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A novel multifunctional cellulolytic enzyme screened from metagenomic resources representing ruminal bacteria

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ABSTRACT

Metagenomic resources representing ruminal bacteria were screened for novel exocellulases using a robotic, high-throughput screening system, the novel *CelEx-BR12* gene was identified and the predicted CelEx-BR12 protein was characterized. The *CelEx-BR12* gene had an open reading frame (ORF) of 1140 base pairs that encoded a 380-amino-acid-protein with a predicted molecular mass of 41.8 kDa. The amino acid sequence was 83% identical to that of a family 5 glycosyl hydrolase from *Prevotella ruminicola* 23. Codon-optimized CelEx-BR12 was overexpressed in *Escherichia coli* and purified using Ni–NTA affinity chromatography. The Michaelis–Menten constant (K_m value) and maximal reaction velocity (V_{max} values) for exocellulase activity were 12.92 μM and 1.55×10^{-4} μmol min^{-1}, respectively, and the enzyme was optimally active at pH 5.0 and 37 °C. Multifunctional activities were observed against fluorogenic and natural glycosides, such as 4-methylumbelliferyl-β-D-cellobioside (0.3 U mg^{-1}), CMC (105.9 U mg^{-1}), birch wood xylan (132.3 U mg^{-1}), oat spelt xylan (67.9 U mg^{-1}), and 2-hydroxyethyl-cellulose (26.3 U mg^{-1}). Based on these findings, we believe that CelEx-BR12 is an efficient multifunctional enzyme as endocellulase/exocellulase/xylanase activities that may prove useful for biotechnological applications.

1. Introduction

Materials containing cellulose, hemicellulose, and lignin (called lignocellulosic materials) are the most abundant type of natural biomass. Cellulose, which is a linear polymer of glucose residues linked by 1,4-β-D-glucosidic bonds, is the main constitute of plants and is the most abundant renewable organic resource in nature [1]. Cellulose can be degraded to glucose through the synergistic action of three classes of glycoside hydrolases (GHs): (1) endo-β-1,4-glucanase (EG; EC 3.2.1.4), which randomly attacks the cellulose polymer by endo action; (2) the exo-β-1,4-cellobiohydrolases, CBH I and CBH II (EC 3.2.1.91), which remove cellobiose from the non-reducing and reducing ends, respectively, of the cellulose chain; and (3) β-glucosidase (BGL; EC 3.2.1.21), which hydrolyzes cello-oligosaccharides and cellobiose to glucose [2,3]. Exocellulase, which is the only enzyme known to degrade highly ordered crystalline regions, is very important for the degradation of crystalline cellulose [4]. Finally, the released cellobiose is converted to glucose by β-glucosidase.

Cellulases are essential enzymes that are widely used in various industrial fields, including the bioethanol, textile, detergent, feedstuff, food, forage, beer-brewing and pulp-and-paper industries [5,6]. During the production of bioethanol, which is one of the most significant alternative energy sources, the release of glucose from cellulose is a crucial step governed by cellulolytic enzymes derived from fungi or bacteria [7]. The current limitations on the cellulosic bioconversion of lignocellulosic biomass include suboptimal enzyme stability and the sensitivities of some cellulases to reaction byproducts and other inhibitory agents [8]. Thus, researchers continue to search for novel and industrially useful enzymes, such as pH-stable, thermostable, high-activity, and/or multifunctional cellulases [9,10].

In this study, we used an high-throughput screening (HTS)-based robotic system to screen metagenomic resources for exocellulase activity, identified a novel gene that appeared to encode such an enzyme (*CelEx-BR12*), overexpressed a codon-optimized CelEx-BR12 in *Escherichia coli*, and characterized this novel multifunctional cellulolytic enzyme.

2. Materials and methods

2.1. Chemicals

The 4-methylumbelliferyl-β-D-cellobioside ($MeUmbG_2$), polysaccharide substrates (avicel, birch wood xylan, α-cellulose, carboxymethyl cellulose, curdlan, 2-hydroxyethyl-cellulose, laminarin, oat spelt xylan, salicin, D-gluconic acid, and starch), and cello-oligosaccharides were all purchased from Sigma–Aldrich

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(St. Louis, MO). All other chemicals were of the highest purity that was commercially available.

