CrossMark

BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Development of a bifunctional xylanase-cellulase chimera with enhanced activity on rice and barley straws using a modular xylanase and an endoglucanase procured from camel rumen metagenome

Kamran Khalili Ghadikolaei[1] · Kambiz Akbari Noghabi[1] · Hossein Shahbani Zahiri[1]

Abstract The camel rumen metagenome is an untapped source of glycoside hydrolases. In this study, novel genes encoding for a modular xylanase (XylC) and a cellulase (CelC) were isolated from a camel rumen metagenome and expressed in *Escherichia coli* BL21 (DE3). XylC with xylanase (Xyn), CBM, and carbohydrate esterase (CE) domains was characterized as a β-1,4-endoxylanase with remarkable catalytic activity on oat-spelt xylan ($K_{cat} = 2919 \pm 57 \text{ s}^{-1}$). The implication of XylC's modular structure in its high catalytic activity was analyzed by truncation and fusion construction with CelC. The resulting fusions including Cel-CBM, Cel-CBM-CE, and Xyn-CBM-Cel showed remarkable enhancement in CMCase activity with K_{cat} values of 742 ± 12, 1289 ± 34.5, and $2799 \pm 51 \text{ s}^{-1}$ compared to CelC with a K_{cat} of $422 \pm 3.5 \text{ s}^{-1}$. It was also shown that the bifunctional Xyn-CBM-Cel with synergistic xylanase/cellulase activities was more efficient than XylC and CelC in hydrolysis of rice and barley straws.

Keywords Xylanase · Cellulase · Fusion protein · Metagenome · Protein engineering

Introduction

Plants as the most abundant renewable biomass resource on the Earth are mainly composed of cellulose and hemicellulose. Therefore, plant materials are considered as prime feedstock candidates for various applications including biofuel production (Anwar et al. 2014; Limayem and Ricke 2012). Cellulose is a linear homopolymer of β-1,4-linked glucose units, while hemicellulose is a short and branched heteromeric mixture of polymers consisting of D-xylose, D-arabinose, D-glucose, D-galactose, and D-mannose. Xylan is the main heteropolymer of hemicellulose accounting for 20 to 30% of the hardwood and herbaceous biomass as well as up to 50% of grass and cereal tissues (Gírio et al. 2010). Xylan consists of a backbone of β-1,4-linked xylose unites with several methylglucuronosyl, α-arabinofuranosyl, and acetyl substitutions (Collins et al. 2005). For many applications of cellulosic materials such as for biofuel production, the enzymatic hydrolysis of plant biomass into fermentable sugars is the major challenge. Complete degradation of cellulose requires three major classes of cellulases including endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (JoBoyce and Walsh 2015; Juturu and Wu 2014; Knowles et al. 1987). Likewise, the degradation of xylan needs cooperation of multiple enzymes including endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) to convert xylan into xylose (Mert et al. 2016). Obviously the endo-acting enzymes, i.e., endo-1,4-β-glucanase and endo-1,4-β-xylanase, are very important for conversion of lignocellulosic materials to fermentable sugars. The enzymes hydrolyzing large polysaccharide molecules to smaller ones provide more substrates for other enzymes involved in complete hydrolysis of cellulosic materials (Van Dyk and Pletschke 2012). The cellulase and xylanase enzymes are of great industrial importance with a variety of applications such as in biobleaching of paper and pulp, bioconversion of lignocellulosic materials such as agricultural wastes to fermentative products like biofuels and beverages, extraction and clarification of juices, and improvement of nutritional value of animal feeds (Gangwar et al. 2014; Golan 2010; Jemli et al. 2016). Bifunctional enzymes, in particular, are of special
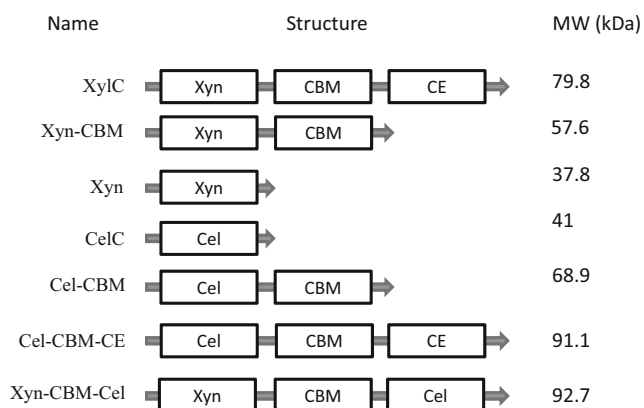
✉ Hossein Shahbani Zahiri
  shahbani@nigeb.ac.ir

[1] Department of Energy and Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran

Springer

interest from both molecular evolution and applied aspects. Such cellulolytic enzymes have been characterized from various sources including metagenomes (Chang et al. 2011; Ghatge et al. 2014; Rashamuse et al. 2013). In addition to the naturally existing bifunctional enzymes, several attempts have been made to engineer synthetic ones (Hong et al. 2006; Rizk et al. 2015). In this study, a camel rumen metagenome was used to discover novel cellulolytic enzymes. In this way, a modular xylanase (XylC) and a single-domain endoglucanase (CelC) were obtained and analyzed to elucidate their biochemical and structural characteristics. The modular XylC was composed of a xylanase (Xyn) domain, a carbohydrate-binding module (CBM), and a carbohydrate esterase (CE) domain. The enzyme was truncated to reveal the impact of its modular structure on the activity and stability of the xylanase domain. In addition, three hybrid fusions including Xyn-CBM-Cel, Cel-CBM, and Cel-CBM-CE were constructed by fusing the XylC's domains to CelC (Fig. 1). Subsequently, the wild type, truncated, and fusion enzymes were analyzed for kinetics and biochemical characteristics. This study provides insights into the engineering of novel glycoside hydrolases with improved catalytic activity for conversion of plant biomass to fermentable sugars.

Materials and methods

Bioinformatics analysis

The homology search was conducted using BlastX program at NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The signal peptide was predicted using SignalP program (http://www.cbs.dtu.dk/services/SignalP/). The protein structural features were analyzed against the PROSITE (http://prosite.expasy.org/) and the NCBI conserved domain (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) databases. The multiple sequence alignment and phylogenetic analysis were

Name	Structure	MW (kDa)
XylC	Xyn CBM CE	79.8
Xyn-CBM	Xyn CBM	57.6
Xyn	Xyn	37.8
CelC	Cel	41
Cel-CBM	Cel CBM	68.9
Cel-CBM-CE	Cel CBM CE	91.1
Xyn-CBM-Cel	Xyn CBM Cel	92.7

Fig. 1 Schematic representation of the molecular structure of wild-type XylC and CelC as well as the truncated and fusion enzymes constructed in this study

conducted by MEGA7 software using neighbor joining method and a bootstrap of 1000 replicates (Kumar et al. 2016).

