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**Fox et al.**

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(45) **Date of Patent: Sep. 29, 2015**

(54) **MULTIFUNCTIONAL CELLULASE AND HEMICELLULASE**

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(73) Assignee: **Wisconsin Alumni Research Foundation**, Madison, WI (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/030,290**

(22) Filed: **Sep. 18, 2013**

(65) **Prior Publication Data**

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**Related U.S. Application Data**

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(51) **Int. Cl.**

*C12N 9/42* (2006.01)
*C07K 14/33* (2006.01)

(52) **U.S. Cl.**

CPC .............. *C12N 9/2437* (2013.01); *C07K 14/33* (2013.01)

(58) **Field of Classification Search**

None
See application file for complete search history.

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| 2008/0286749 | A1 | 11/2008 | Fox |
| 2010/0304405 | A1 | 12/2010 | Fox et al. |

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| | | | |
|---|---|---|---|
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| WO | 2004/096999 | A2 | 11/2004 |
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| WO | 2008/028147 | | 3/2008 |
| WO | 2008/127997 | | 10/2008 |
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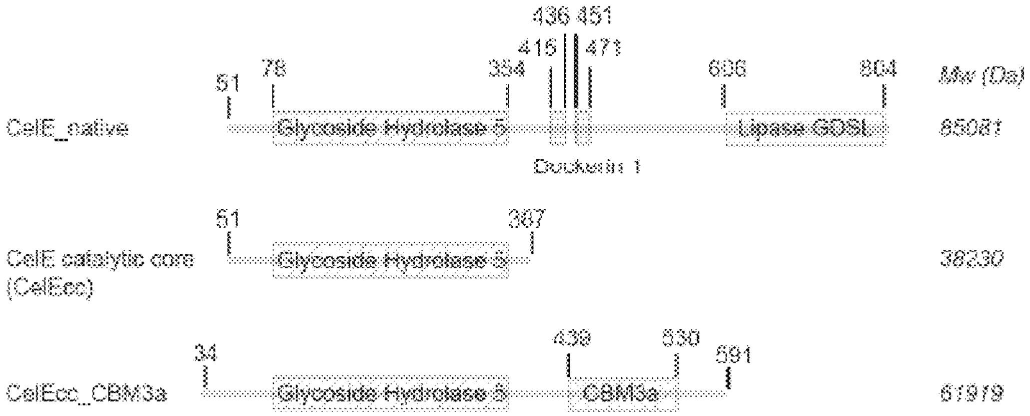
*Primary Examiner* — Christian Fronda
(74) *Attorney, Agent, or Firm* — Quarles & Brady LLP

(57) **ABSTRACT**

A multifunctional polypeptide capable of hydrolyzing cellulosic materials, xylan, and mannan is disclosed. The polypeptide includes the catalytic core (cc) of *Clostridium thermocellum* Cthe_0797 (CelE), the cellulose-specific carbohydrate-binding module CBM3 of the cellulosome anchoring protein cohesion region (CipA) of *Clostridium thermocellum* (CBM3a), and a linker region interposed between the catalytic core and the cellulose-specific carbohydrate binding module. Methods of using the multifunctional polypeptide are also disclosed.

**20 Claims, 22 Drawing Sheets**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415
471
51
78
354
606
804
Mw (Da)
CelE_native
Glycoside Hydrolase 5
Lipase GDSL
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Dockerin 1
51
367
CelE catalytic core (CelEcc)
Glycoside Hydrolase 5
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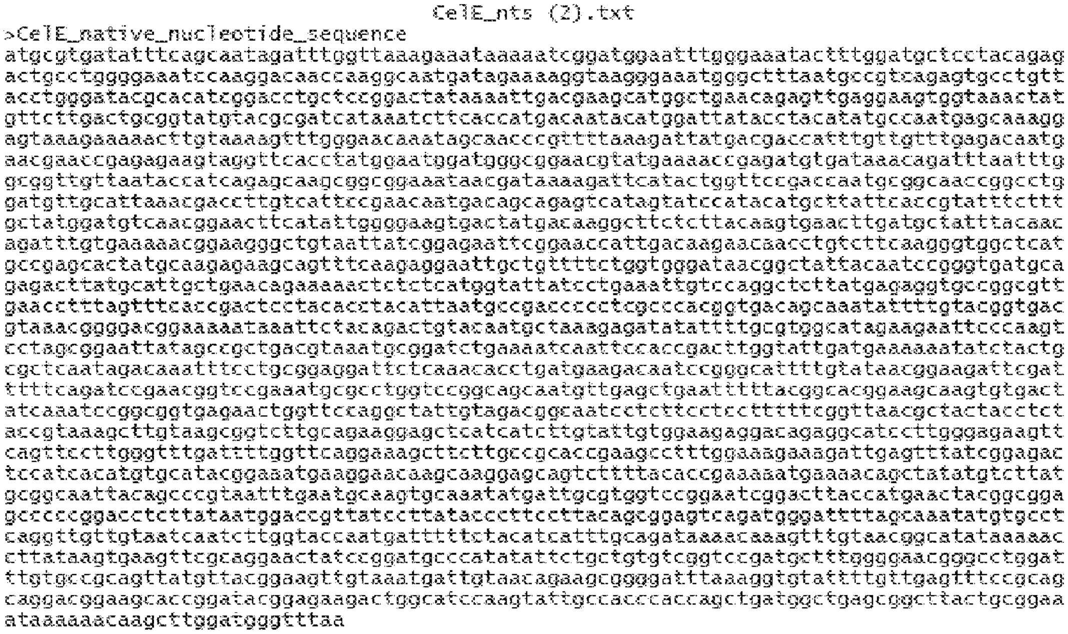
34
439
530
591
CelEcc_CBM3a
Glycoside Hydrolase 5
CBM3a
61919

Figure 1



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AVVNTIRASGGNNDKRFILVPTNAATGLDVALNDLVIPNNDSRVIVSIHAYSPYFFAMDVNGTSYWGSDYDKASLTSELDAIYN
RFVKNGRAVIIGEFGTIDKNNLSSRVAHAEHYAREAVSRGIAVFWWDNGYYNPGDAETYALLNRKTLSWYYPEIVQALMRGAGV
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aggagtaaagaaaaacttgtaaaagtttgggaacaaatagcaacccgttttaaagattatgacgaccatttgttgtttgagaca
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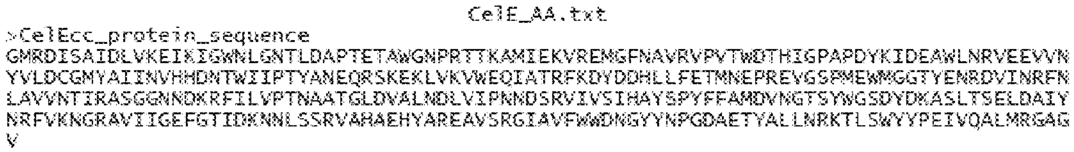

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LAVVNTIRASGGNNDKRFILVPTNAATGLDVALNDLVIPNNDSRVIVSIHAYSPYFFAMDVNGTSYWGSDYDKASLTSELDAIY
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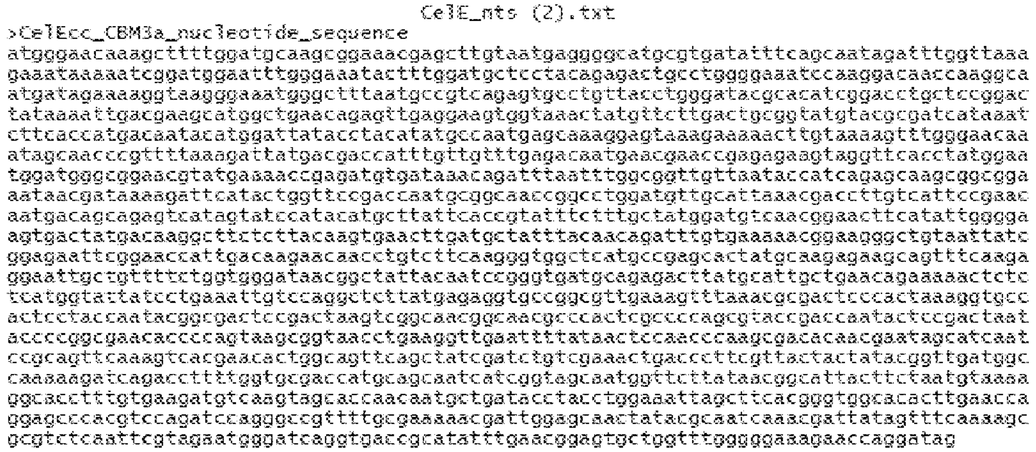

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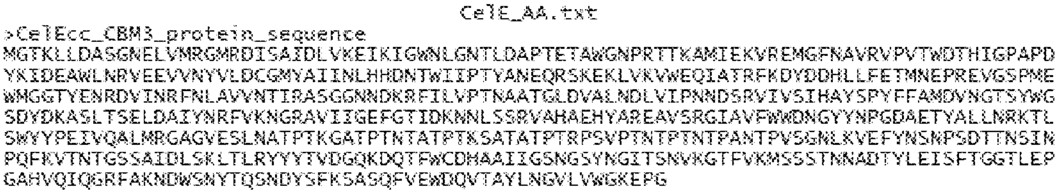

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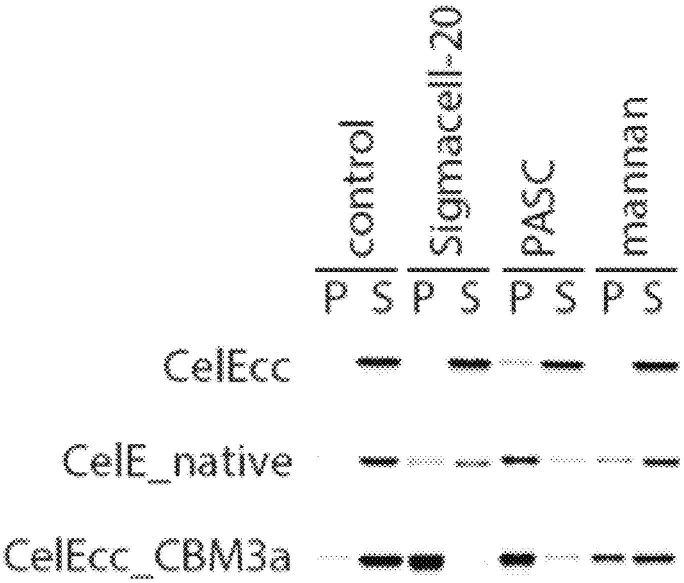
Figure 4B (SEQ ID NO:6)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PASC
mannan
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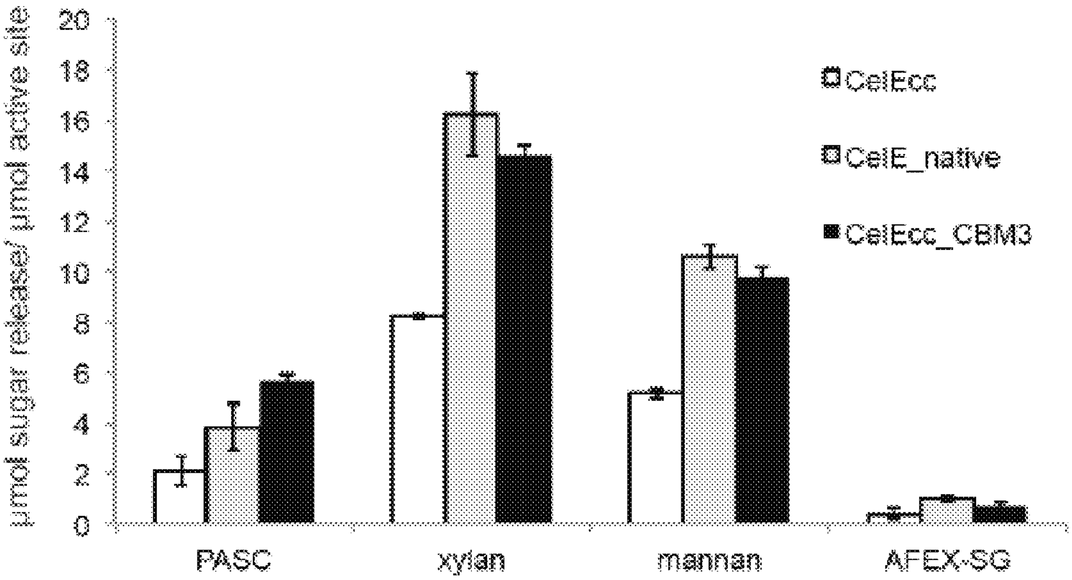
CelE_native
CelEcc_CBM3a