2.2. Screening the metagenomic library using a robotic, HTS system, and shotgun cloning of hit clones for exocellulase

Metagenomic DNA was isolated from the microorganisms of rumen fluid from a rumen-fistulated Korean cow (Hanwoo) using a modified sodium dodecyl sulfate (SDS)-based DNA extraction protocol [11] and a metagenomic library was constructed [12]. Positive samples ("hits") for novel exocellulases were identified using a robotic, HTS system running a protocol for screening metagenomic resources [12]. A potential exocellulase-encoding clone was isolated from the metagenomic library and cloned using a shotgun method [13,14]. The relevant plasmids were purified using a Plasmid Midi kit (Qiagen, Valencia, CA), recombinant fragments were sequenced by SolGent (Daejeon, Korea), and BLAST analyses were performed using the NCBI server [15]. The obtained sequences were aligned using the multialignment program of BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the predicted amino acid sequence was analyzed for potential functional domains using the InterProScan tool (http://www.ebi.ac.uk/InterProScan/).

2.3. Construction of a vector expressing a codon-optimized CelEx-BR12 gene in E. coli

The amino acid sequence of CelEx-BR12 (GenBank Accession No. KC963960) was subjected to codon optimization, by GenoTech (Daejeon, Korea), using a proprietary algorithm, and the optimized gene (*CelEx-BR12opti*) was directionally cloned into the His-tagging vector, pET-22b(+) (Novagen, Madison, WI). DNA sequence of codon-optimized *CelEx-BR12opti* was shown in Supplementary data. More specifically, oligonucleotide primers (CBR12opti-F, 5′-GGA ATT CCA T**AT G**CG GAA GAA TTC CTT TAA A-3′ and CBR12opti-R, 5′-CCG CTC GAG TTT CTC TAG GGG CTT TCC T-3′; underlining indicates an *Nde*I or *Xho*I site, and the bold text shows the start codon) amplified a ~1.2 kb fragment when used for *Taq* polymerase chain reaction (PCR). The amplified DNA fragments were digested with *Nde*I and *Not*I, and ligated into the corresponding sites of pET-22b(+) to generate pET-CBR12. *E. coli* XL1-Blue was used as a host strain for the cloning and maintenance of plasmids. The recombinant plasmid was introduced into *E. coli* BL21 (DE3) for gene characterization and recombinant protein production.

2.4. Expression and purification of recombinant CelEx-BR12

To prepare recombinant CelEx-BR12 cellulase, the above-described *E. coli* were grown at 30 °C in LB (Luria–Bertani) broth containing 50 μg/ml ampicillin and transcription of the recombinant gene was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). After incubation for 4 h at 30 °C, cells were harvested by centrifugation at 7000*g* for 10 min and washed with ice-cold PBS buffer (200 mM NaCl, 3 mM KCl, 2 mM KH_2PO_4, and 1 mM Na_2HPO_4; pH 7.5). Cells were disrupted using a VCX750 sonicator (Sonics Materials, Newtown, CT) and the His-tagged fusion protein was purified using a HiTrap chelating HP column (GE Healthcare, Piscataway, NJ) employing a gradient of 0–500 mM imidazole in PBS buffer containing 500 mM NaCl. The purified protein was desalted on a HiPrep 26/10 desalting column (GE Healthcare). All purification steps were performed using a fast protein liquid chromatography (FPLC) system (ÄKTA Explorer; GE Healthcare). The protein composition was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and the protein concentration was measured by the Bradford method (Bio-Rad protein assay kit; Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin as a standard [16].

2.5. Substrate specificity and determination of cellulolytic enzyme activity

Substrate specificity was examined using $MeUmbG_2$ and various carbohydrates (e.g., avicel, birch wood xylan, α-cellulose, carboxymethyl cellulose, curdlan, 2-hydroxyethyl-cellulose, laminarin, oat spelt xylan, salicin, and starch).