Gene cloning, expression, and purification of wild and engineered enzymes

The camel rumen metagenome used in this study was available from our previous study (Gharechahi et al. 2015). The metagenome served as template for isolation of *xylC* encoding a multidomain xylanase and *celC* encoding an endoglucanase. The genes were isolated by PCR using primers 1 and 2 for *xylC* and primers 3 and 4 for *celC* (see Table 1 for all primers and restriction enzymes used in this study). The amplified genes were purified and separately cloned into pET-26b (Novagen, Madison, WI, USA) giving rise to plasmids pExylC and pEcelC. The correct recombinant plasmids were identified by sequencing and then were used for transformation of *Escherichia coli* BL21 (DE3) (Novagen, Madison, WI, USA) for protein expression. The transformed cells were grown at 37 °C in Luria-Bertoni (LB) broth containing 50 μg ml^{-1} kanamycin, and when the optical density of the cell culture was 0.8 (absorbance 600 nm), the cells were induced with 0.2 mM isopropyl-β-thiogalactopyranoside (IPTG) for gene expression. The growing cells were then incubated at 20 °C for 22 h and finally were harvested by centrifugation at 7000×*g* for 15 min. The histidine-tagged recombinant proteins were purified by Ni-NTA affinity chromatography as recommended by the manufacturer (Qiagen, Hilden, Germany). The purity and concentration of the enzymes were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the Bradford method, respectively (Laemmli 1970; Bradford 1976). The truncated forms of XylC and the fusion hybrids of XylC and CelC were engineered as follows: the gene segment *xyn* encoding for the xylanase domain of XylC (residues 2–229) was isolated from pExylC using primers 1 and 5 and subcloned into pET26b using *Bam*HI and *Xho*I restriction sites to make pExyn. The gene segment *xyn-cbm* encoding for the xylanase domain plus CBM module of XylC (residues 2–486) was isolated from pExylC using primers 1 and 6 and subcloned into pET26b using *Bam*HI and *Sac*I restriction sites to make pExyn-cbm. Subsequently, *celC* was amplified from pEcelC using primers 7 and 4 and inserted into pExyn-cbm using *Sac*I and *Xho*I restriction sites to make pExyn-cbm-cel.

Likewise, the gene segment *cbm-ce* encoding the CBM plus CE domain of XylC (residues 221–687) was isolated from pExylC using primers 8 and 2, and subcloned into pET26b using *Sac*I and *Xho*I restriction sites to make pEcbm-ce. Subsequently, *celC* was amplified from pEcelC using primers 3 and 9 and inserted into pEcbm-ce using BamHI and *Sac*I restriction sites to produce pEcel-cbm-ce. Finally, the fusion gene *cel-cbm* encoding for CelC plus XylC's CBM was isolated from pEcel-cbm-ce using primers

Table 1 List of primers and restriction enzymes used in this study

No.	Sequence	Restriction enzyme
1	AAGGATCCCAAAAACGGTTTAAAAGTAGC	*Bam*HI
2	AACTCGAGATATGTTGCTGACCAG	*Xho*I
3	ATGGATCCCACACCGTTTGAAAAATAC	*Bam*HI
4	TACTCGAGTCTTCCCATGATGTTCTTC	*Xho*I
5	AAGAGCTCCGTCTATAAGAATAACCTGATC	*Sac*I
6	AAGAGCTCAACTGGTTCTTGTTATTATTCTG	*Sac*I
7	ATGAGCTCCACACCGTTTGAAAAATAC	*Sac*I
8	AACTCGAGGGAATTCTGAGCACTG	*Xho*I
9	TAGAGCTCAATCTTCCCATGATGTTC	*Sac*I
10	TAAAGCTTCTGGTTCTTGTTATTATTCTG	*Hin*dIII

3 and 10 and subcloned into pET26b using *Bam*HI and *Hin*dIII restriction sites to make pEcel-cbm. All the recombinant plasmids were expressed in *E. coli* BL21 (DE3), and the engineered proteins were purified by Ni-NTA affinity chromatography.

Enzyme activity assays

The activities of native, truncated, and fusion enzymes were assayed by determination of reducing sugars using 3,5-dinitrosalicylic acid (DNS) reagent (Miller 1959). Cellulase and xylanase activity were measured, respectively, using carboxymethyl cellulose (CMC) and oat spelt xylan (OSX) as standard substrates. Assay mixtures containing either 1% CMC or 1% OSX in 0.1 M citrate buffer (pH 5) were incubated for 45 min at 35 °C. The reactions were stopped by adding three volumes of DNS reagent and heating at 100 °C for 10 min. Finally the absorbance of the developed color was measured at 540 nm to determine enzyme activity. The effect of temperature on the activity of the wild-type and engineered enzymes was analyzed by incubating the assay mixtures at various temperatures. Then, the pH effect was studied by assays conducted in various buffers including citrate-phosphate buffer (pH 3–6), phosphate-buffered saline (pH 6–9), and glycine NaOH (pH 9–11) at the optimum temperature. The kinetic parameters including V_{max} and K_m were determined by fitting the initial velocities at different substrate concentrations with the Michaelis-Menten equation using SigmaPlot software (Systat, San Jose, CA, USA).

Denaturing and non-denaturing protein electrophoresis

The purity of the enzymes was analyzed by SDS-PAGE using 12% polyacrylamide gels by a BioRad system (Bio-Rad, Mississauga, ON, Canada) at 85 v for 2 h. The protein bands were visualized by Coomassie Blue staining. The functional activity of the purified enzymes was assayed by zymography using non-denaturing gel electrophoresis with SDS-free polyacrylamide gels. Purified enzymes were electrophoresed at 4 °C on 12% gels containing either CMC or OSX (0.2%). At the end, the gels were soaked first in deionized water for 15 min at room temperature and then in citrate phosphate buffer (0.1 M, pH 5) for 45 min at 37 °C. Finally the gels were stained with 0.1% Congo Red solution (15 min) and then destained in 1 M NaCl to detect the functional activity of the enzymes.

TLC analysis of hydrolysis products

The enzymatic hydrolysis of CMC and OSX was analyzed by thin layer chromatography (TLC). The enzyme reactions were performed under the standard conditions, and after 24 h, 2 μl of each reaction were spotted on a 60 F254 silica gel plate (Merck, Darmstadt, Germany). The chromatography was conducted using a mixture of acetic acid/2-butanol/water at a ratio of 1:2:1 as mobile phase. At the end, the TLC plate was dried at room temperature, sprayed with 10% solution of H_2SO_4 in methanol, and developed by heating at 120 °C for 10 min.

GenBank accession numbers

The genes encoding for XylC and CelC were submitted to GenBank with accession numbers KX644152 and KX644147, respectively.

Results

Findings from bioinformatics analysis

The translated amino acid sequence of *xylC* and *celC* was used for in silico analyses. Using SignalP server a putative signal peptide of 27 amino acids was identified in XylC that seems to be recognized and cleaved between two adjacent alanines within the VQAAT recognition sequence. The structural features of XylC were analyzed by the PROSITE as well as the NCBI conserved domain databases. The results indicated that

XylC has modular structure consisting of a family 11 glycosyl hydrolase (Xyn) domain (30–224 residues), a family 36 carbohydrate binding module (CBM) domain (313–430 residues), and a family 4 carbohydrate esterase (CE) domain (487–668 residues). The modular enzyme shared homology to some sequences predicted as encoding for peptidoglycan/xylan/chitin deacetylase from genome projects of *Butyrivibrio* and *Pseudobutyrivibrio* species (Fig. 2a–d). In particular, the amino acid sequence of XylC showed significant identity (97%) with a sequence from *Butyrivibrio hungatei* (accession no. SCX74996.1). Otherwise, the identity of XylC with other sequences in NCBI database was below 65%. The phylogenetic analysis of XylC was conducted by the MEGA software using the homologous sequences obtained from BLAST search (blastp suite of NCBI) (Kumar et al. 2016). The phylogenetic tree confirmed the evolutionary relatedness of XylC with the sequences predicted as peptidoglycan/xylan/chitin deacetylase from the ruminal and termite hindgut bacteria of *Lachnospiraceae* family (Fig. 2a). The xylanase domain of XylC is distantly related with xylanases from *Bacillus* species (Fig. 2b), while the CBM and CE domains are more specific and evolutionarily quite distinct from their homologs (Fig. 2c, d).

