Biochemical Characterization of Two Thermostable Xylanolytic Enzymes Encoded by a Gene Cluster of *Caldicellulosiruptor owensensis*

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**Abstract**

The xylanolytic extremely thermophilic bacterium *Caldicellulosiruptor owensensis* provides a promising platform for xylan utilization. In the present study, two novel xylanolytic enzymes, GH10 endo-β-1,4-xylanase (Coxyn A) and GH39 β-1,4-xylosidase (Coxyl A) encoded in one gene cluster of *C. owensensis* were heterogeneously expressed and biochemically characterized. The optimum temperature of the two xylanolytic enzymes was 75°C, and the respective optimum pH for Coxyn A and Coxyl A was 7.0 and 5.0. The difference of Coxyn A and Coxyl A in solution was existing as monomer and homodimer respectively, it was also observed in predicted secondary structure. Under optimum condition, the catalytic efficiency ($k_{cat}/K_m$) of Coxyn A was 366 mg ml⁻¹ s⁻¹ on beechwood xylan, and the catalytic efficiency ($k_{cat}/K_m$) of Coxyl A was 2253 mM⁻¹ s⁻¹ on pNP-β-D-xylopyranoside. Coxyn A degraded xylan to oligosaccharides, which were converted to monomer by Coxyl A. The two intracellular enzymes might be responsible for xylooligosaccharides utilization in *C. owensensis*, also provide a potential way for xylan degradation in vitro.

**Introduction**

Xylan is the main hemicellulose component constituting about 20–30% of the biomass of dicotyl plants (hardwoods and herbaceous plants) [1]. Thus xylan is considered as an abundant renewable resource for biofuels and industrial chemicals production. The backbone chain of xylan is formed by β-1,4-linked D-xylosyl residues, and decorated with branched heteropolysaccharides [2]. Endo-β-1,4-xylanase, mostly present in the glycoside hydrolase (GH) families 10 and 11, hydrolyzes the endo-β-1,4-xylosidic linkages in a random fashion, while the hydrolysis is influenced by sidechain substituents [3]. The β-xylanase, found in GH families 3, 30, 39, 43, 51, 52, and 54, catalyzes the conversion of xylobiose and other short chain xylooligosaccharides to D-xylose [3]. Hence endo-β-1,4-xylanase [EC 3.2.1.8] and β-1,4-xylosidase [EC 3.2.1.37] take primary responsibility for xylan backbone degradation. To date, an increasing number of xylanases and xylosidases have been isolated [3–8], while the knowledge of synergism of extreme xylanolytic enzymes for natural xylan degradation is still limited. Therefore, developing novel thermostable xylanase and xylosidase active at even higher temperature is desirable.

Application of extremely thermophilic microorganisms for biomass conversion is a way to break through the barriers of feedstock fluctuations and process-operating challenges [9]. Genus *Caldicellulosiruptor*, obligatory anaerobic, extreme thermophiles, produces heat-stable extracellular enzyme for biomass degradation. Several xylanases and xylosidases have already been characterized from *Caldicellulosiruptor* [10–14], while there are still many predicted xylanolytic enzymes encoded by *Caldicellulosiruptor* un-characterized.

The optimum culture condition for *Caldicellulosiruptor owensensis* OL is 75°C and pH 7.5. It grows on a wide variety of carbon sources including pentose, hexose, oligosaccharide, and polysaccharides [13]. The physiological characteristics of *C. owensensis* make it a promising candidate for pentose utilization. The genome sequence of *C. owensensis* has been reported, and the diversity of encoded glycoside hydrolases were analyzed thereafter [16–18].

In present study, two novel genes of endo-β-1,4-xylanase and β-1,4-xylosidase encoded by a gene cluster of *C. owensensis* were investigated. The two xylanolytic enzymes were heterogeneously expressed, purified and biochemically characterized. The catalytic model of endo-β-1,4-xylanase and β-1,4-xylosidase versus xylooligosaccharides were investigated, and the synergism of the two enzymes for xylan backbone hydrolysis were also studied.