Enzyme activity was assayed by measuring the release of the MeUmb group when an aliquot of the enzyme was incubated with various MeUmb glycosides (final concentration, 0.1 mM) in 100 mM sodium acetate buffer (pH 5.0) for 20 min at 37 °C, along with the appropriate 4-methylumbelliferylliferone standards (0.05–1 nM). The fluorescence intensities of the released MeUmb groups were determined using a 1420 VICTOR multilabel counter (PerkinElmer Life Sciences, Wallac Finland Oy, Turku, Finland) with $\lambda_{excitation}$ = 365 nm and $\lambda_{emission} \geqslant$ 460 nm. Assays were performed in 100 μl reaction volumes of 100 mM sodium acetate buffer (pH 5.0) containing 0.1 mM of MeUmb glycoside. The reaction was terminated by addition of 100 μl of 500 mM glycine buffer (pH 10.4). One unit of enzyme activity was defined as the amount of enzyme that produced the equivalent of 1 μmol of product (reducing sugar or MeUmb) under optimal conditions in 1 min.

The endocellulase and xylanase activities of CelEx-BR12 for polysaccharide substrates were measured using the 3,5-dinitrosalicylic acid (DNS) reagent, as described by [17]. The reaction mixture consisted of 50 μl of 2% (w/v) carboxymethyl cellulose (molecular weight, 90 kDa; degree of carboxymethyl substitution, 0.7) (Sigma–Aldrich) in 100 mM sodium acetate buffer (pH 5.0); 50 μl of enzyme solution was added to the reaction mixture, followed by incubation at 40 °C for appropriate durations. All natural glycosides were assayed under the same conditions. To stop the reaction, 100 μl of DNS reagent was added and the reaction mixture was heated at 100 °C for 10 min. The mixture was then cooled at room temperature for 10 min and centrifuged at 10,000*g* for 10 min, and the absorbance of the supernatant at 540 nm was measured using a microplate reader (Model 680, Bio-Rad Laboratories).

The Michaelis–Menten constants (K_m values) and the maximal reaction velocities (V_{max} values) of purified CelEx-BR12 were calculated from double-reciprocal plots according to the Lineweaver–Burk method, and the kinetic constants K_m, V_{max}, k_{cat}, and k_{cat}/K_m were estimated using standard formulae [18].

2.6. Characterization of recombinant CelEx-BR12

The optimum pH of CelEx-BR12 was determined by incubation with 0.1 mM $MeUmbG_2$ in various buffers (pH 3.0–13.0) at 37 °C for 20 min. The buffers used to explore the optimum pH were as follows: 100 mM sodium acetate buffer (pH 3.0–6.0), 100 mM sodium phosphate buffer (pH 6.0–8.0), 100 mM Tris–HCl buffer (pH 8.0–9.0), 100 mM sodium bicarbonate buffer (pH 9.0–11.0), and 100 mM potassium chloride buffer (pH 11.0–13.0). The pH stability of the enzyme was assessed by incubating the enzyme at 4 °C for 24 h at various pH values, followed by assessment of residual activity. The optimum temperature for hydrolysis of 0.1 mM $MeUmbG_2$ in 100 mM sodium acetate buffer (pH 5.0) was determined by incubating a mixture of cellulase and 1% (w/v) carboxymethyl cellulose (CMC) for 20 min at temperatures ranging from 20 to 70 °C. Thermostability was evaluated by incubating the enzyme in 100 mM sodium acetate buffer (pH 5.0) for 0–120 min at temperatures ranging from 20 to 60 °C. The effects of metal ions were determined at a final ion concentration of 1 mM. All assays were performed at the optimum pH and