In the case of CelC, no signal sequence was identified by the SignalP server. The enzyme with 313 amino acids was predicted using the NCBI BlastP search engine to be an endoglucanase belonging to the family 5 glycosyl hydrolases. Also, CelC showed 89% identity with a predicted endoglucanase sequence from the *Lachnospiraceae* family of bacteria (accession no. SDJ16454). Other sequences in NCBI database showed an identity of less than 60% with the amino acid sequence of CelC (Fig. 2e). The phylogenetic tree in Fig. 2e exhibits a clustering pattern of CelC among predicted endoglucanases from various bacteria of the *Lachnospiraceae* family.

Purification and characterization of wild and engineered enzyme variants

The genes encoding for XylC and CelC were PCR amplified from the metagenomic template and successfully expressed in *E. coli* BL21 (DE3) using pET-26b as the expression vector. In addition to the wild-type genes, the truncated ones coding for Xyn-CBM and Xyn as well as the fusion genes coding for Xyn-CBM-Cel, Cel-CBM, and Cel-CBM-CE were constructed and successfully expressed in the same way as the wild-type enzymes. The expressed proteins with a C-terminal His-tag were purified to homogeneity using Ni-NTA resin. The purity and activity of the proteins were checked by SDS-PAGE (Fig. 3a, b) and zymography (Fig. 3c, d). The purified proteins were of the expected molecular weights as given in Fig. 1. The functional activity of the proteins was analyzed by zymography using OSX or CMC as substrate. As it can be seen in Fig. 3, both the wild and engineered enzymes all were functional. Importantly, the fusion Xyn-CBM-Cel was

bifunctional with both xylanase activity on OSX and cellulose activity on CMC. Subsequently, the kinetics of the wild-type, truncated, and fusion enzymes were investigated (Table 2). The wild-type XylC was shown to be a β-1,4-endoxylanase with a K_{cat} value of 2919 s^{-1} on OSX. The end products of OSX hydrolysis were tetra-, tri-, di-, and mono-saccharides as revealed by TLC (Fig. 4a, b). On the other hand, CelC was shown to be a β-1,4-endoglucanase with a K_{cat} of 422 s^{-1} on CMC and formation of tetra-, di-, and mono-saccharides as the end products (Fig. 4c).

In this study, the impact of the modular structure of XylC on its xylanase activity was investigated by domain truncation. The removal of the CE domain decreased the specific activity and the K_{cat} of the truncated Xyn-CBM by 14 and 40% respectively, as compared with XylC. A further truncation by removing the CBM domain decreased the specific activity and the K_{cat} of the truncated Xyn much more drastically by 88 and 95%, respectively. The impact of the truncations was also reflected by the rise in the K_m values of Xyn-CBM and Xyn by 2.1 and 2.7 times. From the observations, it seems obvious that the CBM has a very important contribution to the high xylanase activity of XylC. Also, it may be assumed that the presence of CE domain is important for optimum configuration of other domains as was indicated by the significant rise in the K_m value of Xyn-CBM as compared to that of XylC.

Additional insights were provided by fusing the XylC's domains to CelC for construction of hybrid fusion proteins (i.e., Cel-CBM, Cel-CBM-CE, and Xyn-CBM-Cel). The analysis of CMCase activity of the fusion proteins indicated that the XylC's domains remarkably improved the kinetic parameters of the cellulase domain (Table 2). By fusing the Xyn-CBM domains to the N-terminus of CelC, the K_{cat} of the bifunctional Xyn-CBM-Cel for CMCase activity enhanced by 6.6 times as compared to CelC. Likewise, by fusing the CBM-CE domains or the CBM alone to the C-terminus of CelC, the resulting Cel-CBM-CE and Cel-CBM fusions showed enhancement of K_{cat} by 3 and 1.6 times, respectively. One interesting observation was that the CBM domain of XylC could also improve the CMC binding affinity of Cel-CBM as was indicated by the K_m. However, the K_{cat} of Cel-CBM-CE and Xyn-CBM-Cel were 1.7 and 3.8 times higher than that of Cel-CBM while their substrate binding affinity was lower. Therefore, it can be assumed that the highly elevated CMCase activity of Cel-CBM-CE and Xyn-CBM-Cel may partly be due to an improved substrate binding affinity and more importantly to an enhanced catalytic activity that occurred by the modular structure of the fusion biocatalysts.

Effect of temperature on the enzyme activity

The activity of XylC was assayed in the range of 5–65 °C using OSX as substrate. The results showed that the optimum

temperature for the enzyme activity was at 35 °C while 25 and 13% of the activity could still be measured at 5 and 65 °C, respectively (Fig. 5a). The truncation of XylC, first by the removal of the CE domain and then the CBM, upshifted the optimum temperature to 40 and 45 °C for the resulting Xyn-CBM and Xyn molecules. When CelC was fused to the C-terminus of Xyn-CBM, the optimum temperature for xylanase activity of the resulting Xyn-CBM-Cel fusion was restored to 35 °C while the temperature range for the enzyme activity was shortened to 20–45 °C.

The effect of temperature on the activity of CelC was studied in the range of 5–50 °C using CMC as substrate (Fig. 5b). The optimum temperature for CelC activity was at 35 °C, and the enzyme showed 38 and 71% of the activity at 5 and 50 °C, respectively. The hybrid fusions including Cel-CBM, Cel-CBM-CE, and Xyn-CBM-Cel showed the same optimum temperature as that of CelC for CMCase activity, but the relative activity of the fusions at other temperatures was significantly reduced in comparison with CelC.

Thermal stability of the enzymes

The thermal stability of XylC and CelC was analyzed during 1-h incubation at 40, 45, and 50 °C. The results showed that XylC was quite stable at 40 and 45 °C but tend to lose activity at 50 °C with a total loss of 64% after 1 h (Fig. 5c). The truncated forms of XylC including Xyn-CBM and Xyn were more stable and lost only 22 and 36% of their original activity after 1-h incubation at 50 °C. It seems that the CE domain has an important impact on the thermal behavior of XylC.

In contrast, CelC was not stable at 45 °C and lost 22% of its activity during 1-h incubation (Fig. 5d). Also, the fusion forms of CelC showed different degrees of thermal stability at 45 °C. In particular, the Cel-CBM-CE was less stable than other fusions exhibiting total loss of activity in 30 min. The CE domain of XylC seemed to impose a severe adverse effect on the stability of Cel-CBM-CE as was the case with the parental XylC.

Effect of pH on the enzyme activity

The effect of pH on the activity of the wild-type and engineered enzymes was studied in the range of 3–10. The activity pH profile of the XylC showed that the enzyme with maximum activity at pH 5 might be classified as an acidic xylanase (Fig. 5e). However, it could remain active all over the tested pH range with about 20% activity at the two extreme pH values. The truncation of XylC upshifted the optimum pH to 6 for Xyn-CBM, but a further truncation by the removal of CBM restored the optimum pH value to 5 for Xyn. Replacement of the CE domain of XylC with CelC raised the optimum pH of the resulting Xyn-CB-Cel fusion to 7.