Figure 5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20
18
16
14
12
10
8
6
4
2
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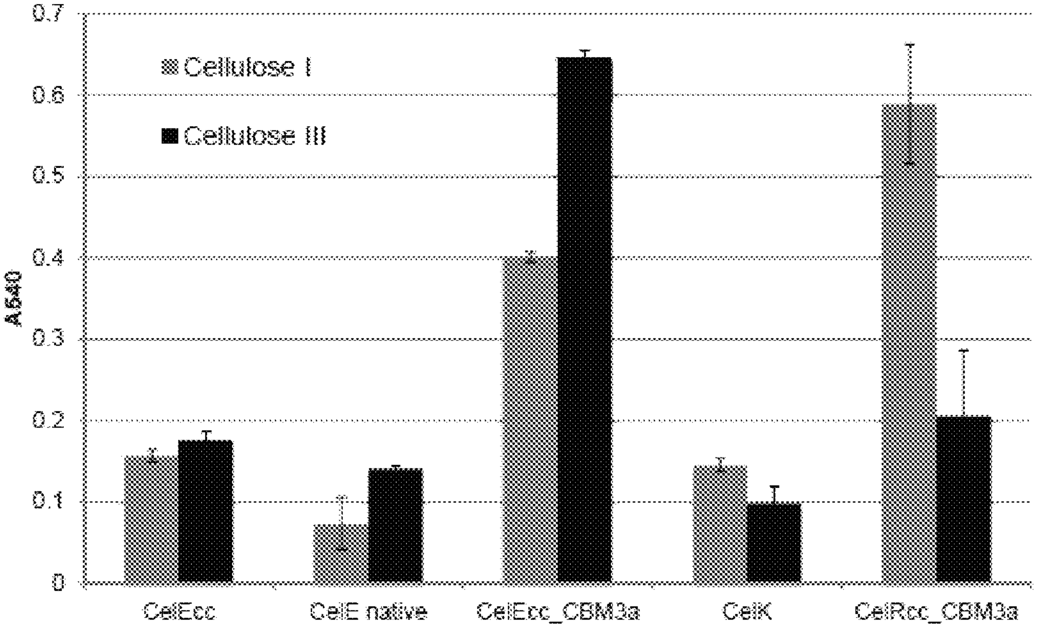
CelEcc_CBM3
PASC
xylan
mannan
AFEX-SG

Figure 6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0.6
0.5
0.4
0.3
0.2
0.1
0
A540
▨ Cellulose I
■ Cellulose III
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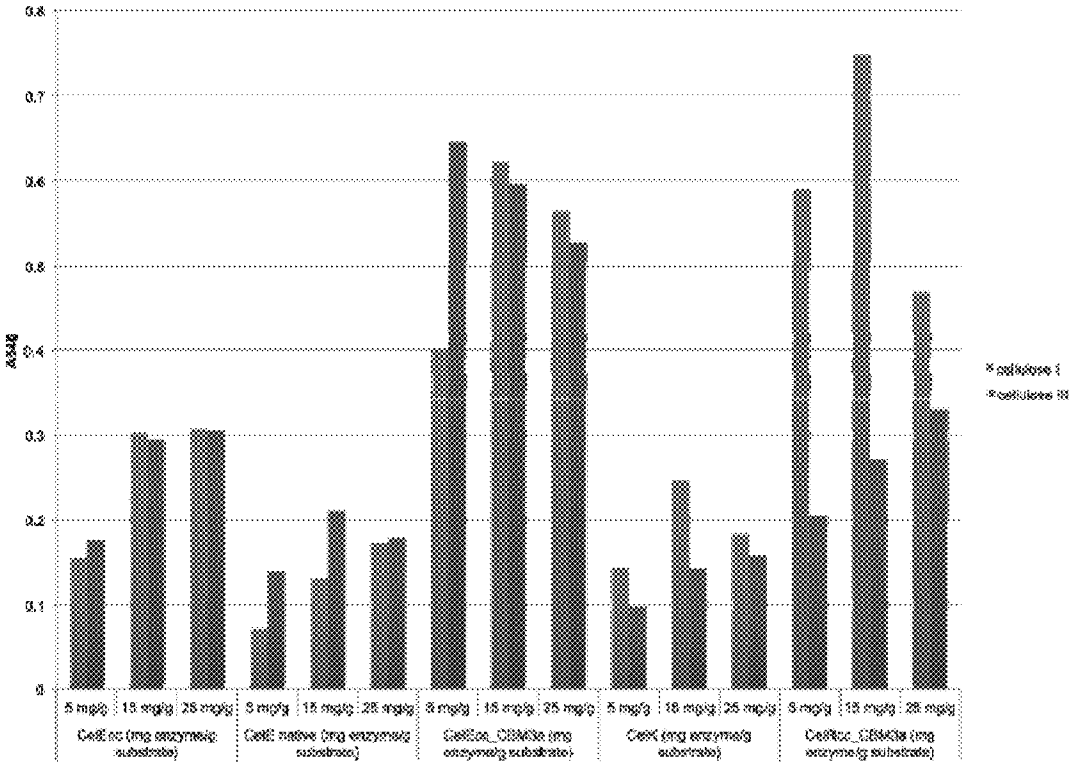
CelE native
CelEcc_CBM3a
CelK
CelRcc_CBM3a

Figure 7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0.3
0.2
0.1
0
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cellulose III
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5 mg/g
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15 mg/g
25 mg/g
5 mg/g
15 mg/g
25 mg/g
5 mg/g
15 mg/g
25 mg/g
CelEcc (mg enzyme/g substrate)
CelE native (mg enzyme/g substrate)
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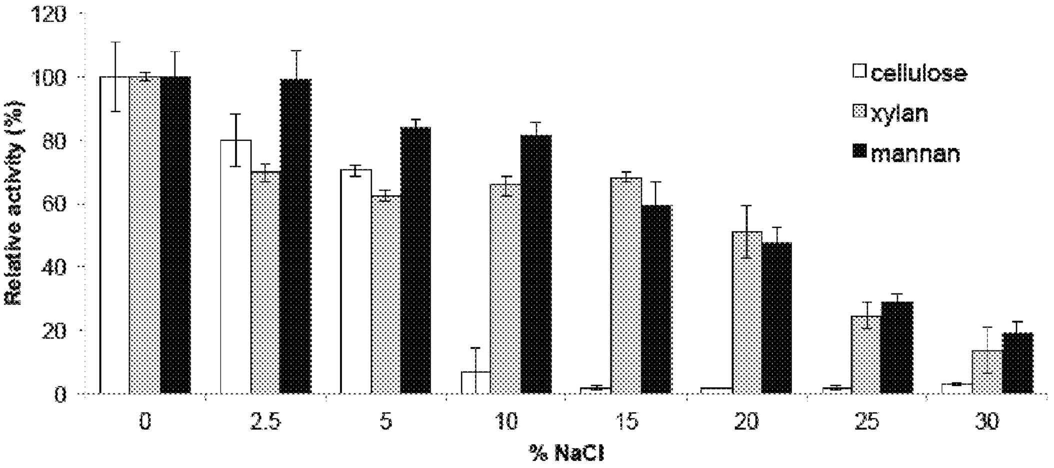
CelK (mg enzyme/g substrate)
CelKcc_CBM3a (mg enzyme/g substrate)

Figure 8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100
80
60
40
20
0
Relative activity (%)
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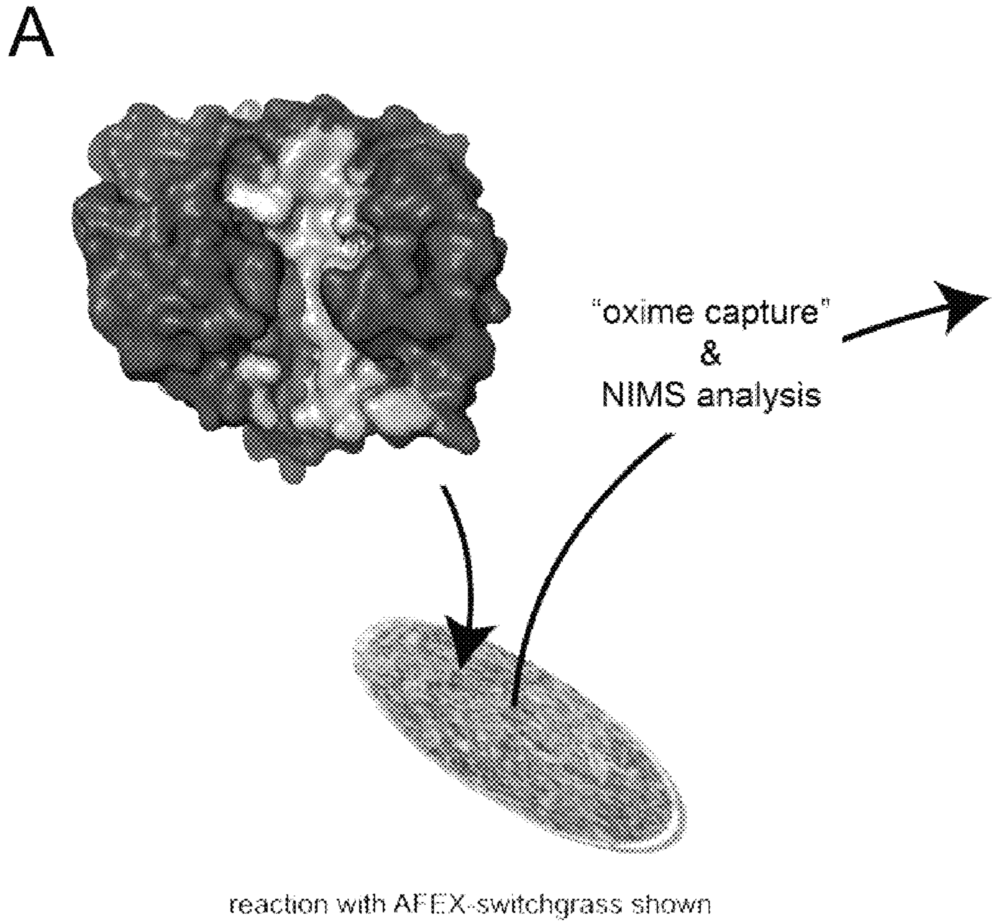
5
10
15
20
25
30
% NaCl

