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Reconstitution of a thermostable xylan-degrading enzyme mixture from the bacterium *Caldicellulosiruptor bescii*

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Running Title: Thermostable xylan-degrading enzyme mixture from *Caldicellulosiruptor bescii*

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ABSTRACT

Xylose, the major constituent of xylans, as well as the side chain sugars such as arabinose, can be metabolized by engineered yeasts into ethanol. Therefore, xylan-degrading enzymes that efficiently hydrolyze xylans will add value to cellulases used in hydrolysis of plant cell wall polysaccharides for conversion to biofuels. Heterogeneous xylan is a complex substrate, and it requires multiple enzymes to release its constituent sugars. However, the components of xylan-degrading enzymes are often individually characterized and therefore leading to a dearth of research that analyzes synergistic actions of the components of xylan-degrading enzymes. In the present report, six genes predicted to encode components of the xylan-degrading enzymes of the thermophilic bacterium *Caldicellulosiruptor bescii* were expressed in *Escherichia coli*, and the recombinant proteins were investigated as individual enzymes and also as a xylan-degrading enzyme cocktail. Most of the component enzymes of the xylan-degrading enzyme mixture had similar optimal pH (5.5~6.5) and temperature (75~90°C) and this facilitated their investigation as an enzyme cocktail for deconstruction of xylans. The core enzymes (two endoxylanases and a β-xylosidase) exhibited high turnover numbers during catalysis, with the two endoxylanases yielding estimated k_{cat} values of ~8000 s^{-1} and ~4500 s^{-1}, respectively, on soluble wheat arabinoxylan. Addition of side chain-cleaving enzymes to the core enzymes increased depolymerization of a more complex model substrate oat spelt xylan. The *C. bescii* xylan-degrading enzyme mixture effectively hydrolyzes xylan at 65°C to 80°C and can serve as a basal mixture for deconstruction of xylans in bioenergy feedstock at high temperatures.

INTRODUCTION

Plant cell wall polysaccharides are the most abundant biomass on earth and represent an important resource for biofuel production (1, 2). Plant cell walls are mainly composed of cellulose (40.6-51.2%), hemicellulose (28.5-37.2%), and lignin (13.6-28.1%) (1). In biofuel production, cellulose can either be hydrolyzed with dilute acids or cellulases into glucose or cellooligosaccharides, which are then fermented by biofuel-producing *Saccharomyces cerevisiae* (3) or other engineered microorganisms such as *Escherichia coli* (4) and *Zymomonas mobilis* (5). In addition, consolidated bioprocessing platforms such as *Clostridium thermocellum* (6) and *S. cerevisiae* (7) can directly convert cellulose into biofuel. Hemicellulose in bioenergy feedstock is mainly xylan, which is a polymer composed of a β-1,4-linked xylose backbone decorated with side-chains such as arabinose, glucuronic acid, acetate, and ferulic acid (8).

Current methods for pretreatment of biomass feedstocks include dilute acid pretreatment, which destroys much of the hemicellulose (9), and ammonia fiber explosion (AFEX), alkali preatreatment, and steam explosion which keep hemicellulose intact (10). Alternatively, the component sugars of hemicellulose can be released by enzymatic treatment; however, enzymatic deconstruction of this polysaccharide requires a set of biocatalysts including the endoxylanases that cleave the xylan backbone, the accessory enzymes that cleave the side chains, and the β-xylosidases that further cleave the xylo-oligosaccharides to xylose. A thermostable xylan-degrading enzyme mixture should be more desirable in the bioconversion of plant cell wall to biofuels, as thermostable enzymes are reported to be superior in their hydrolytic activity to their mesophilic counterparts (11).

In the large-scale production of xylan-degrading enzymes for application in the biofuel industry, expression of the enzymes may rely on different optimized protein-expressing

platforms such as filamentous fungi, *Escherichia coli* and different strains of the genus *Bacillus*. Subsequently the recombinant enzymes can be reconstituted into a functional xylan-degrading enzyme mixture. Surprisingly, although there are numerous biochemical characterizations of individual components of xylan-degrading enzymes, only limited reports address reconstitution of either simple (12, 13) or complex (14, 15) xylan-degrading enzyme mixtures. The number of reports in the literature are even less for thermostable xylan-degrading enzyme cocktails (16).

Caldicellulosiruptor bescii is a cellulose- and xylan-degrading bacterium that grows at an optimal temperature of 75°C (17). The genome of *C. bescii* encodes an arsenal of cellulase-like and xylan-degrading enzymes-like polypeptides that are likely involved in the degradation of plant cell wall polysaccharides (18). Therefore, *C. bescii*, as well as its glycoside hydrolases can be of much utility to the biofuel industry. To our knowledge, only a few *C. bescii* plant cell wall degrading enzymes have been biochemically characterized and published (19-22). Most of the *C. bescii* plant cell wall-degrading enzymes await detailed biochemical characterization to assess their application in the biofuel industry or for production of other value added products. In this study, we present biochemical analyses of six of the components of the xylan-degrading enzymes present in the *C. bescii* genome. Furthermore, we demonstrate the reconstitution of the enzymes into a functional enzyme cocktail. It is anticipated that the *C. bescii* xylan-degrading enzymes, which functions at a broad temperature range, will serve as a minimal enzyme mixture that can be further enhanced based on the structure of the bioenergy feedstock targeted for complete hydrolysis.

MATERIALS AND METHODS

Cloning of the constituents of a *C. bescii* xylan-degrading enzymes. The Rapid Annotation using Subsystem Technology (RAST) server (http://rast.nmpdr.org/) (23) was used for analyzing the *C. bescii* genome sequence. The *C. bescii* genes expressed for biochemical characterization in this study were amplified from the genomic DNA of the bacterium by use of PrimeSTAR HS DNA Polymerase (Takara, Shiga, Japan). The primers for cloning the genes are listed in Supplemental Table 1. For *cbXyn10A* and *cbCBM-Xyn10B*, the genes were first cloned into the TA-cloning vector pGEM-T (Promega, Madison, WI). The *cbCBM-Xyn10B* gene was obtained by performing two independent PCR reactions using primers shown in the Supplemental Table 1. The two PCR products were then combined and another round of PCR was carried out for fusion of the two genes. The gene was then excised from the vector by digestion with NdeI and XhoI and subcloned in-frame with the hexahistidine (His_6) encoding sequence of a modified pET28a (Merck, Darmstadt, Germany) gene expression vector at the NdeI/XhoI restriction sites. The pET28a vector was modified by replacing the gene encoding kanamycin resistance with that coding for ampicillin resistance (24). The other genes, i.e., *cbXyn10B*, *cbXyn10ATM1*, *cbXyn10ATM2*, *cbAra51A*, *cbXyl3A*, *cbAgu67A*, and *cbAxe1A* were PCR amplified and treated with the exonuclease activity of T4 DNA polymerase and annealed to a pET-46 Ek/LIC vector as described by the manufacturer (Merck, Darmstadt, Germany). The annealed products were transformed into an *E. coli* JM109 strain by electroporation and the recombinant plasmids were selected on Lysogeny broth (LB) agar plates infused with ampicillin at 100 µg/ml. The inserts in the recombinant plasmids (pET28a or pET-46 Ek/LIC) were sequenced (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois) to confirm the integrity of the genes.

Gene expression. For expression of the genes, the recombinant plasmids were transformed into *E. coli* BL-21 CodonPlus (DE3) RIPL (Stratagene, La Jolla, CA) competent cells by heat shock. The transformed cells were grown overnight on LB agar plates supplemented with ampicillin (100 µg/mL) and chloramphenicol (50 µg/ml) at 37°C. After 12 h, single colonies were picked and inoculated into fresh LB medium (10 ml) supplemented with the same antibiotics (ampicillin and chloramphenicol) at the same concentrations and cultured for 8 h at 37°C. The pre-cultures were then used to inoculate fresh LB medium (1 L) supplemented with the two antibiotics and the cultures were incubated at 37°C with vigorous shaking (225 rpm). At an optical density of 0.3 at 600 nm (OD_{600}), isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, the temperature was shifted to 16°C, and the culturing was continued for another 16 h. In the case of the expression of CbAra51A, the final concentration of IPTG was 10 µM. The cells were then harvested by centrifugation at 2,575 × *g* for 15 min, and the cell pellets were re-suspended in 35 ml of lysis buffer (50 mM Tris-HCl, 300 mM NaCl, pH 7.0) and ruptured by two passages through an EmulsiFlex C-3 cell homogenizer (Avestin, Ottawa, Canada). For CbAra51A, the cell pellet was re-suspended in a different lysis buffer (25 mM Tris-HCl, 5% Glycerol, 750 mM NaCl, 20 mM imidazole, and 1.25% Tween-20, pH7.5). Each of the cell lysates was clarified by centrifugation at 20,000 × *g* for 30 min at 4°C to remove the cell debris.