**Materials and Methods**

**Bacterial strains, plasmids, chemicals and growth conditions**

*Caldicellulosiruptor owensensis* was purchased as free-dried culture from the German Collection of Microorganisms and Cell
Cultures (DSMZ), DSM number is 13100. *Escherichia coli* Top10 and BL21(DE3) were respectively used for gene cloning and expression. The plasmid, pET-28b(+) (Novagen, USA), was used for recombinant plasmid construction and gene expression. Beechwood xylan and 4-nitrophenyl β-D-xylopyranoside (pNPX) were purchased from Sigma-Aldrich (St louis, MO, USA). All of the other chemicals were of analytical grade and purchased from Sinopharm Chemical Regent Co., Ltd.

*C. owensensis* cells were resuspended and subcultured in anaerobic modified DSMZ medium 640 (cellobiose was replaced by xylose), and grown at 75°C with orbital shaking (75 rpm). *E. coli* Top10 and BL21(DE3) were cultured in aerobic Luria–Bertani (LB) broth at 37°C with orbital shaking (220 rpm).

**Genomic DNA isolation and recombinant vector construction**

For preparation of genomic DNA from *C. owensensis*, cultured cells were harvested by centrifugation at 10,000×g for 1 min, then resuspended in 180 μl lysozyme buffer (20 mM Tris-HCl, pH 8.0, 2 mM Na₂EDTA, 1.2% Triton, lysozyme 20 mg/ml), and incubated at 37°C for 30 min. This suspension was mixed with 5 μl Rnase A (100 mg/ml), shaking for 15 s, then incubated for 5 min at room temperature. Isolation of genomic DNA was carried out according to the instruction of TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China).

**Protein expression and purification**

To express *Coxyn A* and *Coxyl A*, the recombinant DNA plasmids were extract using TIANprep mini plasmid kit (Tiangen Biotech, Beijing, China), and transformed into *E. coli* BL21(DE3) competent cells. The transformed strain was cultured in LB broth with 50 μg/ml kanamycin overnight at 37°C. The culture was diluted 100-fold in 500 ml fresh LB broth with 50 μg/ml kanamycin and to OD₆₀₀ 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) [final concentration of 0.4 mM] was added to

**Figure 1.** Gene cluster encoding xylanase, xylosidase, and esterase in genome of *C. owensensis*, *C. kronotskyensis*, *C. bescii*, and *C. saccharolyticus*.

**Table 1.** Characterization of *Coxyn A* and *Coxyl A*.

| Protein name | Genome locus tag | Length (aa) | Mw (kDa) | Signal peptide | GH family | Predicted secondary structure | Alpha helix | β-sheet | Random coil |
|--------------|-----------------|-------------|----------|----------------|------------|-----------------------------|-------------|---------|-------------|
| *Coxyn A*    | Calow_0124      | 337         | 40.2     | No             | GH10       | 160 aa                      | 69 aa       | 108 aa  |
| *Coxyl A*    | Calow_0126      | 482         | 55.4     | No             | GH39       | 144 aa                      | 109 aa      | 229 aa  |

a: Predicted in The National Center for Biotechnology Information (NCBI).
b: Predicted by SignalP 4.1 server.
c: Predicted by GOR4 software.
induce protein expression, and the culture was cultivated for another 6 hours at 37°C. To purify the protein, the culture was centrifuged at 6,000×g for 15 min and resuspended in 25 ml binding buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl). The cells were broken by sonication, the mixture was then incubated at 65°C for 30 min, and centrifuged at 13,000×g for 15 min. The supernatant was passed five times through the Ni²⁺-NTA column to binding the histidine-tagged target protein. After washing three times to remove the non-specific protein, bound proteins was eluted from column with elution buffer (500 mM imidazole, 50 mM Tris-HCl, pH 7.5, 300 mM NaCl), and analyzed by 12% SDS-PAGE electrophoresis. To determine protein quaternary structure, gel filtration chromatography with Superdex 200 column was used. The column was pre-equilibrated with citrate buffer (50 mM sodium citrate, pH 6.0, 150 mM NaCl), and calibrated using carbonic anhydrase (30.0 kDa), Albumin (66.0 kDa), lactate dehydrogenase (140 kDa) catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) as standards, the molecular masse (Mw) of proteins was estimated from a regression curve by plotting log of Mw standards against their elution volumes of the column. After gel filtration, fractions were analyzed by 12% SDS-PAGE electrophoresis, and the targeted proteins were collected and concentrated by ultrafiltration.