Figure 9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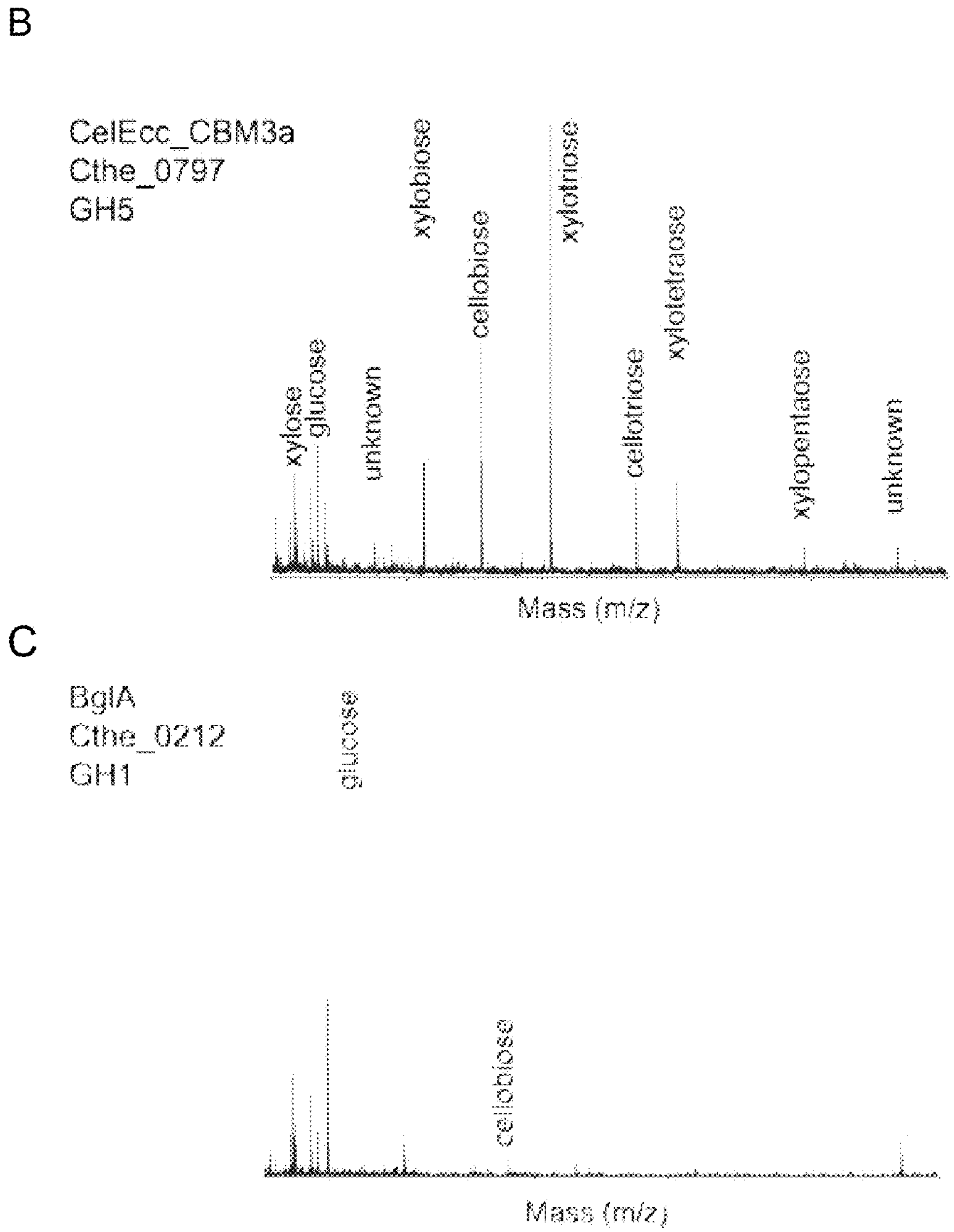
&
NIMS analysis
reaction with AFEX-switchgrass shown

Figure 10A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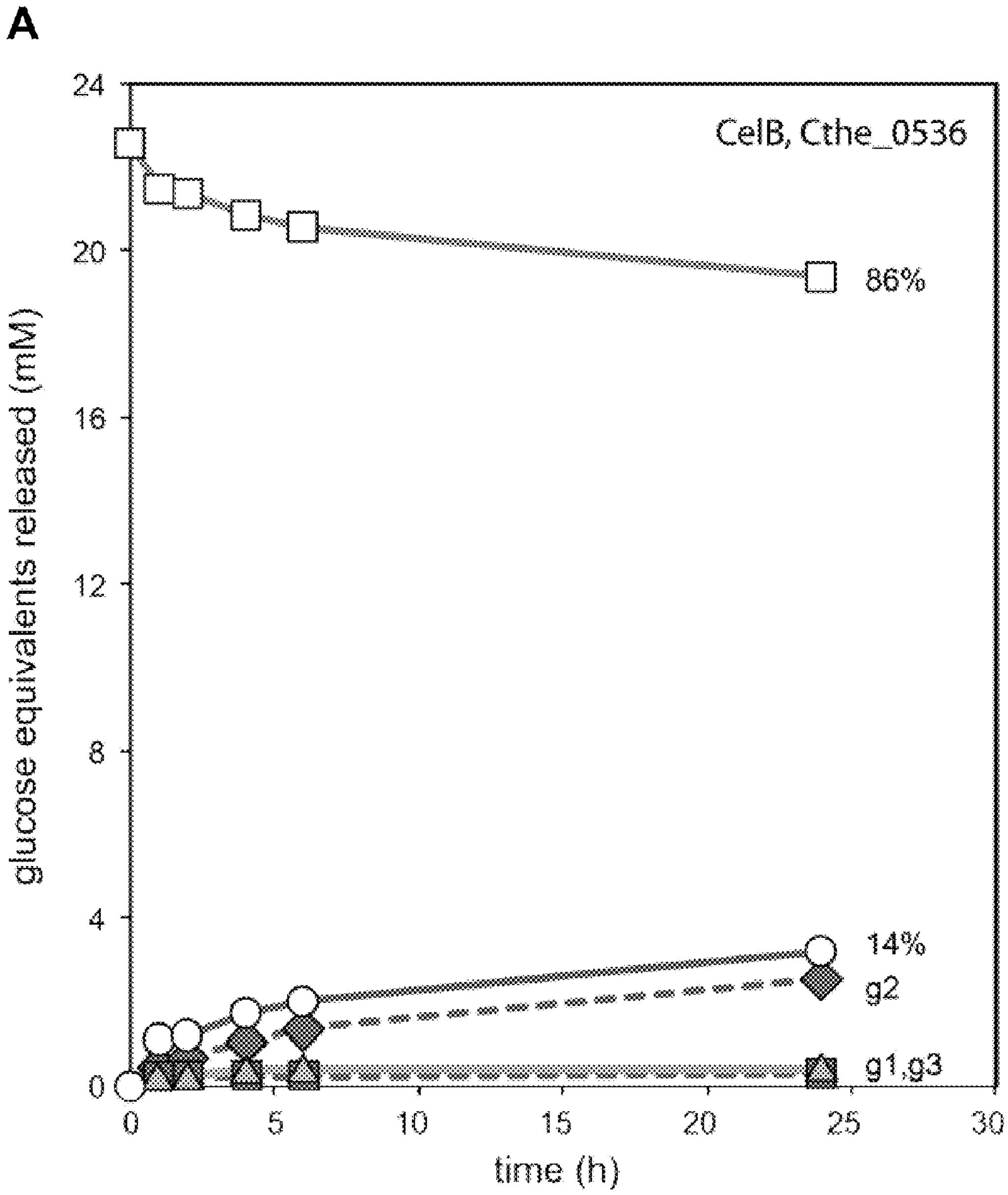
Mass (m/z)

Figure 10 B, C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CelB, Cthe_0536
glucose equivalents released (mM)
time (h)
0
4
8
12
16
20
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25
30
86%
14%
g2
g1,g3

Figure 11 A

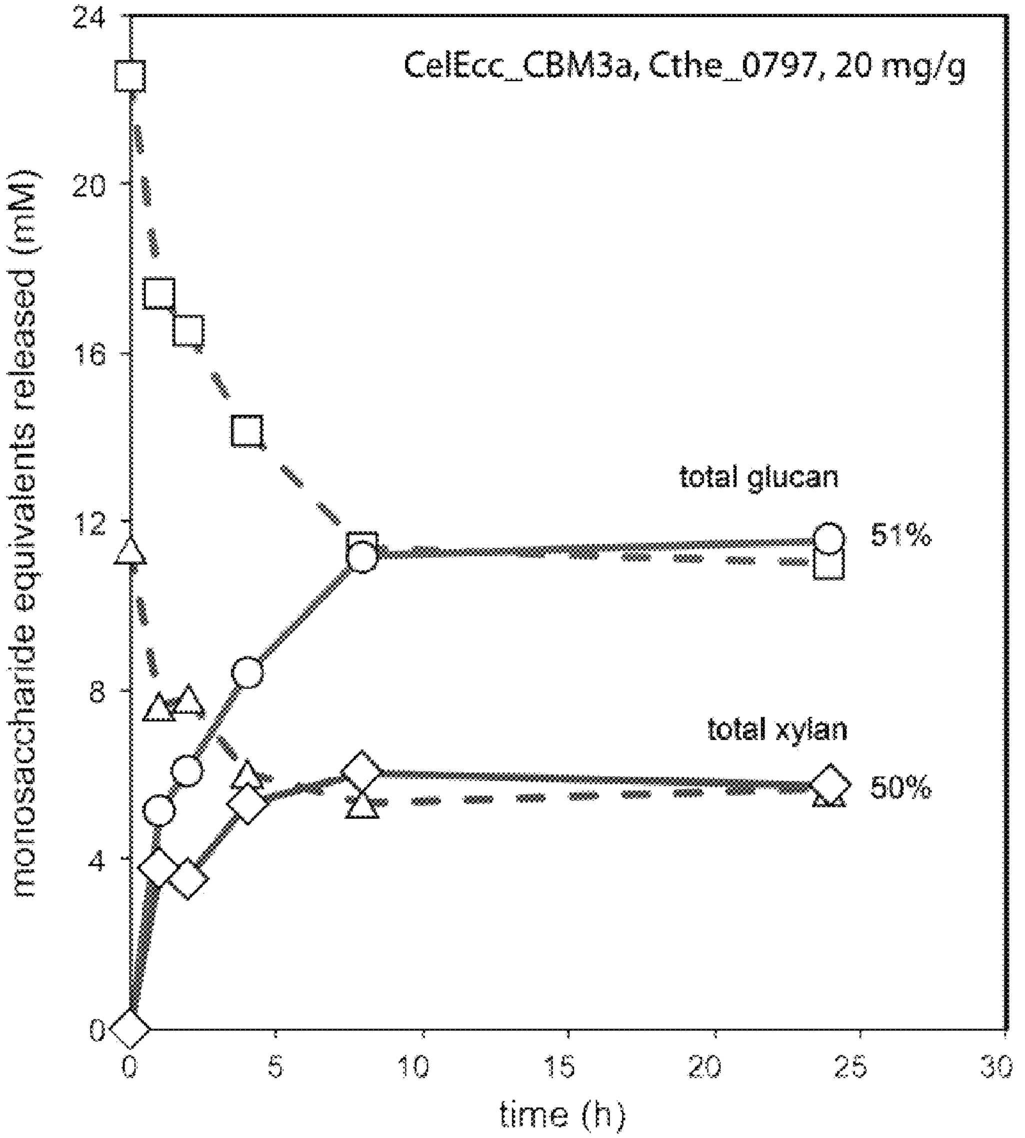
CelEcc_CBM3a, Cthe_0797, 20 mg/g
total glucan
51%
total xylan
50%
monosaccharide equivalents released (mM)
time (h)
0
4
8
12
16
20
24
0
5
10
15
20
25
30