Purification of CbXyn10A and CbXyn10B. The recombinant proteins were purified using Talon Metal Affinity resin (Clontech, Mountain View, CA) according to the supplier's instruction. The proteins that bound to the resin were eluted using an elution buffer composed of

50 mM Tris-HCl, pH 7.5, 300 mM NaCl and 250 mM imidazole. CbXyn10A and CbXyn10B were further purified by ion exchange chromatography followed by gel filtration. CbXyn10A and CbCBM-Xyn10B were purified by cation exchange chromatography using an AKTAxpress fast protein liquid chromatography (FPLC) equipped with a 5 ml HiTrap SP HP column (GE Healthcare, Piscataway, NJ) with a binding buffer (50 mM Tris-HCl, pH 7.0) and an elution buffer (50 mM Tris-HCl, 1M NaCl, pH 7.0). CbXyn10B, CbXyn10A-TM1, and CbXyn10A-TM2 were purified by anion exchange chromatography using a 5 ml HiTrap Q HP column (GE Healthcare, Piscataway, NJ) with a binding buffer (50 mM Tris-HCl, pH 7.0) and elution buffer (50 mM Tris-HCl, 1 M NaCl, pH 7.0). Finally, the eluted proteins were loaded onto a HiLoad 16/60 Superdex 200 gel filtration column (GE Healthcare, Piscataway, NJ) and the eluted proteins were stored in the gel filtration buffer composed of 50 mM Tris-HCl, 150 mM NaCl, pH7.5.

Purification of CbXyl3A and the accessory xylan-degrading enzymes. CbXyl3A and the accessory xylan-degrading enzymes (CbAra51A, CbAgu67A, and CbAxe1A) were also purified using the Talon Metal Affinity resin. For CbXyl3A, the protein was purified by gel filtration by passing the proteins through a HiLoad 16/60 Superdex 200 column using the protein storage buffer as a mobile phase. For CbAra51A, the protein was further purified by anion exchange using a 5 ml HiTrap Q HP column with a binding buffer composed of 20 mM Tris-HCl, pH7.5 and an elution buffer composed of 20 mM Tris-HCl, 1 M NaCl, pH7.5.

158 **Analysis of purified proteins.** Samples from eluted fractions were analyzed by sodium dodecyl
159 sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli's method (25)
160 and protein bands were visualized by staining with Coomassie brilliant blue G-250. Eluted
161 fractions were pooled and the proteins were exchanged into a protein storage buffer (50 mM
162 Tris-HCl, 150 mM NaCl, pH 7.5) by three successive concentration and dilution cycles with
163 Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA).
164
165 **Screening for the activities of *C. bescii* xylan-degrading enzymes on polysaccharides and**
166 **oligosaccharides.** The activities of CbXyn10A and CbXyn10B on different polysaccharides
167 were screened by incubating 0.5 μM of each enzyme with 2.5 mg/ml xylan substrates (wheat
168 arabinoxylan, WAX; oat spelt xylan, OSX; birchwood xylan, BWX), cellulose substrate (sodium
169 carboxymethyl cellulose, CMC), 1,4-β-D-mannan, konjac glucomannan (KGM), and arabinan at
170 75°C for 14 h. The WAX, 1,4-β-D-mannan, and KGM were purchased from Megazyme
171 (Wicklow, Ireland), OSX and BWX were from Sigma-Aldrich (St. Louis, MO), and CMC was
172 from Acros Organics (Geel, Belgium). The reducing sugars released from the reaction mixtures
173 were measured by a *p*HBAH (*p*-hydroxybenzoic acid hydrazide, Sigma-Aldrich, St. Louis, MO)
174 method (26), and the components of the end products were analyzed by thin-layer
175 chromatography (TLC). In the case of CbXyl3A, 0.5 μM of the enzyme was incubated with 1
176 mg/ml of xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), and
177 xylohexaose (X6) in a phosphate buffer (50 mM sodium phosphate, 150 mM NaCl, pH 6.5) at
178 75°C for 14 h. The end products of the reactions were analyzed through TLC with xylose (X1),
179 X2, X3, and X4 as the standards. The xylose was purchased from Sigma-Aldrich (St. Louis, MO),
180 and the xylooligosaccharides were purchased from Megazyme (Wicklow, Ireland). In addition,

the activities of CbXyl3A and CbAgu67A on aldouronic acid mixture (Megazyme, Wicklow, Ireland) were determined by incubation of 0.5 µM of CbXyl3A or 0.5 µM of CbAgu67A or both enzymes with 1 mg/ml aldouronic acid mixture at 75°C for 14 h. The end products of the reactions were analyzed by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) described below. The xylose and xylooligosaccharides (X2-X3) were used as standards. The activities of CbAra51A on arabinose-containing polysaccharides were determined by incubating 0.5 µM of CbAra51A with 2.5 mg/ml WAX, RAX (rye arabinoxylan), OSX, debranched arabinan, and arabinan at 75°C for 14 h. The end products of the reactions were also analyzed by HPAEC-PAD.

Determination of optimal pH and temperature. The following buffers were used throughout the study for pH profiling of the *C. bescii* enzymes: 50 mM sodium citrate, 150 mM NaCl (pH 4.0~pH 6.0) and 50 mM sodium phosphate, 150 mM NaCl (pH 6.5~pH 8.0). For the endoxylanases CbXyn10A and CbXyn10B, each enzyme at a final concentration of 0.5 µM was incubated with 10 mg/ml WAX at 75°C in 10 min. The reducing sugars released were measured using the *p*HBAH method and the initial velocities were calculated. The optimal temperatures were determined by incubating each enzyme at a final concentration of 0.5 µM with 10 mg/ml WAX in a buffer of optimal pH at temperatures ranging from 40°C to 95°C with an interval of 5°C.

The CbXyl3A, CbAra51A, and the CbAxe1A were each incubated at an appropriate concentration with a specific *p*NP-substrate at 75°C and the released *p*NP was continuously monitored by determining the absorbance at 400 nm using the Cary 300 UV-Vis spectrophotometer (Agilent, Santa Clara, CA). The substrate was *p*NP-β-D-xylopyranoside

(*p*NPX) for CbXyl3A, *p*NP-α-L-arabinofuranoside (*p*NPA) for CbAra51A, and *p*NP-acetate (*p*NPAc) for CbAxe1A. The optimal temperature of each enzyme was measured in its corresponding optimal buffer at temperatures ranging from 40°C - 95°C for CbXyl3A and CbAra51A, from 50°C - 90°C for CbAgu67A, and from 40°C - 75°C for CbAxe1A at 5°C intervals. The aldouronic acid mixture was used as the substrate for the determination of optimal pH and temperature for CbAgu67A and the end products of the reactions were analyzed by HPAEC-PAD. Relative activities were calculated based on the amount of released xylose.

Analysis of the components of xylan hydrolysis. TLC and HPAEC-PAD were used for the analysis of the contents of end products of hydrolysis of xylan. The TLC analysis of the end products was carried out by spotting on a 10-cm by 20-cm Whatman silica gel 60A with a thickness of 250 μm (Maidstone, England) and air-dried at room temperature. The plate was developed with a mobile phase composed of *n*-butanol-acetic acid-H_2O with a volumetric ratio of 10:5:1. After development, the plate was dried at room temperature, and visualization of the products was initiated by spraying the plate with a mixture of methanolic orcinol (0.2% [wt/vol]) and sulfuric acid (20% [vol/vol]) with a volumetric ratio of 1:1. The plate was incubated at 80°C until the spots representing the sugars could be clearly seen.

The HPAEC-PAD analysis was carried out as described in our previous report (14). Briefly, a fixed volume of 100 μl of appropriately diluted samples was injected into a System Gold high-performance liquid chromatography (HPLC) instrument from Beckman Coulter (Fullerton, CA) equipped with CarboPac PA1 guard (4 by 50 mm) and analytical (4 by 250 mm) columns from Dionex Corporation (Sunnyvale, CA) and a Coulochem III electrochemical

detector from ESA Biosciences (Chelmsford, MA). Arabinose, xylose, xylobiose, and xylotriose were injected as standards.

Kinetic analysis of *C. bescii* xylan-degrading enzymes. To determine the kinetic parameters of the components of the *C. bescii* xylan-degrading enzymes, reactions were carried out at the optimal pH and temperature of each enzyme. An appropriate concentration of each enzyme was initially determined where the relationship of released product versus time was linear. Then the enzyme was reacted with a range of concentrations of the substrate. For CbXyn10A and CbXyn10B, xylan substrates (WAX, OSX, and BWX) were used. The released reducing sugars were measured using the *p*HBAH method. For the other enzymes, *p*NP-substrates stated above were used as substrates. The release of *p*NP was continuously measured with a Cary 300 UV-Vis spectrophotometer by monitoring the change of absorbance at 400 nm. The initial velocities of each enzyme were plotted against the substrate concentrations and the kinetic parameters were estimated by fitting the data to the Michaelis-Menten equation using the software GraphPad Prism 5.01 (GraphPad, San Diego, CA).

Thermostability of the *C. bescii* enzymes. To determine the thermostability of the *C. bescii* enzymes, each protein in its storage buffer was incubated at different temperatures for 24 h. At different time points, samples were taken and the residual activities were measured. For CbXyn10A and CbXyn10B, the residual activities were analyzed by measuring the xylanase activity with WAX as the substrate. The released reducing sugars were measured through the *p*HBAH assay. For CbXyl3A, CbAra51A, and CbAxe1A, the substrates used were the *p*NP-linked substrates, and the released *p*NP was measured. In the case of CbAgu67A, the aldouronic

acid mixture was used as the substrate, and the amounts of released xylose were analyzed by HPAEC-PAD. The residual relative activities were calculated by dividing the initial velocity of each enzyme sample against the initial velocity at time zero (unheated enzyme) and presented as a percentage.