The optimum pH for CMCase activity of CelC was at 5 which remained unaffected for all CelC fusions (Fig. 5f).

However, the pH range for CMCase activity was significantly shortened to pH 5–7 in the case of Cel-CBM-CE. The bifunctional Xyn-CBM-Cel showed more than 80% of both xylanase and CMCase activities in the range of pH 5–6. Given that pH 5 is optimum for cellulase activity of Xyn-CBM-Cel, it seems reasonable to take the same pH as optimum for the bifunctional enzyme.

The effect of salt on enzyme activity

The effect of salt on the activity of XylC and CelC as well as the truncated and fusion enzymes was studied by enzyme assays conducted in the presence of varying concentrations of NaCl at optimum temperature and pH. The salt in the range of 1–4 M was shown to have stimulating effect on XylC improving the enzyme activity between 78 and 163% (Fig. 6a). The xylanase activity of all truncated and fusion enzymes, especially Xyn-CB and Xyn-CB-Cel, was also stimulated by NaCl. In contrast, the activity of CelC and its derivatives was inhibited by a high salt concentration (Fig. 6b). However, the CMCase activity of CelC and Xyn-CBM-Cel could be stimulated by low salt concentrations up to 1 M. Compared to CelC, its fusions were generally more susceptible to high salt inhibition especially Cel-CBM and Cel-CBM-CE that were completely inactivated by 2.5 and 3 M NaCl, respectively.

Hydrolysis of rice and barley straw by wild, truncated, and fusion enzymes

The rice and barley straws were used to perform an evaluation on the hydrolysis of plant biomass by the enzymes. The straws were ground with a grinder, autoclaved, washed with water, and dried before being used in enzyme assays. The enzyme activities were assayed in citrate-phosphate buffer (pH 5) containing 20 mg ml^{-1} of each straw at 35 °C for 2 h. The concentration of the reducing sugars released as a function of enzyme activity was determined by the DNS method. The results showed that XylC was 24 and 37 times more efficient than CelC in the hydrolysis of rice and barley straws, respectively (Table 3). In agreement with data presented in Table 2, the CelC fusions showed enhanced activity on both straws in comparison with the parental CelC. On the other hand, the XylC-truncated forms were less efficient than XylC. The results also showed that the fusion Xyn-CBM-Cel was 2 and 1.7 times more efficient than XylC in solubilization of rice and barley straws, respectively.

An experiment was also designed with the 1:1 stoichiometric mixtures of XylC plus CelC as well as Xyn-CBM plus CelC to compare the efficiency of the mixtures against Xyn-CBM-Cel in the hydrolysis of rice and barely straws. The results showed that the XylC plus CelC and the Xyn-CBM plus CelC mixtures exhibited, respectively, 51 and 43% activities on rice straw and 62 and 58% activities on barely straw as
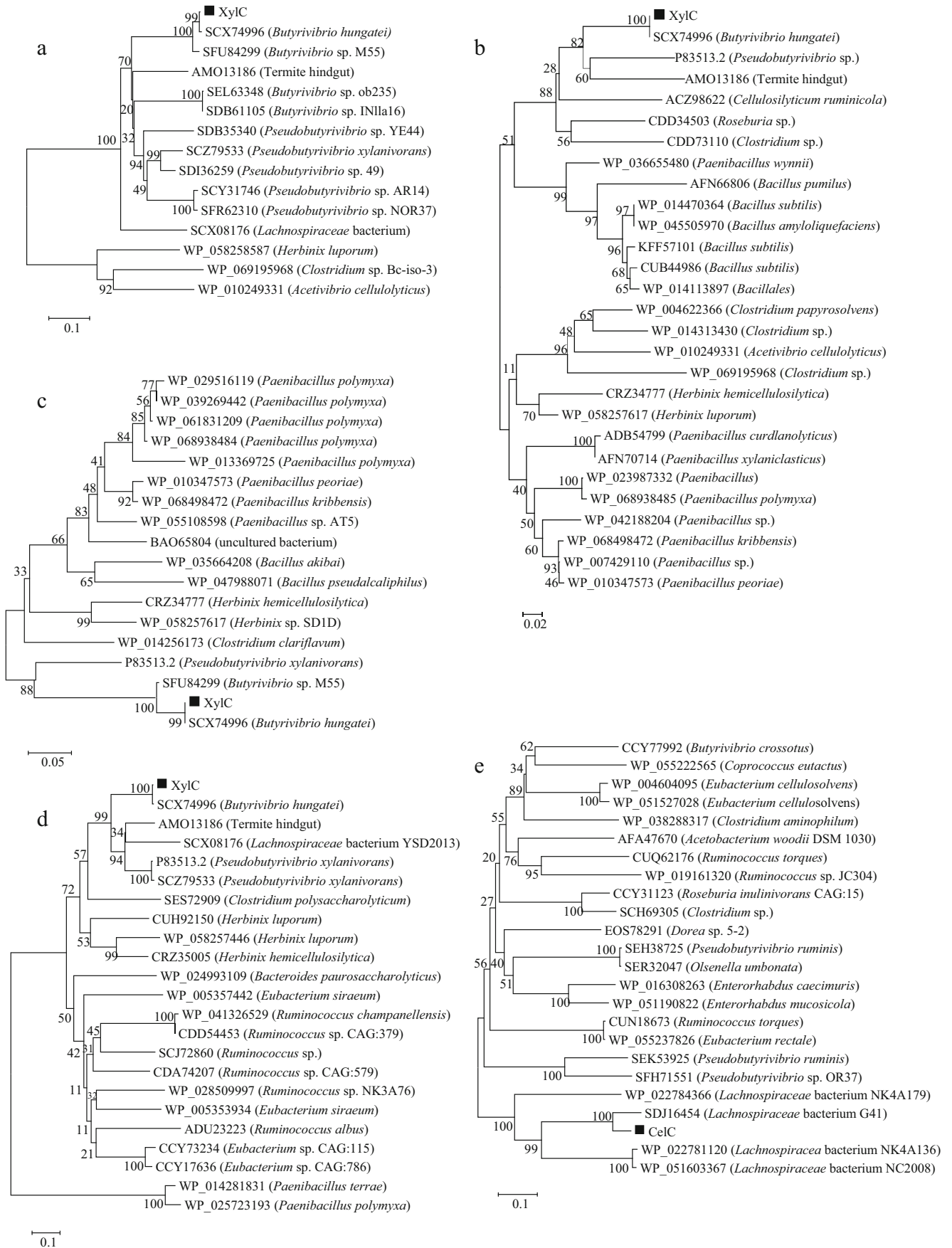
◀ **Fig. 2** Phylogenetic analysis of the multidomain xylanase XylC (**a**), its individual domains including xylanase domain (**b**), carbohydrate binding module (**c**), and carbohydrate esterase domain (**d**) as well as the endoglucanase CelC (**e**). The phylogenetic trees were constructed by Mega software using neighbor joining method with 1000 bootstrapping. The percentage of replicate trees in which the related taxa clustered together in the bootstrap test is shown next to the branches. The *scale bars* indicate the number of substitutions per amino acid site

compared to the activities of Xyn-CBM-Cel on the same substrates. Therefore, the fusion Xyn-CBM-Cel proved to be by far more efficient than not only either of the parental enzymes but also their mixture.