Figure 11 B

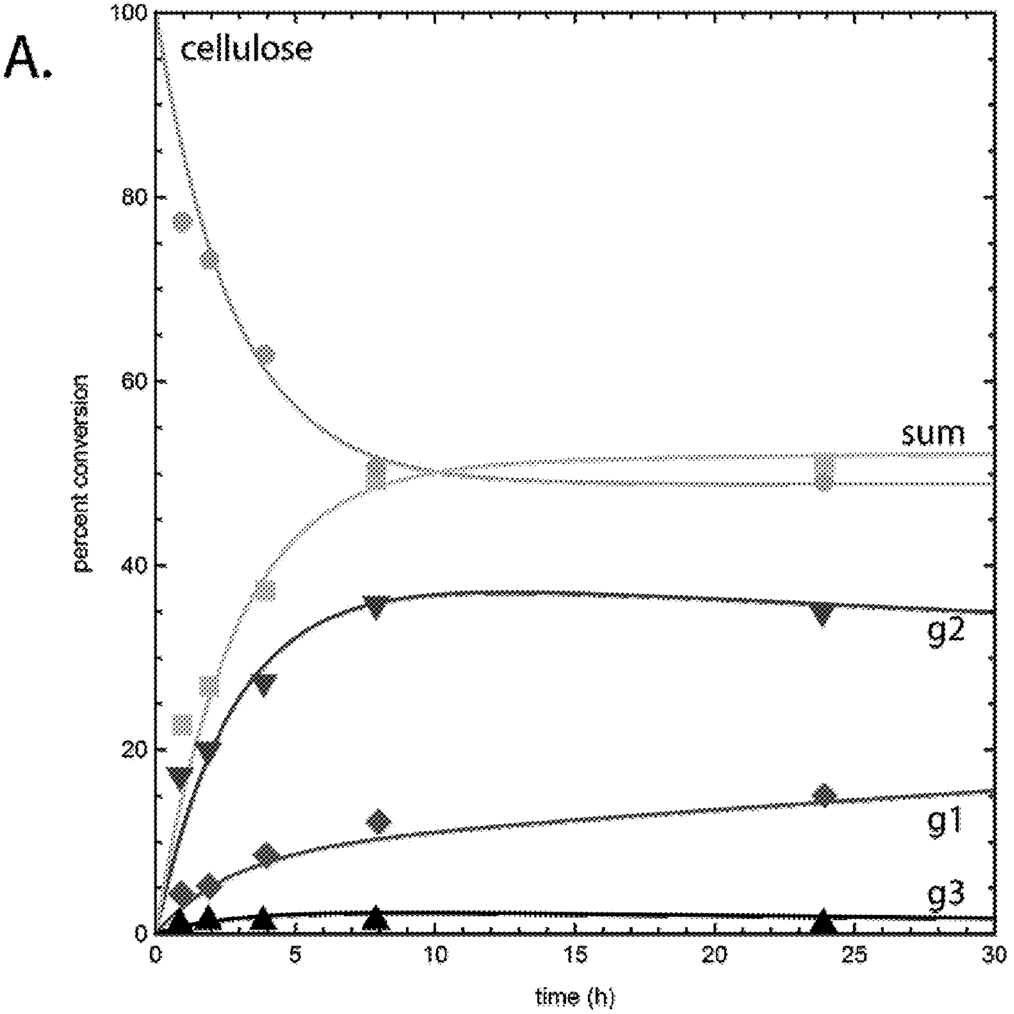
A.
cellulose
sum
g2
g1
g3
percent conversion
time (h)
0
20
40
60
80
100
0
5
10
15
20
25
30

Figure 12 A

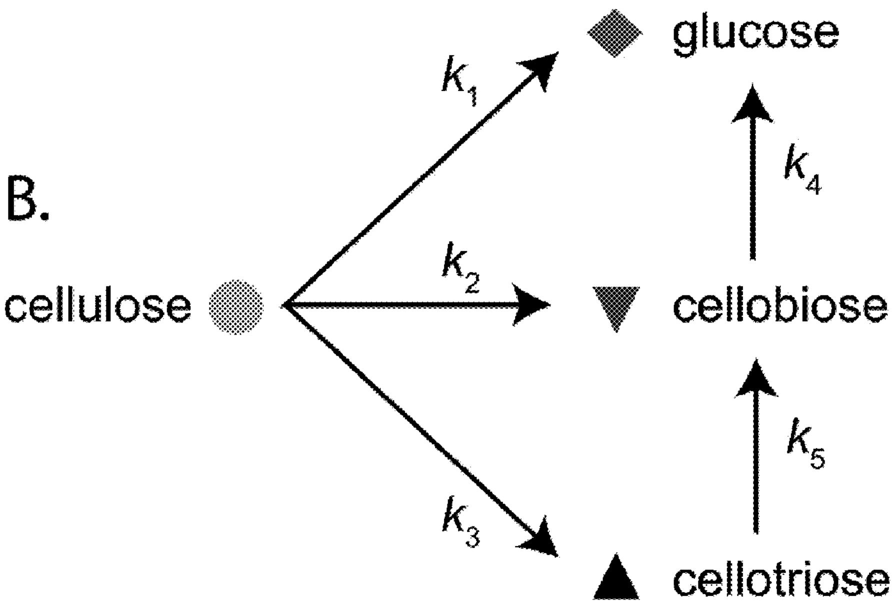

Figure 12 B

y[1] = cellulose
y[2] = glucose
y[3] = cellobiose
y[4] = cellotriose

$dy[1]/d[t] = -(k1 + k2 + k3)\ y[1][t]$
$dy[2]/d[t] = k1\ y[1][t] + k4\ y[3][t] + k5\ y[4][t]$
$dy[3]/d[t] = k2\ y[1][t] + k5\ y[4][t] - k4\ y[3][t]$
$dy[4]/d[t] = k3\ y[1][t] - k5\ y[4][t]$

Figure 12 C

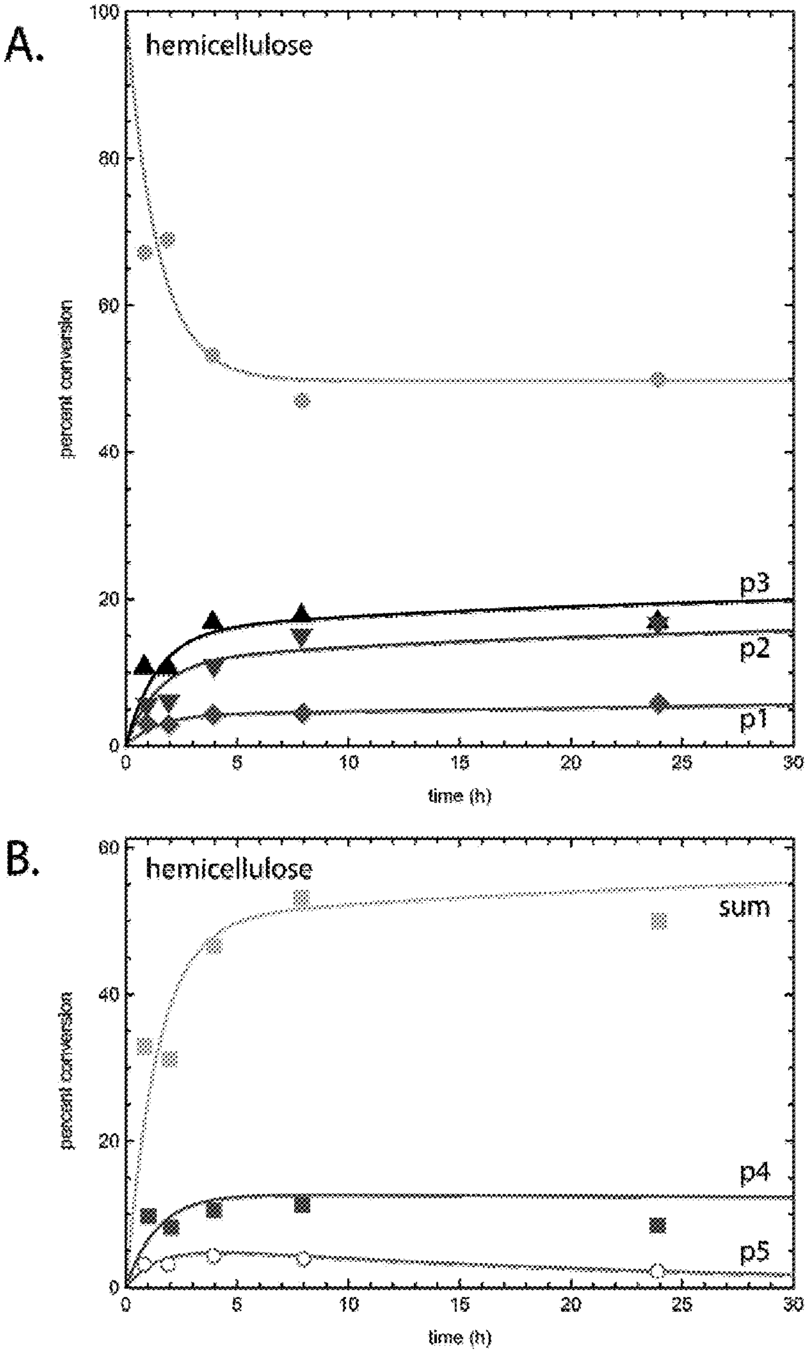
A.
hemicellulose
percent conversion
100
80
60
40
20
0
p3
p2
p1
0
5
10
15
20
25
30
time (h)
B.
hemicellulose
sum
percent conversion
60
40
20
0
p4
p5
0
5
10
15
20
25
30
time (h)