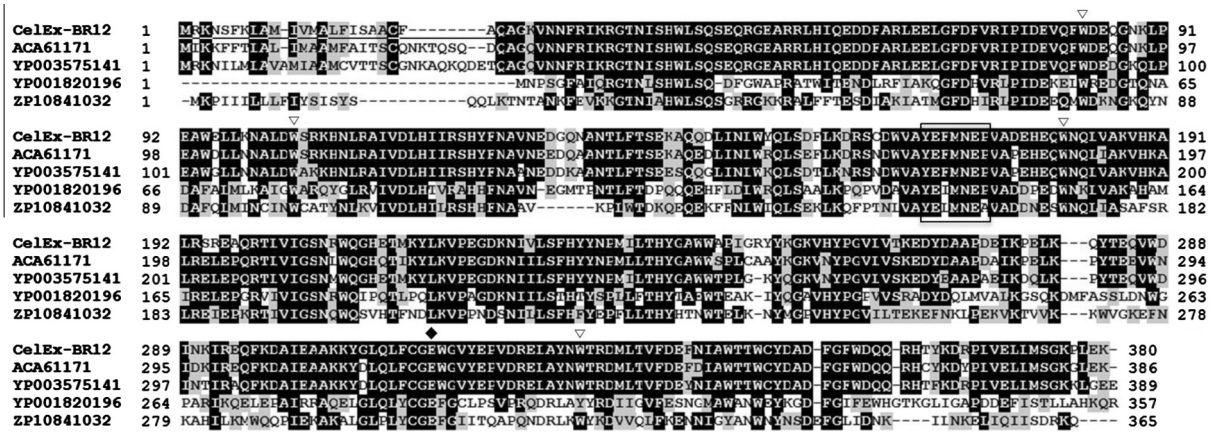

Fig. 1. Comparison of the amino acid sequence of CelEx-BR12 with the catalytic domains of cellobiosidase (ACA61171, uncultured microorganism), family 5 glycosyl hydrolase (YP003575141, *Prevotella ruminicola* 23), glycoside hydrolase (YP001820196, *Opitutus terrae* PB90–1), and cellulase (ZP10841032, *Aquimarina agarilytica* ZC1). The GenBank accession numbers are shown. Regions of identity or high similarity among sequences are shown as black or gray columns, respectively. Signal peptide sequences, probable proton donors, and nucleophilic residues are indicated by underlines, inverted closed triangles and diamonds, respectively. Conserved glutamic acid and tryptophan residues are shown using inverted open triangles. The putative partial catalytic region (FEGLNEP) of family 5 glycosyl hydrolase (Qiu et al. 2000) is boxed. Alignments were performed using BioEdit version 7.0.9.0.

temperature, and the enzyme solution was treated with 1 mM EDTA prior to assays in order to remove any preexisting metal ions.

2.7. TLC analysis

Hydrolytic products from cello-oligosaccharides (ranging from cellobiose to cellopentaose in size) and CMC were analyzed by thin-layer chromatography (TLC) using silica gel 60 plates (Merck, Darmstadt, Germany) developed with 1-butanol/acetic acid/water (2:1:1, v/v/v). For detection of sugars, plates were sprayed with freshly prepared 5% (v/v) H_2SO_4 in ethanol and subjected to heat treatment at 200 °C for 5 min.

3. Results

3.1. Construction of a metagenomic library and screening for exocellulolytic active clones from metagenomic library using HTS based on robot system

We constructed a metagenomic library from rumen-fluid microbes using a fosmid library kit (see Section 2). The metagenomic library consisted of about 20,000 fosmid clones, three of which were randomly chosen and subjected to restriction analysis for verification of bank quality and insert size (data not shown). The insert DNA size was 30–40 kb and the library covered approximately 600–800 Mbp of total metagenomic DNA. To screen for exo-type cellulase activity, a robotic, HTS system was used to assay fosmid clones with $MeUmbG_2$ as the substrate. The exocellulase activity of the metagenomic library was assayed by measuring the release of the methylumbelliferyl (MeUmb) group when an aliquot of enzyme was incubated with $MeUmbG_2$ (final assay concentration of 10 μM) in the HTS system. The reaction was terminated by addition of glycine buffer and the fluorescence of the released MeUmb groups was determined. A positive clone (pFOS–CBR12) was identified by measuring the release of the MeUmb group from $MeUmbG_2$ [12].