Discussion

The mammalian herbivores like camels are thoroughly dependent on their symbiotic microorganisms to obtain carbon and energy from plant biomass. Therefore, the rumen environment is deemed to contain specialized lignocellulose degrading enzymes which are produced by a variety of cultured and uncultured microorganisms. In the current study, a metagenome of camel rumen was investigated for novel cellulose and xylan degrading enzymes. Among the procured metagenomic sequences, two novel genes—one predicted to encode a modular multidomain xylanase (XylC) and the other to encode an endoglucanase (CelC)—were picked for subsequent characterization. XylC was proved to be an endoxylanase exhibiting remarkable activity on OSX with a V_{max} and K_{cat} value of

2299.4 U mg^{-1} and 2919 s^{-1}, respectively. This remarkable xylanase activity of XylC is quite interesting as compared to other characterized xylanases. For instance, the modular endo-1,4-β-xylanase XynA from *Clostridium cellulovorans* has an activity of 155.3 U mg^{-1} on OSX at 37 °C (Kosugi et al. 2002). The bifunctional endoxylanase XynS20E from the ruminal fungus *Neocallimastix patriciarum* shows an activity of 131.3 U mg^{-1} on OSX under optimized conditions at 49 °C (Pai et al. 2010). The endoxylanase XynG1-1 from *Paenibacillus campinasensis* with a family 6-36 CBM exhibits a K_{cat} value of 826.12 s^{-1} (Liu et al. 2015). XylC with maximum activity at about 35 °C, keeping 25% of the activity at 5 °C, as well as instability at temperatures above 45 °C shares similarity with cold-adapted enzymes such as *Pseudoalteromonas haloplanktis* xylanase (Collins et al. 2002). In general, cold-adapted enzymes show higher rates of catalytic activity at moderate and cold temperatures compared to their mesophilic and thermophilic counterparts (Siddiqui and Cavicchioli 2006). The high catalytic activity of cold-adapted enzymes has been ascribed to a more flexible structure that allows a faster substrate/product diffusion rate. However, it is noteworthy that the elevated catalytic activity of cold-adapted enzymes has been obtained at the expense of a lowered thermal stability (Van Den Burg 2003). In agreement, the removal of CE and CBM-CE domains from XylC resulted in an increased thermal stability of truncated Xyn-CBM and Xyn with concomitant decrease in xylanase activity.

It has been suggested that CBMs can enhance the catalytic activity of plant biomass hydrolases by mediating a prolonged

Fig. 3 SDS-PAGE and zymogram analysis of wild-type and engineered enzymes. Purified enzymes were checked on 12% acrylamide gels. **a** *1*—Xyn, *2*—Xyn-CBM, *3*—XylC, *4*—Xyn-CBM-Cel, *M*—protein marker. **b** *1*—Cel-CBM-CE, *2*—Cel-CBM, *3*—CelC, *M*—protein marker. The activity of the purified proteins was analyzed by zymography using native PAGE gels containing either OSX (Fig. 2c) or CMC(Fig. 2d) as follows. **c** *1*—Xyn, *2*—Xyn-CBM, *3*—Xyn-CBM-Cel, *4*—XylC and **d** *1*—CelC, *2*—Cel-CBM-CE, *3*—Xyl-CBM-Cel, *4*—Cel-CBM
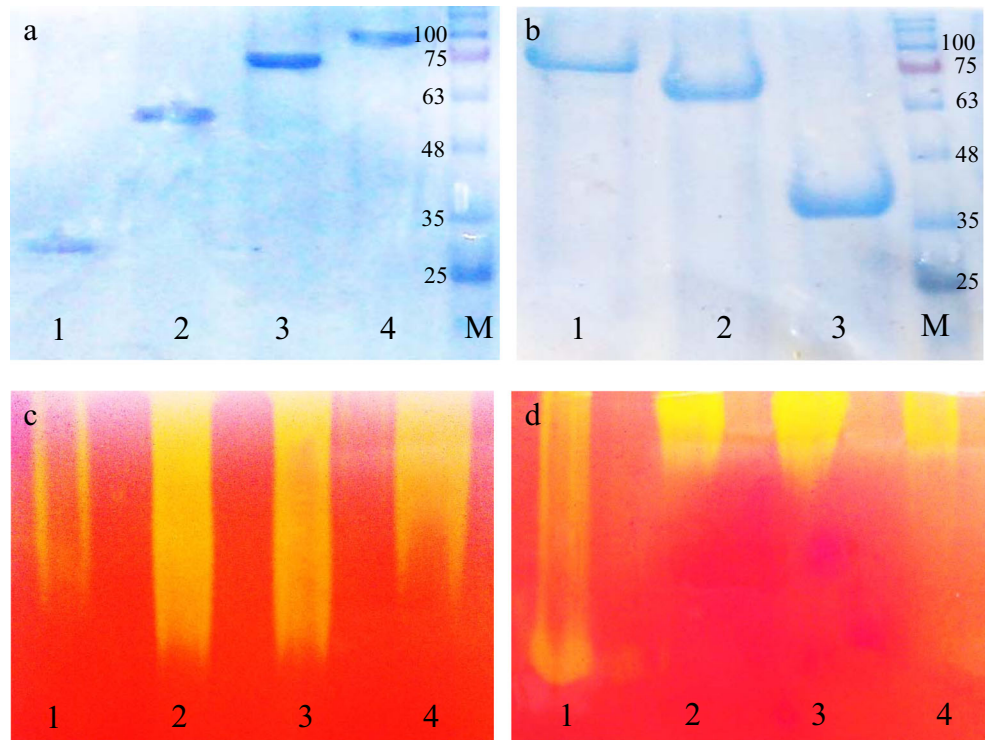
Table 2 Kinetics parameters of the wild-type (XylC and CelC), truncated, and fusion enzymes for oat spelt xylan (OSX) and carboxymethyl cellulose (CMC) hydrolysis

Enzyme name	Substrate	K_m (mg ml^{-1})	Specific activity (U mg^{-1})	K_{cat} (s^{-1})	K_{cat}/K_m
XylC	OSX	4.55 ± 0.55	2299.4 ± 45	2919 ± 57	641.6 ± 66
	CMC	–	–	–	–
Xyn-CBM	OSX	9.66 ± 0.34	1980.5 ± 46	1761 ± 20	182.3 ± 4
	CMC	–	–	–	–
Xyn	OSX	12.2 ± 0.95	276.5 ± 14.5	161 ± 8.5	13.2 ± 1.7
	CMC	–	–	–	–
Xyn-CBM-Cel	OSX	3.16 ± 0.14	922.4 ± 19	1375.4 ± 28	435 ± 10.5
	CMC	9.8 ± 0.7	1979 ± 35	2799 ± 51	285.6 ± 25
Cel-CBM-CE	OSX	8.56 ± 0.61	2.3 ± 0.021	3.36 ± 0.3	0.39 ± 0.06
	CMC	10.3 ± 0.6	883 ± 23.5	1289 ± 34.5	125 ± 10.5
Cel-CBM	OSX	2.45 ± 0.35	4.25 ± 0.34	4.64 ± 0.37	1.9 ± 0.1
	CMC	6.8 ± 0.5	680 ± 11	742 ± 12	109 ± 10
CelC	OSX	20 ± 1.7	3.3 ± 0.46	2.01 ± 0.28	0.1 ± 0.06
	CMC	12.3 ± 0.9	688 ± 6	422 ± 3.5	34 ± 2.2

and intimate association between the enzyme and its target substrate (Henshaw et al. 2004). In accordance, Liu et al. (2015) have reported that the removal of the xylanase binding domain from XynG1-1 decreased the catalytic activity of the enzyme on OSX by 25%. In our study, a similar approach by the removal of CBM-CE domains from XylC resulted in a more severe loss of activity (88%) on the same substrate. Given the high catalytic activity of XylC, it seems that the specific type of CBM can efficiently mediate binding of catalytic domain to substrate.