**Enzyme assay**

The concentration of purified protein was determined by the Coomassie Brilliant Blue G250 binding method using bovine serum albumin solution to make standard curve [19]. To measure the xylanase activity of Coxyn A, 99 µl beechwood xylan (2.5 mg/ml) in citrate buffer (50 mM sodium citrate, pH 6.0, 150 mM

**Figure 2.** The phylogenetic trees of Coxyn A with similar enzymes. A The relationship of Coxyn A (∆) from C. owensensis with other similar GH10 xylanases retrieved from NCBI database. B Sequence alignment of Coxyn A with those structure elucidated xylanses. The phylogenetic trees were constructed by MUSLE program and MEGA 6 Neighbor-joining analysis. Bootstrap values obtained with 1000 resamplings were indicated as percentages at all branches, and the scale bars represent 0.05 substitutions per amino acid position. Numbers followed by the names of the strains were accession numbers of NCBI.
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NaCl) was incubated at 75°C for 5 min, then add 1 μl enzyme (24 μg/ml) into solution to react for 90 s. The reaction was terminated by heating for 10 min at 100°C (boiling). Then the reaction mixture was centrifuged at 13,000 rpm for 10 min. The released reducing sugar in supernatant was measured with PAHBAH (p-Hydroxy benzoic acid hydrazide) method [14,20] and was monitored at the absorbance at 410 nm. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of reducing sugar from beechwood xylan per minute. To measure the activity of Coxyl A, 99 μl pNP-B-D-xylopyranoside (pNPX) (0.5 mM) in citrate buffer (50 mM sodium citrate, pH 6.0, 150 mM NaCl) was incubated at 75°C for 5 min, 1 μl enzyme (46 μg/ml) was added into solution to react for 2 min. The reaction was terminated by adding 200 μl 2 M Na₂CO₃ (pH 10) and monitored at the absorbance at 400 nm. One unit of β-Coxyl Activity was defined as the amount of enzyme capable of releasing 1 μmol pNP from the substrates per minute.

The optimum temperature for Coxyn A and Coxyl A was determined over the range of 40-100°C. The optimum pH of enzyme, different buffer pH 4.0-6.5 (citrate buffer, 50 mM, 150 mM NaCl), pH 7.0-8.5 (phosphate buffer, 50 mM, 150 mM NaCl) were prepared. At the optimum temperature, the specific activity of enzyme was checked at different pH. Thermal stability of enzyme was monitored based on optimum temperature and pH, the enzyme residual activity was determined using standard methods after incubating the enzyme at 70, 75 and 80°C without substrate for different time (0.5, 1, 2, 3, 4, 6 h). The relative activity of enzyme was calculated with the initial activity as 100%.

In citrate buffer (50 mM sodium citrate, 150 mM NaCl) and at 75°C, the effects of different chemicals on the Coxyn A and Coxyl A activity were measured with pH value of 7.0 and 5.0, respectively. The chemicals NaCl, KCl, CaCl₂, FeCl₂, NiCl₂, CoCl₂, MnCl₂, CuCl₂, and MgCl₂ were added at concentrations of 1 mM and 5 mM individually [21]. Ethanol, n-butanol, and isopropanol were added individually to the reaction at concentration of 1% (v/v) and 5% (v/v). 0.1% or 0.5% of SDS and Tween-20 were added to the reaction respectively. Enzyme activity was measured by standard methods as described above at optimum react conditions. The relative activity of enzyme was calculated with untreated control as 100%.