3.2. Cloning, sequencing, and analysis of an exo-type cellulase-positive clone

After shotgun cloning, an HTS-based preliminary search for recombinants expressing exocellulase activity identified only a single positive strain (representing pFOS–CBR12). To identify other genes that could encode proteins with exocellulase activity, further subcloning and activity-based cellulase screenings were performed on LB medium containing 10 μM $MeUmbG_2$.

We selected the initial positive clone for additional analysis; sequencing of plasmid pHSG–CBR12 (harboring a 4.5 kb recombinant DNA fragment) yielded two open reading frames (ORFs): one that showed 84% amino acid sequence identity to a penicillin-binding protein of *Prevotella ruminicola* 23, and one that showed 83% identity to a family 5 glycosyl hydrolase from *Prevotella ruminicola* 23 (GenBank Accession No. YP003575141). The latter ORF, which we designated *CelEx-BR12*, encoded a 380-amino-acid sequence that included a 23-amino-acid N-terminal signal peptide (MRKNSFKLAMIVMALFISAACFA).

BLAST analysis of the deduced amino acid sequence against the GenBank protein database revealed that mature CelEx-BR12 (Fig. 1) has 85%, 83%, 46%, and 42% identity with the catalytic domains of uncultured microorganism cellobiosidase (GenBank Accession No. ACA61171), *Prevotella ruminicola* 23 family 5 glycosyl hydrolase (GenBank Accession No. YP003575141), *Opitutus terrae* PB90-1 glycoside hydrolase (GenBank Accession No. YP001820196), and *Aquimarina agarilytica* ZC1 cellulase (GenBank Accession No. ZP10841032), respectively. On the basis of sequence homology with other family 5 glycosyl hydrolases, a conserved catalytic residue (Glu^{314}) was predicted to serve as the putative nucleophilic residue of the active site.

3.3. Expression and purification of CelEx-BR12 exocellulase

The codon-optimized *CelEx-BR12* gene was amplified and cloned into the pET-22b(+) expression vector, as described in the Section 2. The resultant plasmid, pET-CBR12, was introduced into *E. coli* BL21 (DE3), and the CelEx-BR12 protein was purified by Ni–NTA affinity chromatography. The estimated molecular mass of purified CelEx-BR12 was approximately 42 kDa (Fig. 2).

3.4. Characterization of purified recombinant CelEx-BR12

Fig. 3 shows the pH and temperature optima of the enzyme, and the effects of these parameters on its stability. The activity of CelEx-BR12 was maximal at 35–45 °C, and the optimal temperature and pH were 37 °C and pH 5.0, respectively (Fig. 3A, B). CelEx-BR12 was stable over a range of pH 7.0–9.0 and more than 75% of its activity was retained after incubation at 37 °C for 20 min. (Fig. 3C). The thermostability of purified CelEx-BR12 was analyzed

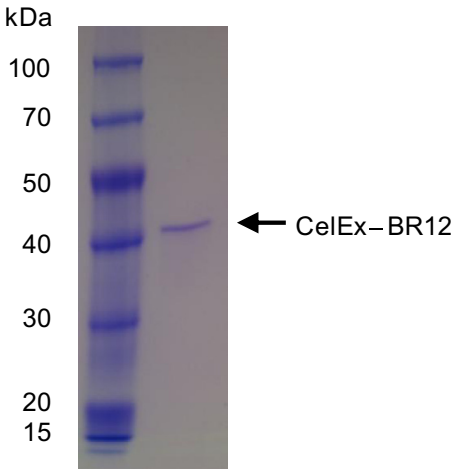

Fig. 2. SDS–PAGE analysis of purified CelEx-BR12. Lanes: 1, molecular mass standards; and 2, purified CelEx-BR12.

by incubation at various temperatures for 2 h at pH 5.0 and the protein was found to be unstable at temperatures above 40 °C (Fig. 3D).