The fusion of proteins is an effective approach to engineer novel enzymes with improved catalytic activity (Yang et al. 2016). When two enzyme are fused together, the catalytic activity may either decrease or increase for both, or even increase for one while decrease for the other (Sun et al. 2011). Also, it has been shown that the order of domains in a fusion protein is decisive for the activity of each domain. For instance, the fusion of a cellulase (Cel5C) and a xylanase (XynA) from *Thermotoga maritima* was bifunctional only when the cellulase domain was at the N-terminus; otherwise, the fusion protein was not functional at all (Hong et al. 2006). In another study, two fusion proteins in reverse orientations were constructed using the cellulase Cel5C and a β-glucosidase (BglB) from *T. maritima*. The Cel5C was functional in both constructs but the BglB retained activity only when it was fused to the N-terminus of Cel5C (Hong et al. 2007). However, the chimeras made from an endoglucanase (Cel5A) from *Fervidobacterium gondwanense* and an endoxylanase (XylT) isolated from hot thermal springs were bifunctional in both orientations (Rizk et al. 2015).

In this study, the hybrid fusions of CelC showed significant enhancement in CMCase activity. By comparison to CelC, the catalytic efficiency (K_{cat}/K_m) of the fusions including Xyn-CBM-Cel, Cel-CBM-CE, and Cel-CBM for CMCase activity was improved by 8.4, 3.7, and 3.2 times, respectively.

In contrast to non-halophilic enzymes that tend to precipitate or become inactivated at high concentrations of salt, halophilic enzymes require a minimum salt concentration of about 2 M for their activity and stability (Madern et al. 2000; Wejse et al. 2003). In this study, not only XylC could tolerate high NaCl concentrations of up to 4 M, but also its xylanase activity was stimulated by more than 2.5 times. The truncation analysis of XylC revealed that the structural integrity of the enzyme is required for maximum stimulation of xylanase activity by salt. The enzymatic behavior of XylC in the presence of high concentrations of salt resembles that of halophilic enzymes. For instance, the halophilic xylanase
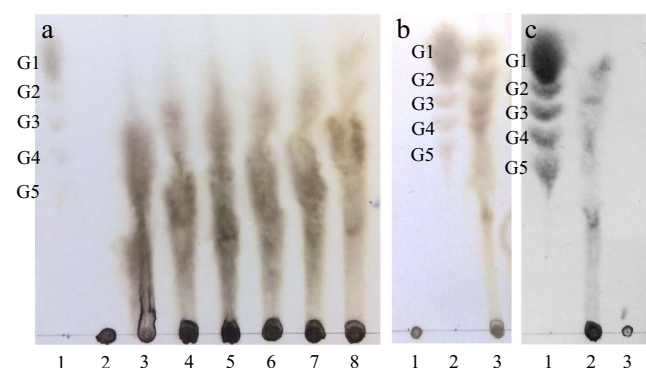
Fig. 4 Thin layer chromatography (TLC) analysis of OSX and CMC hydrolysis by XylC and CelC. Reactions were conducted under standard conditions, and the resulting hydrolysis products were analyzed by TLC. **a** OSX hydrolysis by XylC: lane *1*, marker containing mono-, di-, tri-, tetra-, and pentose sugars; lane *2*, control without enzyme; and lane *3–8*, XylC-treated OSX after 1, 2, 4, 6, 8, and 24 h incubation. **b** OSX hydrolysis by XylC after 48 h incubation: lane *1*, control without enzyme; lane *2*, marker; and lane *3*, XylC-treated OSX after 48 h incubation. **c** CMC hydrolysis by CylC: lane *1*, marker; lane *2*, CelC treated CMC after 48 h incubation; and lane *3*, control without enzyme
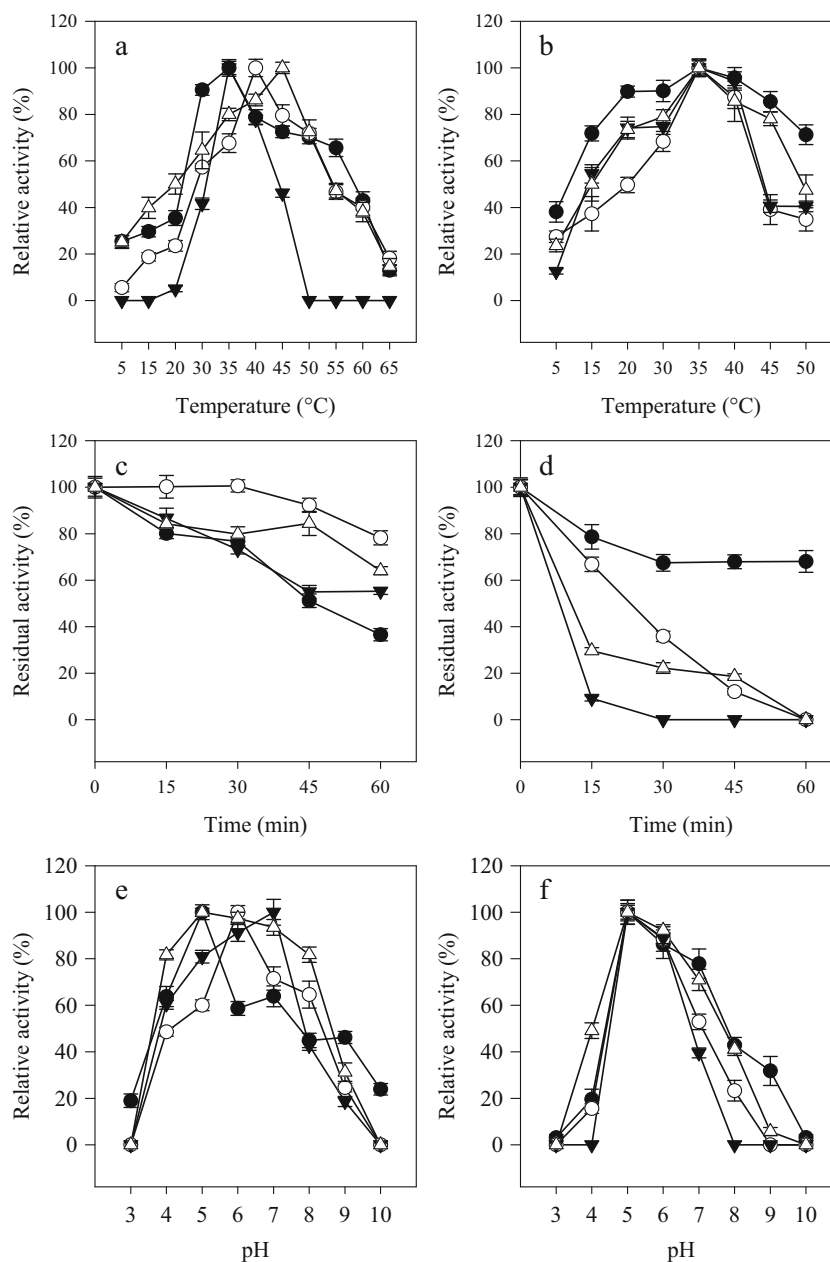
Fig. 5 Impact of temperature and pH on enzyme activity. **a** Impact of temperature on the xylanase activity of XylC (*filled circle*), Xyn-CBM (*empty circle*), Xyn-CBM-Cel (*filled inverted triangle*), and Xyn (*empty triangle*) was studied using OSX as substrate in citrate-phosphate buffer (pH 5). **b** Impact of temperature on the cellulase activity of CelC (*filled circle*), Xyn-CBM-Cel (*empty circle*), Cel-CBM-CE (*filled inverted triangle*), and Cel-CBM (*empty triangle*) was studied using CMC as substrate in citrate-phosphate buffer (pH 5). **c** Impact of temperature on the stability of XylC (*filled circle*), Xyn-CBM (*empty circle*), Xyn-CBM-Cel (*filled inverted triangle*), and Xyn (*empty triangle*). The enzymes were incubated at 50 °C in citrate-phosphate buffer (pH 5) for 1 h, and the residual activity was determined at 15-min intervals at 35 °C. **d** Impact of temperature on the stability of CelC (*filled circle*), Xyn-