Reconstitution of a *C. bescii* xylan-degrading enzyme mixture. The endoxylanase CbXyn10A (0.5 μM) was incubated with OSX (8.0%, w/v) at 75°C for 15 h in a citrate buffer (50 mM sodium citrate, 150 mM NaCl, pH 6.0). The accessory enzymes were successively added to CbXyn10A (final concentration: 0.5 μM for CbAra51A, CbAgu67A, and CbAxe1A, and 4 μM for CbXyl3A) and the enzymatic reactions were carried out under the same condition for 15 h. The end products of the reactions were applied to HPAEC-PAD analysis. CbXyn10A was replaced with equimolar concentration of CbXyn10B (0.5 μM) or a combination of CbXyn10A and CbXyn10B (0.25 μM each) to compare xylan hydrolysis in the presence of either endoxylanase or in the presence of the two enzymes. To gain insight into the activities of the *C. bescii* xylan-degrading enzyme mixture (CbXyn10A, CbAra51A, CbXyl3A, CbAgu67A, and CbAxe1A) at different temperatures, the enzyme mixture was incubated with OSX (8.0%, w/v) in the citrate buffer at different temperatures (65°C, 70°C, 75°C, and 80°C) for 15 h. The released reducing sugars were determined using the *p*HBAH assay.

RESULTS AND DISCUSSION

Identification of genes encoding xylan-degrading enzymes from the genome of *C. bescii*. The bacterium *C. bescii* is known for its ability to grow on both cellulose and xylan at high temperatures (17). The genome of *C. bescii* was initially uploaded onto the Rapid Annotation using Subsystem Technology (RAST) server (23). Cellulase genes in *C. bescii* have already been reported as located in a gene cluster (18) that encodes nine multi-modular glycoside hydrolases (20). The *C. bescii* genome was analyzed for genes encoding proteins with amino acid sequence homology to carbohydrate active enzymes (CAZymes) from glycoside hydrolase families with demonstrated xylan-degrading activity (GH 3, 5, 10, 11, 43, 51, 67; CE 1, 6). To perform these analyses, representative enzymes from each CAZy family were used as queries in a BLASTp search of the *C. bescii* genome. These analyses identified six genes in a cluster encoding putative xylan-degrading enzymes. The genes coded for two putative endoxylanases (Cb193, GenBank accession number ACM59335; Cb195, GenBank accession number ACM59337), two putative xylosidases (Cb194, GenBank accession number ACM59336; Cb197, GenBank accession number ACM59339), a putative esterase (Cb196, GenBank accession number ACM59338), and a GH43 protein with two GH43 catalytic domains and two carbohydrate-binding modules (CBMs) (Cb192, GenBank accession number ACM59334). In addition, several genes encoding putative glycoside hydrolases and carbohydrate esterases were found elsewhere in the genome. These genes coded for a putative xylosidase (Cb2487, GenBank accession number ACM61424), a putative α-L-arabinofuranosidase (Cb1172, GenBank accession number ACM60204), a putative α-glucuronidase (Cb909, GenBank accession number ACM59969), and a putative acetyl xylan esterase (Cb162, GenBank accession number ACM59304). After preliminary screening of the enzymatic activities present in several of the gene products listed above, Cb193, Cb195,

Cb1172, Cb2487, Cb909, and Cb162 were designated CbXyn10A, CbXyn10B, CbAra51A, CbXyl3A, CbAgu67A, and CbAxe1A, respectively. These enzymes were selected mainly because they exhibited robust activities against critical linkages in xylan in the preliminary screening. Furthermore some of the remaining enzymes were either difficult to express in *E. coli* (such as Cb192) or showed undesirable side reactions, such as the transglycosylation activities of Cb194. Each protein was then subjected to biochemical analyses.

Modular arrangement of CbXyn10A and CbXyn10B. The *C. bescii* CbXyn10A and CbXyn10B contained amino acid sequences that suggested that they are members of the GH10 family of endoxylanases. Whereas only a GH10 catalytic module was found in the polypeptide of CbXyn10B, the CbXyn10A contained two additional modules in its N-terminal half of the polypeptide. The two modules were identified as a tandem repeat of family 22 carbohydrate-binding modules (CBM22s) (Figure 1A). The amino acid sequences of the two CBM22s exhibited only 19.4% identity. In contrast, the polypeptide sequences of the GH10 catalytic modules of the two proteins shared 43.5% identity. CbXyn10A was also predicted to harbor a signal peptide at the extreme N-terminus, suggesting that it is a secreted protein. The genes encoding CbXyn10A and CbXyn10B were expressed in *E. coli*, and the recombinant proteins were purified to near homogeneity (Figure 1B).

Biochemical activities of the *C. bescii* endoxylanases. The optimal pH values and temperatures of the *C. bescii* endoxylanases were determined to be 6.0 and 85°C for CbXyn10A and 6.5 and 80°C for CbXyn10B, respectively (Supplemental Table 2). To understand the roles of the two CBM22s in the enzymatic degradation of xylans by CbXyn10A, we serially deleted

the two CBM22s from CbXyn10A. In addition, the two CBM22s were appended to the N-terminus of CbXyn10B. The schematic structures of these truncational mutants are shown in Supplemental Figure 1A. These proteins were successfully expressed, purified, and subsequently analyzed on an SDS-PAGE gel (Supplemental Figure 1B). Deletion of the CBM22s from CbXyn10A negatively impacted the optimal temperatures of the mutants. The optimal pHs and temperatures of all these truncation mutants were also measured and are listed in Supplemental Table 2. CbXyn10A-TM1 (with one CBM22 deleted) and CbXyn10A-TM2 (with two CBM22s deleted) had optimal pHs of 6.5 and 6.0, respectively, and optimal temperatures of 75°C and 70°C (Supplemental Figure 1C), respectively. Adding the two CBM22s did not change the optimal pH significantly or increase the optimal temperature of CbXyn10B; on the contrary, the mutant CbCBM-Xyn10B had an optimal temperature of 55°C, which was 25°C lower than the wild-type CbXyn10B. Both CbXyn10A and CbXyn10B were screened for their ability to hydrolyze different polysaccharides. As shown in Figure 2A, the two enzymes only released significant end products or oligosaccharides from the xylan substrates. Measurements of reducing ends demonstrated that while large amounts of reducing ends were released from WAX, OSX, and BWX by each of the two endoxylanases, reducing ends released from CMC (glucose-configured), KGM (mixed linkage of glucose and mannose), 1,4-β-D-mannan (mannose-configured), and arabinan (arabinose-configured) were very low (Fig. 2A). On TLC plates, CbXyn10A (Fig. 2B) and CbXyn10B (Fig. 2C) displayed nearly identical patterns of hydrolysis of the three xylan substrates. Xylose and xylo-oligosaccharides including xylobiose, xylotriose, xylotetraose, and xylopentaose were released from all these substrates. In contrast, loading of similar amounts of reaction mixtures that contained CMC, KGM, 1,4-β-D-mannan, and arabinan as substrates failed to show products on the TLC plates. However, based on the *p*HBAH assay,

reducing sugars appeared to be released from KGM by CbXyn10A and from 1,4-β-D-mannan by CbXyn10B. The inability to detect end products from substrates, through the TLC analysis, is likely to be due to formation of products that were not well resolved by this method.

The CbXyn10B does not possess a signal peptide; however, this enzyme was still able to degrade complex heterogeneous xylans. Although intracellular xylan-degrading enzymes are generally thought to degrade oligosaccharides of low degree of polymerization (27, 28), there is evidence that these enzymes, from both Gram positive and Gram negative bacteria, can hydrolyze branched or long chain oligosaccharides, and even intact polysaccharides. Reported enzymes with such activities are the *Cellvibrio mixtus* XylC (29), *Paenibacillus* sp. BP-23 Xylanase B (30), *Clostridium thermocellum* arabinofuranosidase Araf51 (31), and a *Geobacillus stearothermophilus* α-glucuronidase (28). It is still unclear why these xylan-degrading enzymes predicted to be intracellularly located possess the capacity to hydrolyze long chain substrates. For Gram negative bacteria, it is suggested that such enzymes reside in the periplasm and act as 'sensor' proteins important for regulation of the expression of plant cell wall degrading genes (29). Interestingly, evidences are emerging that the Gram positive bacteria also possess such a periplasmic space (32, 33). We searched the upstream nucleotide sequence of *cbXyn10B* and could not find an alternate translational start site, which might result in a putative signal peptide. Knowledge of the physiology of *C. bescii* cell is very limited. Therefore, it is not known if other alternative secretion pathways are present in this organism.

The estimated kinetic parameters of CbXyn10A and CbXyn10B on the different xylan substrates were presented in Supplemental Table 2. CbXyn10A was most active on WAX, with a k_{cat} of 7864.0±944.0 s^{-1} and a K_m of 14.0±4.2 mg/ml, respectively. The k_{cat} and K_m were 123.2±6.6 s^{-1} and 1.3±0.5 mg/ml for BWX, and 102.4±7.4 s^{-1} and 3.5±1.1 mg/ml for OSX,

360 respectively. Compared with *C. bescii* CbXyn10A, CbXyn10B exhibited a lower k_{cat} for the
361 simpler substrate WAX and higher k_{cat} for the more complex substrates BWX and OSX. Thus,
362 CbXyn10B exhibited k_{cat} and K_m of 4598.0±163.0 s^{-1} and 5.9 ±0.7 mg/ml, respectively, for WAX,
363 and 198.1±7.6 s^{-1} and 4.0±0.7 mg/ml for BWX, and 400.0±20.9 s^{-1} and 13.3±2.0 mg/ml for OSX,
364 respectively. Note that the K_m values of CbXyn10B for the more complex substrates were always
365 higher than those for CbXyn10A, which harbors the two CBMs.

