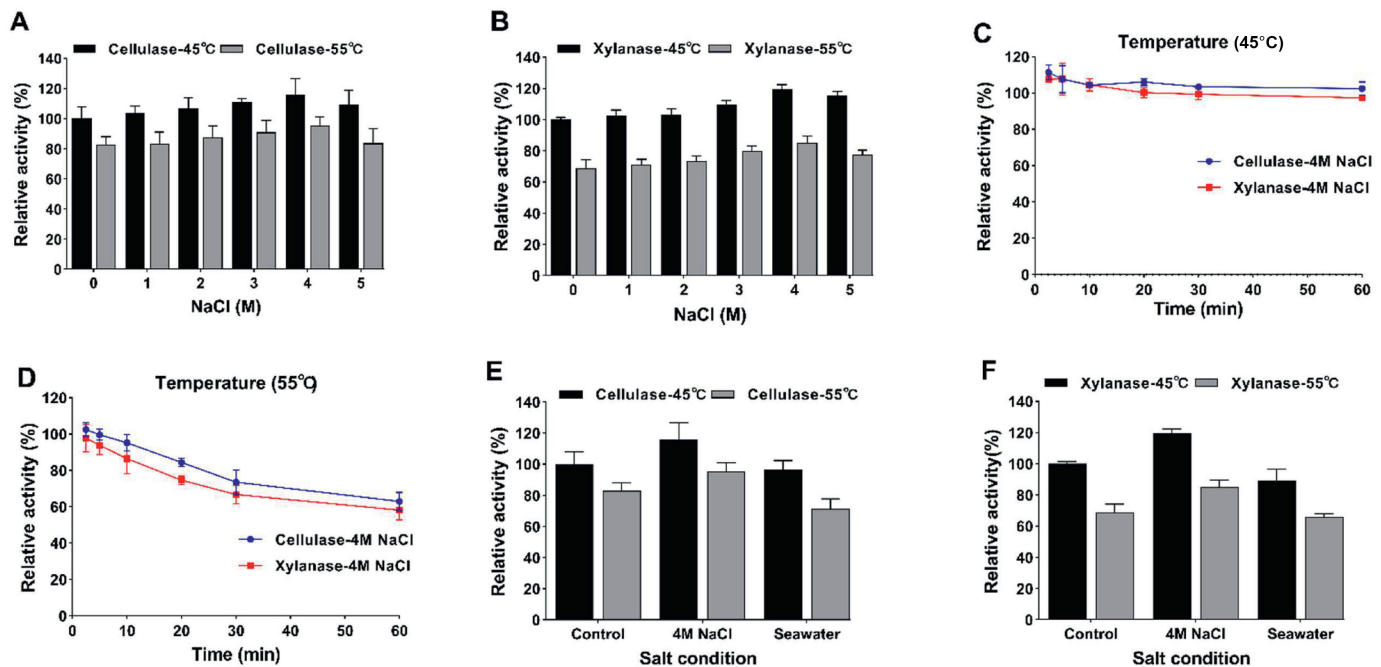


Fig. 5. Impact of salt on CelXyn2 activity. Effect of different concentrations (1 to 5 M) of NaCl solution on (A) cellulase and (B) xylanase activities of CelXyn2. Thermostability of CelXyn2 under the influence of 4 M NaCl during 60 min incubation at (C) 45 °C and (D) 55 °C. The (E) cellulase and (F) xylanase activities of CelXyn2 in artificial seawater compared with that in 4 M NaCl and salt-free control conditions. The CelXyn2 activity at 45 °C in a salt-free buffer was set as 100%. Data are shown as mean \pm standard deviation.

Table 1
Substrate spectrum of CelXyn2 against various substrates.

Substrates	Main linkage type	Specific activity, U/mg
CMC	(β -1,4) glucan	205.03 \pm 5.42
Beechwood xylan	(β -1,4) glucan	274.40 \pm 6.39
Oat spelt xylan	(β -1,4) glucan	16.72 \pm 0.28
Sugarcane xylan	(β -1,4) glucan	55.59 \pm 1.89
Wheat arabinoxylan	(β -1,4) glucan	17.48 \pm 0.60
β -Glucan	(β -1,3) and (β -1,4) glucan	244.74 \pm 3.59
Filter paper	(β -1,4) glucan	13.03 \pm 0.98
Locust bean gum	(β -1,4) glucan	7.05 \pm 0.48
Avicel	(β -1,4) glucan	3.85 \pm 0.14
Lichenan	(β -1,3) and (β -1,4) glucan	906.26 \pm 21.90
Laminarin	(β -1,4) glucan	14.82 \pm 0.54
Xyloglucan	(β -1,4) glucan	72.33 \pm 2.68
α -pNPG	–	ND
β -pNPG	–	ND

CMC = carboxymethylcellulose; α -pNPG = α -p-Nitrophenyl- β -D-glucopyranoside; β -pNPG = β -p-Nitrophenyl- β -D-glucopyranoside; ND = not detected, indicating no enzyme activity.