CBM-Cel (*empty circle*), Cel-CBM-CE (*filled inverted triangle*), and Cel-CBM (*empty triangle*). The enzymes were incubated at 45 °C in citrate-phosphate buffer (pH 5) for 1 h, and the residual activity was determined at 35 °C at 15-min intervals. **e** Impact of pH on the xylanase activity of XylC (*filled circle*), Xyn-CBM (*empty circle*), Xyn-CBM-Cel (*filled inverted triangle*), and Xyn (*empty triangle*) was studied using OSX as substrate at 35 °C in various buffers including citrate-phosphate buffer (pH 3–6), phosphate-buffered saline (PBS) (pH 7–8), and glycine NaOH (pH 9–10). **f** Impact of pH on the cellulase activity of CelC (*filled circle*), Xyn-CBM-Cel (*empty circle*), Cel-CBM-CE (*filled inverted triangle*), and Cel-CBM (*empty triangle*) was studied using CMC as substrate at 35 °C in the same buffers as in **e**

XynA from *Zunongwangia profunda* is stimulated by NaCl in the range of 1–5 M with maximum activity at 3 M (Liu et al. 2014). In contrast, the halotolerant xylanases, Xyl 1 and Xyl 2, from a halophilic bacterium have been reported to be stimulated by 1 M NaCl while they were inhibited at higher salt concentrations (Wejse et al. 2003). Therefore, compared to the
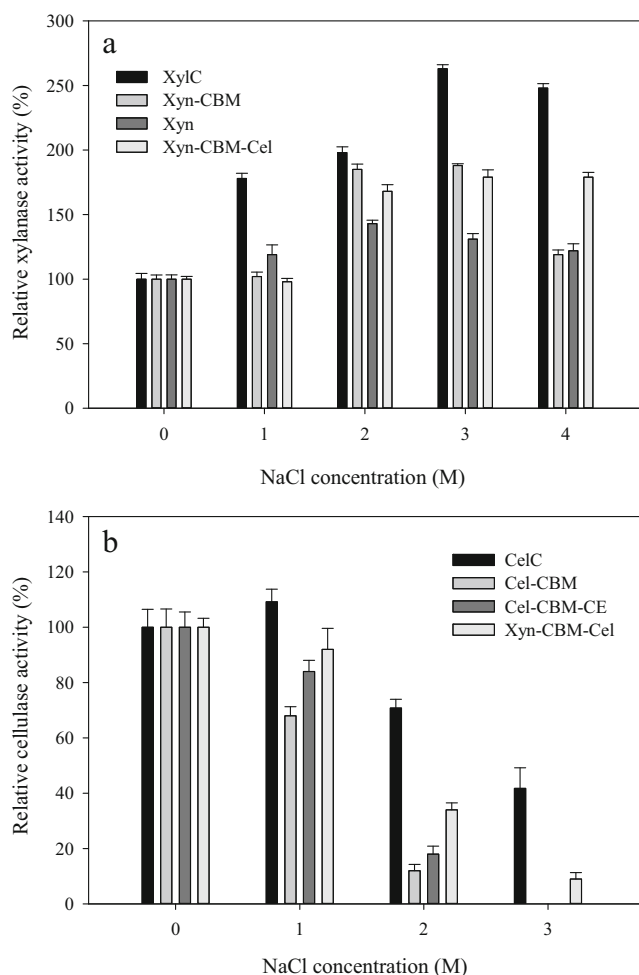
Fig. 6 Impact of NaCl on the xylanase **a** and cellulase **b** activity of the wild-type XylC and CelC as well as the truncated and fusion enzymes constructed in this study. Reactions were conducted at 35 °C in citrate-phosphate buffer (pH 5) in the presence of various salt concentrations

Table 3 Activity of the wild-type (XylC and CelC), truncated, and fusion enzymes for the hydrolysis of rice straw (RS) and barley straw (BS)

Enzyme name	Substrate	Molar activity (U μmol^{-1})
XylC	RS	197 ± 3.3
	BS	210.6 ± 6.5
Xyn-CBM	RS	168.3 ± 6.9
	BS	207.8 ± 7.8
Xyn	RS	128.3 ± 4.5
	BS	161.4 ± 3.9
Xyn-CBM-Cel	RS	398.4 ± 20.9
	BS	350.3 ± 8.4
Cel-CBA-CE	RS	24 ± 1
	BS	21.63 ± 1.22
Cel-CBM	RS	21.6 ± 1.3
	BS	19.8 ± 1.2
CelC	RS	8.37 ± 0.34
	BS	5.64 ± 0.44

In this study, we tried to mimic the architecture of the naturally evolved XylC in the construction of novel fusion enzymes with CelC (Xyn-CBM-Cel and Cel-CBM-CE). In this way, we managed to study the effects of XylC's modules in their original orientations on the activity of the fused cellulase module. Fortunately, this strategy proved to be rewarding. The results of this study showed that camel rumen metagenome is an important source of glycosyl hydrolases. The wild-type XylC and the engineered bifunctional Xyn-CBM-Cel proved promising for conversion of plant biomass to soluble fermentable sugars in various industrial applications such as biofuel production.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

salt-active enzymes, XylC and CelC may be described respectively as halophilic and halotolerant enzymes.

Cellulolytic enzymes with the ability to hydrolyze natural substrates such as straws gain privilege when it comes to conversion of plant biomass to soluble fermentable sugars. The challenge is that the cellulose, hemicellulose, and lignin polymers with silica combine together to form an intricate structure that hinders degradation of straw (Van Soest 2006). It has been suggested that xylanase activity can make a significant contribution to biomass solubilization using enzymatic cocktails composed of various glycoside hydrolases (Goncalves et al. 2015; Thomas et al. 2016). In this study, XylC showed a promising potential for hydrolysis of rice and barley straws. Interestingly, the straw hydrolysis was significantly enhanced using Xyn-CBM-Cel instead of XylC. The remarkable enhancement of straw hydrolysis by the bifunctional fusion biocatalyst may be due to the integration of synergistic xylanase/cellulase activities in one enzyme and also to the elevated cellulase activity of Xyn-CBM-Cel.