366 The predicted intracellularly located CbXyn10B had a higher catalytic efficiency (k_{cat}/K_m:
367 779.3 s^{-1} ml/mg) than CbXyn10A (561.7 s^{-1} ml/mg) on the simplest substrate WAX. In contrast,
368 the catalytic efficiency of CbXyn10B on the more complex substrate OSX (30.1 s^{-1} ml/mg) was
369 similar to that of CbXyn10A on OSX (29.3 s^{-1} ml/mg), and on BWX the estimated catalytic
370 efficiency of CbXyn10A was about twice that of Xyn10B (94.8 s^{-1} ml/mg versus 49.5 s^{-1} ml/mg).
371 The two CBM22s likely facilitate binding of CbXyn10A to the complex xylans, since their
372 removal drastically increased the K_m of CbXyn10A for OSX and BWX, but not for WAX
373 (Supplemental Table 2). In addition, removal of the CBM22s had deleterious effect on the
374 specific activity of CbXyn10A (Supplemental Figure 1D). Appending the two CBM22s,
375 however, did not increase either the catalytic efficiency or the specific activity of CbXyn10B
376 (Supplemental Table 2, Supplemental Figure 1D), indicating that the role of the CBM22s was
377 specific to CbXyn10A and not for CbXyn10B. Therefore, despite the nearly identical hydrolysis
378 patterns of CbXyn10A and CbXyn10B, they differed in their catalytic properties on complex or
379 simple xylan substrates, supporting the hypothesis that multiple glycoside hydrolases within the
380 same family are not necessarily redundant in function (14, 34). We hypothesized that
381 heterogeneous xylans are mainly degraded by the extracellular CbXyn10A into simpler

intermediates. These intermediates are then transported into *C. bescii* for further hydrolysis by CbXyn10B.

Accessory xylan-degrading enzymes from *C. bescii.* Heterogeneous xylans contain side chains that tend to sterically impede complete hydrolysis of xylan substrates by the combined action of endoxylanases and β-xylosidases. It was anticipated that by adding accessory enzymes that cleave the side chain linkages, a synergistic hydrolysis of xylan will be observed among the enzymes. The modular or domain architecture of CbXyl3A, CbAra51A, CbAgu67A, and CbAxe1A was shown in Fig. 1A, and the recombinant proteins purified from *E. coli* were shown in the SDS-PAGE in Fig. 1B. The optimal pH values and temperatures for the *C. bescii* accessory xylan-degrading enzymes were pH6.0 and 90°C for CbXyl3A, pH6.0 and 90°C for CbAra51A, and pH5.5~6.0 and 70~75°C for CbAgu67A, respectively (Supplemental Table 2). CbAxe1A was highly active at pH6~8 and 75°C; however, the assays at higher pH and temperatures were not successful because the substrate, *p*NP-acetate, was very unstable under such conditions (data not shown).

The two endoxylanases of *C. bescii* have similar reaction optima to the accessory xylan-degrading enzymes. An advantage of formulating a xylan-degrading enzyme mixture from a single microorganism is that the enzymes are likely to have similar optimal reaction conditions. Indeed, the optimal pHs of the *C. bescii* xylan-degrading enzymes were within a range of 5.5 - 6.5 (except for the acetyl xylan esterase CbAxe1A). Furthermore, the optimal temperatures were within a range of 75 -90°C. We anticipate that enzymes from microorganisms that live in an environment similar to that of *C. bescii* may be added for further improvement of the current

enzyme mixture. Appropriate candidate microbes may be other species of the genus *Caldicellulosiruptor* (17).

The *C. bescii* CbAra51A released arabinose from a range of arabinose-containing plant polysaccharides including WAX, RAX, OSX, debranched arabinan, and arabinan (Fig. 3A). Therefore, although predicted to be an intracellular enzyme, CbAra51A was also able to hydrolyze complex polysaccharides, which was similar to CbXyn10B. Incubation of xylooligosaccharides, from xylobiose to xylotetraose, with CbXyl3A led to complete hydrolysis of these substrates to xylose. CbXyl3A appeared to prefer short xylo-oligosaccharides with degrees of polymerization of 5 or less, as a longer xylooligosaccharide (xylohexaose) was incompletely degraded (Fig. 3B).

Incubation of CbAgu67A with aldouronic acid mixture resulted in cleavage of α-glucuronic acid from this substrate with concomitant release of xylose and xylo-oligosaccharides (such as xylobiose and xylotriose) (Fig. 3C). In addition, CbAgu67A and CbXyl3A synergistically released xylose from the aldouronic acid mixture (Fig. 3C). CbAxe1A showed activity on *p*NP-acetate by releasing *p*NP, which could be monitored spectroscopically (data not shown). Based on the results above, it was concluded that CbAra51A is an α-L-arabinofuranosidase (Fig. 3A), CbXyl3A is a β-xylosidase (Fig. 3B), CbAgu67A is an α-glucuronidase (Fig. 3C), and CbAxe1A is an acetyl-xylan esterase.

Except for CbAgu67A, the two kinetic parameters k_{cat} and K_m were estimated for each of the accessory xylan degrading enzymes, although in each case an artificial substrate was used. The k_{cat} and K_m of CbXyl3A with *p*NPX as substrate were 620.0±8.0 s^{-1} and 8.2±0.2 mM, respectively; the parameters for CbAra51A with *p*NPA as substrate were a k_{cat} of 1458.0±66.9 s^{-1} and a K_m of 1.3±0.2 mM, respectively; and for CbAxe1A with *p*NPAc as substrate a k_{cat} of

427 170.0±4.0 s^{-1} and 0.3±0.0 mM, respectively (Supplemental Table 2). Aldouronic acid is a
428 mixture of different substrates, and its hydrolysis involves multiple heterogeneous reactions with
429 end products not easily measured. For this reason, the kinetic parameters of CbAgu67A were not
430 determined on the aldouronic acid mixture.
431
432 **_C. bescii_ xylan-degrading enzymes are thermostable enzymes.**
433 *C. bescii* is a hyperthermophilic bacterium, and its thermostable xylan-degrading enzymes
434 should be of interest for industrial application, including in the biofuel industry. The capacity to
435 function at high temperatures over an extended period of time will enhance the utility of such an
436 enzyme cocktail. Thus, the thermostabilities of the six enzymes were investigated (Fig. 4A-F).
437 CbXyn10A appeared to be more thermostable than CbXyn10B. At 80°C, CbXyn10A had a half-
438 life of ~10.5 h (Fig. 4A), while CbXyn10B had a half-life of ~3 h (Fig. 4B). When the
439 temperatures were decreased to 70°C or 75°C, both enzymes were quite thermostable.
440 CbXyn10A had 61.8±4.2% and 61.1±4.0% residual activities after 24 h incubation at 70°C and
441 75°C, respectively, while CbXyn10B had 70.0±6.2% and 47.5±1.9% residual activities at 70°C
442 and 75°C, respectively. CbXyl3A had 54.5±7.1% and 19.9±8.2% residual activities after 24 h
443 incubation at 70°C and 75°C, respectively, but rapidly lost its activity at 80°C and higher
444 temperatures (Fig. 4C). CbAra51A retained 57.2±4.9%, 45.2±1.1%, 35.1±3.7%, and 22.1±0.8%
445 residual activities after 24 h of incubation at 70°C, 75°C, 80°C, and 85°C, respectively (Fig. 4D).
446 The arabinofuranosidase rapidly lost all enzymatic activity after 30 min incubation at 90°C (Fig.
447 4D). CbAgu67A had 20.5±7.0% and 8.1±4.1% residual activities after 24 h at 65°C and 70°C,
448 respectively (Fig. 4E). CbAxe1A did not lose any activity after 24 h incubation at 60°C. At 65°C,

it had 58.5±1.3% residual activity after 24 h incubation. CbAxe1A gradually lost all its activity at 70°C between 19 and 20 h (Fig. 4F).

The thermostability and kinetic parameters of the *C. bescii* xylan-degrading enzymes as well as those of thermophilic xylan-degrading enzymes from other microbes were listed and compared in Supplemental Table 2. Although most of the *C. bescii* xylan-degrading enzymes investigated in this study were thermostable at 70~75°C, the degree of thermostability differed among the enzymes (Fig. 4, Supplemental Table 2). The extracellular CbXyn10A and the intracellular CbXyn10B were encoded in the same gene cluster. Homologs of both CbXyn10A and CbXyn10B with amino acid sequence identities above 80%, as well as a similar synteny of a xylan-degrading enzymes gene cluster is found in *Caldicellulosiruptor saccharolyticus*, *Caldicellulosiruptor owensensis*, and *Caldicellulosiruptor kronotskyensis* (data not shown), suggesting that the two xylan-degrading enzymes may play an important role in xylan utilization by these bacteria (35-37). One may postulate that the enzymes encoded within the same gene cluster will exhibit similar thermostabilities. However, CbXyn10A was more thermostable than CbXyn10B. The presence of the two CBM22s may help stabilize CbXyn10A at high temperatures, as deletion of the CBM22s decreased the temperature optimum by 15°C (Supplemental Table 2). This stabilizing effect has also been observed for other CBM22s (38, 39). The stabilities of these truncation mutants in the 24-h incubation were not determined in this study. In addition, since CbXyn10B (and also CbAgu67A and CbAxe1A) does not possess a signal peptide, it is possible that the intracellular factors help to stabilize the proteins (40). It is important to note that post-translational modification may also influence the thermostabilities of these polypeptides produced in the native organism. Furthermore, binding of the enzymes to substrate may further enhance their thermostability.