Data is shown as mean \pm standard deviation.

xylan (55.59 \pm 1.89 U/mg). These results indicated that CelXyn2 has the ability to hydrolyze cellulosic substrates with β -1,3/4 glucan and hemi-cellulosic substrates with β -1,4 glucan. We mentioned that CelXyn2 exhibited higher enzyme activity against β -glucan (244.74 \pm 3.59 U/mg) than CMC (205.03 \pm 5.42 U/mg). Because CMC contains high methoxy substituents within side chains, which could hinder the enzyme activity towards substrates. The CelXyn2 showed relatively higher enzymatic activity against filter paper due to its bifunctional property. Purified CelXyn2 also showed the ability to hydrolyze crystalline cellulosic materials like Avicel.

Substrate specificity of CelXyn2 compared with published bifunctional enzymes, including single enzymes and fusion enzyme, is published in Table 2. The PersiCelXyn1 from cow rumen metagenome showed the highest specific activity towards CMC (5,412.77 U/mg) and beechwood xylan (7,845.33 U/mg) (Ariaenejad et al., 2021) followed by cbGH5 from a new *Chryseobacterium* strain (Tan et al., 2018). Notably, the specific activity of CelXyn2 was similar to Xyl10g-GS-Cel5B, a fusion protein with a glycine–serine linker, which also showed moderate cellulase and xylanase activities (Kim et al., 2015). The kinetic parameters of CelXyn2 were determined with CMC and Beechwood xylan. The results are shown in Table 3. CelXyn2 exhibited V_{max} values of

Table 3
The kinetic parameters of CelXyn2.

Substrate type	Kinetic properties	
	V_{max} , U/mg	K_m , mg/mL
CMC	378.92 \pm 39.82	9.25 \pm 1.43
Beechwood xylan	395.68 \pm 19.15	12.62 \pm 0.80

CMC = carboxymethylcellulose.

378.92 \pm 39.82 U/mg and 395.68 \pm 19.15 U/mg against CMC and beechwood xylan respectively, indicating high catalytic efficiency for beechwood xylan. Accordingly, CelXyn2 presented K_m values of 9.25 \pm 1.43 mg/mL and 12.62 \pm 0.80 mg/mL against CMC and beechwood xylan, respectively, which suggested a high affinity for the β -1,4-D-glucan and β -1,4-D-xylan substrates (Hua et al., 2018). The K_m value towards CMC of CelXyn2 was consistent with the K_m value (9.8 \pm 0.7 mg/mL) of Xyn-CBM-Cel (Khalili et al., 2017). The K_m value against β -1,4-D-xylan of CelXyn2 was also consistent with the K_m value (10.28 \pm 0.82 mg/mL) of CTendo7 from *C. thermophilum* against β -1,4-D-xylan substrate (Hua et al., 2018).

3.6. Enzymatic hydrolysis of lignocellulosic biomass

Agricultural residues are major lignocellulosic wastes in the world has and they have the potential to be valuable feedstuffs for ruminants. However, the cross-linked structure of cellulose, hemicellulose and lignin leads to a recalcitrant three-dimensional structure, which prevents breakdown by ruminal microbiota and enzymes. To improve the ruminal digestibility of lignocellulosic fiber, enzymatic pretreatment is used to enhance fiber structural disruption (Li et al., 2018a; Zhang et al., 2020). Before the enzyme is applied in livestock as a feedstuff additive, the cellulase/xylanase activity of CelXyn2 towards agricultural residues should be firstly evaluated.