**Kinetic parameter assay**

The activity of Coxyn A was measured in citrate buffer (pH 7.0, 75°C) containing 0.5 to 5 mg/ml beechwood xylan, and activity of Coxyl A was measured in citrate buffer (pH 5.0, 75°C) containing 0.03 to 2.4 mM pNPX by methods described above. Using
software Graphpad Prism 6, the Michaelis-Menten constant ($K_m$) and the maximum velocity ($V_{max}$) were estimated with a nonlinear regression (curve fit) and Michaelis-Menten equation. The $k_{cat}$ value was calculated as the ratio of $V_{max}$ to the concentration of enzyme used in the reaction.

**Hydrolytic properties on xylooligosaccharides and xylan**

To determinate the hydrolytic products, substrate of 0.6 mg/ml beechwood xylan or 4 mg/ml xylooligosaccharides were prepared. The substrate was mixed with 100 μg/ml Coxyn A, or 3 mg/ml Coxyl A, or 100 μg/ml Coxyn A and 3 mg/ml Coxyl A respectively. The reaction mixture in citrate buffer (50 mM sodium citrate, pH 6.0, 150 mM NaCl) was incubated at 75°C for 12 h (with beechwood xylan) or 3 h (with xylooligosaccharides). After incubation, hydrolytic products were analyzed by thin-layer chromatography (TLC) assay, reducing sugar assay and high-performance liquid chromatography (HPLC) assay. For TLC assay, aliquots were spotted onto silica gel plates at intervals, xylose (X1) and xylooligosaccharides (X2-X4) were used as standards. The plates were developed with 1-butanol: acetic acid: water (10: 5: 1, v/v/v) as a mobile phase. For visualization of hydrolytic products, TLC plates were sprayed with a staining solution of methanolic orcinol (0.05%, w/v) and sulfuric acid (5%, v/v), and then heated for 10 min at 75°C for color development. Reducing sugar assay was carried out by PAHBAH method described above. Before HPLC assay, diluted hydrolytic products were filtered through a 0.2 μm filter, isolation of xylose and xylooligosaccharides was performed with Hi-Plex Ca column (7.7 × 300 mm, Agilent Technology, USA) using LC-20AT pump (Shimadzu, Japan) with water at a flow rate of 0.6 ml/min at 85°C. Samples were injected with 10 μl volume, and monitored using RID10A refractive index detector (Shimadzu, Japan). The xylose content of hydrolytic products was measured by quantifying its peak area with D-xylose standard curve ($R^2 = 0.99999868$).
Phylogenetic tree and sequence alignment of Coxyn A and Coxyl A with other thermostable enzymes

Amino acids sequences of Coxyn A and Coxyl A were submitted to NCBI database Blastp (Basic Local Alignment Search Tool for Protein, version 2.2.29+) respectively, and the characterized thermostable proteins with high identity to Coxyn A or Coxyl A were selected to prepare phylogenetic trees. The MUSCLE (multiple sequence comparison by log-expectation) program was used for multiple sequences alignments, and MEGA 6 software was used to plot phylogenetic trees of alignments by the neighbor-joining method.

Statistical analysis

Experiments were repeated three times, results were shown as mean ± SD, and student’s t-test for comparisons were carried out by EXCEL T.TEST function. Values of P<0.01 were considered significant.