Assessment of the substrate specificity of CelEx-BR12 showed that tested polysaccharides could be hydrolyzed to different extents (Table 1). Various carbohydrates (e.g., CMC, avicel, birch wood xylan, oat spelt xylan, laminarin, starch, 2-hydroxyethyl-cellulose, curdlan, α-cellulose, salicin, and $MeUmbG_2$) were also tested. The highest activities were observed against natural glycosides, such as birch wood xylan (132.3 U mg^{-1}), CMC (105.9 U mg^{-1}), oat spelt xylan (67.9 U mg^{-1}), and 2-hydroxyethyl-cellulose (26.3 U mg^{-1}). Avicel pH101, laminarin, starch, curdlan, α-cellulose, salicin, and D-gluconic acid were not susceptible to the effects of CelEx-BR12. We also observed exocellulase activity (0.3 U mg^{-1}) using a fluorogenic glycoside as a substrate.

CelEx-BR12 activity was investigated in terms of catalytic properties and by estimation of kinetic constants, using $MeUmbG_2$ as the substrate for exocellulase activity. As shown in Table 2, the Michaelis–Menten constants (K_m values) and the maximal reaction velocities (V_{max} values) of CelEx-BR12 were 12.92 μM and 1.55×10^{-4} μmole min^{-1}, respectively, for exocellulase activity.

The effects of metal ions on enzyme activity are shown in Table 3. Zn^{2+} strongly stimulated exocellulase activity (to 177% of the control value), whereas Fe^{2+} and Hg^{2+} dramatically reduced enzyme activity (to 0% and 45% of the control value, respectively). No metal ion significantly enhanced exocellulase activity, whereas Cu^{2+}, Hg^{2+}, and Zn^{2+} dramatically reduced enzyme activity to 16%, 15%, and 67% of the control value, respectively.

To investigate the activity of CelEx-BR12 against different cello-oligosaccharides, we used TLC to qualitatively analyze the hydrolysis products obtained from various substrates. As shown in Fig. 4, cellotriose (G3), cellotetraose (G4), cellopentaose (G5), and CMC were fully degraded to glucose, cellobiose, and cellotriose. Notably, glucose was produced to an extent similar to that seen when other cellulases were tested. These findings indicate that CelEx-BR12 cuts randomly along the cello-oligosaccharide chain and has both endo- and exo-glycolytic modes of action.

4. Discussion

Many cellulolytic enzymes have been isolated from various organisms and the genes encoding these enzymes have been cloned and characterized. However, new cellulose-degrading enzymes still are required for specific biotechnological needs.