Figure 13 A, B

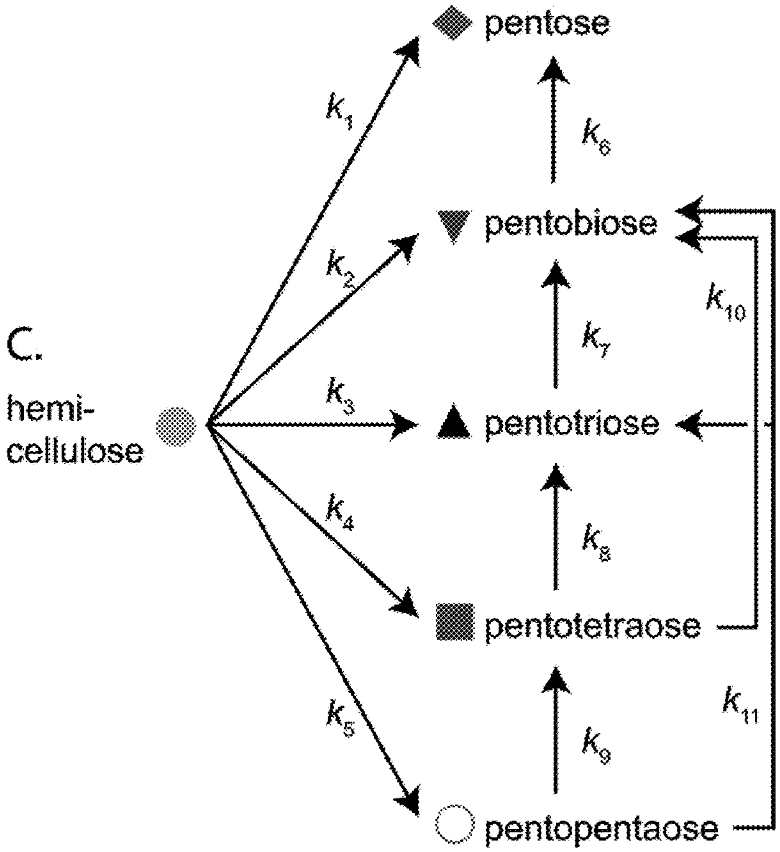
C.
hemi-
cellulose
pentose
pentobiose
pentotriose
pentotetraose
pentopentaose
$k_1$
$k_2$
$k_3$
$k_4$
$k_5$
$k_6$
$k_7$
$k_8$
$k_9$
$k_{10}$
$k_{11}$

Figure 13 C

y[1] = hemicellulose
y[2] = pentose
y[3] = pentobiose
y[4] = pentotriose
y[5] = pentotetraose
y[6] = pentopentaose

$dy[1]/d[t] = -(k1 + k2 + k3 + k4 + k5)\ y[1][t]$
$dy[2]/d[t] = k1\ y[1][t] + 2\ k6\ y[3][t] + k7\ y[4][t] + k8\ y[5][t] + k9\ y[6][t]$
$dy[3]/d[t] = k2\ y[1][t] + 2\ k10\ y[5][t] + k11\ y[6][t] + k7\ y[4][t] - k6\ y[3][t]$
$dy[4]/d[t] = k3\ y[1][t] + k8\ y[5][t] + k11\ y[6][t] - k7\ y[4][t],$
$dy[5]/d[t] = k4\ y[1][t] + k9\ y[6][t] - (k8 + k10)\ y[5][t]$
$dy[6]/d[t] = k5\ y[1][t] - (k9 + k11)\ y[6][t]$

Figure 13 D

# MULTIFUNCTIONAL CELLULASE AND HEMICELLULASE

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. provisional Application No. 61/703,063, filed Sep. 19, 2012, which is incorporated herein by reference for all purposes.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under DE-FC02-07ER64494 awarded by the US Department of Energy and GM094584 awarded by the National Institutes of Health. The government has certain rights in the invention.

## BACKGROUND

Conversion of the insoluble cellulose and hemicellulose polymers found in plant biomass into soluble sugars represents a major bottleneck for the biofuel industry. In order to accomplish this conversion, chemical pretreatment and enzyme hydrolysis are usually required.

Among chemical pretreatments, ammonia fiber expansion (AFEX) alkaline pretreatment has many advantages, such as (1) being a dry to dry process that does not produce a wash or liquid stream, (2) producing a chemically similar biomass composition after pretreatment, and (3) resulting in cleaved lignin-carbohydrate complexes without physically extracting hemicelluloses or lignin into separate process streams. Because alkaline pretreatments retain all constituent fractions of biomass, these pretreatments must be paired with the use of a complex mixture of cellulases and hemicellulases to achieve effective hydrolysis. It would be desirable to decrease the number of enzymes required to provide high yield conversion of polysaccharides present in AFEX-pretreated biomass.

Furthermore, a new variant chemical pretreatment called extractive AFEX (E-AFEX) leads to the production of cellulose III, an unnatural form of cellulose (Chundawat, et al., 2011). The extractive AFEX process not only generates cellulose III as a potentially more easily hydrolyzed allomorph, but also partially removes lignin. Both of these beneficial effects should promote enzymatic processing. Enzyme cocktails which hydrolyze cellulose III may have unique complementarity and thus corresponding utility when combined with the E-AFEX process.

In nature, the complex ultra-structure of the plant cell wall requires the participation of a mixture of many different enzymes to efficiently carry out the conversion of biopolymers into soluble sugars. This mixture is called an enzyme cocktail. An enzyme cocktail simultaneously provides cellulase, xylanase, mannanase, and enzymes having other catalytic activities. Cocktails of enzymes that have high hydrolyzing efficiency for either AFEX treated or extractive AFEX (E-AFEX) treated biomass will have great potential in the biofuel industry.

Chemical pretreatments and enzymes must be used together in order to achieve a high-yield deconstruction of biomass into fermentable sugars. As many as 18 different purified enzymes may be required to give a high yield conversion of AFEX pretreated biomass into soluble sugars, because AFEX pretreatment does not destroy the many types of bonds found in hemicellulose. Approaches that result in a reduced number of enzymes required for an efficient biomass hydrolysis would be beneficial to the biofuel industry, because it would be easier to manufacture a smaller number of enzymes, and the total weight of enzyme required may also be decreased.

One way to simplify the composition of enzyme cocktails is to use multifunctional enzymes, whereby a single multifunctional enzyme can replace two or more monofunctional enzymes in the cocktail. As a result of such substitutions, a less complex enzyme cocktail containing fewer enzymes may be used.

Accordingly, there is a need in the art for a multifunctional enzyme which can hydrolyze the three major materials of plant cell walls: cellulose, xylan, and mannan. Such a multifunctional enzyme could replace two or more enzymes in a conventional enzyme cocktail, while providing advantages in specific activity and stability of the cocktail.

## BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention encompasses a multifunctional polypeptide capable of hydrolyzing a cellulosic material, xylan, and mannan. The multifunctional polypeptide comprises the catalytic core (cc) of *Clostridium thermocellum* Cthe_0797 (CelE), a cellulose-specific carbohydrate-binding module (CBM), wherein the CBM is CBM3 of the cellulosome anchoring protein cohesion region (CipA) of *Clostridium thermocellum* (CBM3a), and a linker region interposed between the catalytic domain and the cellulose-specific carbohydrate binding module. In one embodiment, the linker region consists of a 15-40 amino acid sequence.

In certain embodiments, the cellulosic material suitable for being hydrolyzed by the polypeptide is a material selected from the group consisting of filter paper, crystalline cellulose allomorph I, and amorphous cellulose. In some embodiments, the cellulosic material suitable for being hydrolyzed by the polypeptide is cellulose III. In some embodiments, the cellulosic material suitable for being hydrolyzed by the polypeptide is pretreated using acidic, basic, or oxidative pretreatment methods. In some embodiments, the cellulose III suitable for being hydrolyzed by the polypeptide is non-natural cellulose and is produced by extractive ammonia fiber expansion treatment of biomasses.

In some embodiments, the polypeptide is capable of hydrolyzing SIGMACELL, beta-glucan, galactan, galactomannan, and lichenan. In some embodiments, the catalytic reactivity of polypeptide is at least 50% higher than that of the native CelE. In some embodiments, the catalytic reactivity of the polypeptide is at least 1.5 times, preferably at least 2 times, higher than that of the catalytic core (cc) of the native CelE. In some embodiments, the polypeptide is most catalytically reactive at 60° C., and the polypeptide retains at least 50% of its maximal reactivity at 45° C. In some embodiments, the polypeptide is most catalytically reactive at pH 6.0 and the polypeptide retains at least 50% of the maximal activity at pH 4.5. In some embodiments, the polypeptide remains active under a salt concentration of at least 20% (weight per volume).

In some embodiments, the polypeptide comprises the amino acid sequence of the protein CelEcc_CBM3a (SEQ ID NO:6). In some such embodiments, the amino acid sequence of SEQ ID NO:6 is encoded by the nucleotide sequence of SEQ ID NO:5.

In some embodiments, a polypeptide composition that includes the polypeptide as described above can be used for increasing the rate and the extent of fiber digestion in a mammal. In some embodiments, the mammal can be selected from the group consisting of human, cattle, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn, nilgai, kangaroo, horse, pig and rabbit.

In a second aspect, the present invention encompasses a method of hydrolyzing a substrate comprising cellulosic materials, xylan, and mannan. The method includes the step of exposing a substrate comprising a cellulosic material, xylan, and mannan to an effective amount of the multifunctional polypeptide as described above, whereby the cellulosic materials, xylan, and mannan in the substrate are at least partially hydrolyzed.

In some embodiments, the cellulosic material is selected from the group consisting of filter paper, crystalline cellulose allomorph I, and amorphous cellulose. In some embodiments, the cellulosic material is cellulose III. In some embodiments, the cellulosic material suitable for the polypeptide is pretreated using acidic, basic, or oxidative pretreatments. In some such embodiments, the cellulose III is non-natural cellulose and is produced by extractive ammonia fiber expansion treatment of biomass.

In some embodiments, one or more of SIGMACELL, beta-glucan, galactan, galactomannan, and lichenan are also hydrolyzed. In some embodiments, the catalytic reactivity of the polypeptide is at least 50% higher than that of the native CelE. In some embodiments, the catalytic reactivity of polypeptide is at least 1.5 times, preferably at least 2 times, higher than that of the catalytic core (cc) of the native CelE. In some embodiments, the polypeptide is most catalytically reactive at 60° C., and the polypeptide retains at least 50% of the maximal reactivity at 45° C. In some embodiments, the polypeptide is most catalytically reactive at pH 6.0 and the peptide retains at least 50% of the maximal activity at pH 4.5. In some embodiments, the polypeptide remains active under a salt concentration of at least 20% (weight per volume).

In some embodiments, the effective amount of the multifunctional polypeptide is in the range of 1-100 mg/g (enzyme/glucan), preferably 1-10 mg/g (enzyme/glucan).