Results and Discussion

Gene cloning and bioinformatic analysis

The genes encoding glycoside hydrolases in C. owensensis genome were screened by using KEGG GENOME and Rapid annotation using subsystem technology (RAST). Five new predicted xylanases and two new xylosidases were identified in genome through amino acids similarity analysis. Among the genes, two of them Calow_0124 and Calow_0126 were identified in a gene cluster predicted for xylan degradation and metabolism. The genes Calow_0124 and Calow_0126 encoded a glycoside hydrolase (GH) 10 endo-β-1,4-xylanase of 337 amino acids and a GH39
The homology of Coxyn A was analyzed with the most similar characterized GH10 xylanase (amino acids sequence identity was equal or greater than 51%) (Fig. 2A). Coxyl A was compared with other GH39 xyllosidase (amino acids sequence identity was equal or greater than 35%) (Fig. 3A). The phylogenetic tree of GH10 xylanase showed that Coxyn A from *C. owensensis* OL was the closest to GH10 xylanase from *C. saccharolyticus* and *C. bescii*, and they were separated from all other similar GH10 xylanases, which were from *Paenibacillus, Geobacillus* and *Bacillus* etc. In the listed GH10 enzymes, three mostly closest xylanase from PDB had been aligned with Coxyn A by ClustalW software. With protein 3EWC (PDB ID) as template, the result of ESPrit 3.0 software showed that Coxyn A aligned with mostly closed xylanase from PDB by ClustalW, and secondary structure of Coxyn A and Coxyl A are special to other enzymes, and β-1,4-xyllosidase of 482 amino acids respectively. In the genes cluster of *C. owensensis*, a gene (*Calow_0125*) predicted as xylan esterase located between the two xylanolytic genes. Similar genes arrangement was observed in *C. kronotskyensis* and *C. bescii* (Fig. 1). In genome of *C. saccharolyticus*, corresponded gene cluster encodes a predicted peptidase (*Csac_2403*) except xyllosidase (*Csac_2405*) and xylanase (*Csac_2404*). Based on amino acids analysis, all of the identity of Calkro_2385, Athe_185, and Csac_2405 with Calow_0124 was 81%. The amino acids identity of Calkro_2383, Athe_187, and Csac_2404 with Calow_0126 was 83–84%.

Both Coxyn A and Coxyl A have no signal peptide predicted by SignalP 4.1 server, suggesting the two enzymes might be intracellular enzymes. The secondary structures of the two enzymes were also predicted by GOR4 software. Results showed that Coxyn A contains 47.5% alpha helix (160 aa), 20.5% β-sheet (69 aa) and 32.0% random coil (108 aa); while Coxyl A has 29.9% alpha helix (144 aa), 22.6% β-sheet (109 aa) and 47.5% random coil (229 aa). That meant that nearly half secondary structure of Coxyn A was alpha helix, while most part of Coxyl A exists as random coil (Table 1). The difference of the two enzymes in secondary structure might contribute to the intermolecular interaction, as Coxyn A and Coxyl A exist as monomer and homodimer in buffer.

### Analysis of homologous sequence and active site prediction

The homologous sequence of Coxyn A and Coxyl A with other xylanases and xyllosidases was analyzed through phylogenetic trees, which were constructed based on amino acids sequences.

| Chemicals | Relative activity (%)\(^1\) |
|-----------|-----------------------------------|
|           | 1 mM | 5 mM | 1 mM | 5 mM |
| **Control** | 100.0 ± 1.1 | 100.0 ± 1.7 | 100.0 ± 0.5 | 100.0 ± 0.8 |
| NaCl      | 112.8 ± 6.3 | 89.8 ± 5.5 | 102.1 ± 0.4 | 98.0 ± 1.6 |
| KCl       | 100.1 ± 2.3 | 32.6 ± 2.9 | 102.4 ± 0.2 | 97.3 ± 1.2 |
| CaCl\(_2\) | 100.2 ± 1.9 | 102.5 ± 1.7 | 102.4 ± 2.7 | 96.1 ± 2.4 |
| FeCl\(_2\) | 77.1 ± 7.7 | 33.0 ± 2.3 | 88.5 ± 0.5 | 48.7 ± 1.2 |
| NiCl\(_2\) | 33.5 ± 3.6 | 82.3 ± 18.4 | 96.2 ± 0.7 | 69.9 ± 3.0 |
| CoCl\(_2\) | 102.0 ± 5.6 | 151.1 ± 6.1 | 98.2 ± 2.2 | 81.3 ± 1.1 |
| MnCl\(_2\) | 44.6 ± 3.2 | 78.8 ± 7.0 | 98.4 ± 2.9 | 86.2 ± 2.6 |
| CuCl\(_2\) | 113.3 ± 5.8 | 168.9 ± 17.4 | 85.7 ± 0.7 | 61.5 ± 0.7 |
| MgCl\(_2\) | 88.9 ± 1.1 | 117.3 ± 1.1 | 102.9 ± 2.2 | 95.0 ± 0.4 |
| 5% | 10% | 5% | 10% |
| Glycerol  | 173.9 ± 2.2 | 185.6 ± 4.2 | 63.5 ± 0.4 | 41.1 ± 1.3 |
| Ethanol   | 34.3 ± 1.1 | 0.3 ± 4.0 | 101.1 ± 1.8 | 94.9 ± 1.5 |
| n-Butanol | 0 | 0 | 104.3 ± 8.3 | 57.4 ± 4.5 |
| Isopropanol | 45.7 ± 7.4 | 0 | 114.0 ± 3.6 | 107.5 ± 6.3 |
| 0.1% | 0.5% | 0.1% | 0.5% |
| SDS       | 5.6 ± 5.9 | 0 | 8.4 ± 1.9 | 11.5 ± 0.6 |
| Tween-20  | 102.8 ± 9.0 | 111.3 ± 13.5 | 151.2 ± 0.5 | 123.3 ± 2.4 |