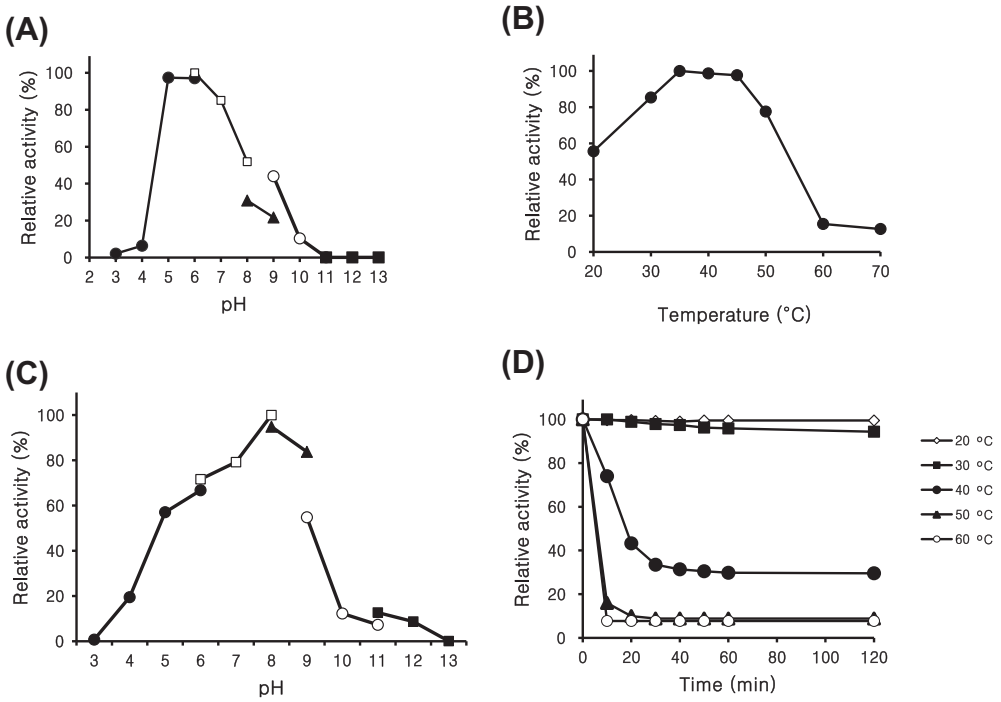

Fig. 3. Biochemical characterization of CelEx-BR12. (A) The optimal pH for activity was determined by incubating the enzyme in buffers of various pH values (see below) at 37 °C for 20 min. (B) Effect of temperature on the activity of CelEx-BR12. Enzyme activity was assayed in 100 mM sodium acetate buffer (pH 5.0) for 20 min at the indicated temperatures. (C) To evaluate pH stability, CelEx-BR12 was incubated in buffers of various pH values (see below) at 4 °C for 24, and $MeUmbG_2$ hydrolysis was assessed under optimal conditions (sodium acetate buffer at pH 5.0, 37 °C for 20 min). The buffers used to establish optimum pH and assess pH stability were as follows: 100 mM sodium acetate buffer (pH 3.0–6.0, filled circles), 100 mM sodium phosphate buffer (pH 6.0–8.0, unfilled squares), 100 mM Tris–HCl buffer (pH 8.0–9.0, filled triangles), 100 mM sodium bicarbonate buffer (pH 9.0–11.0, unfilled circles), and 100 mM potassium chloride buffer (pH 11.0–13.0, filled squares). (D) The thermal stability of CelEx-BR12 was explored by incubation at different temperatures for 0–120 min.

Table 1
Substrate specificity of the CelEx-BR12 cellulase.

Substrate	Specific activity (U mg^{-1})
CMC	105.9
Avicel pH101	LA
Birch wood xylan	132.3
Oat spelt xylan	67.9
Laminarin	NA
Starch	NA
2-Hydroxyethyl-cellulose	26.3
Curdlan	NA
α-Cellulose	NA
Salicin	NA
D-Gluconic acid	NA
$MeUmbG_2$	0.3

Purified protein was added to 100 μl sodium acetate buffer containing 0.5% (w/v) salicin, 50 μM D-gluconic acid, 100 μM $MeUmbG_2$, or 1% (w/v) of the other substrates. The reaction times were 120 min for avicel pH101, starch, curdlan, and α-cellulose (insoluble substrates), and 20 min for the other substrates. Specific activities are shown in units per milligram of purified enzyme. One unit of enzyme activity was defined as the amount of enzyme that produced the equivalent of 1 μM of product (reducing sugar or 4-methylumbelliferyl) per minute. LA and NA indicates low and no activity, respectively.

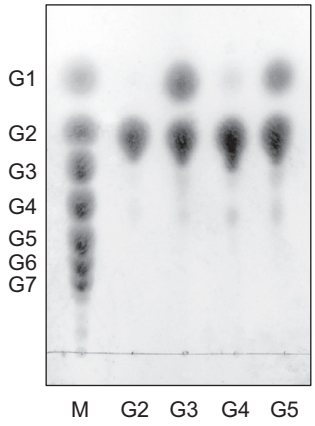

Fig. 4. TLC analysis of products upon hydrolysis of cello-oligosaccharides by CelEx-BR12. Each cello-oligosaccharide was incubated with CelEx-BR12 for 12 h and the hydrolysate was analyzed by TLC. Lanes: M, authentic oligosaccharides; G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; and CMC, carboxymethyl cellulose. TLC was performed using a butanol/acetic acid/water (2:1:1, v/v/v) solvent. For visualization of sugars, the TLC plates were sprayed with 5% (v/v) sulfuric acid in ethanol and incubated at 200 °C for 5 min.

Table 2
Kinetic values for the exo-type cellulase activity of CelEx-BR12.