References

Anwar Z, Gulfraz M, Irshad M (2014) Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. J Radiat Res Appl Sci 7(2):163–173

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72(1–2):248–254

Chang L, Ding M, Bao L, Chen Y, Zhou J, Lu H (2011) Characterization of a bifunctional xylanase/endoglucanase from yak rumen microorganisms. Appl Microbiol Biotechnol 90(6):1933–1942

Collins T, Meuwis M-A, Stals I, Claeyssens M, Feller G, Gerday C (2002) A novel family 8 xylanase, functional and physicochemical characterization. J Biol Chem 277(38):35133–35139

Collins T, Gerday C, Feller G (2005) Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev 29(1):3–23

Gangwar AK, Prakash NT, Prakash R (2014) Applicability of microbial xylanases in paper pulp bleaching: a review. Bioresources 9(2): 3733–3754

Gharechahi J, Zahiri HS, Noghabi KA, Salekdeh GH (2015) In-depth diversity analysis of the bacterial community resident in the camel rumen. Syst Appl Microbiol 38(1):67–76

Ghatge SS, Telke AA, Kang S-H, Arulalapperumal V, Lee K-W, Govindwar SP, Um Y, Oh D-B, Shin H-D, Kim S-W (2014) Characterization of modular bifunctional processive endoglucanase Cel5 from Hahella chejuensis KCTC 2396. Appl Microbiol Biotechnol 98(10):4421–4435

Gírio FM, Fonseca C, Carvalheiro F, Duarte LC, Marques S, Bogel-Łukasik R (2010) Hemicelluloses for fuel ethanol: a review. Bioresour Technol 101(13):4775–4800

Golan AE (2010) Cellulase: types and action, mechanism, and uses. Nova Science Publishers, New York

Goncalves GA, Takasugi Y, Jia L, Mori Y, Noda S, Tanaka T, Ichinose H, Kamiya N (2015) Synergistic effect and application of xylanases as accessory enzymes to enhance the hydrolysis of pretreated bagasse. Enzym Microb Technol 72:16–24

Henshaw JL, Bolam DN, Pires VM, Czjzek M, Henrissat B, Ferreira LM, Fontes CM, Gilbert HJ (2004) The family 6 carbohydrate binding module CmCBM6-2 contains two ligand-binding sites with distinct specificities. J Biol Chem 279(20):21552–21559

Hong SY, Lee JS, Cho KM, Math RK, Kim YH, Hong SJ, Cho YU, Kim H, Yun HD (2006) Assembling a novel bifunctional cellulase–xylanase from Thermotoga maritima by end-to-end fusion. Biotechnol Lett 28(22):1857–1862

Hong S-Y, Lee J-S, Cho K-M, Math RK, Kim Y-H, Hong S-J, Cho Y-U, Cho S-J, Kim H, Yun H-D (2007) Construction of the bifunctional enzyme cellulase-β-glucosidase from the hyperthermophilic bacterium Thermotoga maritima. Biotechnol Lett 29(6):931–936

Jemli S, Ayadi-Zouari D, Hlima HB, Bejar S (2016) Biocatalysts: application and engineering for industrial purposes. Crit Rev Biotechnol 36(2):246–258

JoBoyce A, Walsh G (2015) Characterisation of a novel thermostable endoglucanase from Alicyclobacillus vulcanalis of potential application in bioethanol production. Appl Microbiol Biotechnol 99(18): 7515–7525

Juturu V, Wu JC (2014) Microbial cellulases: engineering, production and applications. Renew Sust Energ Rev 33:188–203

Knowles J, Lehtovaara P, Teeri T (1987) Cellulase families and their genes. Trends Biotechnol 5(9):255–261

Kosugi A, Murashima K, Doi RH (2002) Xylanase and acetyl xylan esterase activities of XynA, a key subunit of the Clostridium cellulovorans cellulosome for xylan degradation. Appl Environ Microbiol 68(12):6399–6402

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227(5259):680–685

Limayem A, Ricke SC (2012) Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog Energy Combust Sci 38(4):449–467

Liu X, Huang Z, Zhang X, Shao Z, Liu Z (2014) Cloning, expression and characterization of a novel cold-active and halophilic xylanase from Zunongwangia profunda. Extremophiles 18(2):441–450

Liu Y, Huang L, Li W, Guo W, Zheng H, Wang J, Lu F (2015) Studies on properties of the xylan-binding domain and linker sequence of xylanase XynG1-1 from Paenibacillus campinasensis G1-1. J Ind Microbiol Biotechnol 42(12):1591–1599

Madern D, Ebel C, Zaccai G (2000) Halophilic adaptation of enzymes. Extremophiles 4(2):91–98

Mert MJ, la Grange DC, Rose SH, van Zyl WH (2016) Engineering of Saccharomyces cerevisiae to utilize xylan as a sole carbohydrate source by co-expression of an endoxylanase, xylosidase and a bacterial xylose isomerase. J Ind Microbiol Biotechnol 43(4):431–440

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31(3):426–428

Pai C-K, Wu Z-Y, Chen M-J, Zeng Y-F, Chen J-W, Duan C-H, Li M-L, Liu J-R (2010) Molecular cloning and characterization of a bifunctional xylanolytic enzyme from Neocallimastix patriciarum. Appl Microbiol Biotechnol 85(5):1451–1462

Rashamuse K, Visser D, Hennessy F, Kemp J, Roux-van der Merwe M, Badenhorst J, Ronneburg T, Francis-Pope R, Brady D (2013) Characterisation of two bifunctional cellulase–xylanase enzymes isolated from a bovine rumen metagenome library. Curr Microbiol 66(2):145–151

Rizk M, Elleuche S, Antranikian G (2015) Generating bifunctional fusion enzymes composed of heat-active endoglucanase (Cel5A) and endoxylanase (XylT). Biotechnol Lett 37(1):139–145

Siddiqui KS, Cavicchioli R (2006) Cold-adapted enzymes. Annu Rev Biochem 75:403–433

Sun J, Wang H, Lv W, Ma C, Lou Z, Dai Y (2011) Construction and characterization of a fusion β-1, 3-1, 4-glucanase to improve hydrolytic activity and thermostability. Biotechnol Lett 33(11):2193

Thomas L, Parameswaran B, Pandey A (2016) Hydrolysis of pretreated rice straw by an enzyme cocktail comprising acidic xylanase from Aspergillus sp. for bioethanol production. Renew Energy 98:9–15

Van Den Burg B (2003) Extremophiles as a source for novel enzymes. Curr Opin Microbiol 6(3):213–218

Van Dyk J, Pletschke B (2012) A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—factors affecting enzymes, conversion and synergy. Biotechnol Adv 30(6):1458–1480

Van Soest P (2006) Rice straw, the role of silica and treatments to improve quality. Anim Feed Sci Technol 130(3):137–171

Wejse PL, Ingvorsen K, Mortensen KK (2003) Purification and characterisation of two extremely halotolerant xylanases from a novel halophilic bacterium. Extremophiles 7(5):423–431

Yang H, Liu L, Xu F (2016) The promises and challenges of fusion constructs in protein biochemistry and enzymology. Appl Microbiol Biotechnol 100(19):8273–8281