Due to different assay conditions, a precise comparison of the *C. bescii* xylan-degrading enzymes with other thermophilic xylan-degrading enzymes may be difficult to achieve. Nevertheless, the data presented in Supplemental Table 2 suggests that the *C. bescii* xylan-degrading enzymes are promising enzymes for deconstruction of xylans into fermentable sugars at high temperatures. As thermophilic biocatalysts, the *C. bescii* xylan-degrading enzymes generally have high catalytic efficiencies (k_{cat}/K_m). For example, CbXyn10A had a catalytic efficiency of 779.3 s^{-1} ml/mg on WAX and CbAra51A had a catalytic efficiency of 1121.5 s^{-1} mM^{-1} on *p*NPA. Many thermophilic enzymes listed in Supplemental Table 2 may either have high catalytic efficiencies (such as the *Neocallimastix patriciarum* CDBFV, with a catalytic efficiency of 339.2 s^{-1} ml/mg on BWX) or thermostability (such as the *Thermotoga maritima* Tm-AFase, which has 100% residual activity after incubation at 90°C for 24 h). However, for the enzymes that have been characterized in detail, only a few, including the *C. bescii* enzymes, appear to possess the two merits of high catalytic efficiency and high thermostability. The bacterium *C. bescii* was isolated from a hot-spring in Kamchatka (Russia) more than 2 decades ago (41); thereafter, several cellulose- and xylan-utilizing *Caldicellulosiruptor* species have also been isolated from thermal springs (37, 42, 43). The high efficiency of the *C. bescii* xylan-degrading enzymes in hydrolyzing xylan, as well as their thermostability, underpins their potential in industrial processes at high temperatures.

Reconstitution of a thermostable xylan-degrading enzyme mixture from *C. bescii*.

CbXyn10A exhibited a higher thermostability compared to CbXyn10B, and as they shared similar hydrolytic activity and pattern on xylan substrates, a xylan-degrading enzyme cocktail was initially reconstituted based on CbXyn10A. The soluble WAX is a simple substrate

composed mainly of xylose linked together in β-1,4-glycosidic bonds as the backbone with arabinose side chains. In contrast, OSX and BWX are more complex substrates, with OSX containing a higher amount (9.7%) of arabinose than BWX (1.0%) (44). Therefore, OSX was chosen as a model xylan to examine the enzymatic activities in the reconstituted xylan-degrading enzyme mixture. The endoxylanase CbXyn10A alone released xylo-oligosaccharides as well as xylose from OSX, as analyzed by HPLC (Fig. 5A). Adding the arabinofuranosidase CbAra51A to CbXyn10A released arabinose in addition to increased amounts of xylose from 15.1±0.1 mM to 16.5±0.0 mM (p<0.01) and xylobiose from 16.7±0.1 mM to 20.9±0.2 mM (p<0.01). Adding the β-xylosidase CbXyl3A to the CbXyn10A/CbAra51A mixture converted nearly all xylo-oligosaccharides to xylose, thereby resulting in a large increase in xylose (92.2±0.5 mM) in the reaction mixture. Upon addition of the α-glucuronidase CbAgu67A to the three-enzyme mixture, a slight but significantly enhanced release of xylose to 96.2±0.5 mM (p<0.01) was observed. Lastly, adding the acetyl xylan esterase CbAxe1A further enhanced release of xylose to 106.0±0.3 mM (p<0.01). Adding accessory enzymes markedly improved the efficiency of xylan degradation by the *C. bescii* endoxylanases. Therefore, other accessory xylan-degrading enzymes such as ferulic acid esterase, galactosidase, and mannanase may be added to the *C. bescii* xylan-degrading enzyme mixture, based on the composition of the heterogeneous xylan to be hydrolyzed. Such rational incorporation of other hemicellulose targeting enzymes will lead to higher yields of fermentable sugars.

Two modifications were made to the *C. bescii* xylan-degrading enzyme mixture by either replacing CbXyn10A completely with CbXyn10B or by replacing half of CbXyn10A with CbXyn10B in the mixture. The activities of the reconstituted xylan-degrading enzyme mixtures were compared to determine their capacities to release reducing ends from OSX. The mixture

containing CbXyn10A alone as the endoxylanase released slightly more reducing sugars than the mixture containing CbXyn10B as the sole endoxylanase. Furthermore, combining CbXyn10A/CbXyn10B with the four accessory enzymes did not lead to a higher release of reducing ends from OSX (data not shown). Therefore, the xylan-degrading enzyme mixture containing only CbXyn10A as the endoxylanase was selected for investigation of its ability to hydrolyze xylan at different temperatures. The hydrolytic activities on OSX based on release of reducing ends at 65°C, 70°C, and 75°C were not different. At 80°C, the enzyme cocktail released lower amounts of reducing ends than at the three lower temperatures (Fig. 5B). The *C. bescii* xylan-degrading enzyme cocktail therefore works over a broad range of high temperatures. The decrease in end products at 80°C may suggest denaturing of the recombinant enzymes, although in the bacterium this may be prevented by post-translational modification.

Aside from this report, there was an attempt in which a thermophilic xylan-degrading enzyme mixture was reconstituted (16). In that study, the source of the enzymes was the thermophilic bacterium *Clostridium stercorarium* and the mixture contained two endoxylanases, one xylosidase, and one arabinofuranosidase. *C. stercorarium* has an optimal growth temperature of 65°C. Consequently, the optimal temperatures of the selected enzymes of *C. stercorarium* were from 55 to 75°C (16). The *C. bescii* xylan-degrading enzyme cocktail is different from that of *C. stercorarium*. The *C. bescii* enzyme mix contains one xylanase (CbXyn10A) and four accessory enzymes (one xylosidase CbXyl3A, one arabinofuranosidase CbAra51A, one α-glucuronidase CbAgu67A, and one acetyl xylan esterase CbAxe1A), and the contents of the xylan-degrading enzyme mixture described here also have higher optimal temperatures. Thus, the enzyme mixture was effective in hydrolysis of xylan at 65°C to 80°C. Xylan-degrading enzymes that act at these high temperatures (from 65°C to 80°C) have not been described in the literature.

Our group has also published a mesophilic xylan-degrading enzyme mixture from *Ruminococcus albus 8* (14). Five endoxylanases were tested in that study. The combination of one endo-xylanase with one β-xylosidase and one α-arabinofuranosidase, at 0.5 μM each, released xylose from 94.9±3.05 mM to 150.5±5.11 mM and arabinose from 10.6±0.54 mM to 21.2±1.01 mM from 8% oat spelt xylan at 37°C for 15 h. These concentrations are comparable to what were released from OSX using the *C. bescii* xylan-degrading enzymes. Therefore, the *C. bescii* xylan-degrading enzyme mix can serve as a basal enzyme mixture for depolymerization of xylans in bioenergy feedstock at high temperatures. However, it should be noted that, from the HPLC analysis, ~25% xylose and arabinose were released under the conditions used in the present study. Furthermore, our previous report demonstrated that xylan degrading enzymes from *R. albus* 8 exhibit end products inhibition (14). Therefore, more detailed analyses of the remaining components of the xylan and the inhibitory effects of the end products of hydrolysis on the *C. bescii* xylan-degrading enzymes are required. Insights gained into these questions will undoubtedly shed light on strategies to improve the basal xylan-degrading enzyme mixture described from *C. bescii* in this report.

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562 **FIGURE LEGENDS**

563 **Figure 1.** Polypeptides characterized as a basal xylan-degrading enzyme mixture of *C. bescii*. A.
564 Schematic diagram for the domain or modular architecture of enzymes combined as a basal
565 xylan-degrading enzyme mixture from *C. bescii*. CBM22: carbohydrate-binding module family
566 22; GH10: glycoside hydrolase family 10; GH3_N: N-terminal part of glycoside hydrolase
567 family 3; GH3_C: C-terminal part of family 3 glycoside hydrolase; Fn3: Fibronectin type 3
568 domain; GH51: glycoside hydrolase family 51; GH67N: N-terminal part of glycoside hydrolase
569 family 67; GH67M: middle part of glycoside hydrolase family 67; GH67C: C-terminal part of
570 family 67 glycoside hydrolase; AXE1: acetyl xylan esterase domain. B: SDS-PAGE analysis of
571 the purified recombinant proteins. Two μg of each of the proteins was resolved on a 12% SDS-
572 polyacrylamide gel.