As shown in Fig. 6, all lignocellulosic substrates were hydrolyzed by CelXyn2, and the highest activity was found in sugar beet pulp. At 24 h, 3.0 mM reducing sugar was produced from sugar beet pulp that increased to 6.2 mM at 168 h. A total of 1.5 μ mol/mL reducing sugar was generated from wheat straw at 24 h, which reached 3.2 mM after 168 h incubation, which was similar to rice straw and sheepgrass. These results demonstrated that CelXyn2 can degrade natural agricultural residues. Efficient hydrolysis of these

Table 2
Property comparison of CelXyn2 with some reported bifunctional cellulase-xylanase enzymes.

Protein	Mw, kDa	Optimal pH and TM, °C	Cellulase activity		xylanase activity		References
			Specific activity, U/mg	K_m , mg/mL	Specific activity, U/mg	K_m , mg/mL	
RuCelA	60.6	5/50, 7/65	54.3	–	264.1	–	Chang et al. (2011)
Endo5A-GS-Xyl11D	61	7/50, 6/50	16.3 \pm 0.02	–	13.7 \pm 0.46	–	Adlakha et al. (2011)
Cel5	60	6.5/65, 5.5/55	77.19 \pm 5.01	1.8	7.52 \pm 0.60	7.1	Ghatge et al. (2014)
CbXyn10C		6.5/85	39 \pm 3.3	4.3 \pm 0.0	39,000 \pm 160	0.3 \pm 0.1	Xue et al. (2015)
Xyl10g-GS-Cel5B	97	4/60, 6/70	193.92 \pm 4.14	13.38	252.86 \pm 2.40	35.16	Kim et al. (2015)
Cel5A-XylT	80	6/80, 6/70	–	–	298 \pm 9.6	–	Rizk et al. (2015)
rCtCel5E		5/50, 6/60	736.2 \pm 12.8	2.1 \pm 0.2	351.6 \pm 2.9	4.6 \pm 0.2	Yuan et al. (2015)
rBhcell-xyl	62	6/60	16.88	3.8	6.7	3.2	Rattu et al. (2016)
cel28a	57	5/50	20.56	0.144	12.11	2.75	Cheng et al. (2016)
Xyn-CBM-Cel	92.7	5/35	1,979 \pm 35	9.8 \pm 0.7	1,979 \pm 35	9.8 \pm 0.7	Khalili et al. (2017)
Xyn10E	90	6/50	1.1 \pm 0.1	–	1,487.1 \pm 16.4	10.4	Sermathanaswadi et al. (2017)
CTendo7	47.0	5/55	1.91 \pm 0.14	79.19 \pm 5.76	3.05 \pm 0.11	10.28 \pm 0.82	Hua et al. (2018)
EG-M-Xyn	75	3.5/50, 7/70	491.0	6.8 \pm 0.7	1585.2	60.6 \pm 3.1	Chen et al. (2018)
KG51	45.9	5/40, 5/50	3.79	–	2.63	–	Lee et al. (2018)
cbGH5	66	9/90, 8/90	3,237.15 \pm 160.23	1.80 \pm 0.03	1,792.83 \pm 115.65	4.52 \pm 0.11	Tan et al. (2018)
PersiCelXyn1	51	5/50	5,412.77	0.1	7,845.33	0.15	Ariaenejad et al. (2021)
CelXyn2	43.5	6/45	205.03 \pm 5.42	9.25 \pm 1.43	274.40 \pm 6.39	12.62 \pm 0.80	This study

Mw = molecular mass; TM = temperature; K_m = Michaelis constant.