\(^1\)Data are means ± SD (n = 3) relative to the control samples. 

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Expression and purification of Coxyn A and Coxyl A

The recombinant Coxyn A and Coxyl A were expressed in *E. coli* BL21 (DE3), then purified with affinity chromatography (Ni2+-NTA resins) and gel filtration chromatography. The SDS-PAGE (12%) analysis of Coxyn A after affinity chromatography was shown in Fig. 4A. The contaminant bands were removed by gel filtration, purified Coxyn A existed as monomer (40 kDa) (Fig. 4B). Similarly, after affinity chromatography, Mw of Coxyl A was about 55 kDa analyzed by 12% SDS-PAGE (Fig. 4C), the enzyme existed as homodimer through gel filtration analysis (Fig. 4D). Most reported β-xylosidase existed as monomer with an exception of *Bacillus pumilus* homodimeric β-xylosidase [25].

Biochemical characterization of Coxyn A and Coxyl A

In temperature profile analysis, Coxyn A showed highest activity at 75°C and retained more than 80% activity at temperature range of 60–75°C (Fig. 5A). Coxyn A retained 50% activity at pH range from 4.0 to 8.5 and optimum pH was 7.0 (Fig. 5B). The temperature feature of Coxyl A activity was similar to Coxyn A, and the optimum temperature was 75°C (Fig. 5C). In contrast, Coxyl A was active at acidic condition (pH range of 4.5 to 6.0), with an optimum pH of 5.0 (Fig. 5D). The genes encoding Coxyn A and Coxyl A were located in same gene cluster, while the optimum pH of the two enzymes is different. As the interesting results, it might be speculated that *C. owensensis* encoded seven xylanases and xylosidase adapting to different condition, while the speculation need to be tested.

For determination of thermostability, Coxyn A and Coxyl A were pre-incubated at different temperature (70, 75, and 80°C) for 0.5–6 h in citrate buffer with optimum pH. The results showed that Coxyn A was not very stable at optimum temperature, the residual activity retained only about 40% at 70°C and 75°C after 2 h's incubation. After incubating for 2 h at 80°C, no activity of Coxyn A was observed (Fig. 5E). However, Coxyl A was stable after incubating for 6 h at 70°C, 75°C and 80°C (Fig. 5F). It might be the homodimeric form of Coxyl A that stabilizes its structure, and reduced the loss of enzyme activity during the incubation. It should be noted that both Coxyn A and Coxyl A were stable at room temperature for several days. The thermostability of Coxyl A from *C. owensensis* makes it useful in biotechnological processing of biomass.