573

574 **Figure 2.** Screening for the activities of CbXyn10A and CbXyn10B on plant polysaccharides
575 with different glycosidic linkages. A: Reducing sugar analysis. B and C: Thin-layer
576 chromatography analysis (B: CbXyn10A, and C: CbXyn10B). The plant polysaccharides were
577 incubated separately with either CbXyn10A or CbXyn10B at 75°C for 14 h. The amounts of
578 released reducing sugar were determined by the *p*HBAH method. To determine the components
579 of the released sugars in the hydrolyzed products, the samples were appropriately diluted and
580 applied to TLC analysis. WAX: wheat arabinoxylan; OSX: oat spelt xylan; BWX: birchwood
581 xylan; CMC: sodium carboxymethyl cellulose; KGM: konjac glucomannan; X1: xylose; X2:
582 xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose.

583

584 **Figure 3.** Activity analyses of accessory xylan-degrading enzymes (CbAra51A, CbXyl3A, and

CbAgu67A) from *C. bescii*. A: CbAra51A released arabinose from five arabinose-containing polysaccharides (WAX, RAX, OSX, debranched arabinan, and arabinan). CbAra51A (0.5 μM) was incubated with each polysaccharide (2.5 mg/ml) at 75°C for 14 h. The samples were heated at 100°C to inactivate the enzyme, and appropriately diluted samples were applied to HPLC analysis. B: Thin-layer chromatography analysis of xylo-oligosaccharides hydrolyzed by CbXyl3A. X1: xylose; X2: xylobiose; X3: xylotriose; X4: xylotetraose; X5: xylopentaose; X6: xylohexaose. C: HPLC analysis of aldouronic acid mixture hydrolyzed by CbAgu67A, CbXyl3A, or a combination of the two enzymes. Aldouronic acid mixture (1 mg/ml) were incubated with CbXyl3A (0.5 μM) and/or CbAgu67A (0.5 μM) in a citrate buffer (50 mM sodium citrate, 150 mM NaCl, pH 6.0) at 75°C for 14 h.

Figure 4. Thermostability analyses of CbXyn10A (A), CbXyn10B (B), CbXyl3A (C), CbAra51A (D), CbAgu67A (E), and CbAxe1A (F). The *C. bescii* xylan-degrading enzymes were incubated at either four (CbXyn10A, CbXyn10B, CbXyl3A, CbAgu67A, and CbAxe1A) or five (CbAra51A) different temperatures for 24 h. At different time intervals, samples were taken out and analyzed for the residual activities. By setting the activity of the enzymes at time zero as 100%, the residual activities were calculated by dividing the activities at different time intervals against the activity at time zero. The residual activities were presented in percentage. The substrates used for residual activity measurement were WAX for CbXyn10A and CbXyn10B, *p*NP-β-D-xylopyranoside for CbXyl3A, *p*NP-α-L-arabinofuranoside for CbAra51A, aldouronic acid mixture for CbAgu67A, and *p*NP-acetate for CbAxe1A.

Figure 5. Reconstitution of a thermostable *C. bescii* xylan-degrading enzyme mixture. A:

608 Hydrolysis of OSX by the core enzyme CbXyn10A alone or in presence of successively added
609 accessory xylan-degrading enzymes. OSX at a final concentration of 8.0% (w/v) was incubated
610 with CbXyn10A alone or in presence of the xylan-degrading enzymes at 75°C for 15 h in the
611 citrate buffer (50 mM sodium citrate, pH 6.0, 150 mM NaCl). The concentrations for the xylan-
612 degrading enzymes alone or in mixtures were CbXyn10A (0.5 µM), CbAra51A (0.5 µM),
613 CbXyl3A (4 µM), CbAgu67A (0.5 µM), and CbAxe1A (0.5 µM). The hydrolysis products were
614 appropriately diluted in H_2O and subjected to HPLC analysis. B: Hydrolysis of OSX by the
615 xylan-degrading enzyme mixture containing CbXyn10A as the only endoxylanase and incubated
616 at different temperatures. The end product yields were analyzed by the reducing sugar assay. The
617 *C. bescii* xylan-degrading enzymes included CbXyn10A (0.5 µM), CbAra51A (0.5 µM),
618 CbXyl3A (4 µM), CbAgu67A (0.5 µM), and CbAxe1A (0.5 µM). The reactions were carried out
619 by incubation of the enzymes with 8% OSX at 65ºC, 70ºC, 75ºC, and 80ºC for 15 h.
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REFERENCES

1. **Pauly M, Keegstra K.** 2008. Cell-wall carbohydrates and their modification as a resource for biofuels. Plant J. **54:**559-568.
2. **Duchesne LC, Larson DW.** 1989. Cellulose and the evolution of plant life. BioScience **39:**238-241.
3. **Galazka JM, Tian C, Beeson WT, Martinez B, Glass NL, Cate JH.** 2010. Cellodextrin transport in yeast for improved biofuel production. Science **330:**84-86.
4. **Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD.** 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature **463:**559-562.
5. **Rogers PL, Jeon YJ, Lee KJ, Lawford HG.** 2007. *Zymomonas mobilis* for fuel ethanol and higher value products. Adv Biochem Eng Biotechnol **108:**263-288.
6. **Lynd LR, van Zyl WH, McBride JE, Laser M.** 2005. Consolidated bioprocessing of cellulosic biomass: an update. Current opinion in biotechnology **16:**577-583.
7. **Wen F, Sun J, Zhao H.** 2010. Yeast surface display of trifunctional minicellulosomes for simultaneous saccharification and fermentation of cellulose to ethanol. Appl. Environ. Microbiol. **76:**1251-1260.
8. **Dodd D, Cann IK.** 2009. Enzymatic deconstruction of xylan for biofuel production. Glob. Change Biol. Bioenergy **1:**2-17.
9. **Kumar P, Barrett DM, Delwiche MJ, Stroeve P.** 2009. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind. Eng. Chem. Res. **48:**3713-3729.
10. **Chiaramonti D, Prussi M, Ferrero S, Oriani L, Ottonello P, Torre P, Cherchi F.** 2012. Review of pretreatment processes for lignocellulosic ethanol production, and development of an innovative method. Biomass & Bioenergy **46:**25-35.
11. **Yeoman CJ, Han Y, Dodd D, Schroeder CM, Mackie RI, Cann IK.** 2010. Thermostable enzymes as biocatalysts in the biofuel industry. Adv. Appl. Microbiol. **70:**1-55.
12. **Dodd D, Kocherginskaya SA, Spies MA, Beery KE, Abbas CA, Mackie RI, Cann IK.** 2009. Biochemical analysis of a β-D-xylosidase and a bifunctional xylanase-ferulic acid esterase from a xylanolytic gene cluster in *Prevotella ruminicola* 23. J. Bacteriol. **191:**3328-3338.
13. **Sorensen HR, Pedersen S, Jorgensen CT, Meyer AS.** 2007. Enzymatic hydrolysis of wheat arabinoxylan by a recombinant "minimal" enzyme cocktail containing β-xylosidase and novel endo-1,4-β-xylanase and α-l-arabinofuranosidase activities. Biotechnol. Prog. **23:**100-107.
14. **Moon YH, Iakiviak M, Bauer S, Mackie RI, Cann IK.** 2011. Biochemical analyses of multiple endoxylanases from the rumen bacterium *Ruminococcus albus* 8 and their synergistic activities with accessory hemicellulose-degrading enzymes. Appl. Environ. Microbiol. **77:**5157-5169.
15. **Bachmann SL, McCarthy AJ.** 1991. Purification and cooperative activity of enzymes constituting the xylan-degrading system of *Thermomonospora fusca*. Appl. Environ. Microbiol. **57:**2121-2130.
16. **Adelsberger H, Hertel C, Glawischnig E, Zverlov VV, Schwarz WH.** 2004. Enzyme system of *Clostridium stercorarium* for hydrolysis of arabinoxylan: reconstitution of the in vivo system from recombinant enzymes. Microbiology **150:**2257-2266.
17. **Blumer-Schuette SE, Lewis DL, Kelly RM.** 2010. Phylogenetic, microbiological, and glycoside hydrolase diversities within the extremely thermophilic, plant biomass-degrading genus *Caldicellulosiruptor*. Appl. Environ. Microbiol. **76:**8084-8092.
18. **Dam P, Kataeva I, Yang SJ, Zhou F, Yin Y, Chou W, Poole FL, 2nd, Westpheling J, Hettich R, Giannone R, Lewis DL, Kelly R, Gilbert HJ, Henrissat B, Xu Y, Adams MW.** 2011. Insights into plant biomass conversion from the genome of the anaerobic thermophilic bacterium *Caldicellulosiruptor bescii* DSM 6725. Nucleic Acids Res. **39:**3240-3254.
19. **Zverlov V, Mahr S, Riedel K, Bronnenmeier K.** 1998. Properties and gene structure of a bifunctional cellulolytic enzyme (CelA) from the extreme thermophile '*Anaerocellum thermophilum*' with separate glycosyl hydrolase family 9 and 48 catalytic domains. Microbiology **144:**457-465.
20. **Su X, Mackie RI, Cann IK.** 2012. Biochemical and mutational analyses of a multidomain cellulase/mannanase from *Caldicellulosiruptor bescii*. Appl. Environ. Microbiol. **78:**2230-2240.
21. **Ye L, Su X, Schmitz GE, Moon YH, Zhang J, Mackie RI, Cann IK.** 2012. Molecular and biochemical analyses of the GH44 module from CbMan5B/Cel44A, a bifunctional enzyme from the hyperthermophilic bacterium *Caldicellulosiruptor bescii*. Appl. Environ. Microbiol. **78:**7048-7059.