In a third aspect, the present invention encompasses a method of increasing the rate and the extent of fiber digestion in a mammal. The method includes the step of administering to a mammal an effective amount of the multifunctional polypeptide as described above, whereby the cellulosic materials, xylan, and mannan in the fiber consumed by the mammal are at least partially hydrolyzed. In some embodiments, the mammal is selected from the group consisting of human, cattle, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn, nilgai, kangaroo, horse, pig and rabbit. In some embodiments, the effective amount of multifunctional polypeptide is in the range of 1-100 mg/g (enzyme/glucan), preferably 1-10 mg/g (enzyme/glucan). In some embodiments, the concentration of the multifunctional polypeptide is in the range of 20-200 nmol/g (enzyme/glucan).

In a fourth aspect, the present invention encompasses a method of making a multifunctional polypeptide. The method includes the step of linking the catalytic core (cc) of *Clostridium thermocellum* Cthe_0797 (CelE) with a cellulose-specific carbohydrate-binding module (CBM) by using a linker region, whereby a linker region is interposed between the catalytic domain and the cellulose-specific carbohydrate binding module, and wherein the CBM is CBM3 of the cellulosome anchoring protein cohesion region (CipA) of *Clostridium thermocellum* (CBM3a).

## DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram illustrating the domain structures of native CelE, CelEcc, and CelE_CBM3a. Molecular weight (Da) and positions of amino acid residues of each domain relative to CelE sequence (NCBI:gi125973315, incorporated by reference herein) are indicated. Truncation of the N-terminus of each construct (missing the first 30 to 50 residues) is due to the presence of undesirable signal peptide in the reported sequence.

FIG. **2**A shows the native CelE gene nucleic acid sequence (SEQ ID NO:1); FIG. **2**B shows the native CelE protein amino acid sequence (SEQ ID NO:2).

FIG. **3**A shows the CelEcc gene nucleic acid sequence (SEQ ID NO:3); FIG. **3**B shows the CelEcc protein amino acid sequence (SEQ ID NO:4).

FIG. **4**A shows the CelE_CBM3a gene nucleic acid sequence (SEQ ID NO:5); FIG. **4**B shows the CelE_CBM3a protein amino acid sequence (SEQ ID NO:6).

FIG. **5** is a graph of sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE) analysis showing the binding affinity for insoluble polysaccharides assessed by pull-down assay for three different constructs of CelE.

FIG. **6** is a diagram demonstrating cellulase and hemicellulase activity of CelE, native CelE, and CelEcc_CBM3a. Hydrolytic activity of CelEcc, native CelE, and CelEcc_CBM3 in μmol sugar release per μmol of active site are shown on phosphoric acid swollen cellulose (PASC), xylan, mannan and AFEX-pretreated switchgrass (AFEX-SG). Reaction was carried out at 8 mg/g enzyme to glucan for 20 hours at 60° C. DNS measurement was performed to determine the reducing end sugar products released in each reaction.

FIG. **7** is a diagram showing a catalytic activity comparison of CelEcc, CelE native, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with either cellulose I (SIGMACELL-20) or cellulose III prepared from SIGMACELL-20 by extractive AFEX.

FIG. **8** is a diagram depicting a catalytic activity comparison of CelEcc, CelE native, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with either cellulose I or cellulose III with different enzyme loading.

FIG. **9** is a set of diagrams demonstrating catalytic activity of CelEcc, CelE native, and CelEcc_CBM3a under various concentrations of NaCl.

FIG. **10** is a set of diagrams and graphs showing the multifunctional catalytic activity of CelEcc_CBM3a. FIG. **10**A is a diagram showing that CelEcc_CBM3a alone can react with biomass, e.g., AFEX-switchgrass. FIG. **10**B is a graph showing the NIMS analysis of the products from the catalytic reaction of CelEcc_CBM3a (Cthe_0797; GH5) with AFEX-switchgrass. FIG. **10**C is a graph showing NIMS analysis of the products from the catalytic reaction of BglA (Cthe_0212; GH1) with AFEX-switchgrass as a control experiment.

FIG. **11** is a set of graphs showing the time-dependent catalytic reactions and kinetics of enzymes with ionic-liquid treated switchgrass (IL-SG). FIG. **11**A is a graph showing the time-dependent catalytic reactions and kinetics of CelB (Cthe_0536) with IL-SG. FIG. **11**B is a graph showing the time-dependent catalytic reactions and kinetics of CelEcc_CBM3a (Cthe_0797) with IL-SG.

FIG. **12** is a set of graphs and diagrams showing time-dependent catalytic reactions and kinetics for the individual products derived from the reaction of CelEcc_CBM3a (Cthe_0797) with the cellulose fraction of biomass, in this case ionic liquid switchgrass (IL-SG). FIG. **12**A is a graph showing time-dependent catalytic reactions and product formation kinetics of CelEcc_CBM3a (Cthe_0797) with cellulose. FIG. **12**B is a diagram showing a predictive kinetic scheme for the catalytic reaction between CelEcc_CBM3a (Cthe_0797) and cellulose. FIG. **12**C shows the differential equations corresponding to FIG. **12**B. These equations are used to perform the analysis of time-dependence of product formation from cellulose hydrolysis.

FIG. **13** is a set of graphs and diagrams showing time-dependent catalytic reactions and kinetics for the individual products derived from the reaction of CelEcc_CBM3a (Cthe_0797) with the hemicellulose fraction of biomass, in this case ionic liquid switchgrass (IL-SG). FIGS. **13**A-B are graphs showing time-dependent catalytic reactions and product formation kinetics of CelEcc_CBM3a (Cthe_0797) with hemicellulose. FIG. **13**C is a diagram showing a predictive kinetic scheme for the catalytic reaction between CelEcc_CBM3a (Cthe_0797) and hemicellulose. FIG. **13**D shows the differential equations corresponding to FIG. **13**C. These equations are used to perform the analysis of time-dependence of product formation from hemicellulose hydrolysis.

## DESCRIPTION OF THE INVENTION

### In General

Typical cellulases and hemicellulases can often cleave only one type of glycosidic bond with high specificity. To effectively hydrolyze the ultrastructure of plant cell walls into soluble sugars, a complex combination of enzymes, including multiple cellulases, xylanases, mannanases, etc., is typically required. It would be helpful to use a single multifunctional enzyme capable of substituting for at least two cellulases, xylanases, or mannanases in a hydrolyzing enzyme cocktail.

The inventors have produced a multifunctional enzyme that can hydrolyze three major components of plant cell walls, including cellulosic materials, mannan, and xylan. In one aspect of the invention, the multifunctional enzyme remains catalytically active under harsh environments. Harsh environments include high temperatures (35-60° C.), acidic solution (pH 4.5), and high concentration of salt (at least 20%).

Cellulose III, an unnatural form of cellulose, has been produced through the chemical pretreatment of extractive ammonia fiber expansion (E-AFEX) (Chundawat, et al., 2011). In one embodiment, the present invention provides an enzyme or a polypeptide showing a specific catalytic reactivity with cellulose III.

### Compositions of the Present Invention

As used herein, the term "multifunctional" refers to an enzyme or a polypeptide capable of hydrolyzing a cellulosic material, xylan, and mannan.

"Cellulosic materials," as used herein, refers to any material or any combination of materials containing various forms of cellulose. Non-limiting examples of cellulosic materials include filter paper, crystalline cellulose allomorph I, amorphous cellulose, cellulose III, hemicelluloses, corn stover, corn stalks, wheat straw, cereal grain, cotton, and other agricultural residues, and industrial biomass, including miscanthus, switchgrass, hemp, poplar, willow, sorghum, sugarcane, bamboo, and any other cellulose-containing materials.

As used herein, the term "stable" refers to an enzyme or a polypeptide that remains catalytically active hydrolyzing biomass for at least 100 hours.

As used herein, the term "highly reactive" refers to an enzyme or a polypeptide retaining more than 70% of its maximal reactivity.

As used herein, the term "high concentration of salt" refers to a salt having a concentration higher than 20% weight per volume.

As used herein, the term "partially active" refers to an enzyme or peptide retaining at least 50% of its maximal activity.

As used herein, the terms "substrate," "solid substrate," or "insoluble substrate" refer to a material comprising one or more cellulosic materials, xylan, and mannan.

The terms "enzyme," "polypeptide," and "protein," are used interchangeably throughout this disclosure.

The term "linker region" refers to a sequence interposed between the CelEcc and the CBM3a. The molecule is preferably an enzyme or a peptide, and may be of any length that permits the connected sequences to function catalytically. Preferably, the linker region is derived from the CipA (Cthe_3077) protein from *Clostridium thermocellum*. The linker used in the construct in the Examples is the amino acid sequence right before the CBM3a domain from *C. thermocellum* CipA (Cthe_3077). The linker includes residues 324-364 of SEQ ID NO:6 (CBM3a includes residues 365-523 of SEQ ID NO:6). This linker region may be susceptible to proteolysis by trypsin-like proteases, which may be a problem in ruminant applications. This problem could be overcome by providing a different amino acid sequence, preferably one lacking Arg and Lys residues, or by a sequence that supports glycosylation.

The natural protein CelR provides an example of a useful alternative linker region. The natural domain structure of CelR is GH9-CBM3a-Dockerin-Dockerin. The form that the inventors described in a previous patent application (U.S. Patent Publication No. 2010/0304405, incorporated by reference herein) was engineered to have the format of GH9-CBM3a-CBM3a, where the second CBM3a was from the CipA gene, which is the same CBM3a that is disclosed herein as linked to CelEcc. The first CBM3 domain also has a linker, and the linker is not hydrolyzed by protease-rich rumen fluid. The short sequence of this linker, which includes residues 178 to 193 of the CelR gene (Cthe_0578) (TVTAGTAAALAATAL (SEQ ID NO:7)), still allows the CelR catalytic domain to function. Other amino acid sequences may also be suitable for use as a linker region. Suitable amino acid sequences may include 5-150 residues, preferably 10-50 residues, most preferably 15-40 residues.

Substrates of cellulosic materials, xylan, and mannan used in the disclosed methods may first be pretreated. The pretreatment process may include any conventional pretreatment methods, including without limitation acidic, basic, or oxidative methods. Preferably, the pretreatment process may be an ammonia fiber expansion (AFEX) process. Most preferably, the pretreatment process may be an extractive ammonia fiber expansion (E-AFEX) process.

In one embodiment of the invention, *Clostridium thermocellum* Cthe_0797, also called CelE, is used as a multifunctional polysaccharide-degrading enzyme. CelE was described in U.S. Patent Publication No. 2010/0304405, which is incorporated by reference herein. As shown in FIG. **1**, the native CelE is composed of four domains, including one glycoside hydrolase 5 (GH5), two dockerin type 1 domains, and a lipase GDSL domain. The amino acid positions where these domains begin and end are indicated in FIG. **1**. Native CelE is capable of hydrolyzing cellulosic materials, including filter paper, crystalline cellulose allomorph I, amorphous cellulose, and others. CelE can also hydrolyze mannan, galactomannan, xylan, and lichenan. With this broad catalytic specificity, CelE has potential utility as a dominant enzyme in new enzyme cocktails for use in biomass hydrolysis.