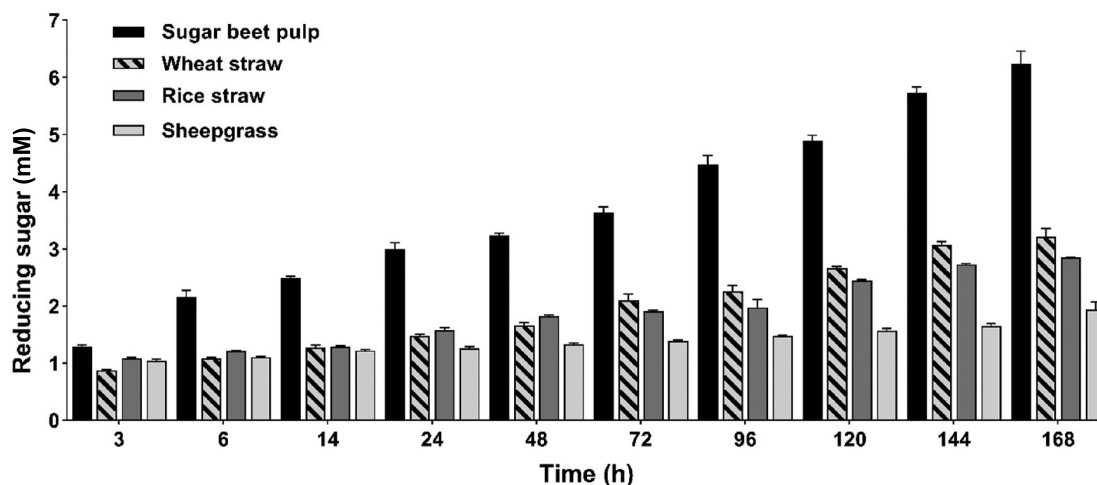


Fig. 6. Hydrolysis of sugar beet pulp, wheat straw, rice straw and sheepgrass by CelXyn2 after 168 h at 45 °C. Data is shown as mean \pm standard deviation.

lignocellulosic materials by enzymes was reported in previous studies (Ariaenejad et al., 2021; Zhang et al., 2020). It was noted that CelXyn2 hydrolyzed sugar beet pulp more easily than rice straw, wheat straw and sheepgrass, which may due to the lower lignin content of sugar beet pulp. Enzymatic hydrolysis could be affected by the high lignin content of lignocellulosic biomass (Ioelovich and Morag, 2012). The results reveal that CelXyn2 might be an excellent candidate for hydrolysis of agricultural residues.

3.7. In vitro ruminal fermentation

The effect of CelXyn2 on in vitro ruminal fermentation was investigated (Table 4). After 48-h incubation, supplementing CelXyn2 did not significantly affect the ruminal pH value, averaging 6.67 (data not shown). CelXyn2 increased total gas of rice straw, wheat straw and sheepgrass by 7.5%, 10.5% and 8.8%, respectively, compared with controls ($P < 0.05$). Compared with the controls, the acetate concentration was greater in CelXyn2-added rice straw and wheat straw ($P < 0.05$), which resulted in higher total VFA concentration ($P < 0.05$). With sheepgrass, CelXyn2 did not affect the acetate concentration ($P > 0.05$). Across all of the treatment groups, CelXyn2 did not significantly affect the concentrations of propionate and butyrate ($P > 0.05$). Additionally, the acetate to propionate ratio was not affected by CelXyn2 ($P > 0.05$). Interestingly, the concentration of other VFA was increased further by CelXyn2 in the treatment groups ($P < 0.05$). An increase in lactate concentration was found for rice straw and wheat straw ($P < 0.05$). The $\text{NH}_3\text{-N}$

concentration and MCP synthesis were not affected during the fermentation of all three agricultural residues with the enzyme added ($P > 0.05$).

Current results showed that CelXyn2 significantly improved the hydrolysis of all agricultural residues, which enhanced total gas and VFA production during in vitro ruminal fermentation. To the best of our knowledge, few studies about the effect of bifunctional enzymes on agricultural residues have been investigated. However, the recombinant xylanase rLeXyn11A from *Lentinula edodes* was found to improve hydrolysis and in vitro ruminal fermentation of rice straw, wheat straw, corn straw (Li et al., 2018a) and soybean straw (Zhang et al., 2020). Briefly, rLeXyn11A improved VFA, DM and NDF digestibility, and MCP synthesis, which was partially in agreement with our results. The rLeXyn11A enzyme was found to reduce ammonia-N concentration and increase MCP synthesis due to fiber digestion by xylanase (Zhang et al., 2020). However, in the present study, CelXyn2 did not affect $\text{NH}_3\text{-N}$ concentration and MCP synthesis, which was consistent with a previous study (Vallejo et al., 2016). Differences in enzyme properties, fermentation substrates and application amount may have resulted in this discrepancy (Vallejo et al., 2016).

3.8. In vitro ruminal microbial community

Based on 16S rRNA high-throughput sequencing, the samples generated 1,324,094 filtered sequences in total (Supplementary Table S2). All of the filtered sequences were further denoised and

Table 4
Effect of CelXyn2 on in vitro ruminal fermentation.