22. **Su X, Zhang J, Mackie RI, Cann IK.** 2012. Supplementing with non-glycoside hydrolase proteins enhances enzymatic deconstruction of plant biomass. PloS one **7:**e43828.
23. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC genomics **9:**75.
24. **Bae B, Ohene-Adjei S, Kocherginskaya S, Mackie RI, Spies MA, Cann IK, Nair SK.** 2008. Molecular basis for the selectivity and specificity of ligand recognition by the family 16 carbohydrate-binding modules from *Thermoanaerobacterium polysaccharolyticum* ManA. J. Biol. Chem. **283:**12415-12425.
25. **Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-685.
26. **Lever M.** 1972. A new reaction for colorimetric determination of carbohydrates. Anal. Biochem. **47:**273-279.
27. **Han Y, Dodd D, Hespen CW, Ohene-Adjei S, Schroeder CM, Mackie RI, Cann IK.** 2010. Comparative analyses of two thermophilic enzymes exhibiting both β-1,4 mannosidic and β-1,4 glucosidic cleavage activities from *Caldanaerobius polysaccharolyticus*. J. Bacteriol. **192:**4111-4121.
28. **Shulami S, Gat O, Sonenshein AL, Shoham Y.** 1999. The glucuronic acid utilization gene cluster from *Bacillus stearothermophilus* T-6. J. Bacteriol. **181:**3695-3704.
29. **Fontes CM, Gilbert HJ, Hazlewood GP, Clarke JH, Prates JA, McKie VA, Nagy T, Fernandes TH, Ferreira LM.** 2000. A novel *Cellvibrio mixtus* family 10 xylanase that is both intracellular and expressed under non-inducing conditions. Microbiology **146:**1959-1967.
30. **Gallardo O, Diaz P, Pastor FI.** 2003. Characterization of a *Paenibacillus* cell-associated xylanase with high activity on aryl-xylosides: a new subclass of family 10 xylanases. Appl. Microbiol. Biotechnol. **61:**226-233.
31. **Taylor EJ, Smith NL, Turkenburg JP, D'Souza S, Gilbert HJ, Davies GJ.** 2006. Structural insight into the ligand specificity of a thermostable family 51 arabinofuranosidase, Araf51, from *Clostridium thermocellum*. Biochem. J. **395:**31-37.
32. **Matias VR, Beveridge TJ.** 2005. Cryo-electron microscopy reveals native polymeric cell wall structure in *Bacillus subtilis* 168 and the existence of a periplasmic space. Mol. Microbiol. **56:**240-251.
33. **Zuber B, Haenni M, Ribeiro T, Minnig K, Lopes F, Moreillon P, Dubochet J.** 2006. Granular layer in the periplasmic space of gram-positive bacteria and fine structures of *Enterococcus gallinarum* and *Streptococcus gordonii* septa revealed by cryo-electron microscopy of vitreous sections. J. Bacteriol. **188:**6652-6660.
34. **Dodd D, Kiyonari S, Mackie RI, Cann IK.** 2010. Functional diversity of four glycoside hydrolase family 3 enzymes from the rumen bacterium *Prevotella bryantii* B14. J. Bacteriol. **192:**2335-2345.
35. **Vanfossen AL, Verhaart MR, Kengen SM, Kelly RM.** 2009. Carbohydrate utilization patterns for the extremely thermophilic bacterium *Caldicellulosiruptor saccharolyticus* reveal broad growth substrate preferences. Appl. Environ. Microbiol. **75:**7718-7724.
36. **Huang CY, Patel BK, Mah RA, Baresi L.** 1998. *Caldicellulosiruptor owensensis* sp. nov., an anaerobic, extremely thermophilic, xylanolytic bacterium. Int. J. Syst. Bacteriol. **48:**91-97.
37. **Miroshnichenko ML, Kublanov IV, Kostrikina NA, Tourova TP, Kolganova TV, Birkeland NK, Bonch-Osmolovskaya EA.** 2008. *Caldicellulosiruptor kronotskyensis* sp. nov. and *Caldicellulosiruptor hydrothermalis* sp. nov., two extremely thermophilic, cellulolytic, anaerobic bacteria from Kamchatka thermal springs. Int. J. Syst. Evol. Microbiol. **58:**1492-1496.
38. **Charnock SJ, Bolam DN, Turkenburg JP, Gilbert HJ, Ferreira LM, Davies GJ, Fontes CM.** 2000. The X6 "thermostabilizing" domains of xylanases are carbohydrate-binding modules: structure and biochemistry of the *Clostridium thermocellum* X6b domain. Biochemistry **39:**5013-5021.
39. **Araki R, Karita S, Tanaka A, Kimura T, Sakka K.** 2006. Effect of family 22 carbohydrate-binding module on the thermostability of Xyn10B catalytic module from *Clostridium stercorarium*. Biosci. Biotechnol. Biochem. **70:**3039-3041.
40. **Bryant P, Kriek M, Wood RJ, Roach PL.** 2006. The activity of a thermostable lipoyl synthase from *Sulfolobus solfataricus* with a synthetic octanoyl substrate. Anal. Biochem. **351:**44-49.
41. **Svetlichnyi VA, Svetlichnaya TP, Chernykh NA, Zavarzin GA.** 1990. *Anaerocellum thermophilum* gen. nov., sp. nov., an extremely thermophilic cellulolytic eubacterium isolated from hot-springs in the valley of Geysers. Microbiology **59:**598-604.

730 42. **Bredholt S, Sonne-Hansen J, Nielsen P, Mathrani IM, Ahring BK.** 1999. *Caldicellulosiruptor*
731 *kristjanssonii* sp. nov., a cellulolytic, extremely thermophilic, anaerobic bacterium. Int. J. Syst. Bacteriol.
732 **49:**991-996.
733 43. **Hamilton-Brehm SD, Mosher JJ, Vishnivetskaya T, Podar M, Carroll S, Allman S, Phelps TJ, Keller**
734 **M, Elkins JG.** 2010. *Caldicellulosiruptor obsidiansis* sp. nov., an anaerobic, extremely thermophilic,
735 cellulolytic bacterium isolated from Obsidian Pool, Yellowstone National Park. Appl. Environ. Microbiol.
736 **76:**1014-1020.
737 44. **Kormelink FJM VA.** 1993. Degradation of different [(glucurono)arabino]xylans by a combination of
738 purified xylan-degrading enzymes. Appl. Microbiol. Biotechnol. **38:**688-695.

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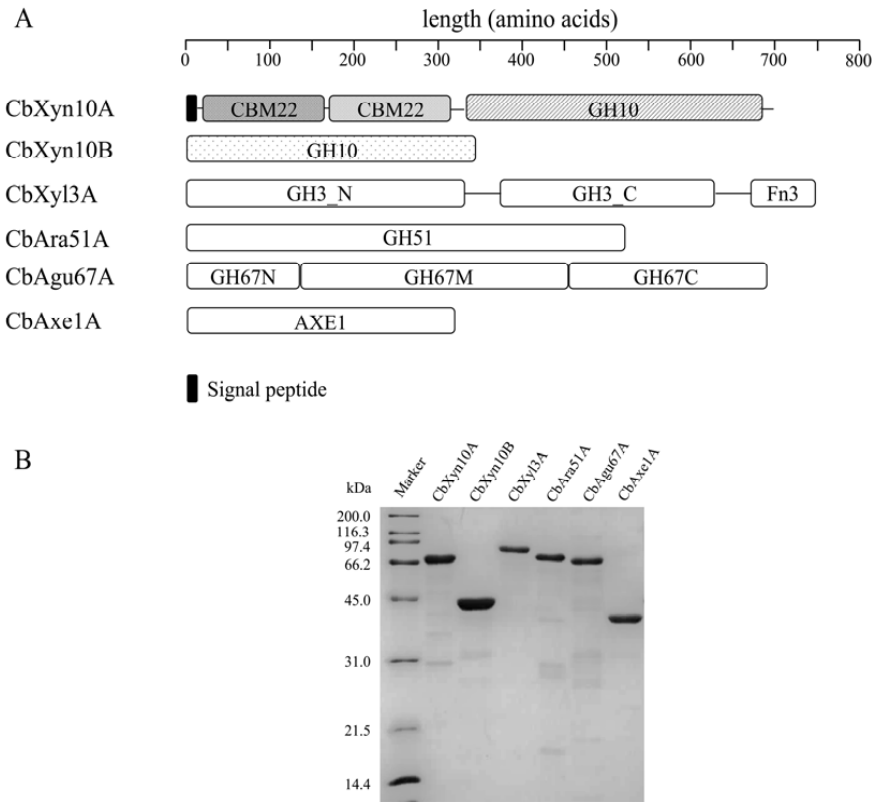