To optimize the condition for catalytic activity, the effects of metal ions and chemicals on enzyme activities were investigated (Table 2). For Coxyn A, its activity was influenced slightly by smaller atomic monovalent and divalent cations and their chelating agent, i.e. Na⁺, Ca²⁺, and Mg²⁺, while K⁺ was an exception. Heavier metal cations had different influence, Co²⁺ and Cu²⁺ had positive influence on Coxyn A activity, especially at 5 mM concentration. Ni²⁺ and Mn²⁺ had negative effect on enzyme activity at 1 mM concentration than at 5 mM, and the negative influence of K⁺ and Fe²⁺ grew with their concentration increased. In organic reagents, ethanol, n-butanol and isopropanol, but not glycerol, had negative effect on Coxyn A activity. Except for SDS, several surfactants had no negative effect on Coxyn A activity. Different from Coxyn A, Coxyl A activity was enhanced by adding surfactants such as tween-20 and Glycerol. While SDS, ethanol, n-butanol and isopropanol showed negative effects on Coxyl A activity. Furthermore, metal ions had no or slightly effect on Coxyl A activity except for the negative influence of Fe²⁺.

Enzyme kinetic parameters of Coxyn A and Coxyl A

The kinetic parameters for Coxyn A were determined under the optimum condition of 75°C and pH 7.0. As shown in Table 3, with
Table 4. Kinetic parameters of Coxyl A compared with other xylosidases.

| Organism                          | $K_m$ (mM) | $V_{max}$ ($\mu$mol mg$^{-1}$ min$^{-1}$) | $k_{cat}$ (s$^{-1}$) | Optimum temperature (°C) | Optimum pH | Reference |
|-----------------------------------|------------|----------------------------------------|----------------------|--------------------------|------------|-----------|
| Caldicellulosiruptor owensensis   | 1.6±0.1    | 3930±132                               | 3628±122             | 75                       | 5.0        | This work |
| Caldicellulosiruptor bescii       | 8.2±0.2    | ND                                     | ND                   | 90                       | 6.0        | [14]      |
| Caulobacter crescentus            | 9.3±0.45   | 402±19                                 | ND                   | 55                       | 6.0        | [38]      |
| Thermotoga thermarum              | 0.27       | 223.2                                  | 316.8                | 95                       | 6.0        | [39]      |
| Blidobacterium animalis subsp. lactis BB-12 | 15.6±4.2 | 166                                    | 60.6±10.8            | 50                       | 5.5        | [40]      |
| Thermoanaerobacterium saccharolyticum | 28       | 189292                                 | 276                  | 65                       | 6.0        | [41]      |
| Aspergillus sp. BCC125            | 1.7        | 211.5                                  | 338                  | 60                       | 4.0–5.0    | [28]      |
| Aspergillus phoenicis             | 2.36±0.54  | ND                                     | 920.75±40.45         | 75                       | 4.0–4.5    | [42]      |

*Substrate: pNPX

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Figure 6. Hydrolytic mode of xylooligosaccharides by Coxyn A and Coxyl A. A Thin-layer chromatography (TLC) assay. lane 1: standards of xylose and xylooligosaccharides (XOS) (X1: xylose, X2: xylobiose, X3: xylotriose, X4: xylotetrose); lane 2: 4 mg/ml XOS; lane 3: 4 mg/ml XOS was incubated with 100 mg/ml Coxyn A; lane 4: 4 mg/ml XOS was incubated with 3 mg/ml xylanase A; lane 5: 4 mg/ml XOS was incubated with 100 μg/ml Coxyn A and 3 mg/ml Coxyl A. The reaction was conducted in citrate buffer (pH 6.0), 75 °C for 3 h. B Reducing sugar assay of hydrolysis. *P<0.01 (n = 3), the number of sample means the same sample loaded on the line in (A). C High Performance Liquid Chromatography assay of products.
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beechwood xylan as substrate, the values for $K_m$, $V_{max}$ and $k_{cat}$ of Coxyn A were 0.62 ± 0.33 mg/ml, 341.4 ± 47.1 μmol mg$^{-1}$ min$^{-1}$ and 227 ± 31.3 s$^{-1}$, respectively. Compared with the similar xylanases characterized and listed in Fig. 5, Coxyn A was a xylanase with high efficiency at high temperature and neutral environment. The kinetic parameters of Coxyl A were shown in Table 4, and the respective values for $K_m$, $V_{max}$ and $k_{cat}$ were 1.6 mM, 3930 ± 50 μmol mg$^{-1}$ min$^{-1}$ and 3628 s$^{-1}$ with pNPX as substrate. Compared to characterized xylosidase listed in Fig. 3, $K_m$ of Coxyl A is lower and the $k_{cat}$ of Coxyl A was the highest.