Item	Rice straw			Wheat straw			Sheepgrass		
	Control	CelXyn2	P-value	Control	CelXyn2	P-value	Control	CelXyn2	P-value
Total gas, mL	102.15 \pm 5.29	109.77 \pm 5.42	0.034	80.65 \pm 2.23	89.15 \pm 2.57	<0.001	87.87 \pm 2.31	95.58 \pm 4.02	0.004
Total VFA, mM	52.55 \pm 1.67	56.92 \pm 1.53	0.029	47.03 \pm 2.64	53.65 \pm 2.00	0.026	50.35 \pm 0.59	54.31 \pm 2.27	0.043
pH	6.61 \pm 0.01	6.60 \pm 0.01	0.116	6.71 \pm 0.03	6.68 \pm 0.02	0.317	6.70 \pm 0.01	6.69 \pm 0.02	0.152
Acetate, mM	35.7 \pm 1.03	38.80 \pm 1.00	0.020	30.73 \pm 1.51	35.39 \pm 1.14	0.013	33.58 \pm 0.52	36.50 \pm 1.94	0.112
Propionate, mM	10.62 \pm 0.37	11.25 \pm 0.40	0.112	9.80 \pm 0.70	10.98 \pm 0.52	0.078	9.78 \pm 0.15	10.32 \pm 0.35	0.071
Acetate to propionate ratio	3.36 \pm 0.04	3.45 \pm 0.04	0.083	3.14 \pm 0.07	3.22 \pm 0.05	0.157	3.43 \pm 0.08	3.54 \pm 0.09	0.215
Butyrate, mM	4.21 \pm 0.18	4.49 \pm 0.16	0.107	4.13 \pm 0.27	4.50 \pm 0.21	0.138	4.47 \pm 0.06	4.68 \pm 0.18	0.125
Other VFA, mM	2.03 \pm 0.11	2.37 \pm 0.07	0.010	2.36 \pm 0.17	2.78 \pm 0.13	0.030	2.52 \pm 0.08	2.81 \pm 0.13	0.031
Ammonia-N, mM	5.15 \pm 1.56	5.42 \pm 0.56	0.793	5.21 \pm 0.72	6.71 \pm 1.12	0.123	6.33 \pm 1.13	7.13 \pm 0.48	0.320
MCP, $\mu\text{g/mL}$	170.26 \pm 24.61	215.03 \pm 37.65	0.160	149.35 \pm 10.32	174.70 \pm 17.83	0.100	157.50 \pm 15.04	208.47 \pm 47.59	0.152
Lactate, mM	0.61 \pm 0.09	0.45 \pm 0.12	0.005	0.27 \pm 0.02	0.39 \pm 0.11	0.010	0.13 \pm 0.02	0.12 \pm 0.01	0.157

VFA = volatile fatty acids (acetic, propionic, butyric, valeric, iso-butyric, 2-me-butyric and iso-valeric); Other VFA = valeric, iso-butyric, 2-me-butyric and iso-valeric; MCP = microbial protein.

Values are represented as the mean \pm standard deviation.

merged, which then generated 1,066,269 non-chimeric sequences (Supplementary Table S2). These sequences were then assigned a total of 905 OTUs with 99% sequence identity. As shown in Supplementary Table S3, the α -diversity was not significantly affected by adding CelXyn2 ($P > 0.05$). Principal coordinate analysis (PCoA) was conducted based on the Bray–Curtis metric to reflect dissimilarities in microbial composition between the control and CelXyn2 groups (Fig. 7). The PCoA result showed that microbes clustered separately between control and CelXyn2 and axes accounted for 90.97% of the total variation. We also mentioned that the samples with different control substrates were also clearly distinguished. The total bacterial OTUs were classified into 18 phyla and 189 genera by QIIME2 based on the Silva taxonomic database. Bacteroidetes (66.16% to 70.19%) and Firmicutes (21.18% to 24.91%) were the dominant bacterial phyla in all samples (Fig. 8A), which was consistent with previous findings (de Oliveira et al., 2013). At genus level, *Bacteroidales_F082* (16.40% to 29.94%), *unclassified_Prevotellaceae* (11.89% to 24.49%) and *Prevotella* (6.95% to 15.87%) were the dominant bacteria (Fig. 8B). To discover the taxon distributions in different groups, STAMP analysis (Parks et al., 2014) was carried out and differentially abundant taxa were present in both CelXyn2 and control groups. At genus level, there were 15, 19 and 13 differentially abundant taxa found for rice straw, wheat straw and sheepgrass, respectively (Fig. 9). For the rice straw group (Fig. 9A), 8 taxa were increased by CelXyn2, and the highest relative abundance was *unclassified_Selenomonadaceae*; 7 taxa were