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744 **Figure 1**

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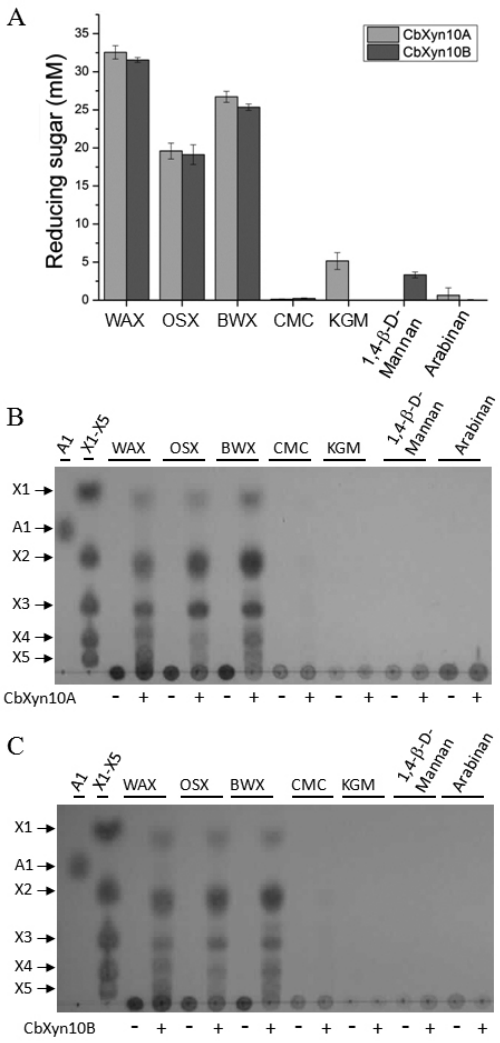

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749 **Figure 2**

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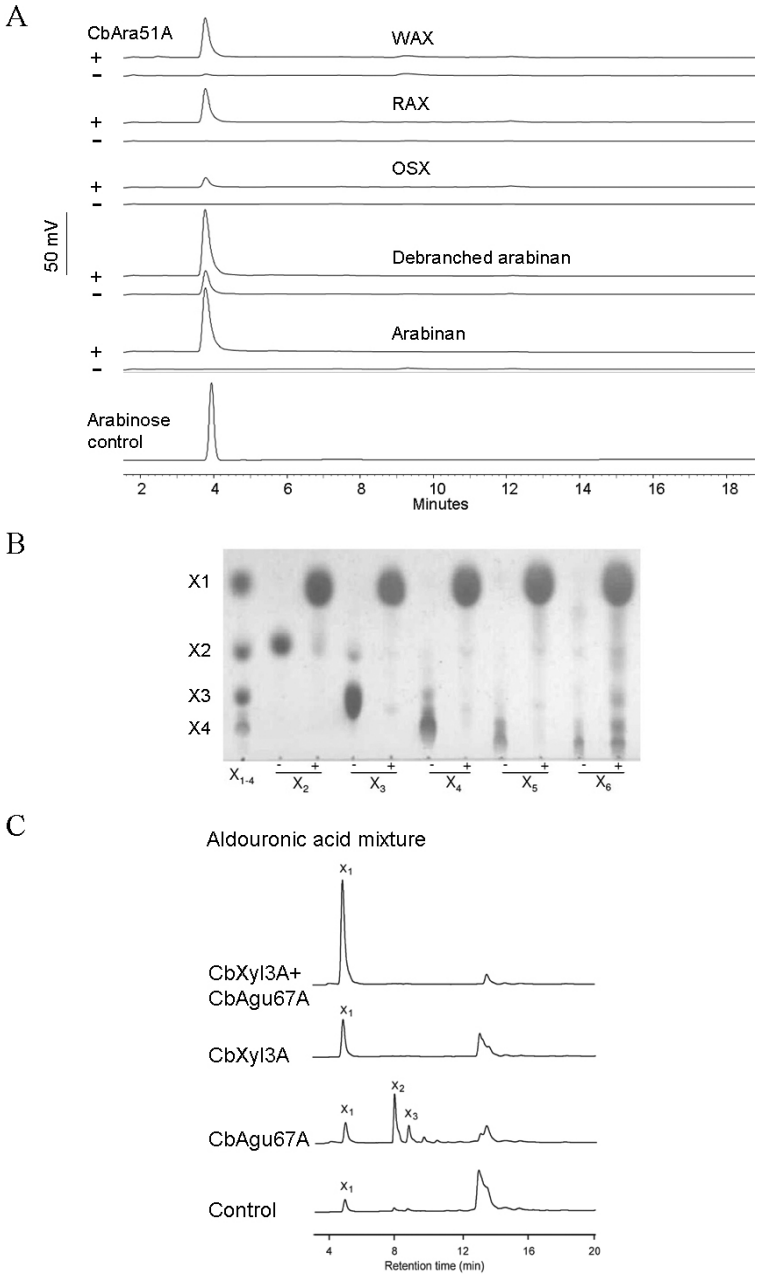

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753 **Figure 3**

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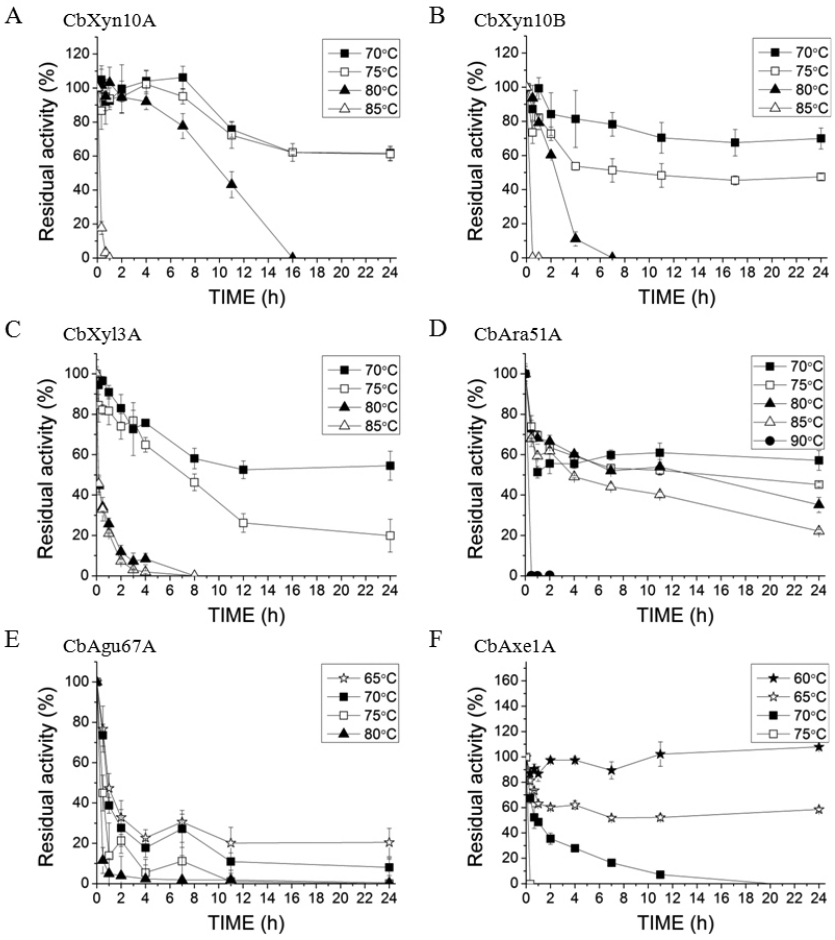

Figure 4

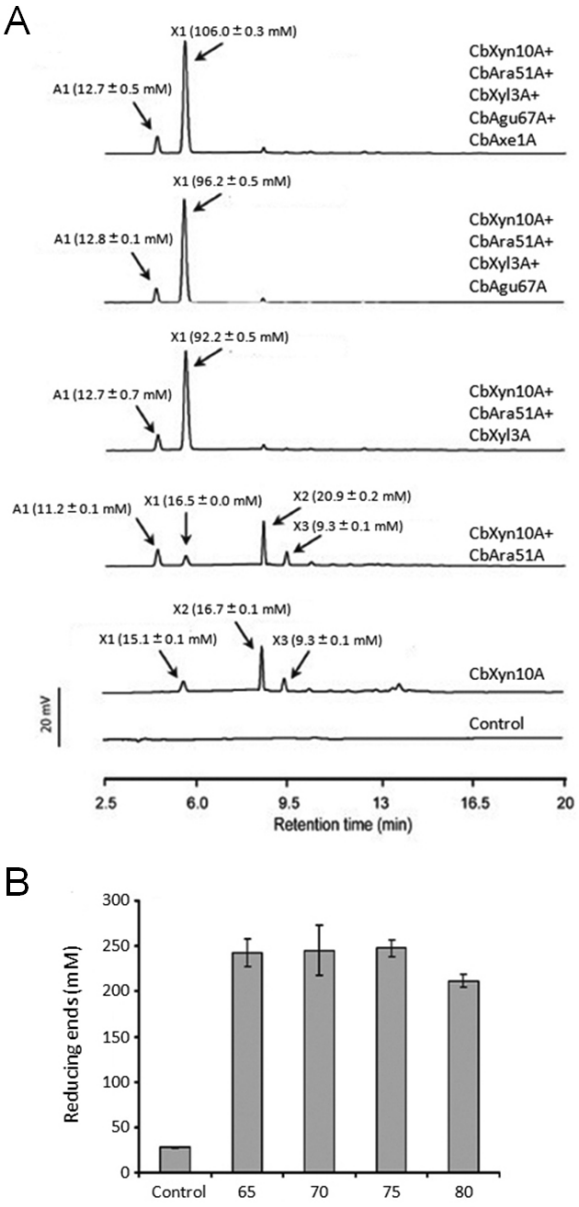

Figure 5