In another embodiment, truncation of the natural dockerin domain from the C-terminus of the native CelE increases its stability upon heterologous expression, leading to the production of the catalytic core (cc) of the native CelE (CelEcc). As shown in FIG. **1**, CelEcc includes only one glycoside hydrolase 5 (GH5) domain. CelEcc is capable of hydrolyzing cellulosic materials, including filter paper, crystalline cellulose allomorph I, amorphous cellulose, and others. CelEcc can also hydrolyze mannan, galactomannan, xylan, and lichenan.

Further, by applying an engineered fusion domain approach, such as that disclosed in U.S. Patent Publication No. 2010/0304405 (incorporated by reference herein), an enzyme with improved catalytic reactivity was produced by introducing a linker region from one protein or polypeptide and a cellulose-specific carbohydrate-binding module from another different protein or polypeptide. CBM3a was used here as the cellulose-specific carbohydrate-binding module, as it has been reported to specifically bind to a planar cellulose surface. CBM3a is encoded as an internal domain of the cellulosome anchoring protein (CipA; Cthe_3077) of *Clostridium thermocellum*. This portion of the protein has been incorporated into the engineered fusion domain approach by PCR amplification and cloning.

As shown in FIG. **1**, the resulting fusion enzyme, CelEcc_CBM3a, includes the catalytic core of native CelE (CelEcc) having one single glycoside hydrolase 5 (GH5) domain, one cellulose-specific carbohydrate-binding module (CBM3a), and a linker region (not labeled) connecting CelEcc and CBM3a. This construct is created to optimize the performance of the enzyme, and the replacement of the dockerin domain with the CBM3a domain abrogates the need for a cellulosomal attachment to obtain maximal catalytic activity from CelEcc_CBM3a on solid substrates. Following similar strategies, one might wish to use other substrate-specific carbohydrate-binding modules instead of CBM3a to create enzymes targeting the corresponding substrates. The resulting enzymes might be specific to beta-glucan, galactan, mannan, galactomannan, xylan, lichenan, cellulosic materials, or any other polysaccharide included in biomass.

Native CelE, the catalytic core of native CelE (CelEcc), and the engineered construct of CelEcc_CBM3a may be expressed in any manner known to those skilled in the art, including utilizing an *Escherichia coli* expression system by cloning corresponding coding sequences into *E. coli* expression vector following a similar method and process as described in U.S. Patent Publication No. 2010/0304405, which is incorporated by reference herein. Gene and protein sequences of native CelE, CelEcc, and CelEcc-CBM3a are shown in FIG. **2**, FIG. **3**, and FIG. **4**, respectively.

In one embodiment of the invention, the engineered construct of CelEcc_CBM3a shows a much stronger binding affinity to insoluble polysaccharide substrates than native CelE and CelEcc. FIG. **5** shows a comparison of binding affinity of CelE, CelEcc, and CelEcc_CBM3a with the substrates of crystalline cellulose (SIGMACELL-20), phosphoric acid amorphous cellulose (PASC) and mannan. As depicted in FIG. **5**, the bound and unbound fraction are indicated as pellet (P) and supernatant (S), respectively. As expected, native CelE, which is a cellulosomal protein lacking a carbohydrate-binding module (CBM) domain, shows only weak binding affinities to all three substrates of SIGMACELL-20, PASC, and mannan. The catalytic core of native CelE (CelEcc) does not bind to the substrates of SIGMACELL-20 and mannan and CelEcc only weakly binds to PASC, compared to a control. In contrast, the engineered construct of CelEcc_CBM3a shows a much stronger binding affinity to the substrates of SIGMACELL-20, PASC, and mannan than native CelE and CelEcc. Fusion of a carbohydrate-binding module (CBM3a) onto the catalytic core of native CelE (CelEcc) appears to increase the binding affinity of the resulting enzyme with solid substrates, thus potentially improving the catalytic reactivity of the enzyme.

In another embodiment of the invention, high pressure liquid chromatography (HPLC) is used to identify (and optionally quantify) soluble sugars and the hydrolysis products of insoluble substrates following enzyme catalysis. For example, HPLC assays of the hydrolysis products demonstrate that the catalysis of CelE produces cellotriose, xylotriose, and mannotriose from oligosaccharide cellohexaose, xylohexaose, and mannohexaose, respectively, as the corresponding major soluble products.

In one preferred embodiment of the invention, fusion of a carbohydrate-binding module (CBM3a) onto the catalytic core of native CelE (CelEcc) has greatly increased the catalytic reactivity of the resulting enzyme (CelEcc_CBM3a) with cellulosic materials. FIG. **6** demonstrates that native CelE, CelEcc, and CelEcc_CBM3a have different rates and relative activities for hydrolyzing insoluble substrates. As shown in FIG. **6**, the engineered construct of CelEcc_CBM3a has a catalytic reactivity with phosphoric acid amorphous cellulose (PASC) of at least 50% higher than that of native CelE. The engineered construct of CelEcc_CBM3a has a catalytic reactivity with phosphoric acid amorphous cellulose (PASC) at least three times over that of CelEcc. While CelEcc_CBM3a shows a comparable reactivity with xylan and mannan to that of native CelE, the presence of CBM3a increases the catalytic reactivity by at least 1.5 times, preferably 2 times compared with that of CelEcc. In another embodiment of the reaction, CelEcc_CBM3a was shown to hydrolyze SIGMACELL, filter paper, beta-glucan, galactan, mannan, galactomannan, xylan, and lichenan.

Further, in another preferred embodiment of the invention, the engineered construct of CelEcc_CBM3a shows significant improvement of catalytic reactivity with specific substrates such as cellulose I and cellulose III. FIG. **7** depicts a catalytic activity comparison of CelEcc, CelE native, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with either cellulose I (SIGMACELL-20) or cellulose III prepared from SIGMACELL-20 by extractive AFEX. The creation and utility of CelKcc and CelRcc_CBM3a was described in U.S. Patent Publication No. 2010/0304405, which is incorporated by reference herein. As shown in FIG. **7**, in addition to the increased activity with PASC, CelEcc_CBM3a appears to have significant improvements of catalytic reactivity with crystalline cellulose I compared with native CelE and CelEcc. Cellulose I is the natural allomorph commonly found in biomass. For example, the catalytic reactivity of CelEcc_CBM3a with cellulose I is at least four times over that of native CelE, and the catalytic reactivity of CelEcc_CBM3a with cellulose I is at least two times over that of CelEcc. Surprisingly, the engineered construct of CelEcc_CBM3a shows even more dramatic improvement in its reaction with cellulose III, a non-natural allomorph formed during extractive AFEX pretreatment.

As shown in FIG. **7**, the catalytic reactivity of CelEcc_CBM3a with cellulose III is almost five times over that of native CelE, and the catalytic reactivity of CelEcc_CBM3a with cellulose III is at least three times over that of CelEcc. Further, CelEcc_CBM3a is more reactive with cellulose III than either CelKcc or CelRcc_CBM3a (FIG. **7**). As a comparison, CelRcc_CBM3a is strongly reactive with cellulose I, but weakly reactive with cellulose III, highlighting the uniqueness and the unanticipated results of the engineered construct of CelEcc_CBM3a for hydrolysis of cellulose III.

One major challenge for the current biofuel industry is to minimize enzyme requirements for biomass hydrolysis, with a goal of using less than 5 mg/g (enzyme/glucan). Due to its characteristic multifunctional properties, CelEcc_CBM3a has great potential in meeting the goal of minimizing enzyme requirements for biomass hydrolysis. FIG. **8** depicts a catalytic activity comparison of CelEcc, CelE native, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with either cellulose I or cellulose III with different enzyme loading. At 5 mg/g (enzyme/substrate), CelEcc_CBM3a reaches almost 80% of its maximal catalytic reactivity with cellulose I. Further, at 5 mg/g (enzyme/substrate), CelEcc_CBM3a reaches its maximal catalytic reactivity with cellulose III. By comparison, at 5 mg/g (enzyme/substrate), native CelE and CelEcc reach 50-60% of their maximal reactivity with cellulose I and cellulose III.

In one embodiment of the invention, one would apply CelEcc_CBM3a as one component enzyme of the enzyme cocktail to degrade extractive-AFEX pretreated biomass by utilizing its extraordinary catalytic reactivity with cellulose I and cellulose III. This same enzyme would also be capable of reacting with the xylan and mannan present in the biomass. Thus, due to the multifunctional property of CelEcc_CBM3a, its use would consequently minimize the number of enzymes needed in the enzyme cocktail.

In one embodiment of the invention, native CelE, CelEcc, and CelEcc_CBM3a are provided for use as enzymes or peptides which remain stable and at least partially active under harsh environments. Harsh environments may include a temperature higher than 40, 50, or even 60° C., an acidic solution with a pH value less than 6.0, 5.0 or even 4.0, and a salt solution with a concentration higher than 20%.

Native CelE, CelEcc, and CelEcc_CBM3a are found to be most catalytically reactive at pH 6.0, and they retain about 50% of the maximal catalytic reactivity at pH 4.5 and pH 7.0. Native CelE, CelEcc, and CelEcc_CBM3a are found to be most catalytically reactive at 60° C., and they retain about 50% of the maximal catalytic reactivity at 45° C. Further, native CelE, CelEcc, and CelEcc_CBM3a do not become inactive in a solution having high concentration of salt. Many of the currently utilized pretreatment processes in the biofuel industry are performed under harsh environments, including at elevated temperatures or pressures, in acidic solutions, in solutions having a high concentration of salt, etc. Thus, the pretreated biomass must be further treated before an enzyme cocktail can be introduced. This additional treatment process may require significant time and/or energy, thus increasing the cost of biofuel production. Further, current enzyme cocktails are mostly produced from mesophilic fungal sources and most of them are not stable under harsh environments.

Thus, it would be beneficial to develop an enzyme cocktail that is stable and could be added to the biomass under harsh environments. Native CelE, CelEcc, and CelEcc_CBM3a are produced by the thermophilic bacterium of *C. thermocellum*. These enzymes are stable and they remain at least partially active under the above harsh environments. Therefore, a designer enzyme cocktail including native CelE, CelEcc, or CelEcc_CBM3a could be added to the pretreated biomass under a much harsher environment than that for the current commercially available enzyme cocktails. This process would not only save time but also could result in a significant reduction in energy. The stability of native CelE, CelEcc, and CelEcc_CBM3a makes them desirable enzymes or polypeptides to add into all of the current pretreatment processes and any future pretreatments that require enzymatic activity under harsh environments.

In one embodiment of the invention, an enzyme or polypeptide composition for a mammal to increase the rate and extent of fiber digestion is disclosed. Due to their multifunctional properties and their stability under harsh environments, native CelE, CelEcc, and CelEcc_CBM3a can be used to assist in fiber digestion in a mammal. Native CelE, CelEcc, or CelEcc_CBM3a may be used individually. Native CelE, CelEcc, or CelEcc_CBM3a may also be used in a composition which includes other enzymes or polypeptides and other ingredients. The composition may be an enzyme cocktail.