reduced by CelXyn2, among which *Christensenellaceae_R-7_group* was the predominant bacteria. For the wheat straw group (Fig. 9B), 3 taxa were increased by CelXyn2, and the highest relative abundance was also *unclassified_Selenomonadaceae*; 16 taxa were reduced by CelXyn2, among which *Anaeroplasma* was the predominant bacteria. For the sheepgrass group (Fig. 9C), 9 taxa were increased by CelXyn2, and the highest relative abundance was *WCHB1-41*; 4 taxa were reduced by CelXyn2, among which *Prevotella* was the predominant bacteria. The count of total bacteria was determined using real-time PCR (Fig. 10A). CelXyn2 significantly affected the 16S rRNA copy numbers of total bacteria in the rice straw group. However, the copy numbers of total bacteria were not affected by CelXyn2 in wheat straw and sheepgrass. In addition, *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* were representative fibrolytic bacteria species in rumen, which were not significantly affected by CelXyn2 (Fig. 10B–D). As we mentioned above, the greater acetate concentrations with CelXyn2 addition might be due to the growth of microbial bacteria, especially fiber-colonizing bacteria, resulting in a shift in metabolic pathways (Almaraz et al., 2010). However, in the present study, CelXyn2 only significantly stimulated the copy numbers of total bacteria in the rice straw group. Besides, the relative abundances of the main types of fibrolytic bacteria were not affected by CelXyn2. The inconsistent microbial results might be a result of enzyme type, activity, dose and stability, and the amount of enzyme added.

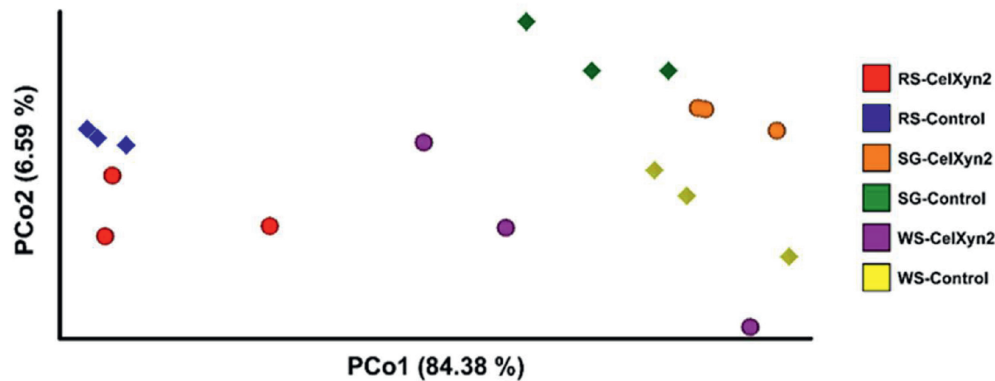


Fig. 7. The PCoA plot of samples from in vitro ruminal incubation of rice straw, wheat straw and sheepgrass based on Bray–Curtis distances. The PCoA analysis was performed using the OTUs obtained. PCoA = principal coordinate analysis; RS = rice straw; WS = wheat straw; SG = sheepgrass.