K_m (μM)	V_{max} (μmol min^{-1})	k_{cat} (min^{-1})	k_{cat} K_m^{-1} (min^{-1} μM^{-1})
12.92	1.55×10^{-4}	1.2×10^{-5}	9.29×10^{-7}

One unit of enzyme activity was defined as the amount of enzyme that produced the equivalent of 1 μM of product (4-methylumbelliferyl) per minute under optimum conditions.

Table 3
Effects of metal ions on enzymatic activity of CelEX-BR12.

Metallic ion	Relative activity (%)
None	100
Ca^{2+}	139
Co^{2+}	147
Cu^{2+}	137
Fe^{2+}	45
Hg^{2+}	0
Mg^{2+}	142
Mn^{2+}	143
Ni^{2+}	149
K^{+}	149
Rb^{+}	149
Zn^{2+}	177

Metal ions were added to a final concentration of 1 mM. All assays were performed at optimum pH and temperature values. The CelEx-BR12 enzyme was treated with 1 mM EDTA prior to experiments, in order to remove any additional metal ions.

The use of bifunctional or multifunctional cellulases has evolved as a strategy for improving the degradation of cellulosic biomasses, overcoming the limited availability of energy for enzyme synthesis, and dealing with the wide variety of substrates that may exist in the biomass [19]. However, this often requires the assembly of a novel bifunctional cellulase–xylanase by end-to-end fusion. For example, researchers generated an artificial bifunctional and thermostable cellulase–xylanase enzyme from *Thermotoga maritima* by gene fusion [20]. However, although bifunctional/multifunctional cellulases are beneficial for industrial applications, most natural cellulases are monofunctional. The few known naturally occurring bifunctional/multifunctional cellulases include the bifunctional chitosanase/cellulase of *Myxobacter* sp. AL-1 [21], the bifunctional endoglucanase/β-glucosidase of *Bacillus subtilis* CD4 [22], the endo/exocellulase Rucel5B of the yak rumen metagenome [23], and an endo/exoglucanase of anaerobic ruminal bacterium called *AN-C16-KBRB* [9].

Here, we offer CelEx-BR12 as a novel multifunctional cellulase, in the hope that it may be used to improve the degradation of cellulosic biomasses for biorefinery. CelEx-BR12 can hydrolyze CMC, $MeUmbG_2$, xylan-type substrates and 2-hydroxyethyl-cellulose. It showed its highest activities against birch wood xylan (132.3 U mg^{-1}), CMC (105.9 U mg^{-1}), oat spelt xylan (67.9 U mg^{-1}), and 2-hydroxyethyl-cellulose (26.3 U mg^{-1}). Furthermore, it showed exocellulase activity against a fluorogenic glycoside (0.3 U mg^{-1}). It might be considered somewhat similar to Rucel5B (GenBank Accession No. GQ849224), which is a bifunctional GH5 enzyme cloned from a yak rumen metagenome (Bao et al. 2011), and bifunctional CelEdx16 cloned from anaerobic ruminal bacteria [9]. However, Rucel5B and CelEdx16 are an endocellulase/exocellulase with endoglucanase activity, but little or no xylanase activity. Thus, CelEx-BR12 has unique characteristics as mutifunctional enzyme showing endocellulase/exocellulase/xylanase activities that might make it industrially useful.

The addition of most metal ions could enhance the activity of CelEx-BR12, but the activities of Rucel5B and CelEdx16 were reduced or unaffected by metal ions.

In sum, we herein firstly isolated the *CelEx-BR12* gene from metagenomic resources representing ruminal bacteria for exocellulase activity using a robotic HTS system, expressed the corresponding protein in *E. coli*, and characterized the recombinant CelEx-BR12. This interesting cellulase is multifunctional and can be stimulated by various metal ions. Further studies are warranted, including crystallization of the protein for structural studies, examination of its cell surface display, and studies on potential protein evolution. We believe that the CelEx-BR12 cellulase will prove to be an efficient multifunctional enzyme for use in biotechnological applications in the environmental, food safety, and medical fields.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.120.

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