Hydrolytic property analysis

To determine the hydrolytic mode of Coxyn A and Coxyl A, xylooligosaccharides and xylan were applied as substrates. When the xylooligosaccharides were hydrolyzed with Coxyn A, the final products were xylose and xylobiose. As a contrast, the main product was xylose when xylooligosaccharides was hydrolyzed with Coxyl A. All above were in citrate buffer (pH 6.0), 75°C for 12 h. The results indicated that Coxyl A was active on oligosaccharides with DP ≥ 2 (Fig. 6A). The products of xylooligosaccharides hydrolysis were also analyzed by HPLC assay. The concentrations of xylose of the control, Coxyn A, Coxyl A, and Coxyn A&Coxyl A were 0, 6.1, 17.7, 22.6 nmol, respectively (Fig. 6C). This result suggested that the combination of Coxyn A and Coxyl A could hydrolyze xylooligosaccharides into xylose more efficiently than they acted alone. Similar results were detected in reducing sugar assay, the final reducing sugars for the control, Coxyn, Coxyl A, and Coxyn&Coxyl A were 20.8 ± 0.2, 26.0 ± 0.1, 32.7 ± 0.3, 35.1 ± 0.1 mM. Significant difference at $P < 0.01$ was observed in statistical analysis between Coxyn and Coxyn&Coxyl A, Coxyl A and Coxyn&Coxyl A (Fig. 6B).

Similar hydrolysis experiments were conducted using beechwood xylan as substrate. In hydrolytic products analysis through TLC assay, xylotriose and even longer xylooligosaccharides decorated with side chains were observed, while no xylobiose and xylose were found in the mixture. With Coxyl A was incubated with xylan, a small quantity of xylose was liberated, which might come from the degradation of small amount of xylooligosaccharides existing in the substrate. With combination of
Coxy A and Coxy A, xylose and longer chain xylobioigosacchar-
ides exist, while no xylobiose or xylotriose were observed (Fig. 7A).
The corresponding reduced sugar in the mixture was shown in Fig. 7B, the final amount of reducing sugar for the control, Coxy, Coxy A, and CoxyX&Coxy A were 0.06±0.01, 2.9±0.2, and 0.06±0.01 μM. In statistical analysis, the difference at P<0.01 was observed between Coxy and CoxyX&Coxy A, Coxy A and CoxyX&Coxy A. The products of beechwood xylan (diluted 10-fold) hydrolysis with different enzyme was also analyzed by HPLC assay. When Coxy A was applied in the mixture, very small amount of xylose (0.6 nmol) was observed, which was under the detection limit of TLC. The amounts of xylose from reaction of control, Coxy A, Coxy A, and CoxyX&Coxy A were 0, 0.6, 1.7 and 2.4 nmol, respectively (Fig. 7C). To date, a increasing number of xylanases and xylosidases were
which was under the detection limit of TLC. The amounts of
enzymes encoded by one gene cluster were rarely reported. It
remained unclear whether the coordinates were from one gene cluster.

**Conclusion**

The genes for two novel xylanolytic enzymes GH10 endo-β-1, 4-xylanase and GH39 β-1, 4-xylosidase located in one gene cluster were found in genome of C. owensensis. Synergism of the two novel xylanolytic enzymes could efficiently convert the backbone of natural xylan to xylose at 75°C. The two xylanolytic enzymes are predicted to be intracellular enzymes and responsible for degrading polysaccharides or oligosaccharides to xylose. The high efficiency and excellent thermostability of Coxy A and Coxy A provide a potential way for xylan degradation in vitro.

**Author Contributions**

Conceived and designed the experiments: SM YH. Performed the experiments: SM XJ WQ. Analyzed the data: SM YH. Contributed to the writing of the manuscript: SM JW YH XJ XP.

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