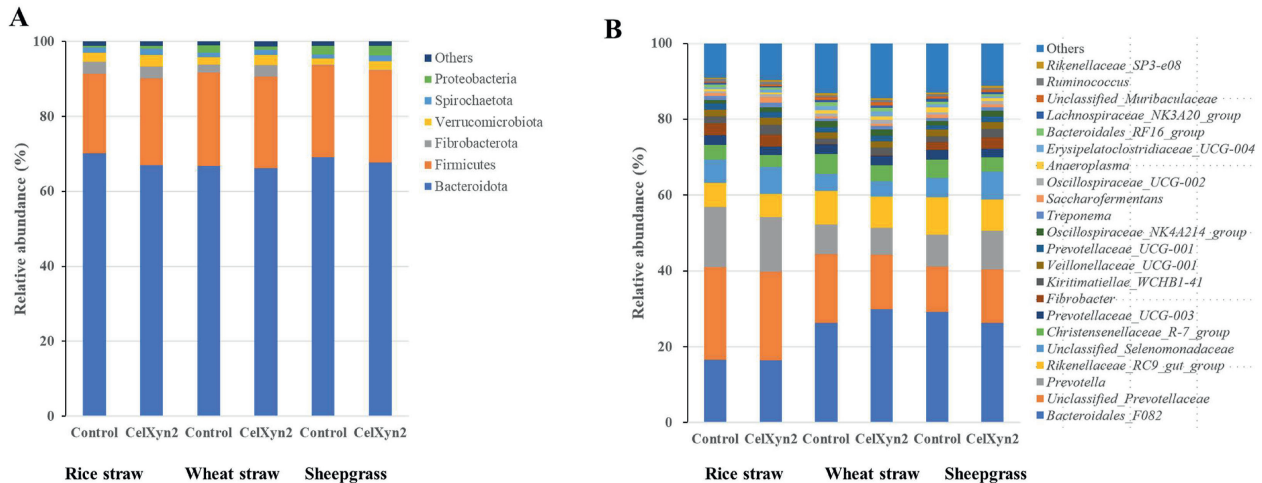


Fig. 8. Distribution of the predominant rumen bacteria at (A) phylum level and (B) genus level.

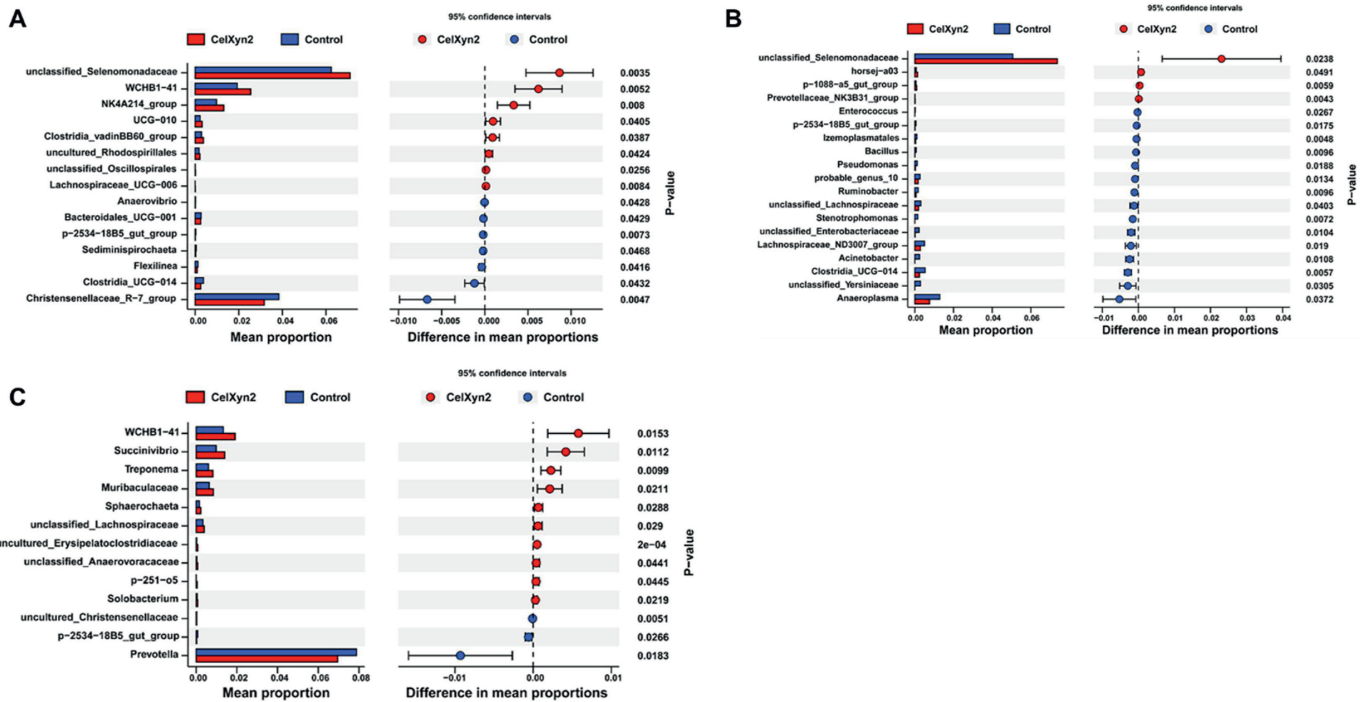


Fig. 9. The STAMP analysis displaying the ruminal bacteria change between the control group and CelXyn2 group in vitro ruminal incubation of (A) rice straw, (B) wheat straw and (C) sheepgrass.

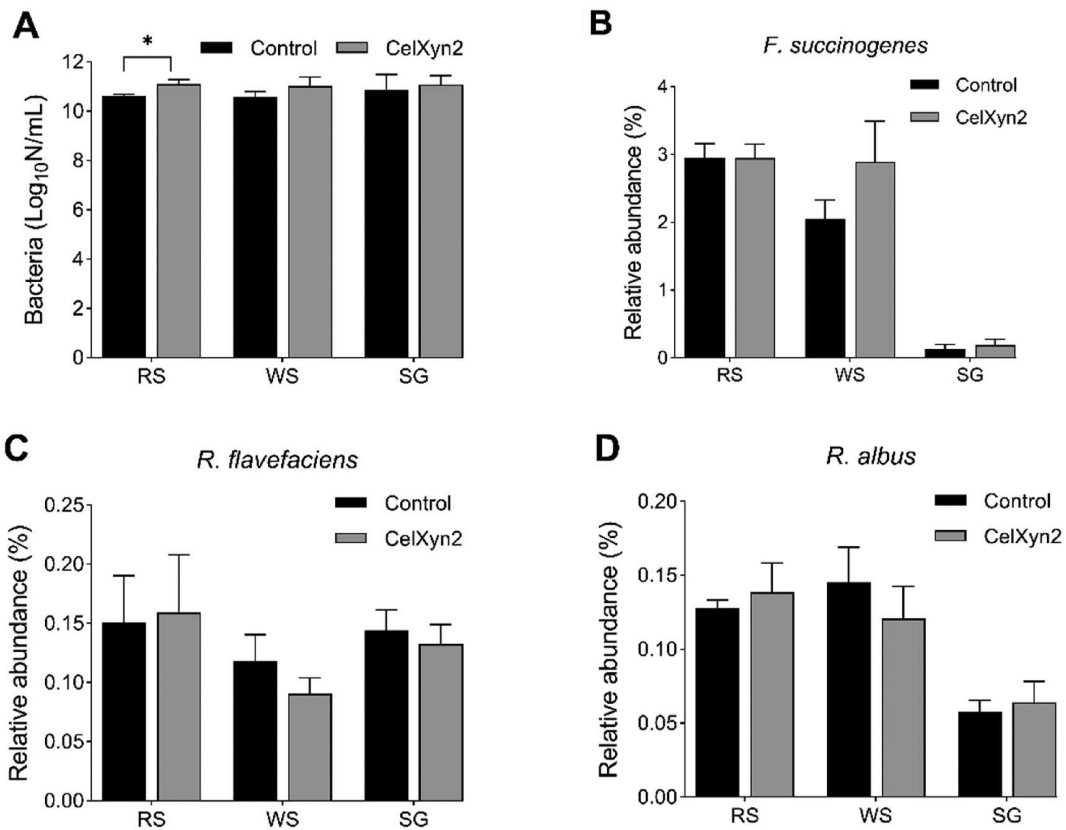


Fig. 10. Analysis of microbial community. (A) Effect of CelXyn2 on 16S rRNA gene copy numbers of total bacteria. The relative abundances of (B) *F. succinogenes*, (C) *R. flavefaciens* and (D) *R. albus*. RS = rice straw; WS = wheat straw; SG = sheepgrass. (* 0.01 < P < 0.05).

4. Conclusions

This study provides an initial insight into the heterologous protein expression and biochemical characterization of a bifunctional cellulase-xylanase enzyme CelXyn2 from buffalo rumen metagenome. This enzyme was found to successfully hydrolyze rice straw, wheat straw, sheepgrass and sugar beet pulp and improve *in vitro* ruminal fermentation of rice straw, wheat straw and sheepgrass. Thus, CelXyn2 may have the potential to be a feedstuff additive to improve agricultural residue utilization in ruminants.

Author contributions

Zhenxiang Meng designed the study. **Jing Ma** and **Zhanying Sun** were involved in the development of the study and the analysis of the samples. **Zhenxiang Meng** performed the statistical analysis of the data. **Zhenxiang Meng** wrote the first draft of the manuscript. **Chengjian Yang**, **Jing Leng**, **Yanfen Cheng** and **Weiyun Zhu** critically revised the manuscript and gave final approval of the document before submission.

Data availability statement

The metagenomic data was submitted to the NCBI as BioProject: PRJNA815894. The sequence of CelXyn2 in this study is available from the NCBI database under accession number MBR6042486.1.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2023.01.004>.

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