A "mammal" includes all mammals, preferably humans, ruminants and monogastric animals. A ruminant includes any animal that digests plant-based food such as cattle, goats, sheep, giraffes, bison, moose, elk, yaks, water buffalo, deer, camels, alpacas, llamas, antelope, pronghorn, nilgai, kangaroos, and others. Monogastric animals that digest plant-based foods include humans, pigs, horses, rabbits, and others.

For a human, an enzyme or polypeptide composition including native CelE, CelEcc, or CelEcc_CBM3a may be used to help reduce the size of phytobezoars. A phytobezoar is a type of bezoar, or a trapped mass in the stomach, that consists of components of indigestible plant material, such as fibers, skins and seeds. While phytobezoars may be discovered incidentally on barium x-ray or endoscopic testing of the stomach, individuals with phytobezoars may develop symptoms such as nausea, vomiting, gastric outlet obstruction, perforation, abdominal pain, and even bleeding. The currently available treatments for phytobezoars include endoscopy with various assisted devices (Roth baskets, snares, or Dormia baskets), electrohydraulic lithotripsy, mechanical lithotripsy, Nd:YAG laser therapy, and even surgical gastrotomy. Native CelE, CelEcc, and CelEcc_CBM3a have multifunctional properties to hydrolyze three major components of plant cell walls, thus, a composition including Native CelE, CelEcc, and CelEcc_CBM3a may provide a cheaper and an alternative treatment solution for phytobezoars.

For a ruminant or a monogastric animal, native CelE, CelEcc, or CelEcc_CBM3a can be utilized to increase the nutritional density of animal feed. The addition of these enzymes or polypeptides to animal feed partially breaks down the recalcitrant polysaccharides of the plant cell walls, thus increasing the amount of readily available digestible sugars. This treatment allows the animal to extract more energy from the feed compared to a feed which was not treated by enzymes or polypeptides. In one embodiment, an enzyme cocktail including native CelE, CelEcc, or CelEcc_CBM3a can be utilized to partially digest the feedstock. In another embodiment, native CelE, CelEcc, or CelEcc_CBM3a could also be utilized individually to release easily digestible sugars. Further, native CelE, CelEcc, or CelEcc_CBM3a is stable and shows high catalytic activity under conditions found in the rumen (40° C. and pH 6.9). Thus, the enzymes would continue to help release sugars even after the feedstock has been ingested. Consequently, by using native CelE, CelEcc, or CelEcc_CBM3a, one would greatly simplify the complexity of the enzymes needed to partially degrade animal feed and one would drastically reduce the cost of producing multiple enzymes needed for feeding to ruminants.

## Methods of the Present Invention

In one embodiment, the present invention is a method of hydrolyzing a substrate by applying native CelE, CelEcc, or CelEcc_CBM3a to the substrate. A substrate may comprise cellulosic materials, xylan, and mannan. Specifically, a substrate may include cellulose III and cellulose I. A substrate may include a material which contains one or more of cellulosic materials, such as xylan, mannan, beta-glucan, galactan, mannan, galactomannan, xylan, lichenan, and any other polysaccharide included in biomass.

Native CelE, CelEcc, or CelEcc_CBM3a is produced following the method and procedure described in U.S. Patent

Publication No. 2010/0304405, which is incorporated by reference herein. The enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may be prepared as a powder or a dried or lyophilized form. Subsequently, a solution of the enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may be prepared by dissolving the powders into a suitable solvent. A suitable solvent may include water, dimethyl sulfoxide (DMSO), alcohols, acetone, and any other suitable inorganic solvents and any other suitable organic solvents. The enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may also be prepared as a solution.

Native CelE, CelEcc, or CelEcc_CBM3a may be applied individually to degrade the substrate. Native CelE, CelEcc, or CelEcc_CBM3a may also be applied in a combination with one or more of other enzymes. The enzymes may include any other celluloses, xylanases, or mannanases. In one embodiment, an enzyme cocktail including native CelE, CelEcc, or CelEcc_CBM3a may be applied to hydrolyze the substrate.

Native CelE, CelEcc, or CelEcc_CBM3a may be applied as a powder or a dried or lyophilized form. A suitable amount of native CelE, CelEcc, or CelEcc_CBM3a may be mixed with the substrate into a mixture solution. In order to effectively mix the substrate with enzymes or polypeptides, the process may include applying a mixing apparatus such as shake flasks (SFs), roller bottle reactors (RBRs), and sonicators, and conventional mixing methods such as shaking, gravitational tumbling, and hand stirring. Agricultural feeds may be mixed with the enzymes using TMR (total mixed ration) mixers, tumble drum mixers, grinder mixers, horizontal mixers, reel mixers, vertical mixers, roller mixers, and hand mixing.

The suitable amount of native CelE, CelEcc, or CelEcc_CBM3a is in the range of 1-100 mg/g (enzyme/glucan), preferably 1-10 mg/g (enzyme/glucan). The catalytic reaction takes place under suitable conditions for hydrolytic enzymes. The suitable condition may include a pH value between 4.5 and 7.0, preferably 6.0, a temperature range of 35-60° C., preferably 60° C., and a reaction time between 10-100 hours, preferably 16-24 hours, most preferably 20 hours.

In one embodiment, native CelE, CelEcc, or CelEcc_CBM3a may be applied as a solution. If one were to use a solution of native CelE, CelEcc, or CelEcc_CBM3a, an equivalent amount of native CelE, CelEcc, or CelEcc_CBM3a protein as described above will be applied.

In another embodiment, the invention is a method to treat a mammal for increasing the rate and the extent of fiber digestion using native CelE, CelEcc, or CelEcc_CBM3a. A mammal may include a human, a ruminant, and a monogastric animal. For a human, who has at least one phytobezoar in his/her stomach, the enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may be applied as a powder or a dried or lyophilized form. The enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may be taken as pills, tablets or any other solid forms. Given the sizes of the phytobezoars, the suitable amount of native CelE, CelEcc, or CelEcc_CBM3a may be in the range of 1-100 mg/g (enzyme/glucan), preferably 1-10 mg/g (enzyme/glucan). Alternatively, the enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a may be applied in a solution. If one were to use a solution of native CelE, CelEcc, or CelEcc_CBM3a, an equivalent amount of native CelE, CelEcc, or CelEcc_CBM3a protein as described above will be applied.

For a ruminant, the invention is a method for increasing the digestibility of ruminant feed with enzymes or polypeptides of native CelE, CelEcc, or CelEcc_CBM3a. In one preferred embodiment, the invention is a method of applying an aqueous mixture of native CelE, CelEcc, or CelEcc_CBM3a to ruminant feed. The suitable amount of native CelE, CelEcc, or CelEcc_CBM3a to effectively digest ruminant feed is in the range of 1-100 mg/g (enzyme/glucan), preferably 1-10 mg/g (enzyme/glucan). Native CelE, CelEcc, or CelEcc_CBM3a may be initially prepared as an aqueous solution, having a concentration of 10-500 nmol/g (enzyme/glucan), preferably between 50-150 nmol/g (enzyme/glucan). In some embodiments, the concentration will be between 20-200 nmol/g (enzyme/glucan). If one were to use a solution of native CelE, CelEcc, or CelEcc_CBM3a, an equivalent amount of native CelE, CelEcc, or CelEcc_CBM3a protein as described above will be applied.

The aqueous solution of enzymes or polypeptides may be applied to the ingestible materials of ruminant feed, such as hay or other forage, immediately prior to ingestion. Preferably, the aqueous solution of enzymes or polypeptides may be sprayed using an applicator tube or a spray nozzle. To effectively mix enzymes or polypeptides with ruminant feed, the ingestible materials of ruminant feed may be mechanically turned as the spray is applied, for example using a TMR mixer, a reel or an auger. To maintain the effective catalytic activity of the enzymes or polypeptides and to increase binding of enzymes or polypeptides to fibers, the aqueous solution of enzymes or polypeptides, the ruminant feed, and the mixture may be heated to an elevated temperature such as 60° C.

The aqueous solution of enzymes or polypeptides may be applied to the ingestible materials of ruminant feed, such as hay or other forage, as part of their preparation for storage in a silo. Preferably, the aqueous solution of enzymes or polypeptides may be sprayed using an applicator tube or a spray nozzle. To effectively mix enzymes or polypeptides with ruminant feed, the ingestible materials of ruminant feed may be mechanically turned as the spray is applied, for example using a TMR mixer, reel or an auger.

## EXAMPLES

### Example 1

### The Binding Affinity Assays of Native CelE, CelEcc and CelEcc_CBM3a

Due to weak binding ability of native CelE and CelEcc to insoluble substrates such as cellulosic materials, xylan, and mannan, it is desirable to produce an enzyme of CelEcc_CBM3a by fusing the catalytic core of the native CelE with a cellulose-specific carbohydrate-binding module (CBM3a) domain. To confirm that the engineered construct of CelEcc_CBM3a has better binding ability with insoluble substrates than native CelE and CelEcc, the experiment of binding affinity assay of native CelE, CelEcc and CelEcc_CBM3a was performed. All constructs of native CelE, CelEcc, and CelEcc_CBM3a were produced by expression in *Escherichia coli* using an auto-inducing medium containing glucose, lactose, tryptone and yeast extract at 25° C. for 24 hours. The cells were harvested by centrifuging the growth media at 3500 g for 20 minutes, as described in the previous references (Blommel, et al. 2007; Jeon, et al. 2005; Blommel and Fox, 2007). The resulting cell pellet was resuspended in 100 mM MOPS at pH 7.5, 500 mM NaCl, and 10 mM imidazole and lysed by sonication. Insoluble cellular material was removed by centrifuging the lysed samples at 75,000 g for 45 minutes. All CelE constructs were purified by immobilized metal affinity chromatography, as described in the previous references (Blommel, Becker, et al. 2007; Jeon, Aceti, et al. 2005). The enzymes were mixed with substrates, and the mixture was incubated for 1 hour at 4° C., and the mixture was then centrifuged to separate bound and unbound fraction, indicated as pellet (P) and supernatant (S), respectively.

Control experiments were carried out in the absence of substrates demonstrating that the protein precipitation did not occur due to protein aggregation or instability. The results were depicted in FIG. **5**, where the substrates included crystalline cellulose (SIGMACELL-20), phosphoric acid amorphous cellulose (PASC) and mannan. As shown in FIG. **5**, native CelE, which was a cellulosomal protein lacking a carbohydrate-binding module (CBM) domain, showed weak binding affinities to all three substrates of SIGMACELL-20, PASC, and mannan. Similarly, the catalytic core of native CelE (CelEcc) did not bind to the substrates of SIGMACELL-20 and mannan and CelEcc only weakly bound to PASC. In contrast, the engineered construct of CelEcc_CBM3a showed a much stronger binding affinity to all the substrates of SIGMACELL-20, PASC, and mannan. Therefore, fusion of a carbohydrate-binding module (CBM3a) onto the catalytic core of native CelE (CelEcc) increased the binding affinity of the resulting enzyme to solid substrates, thus improving the catalytic reactivity of the enzyme.

## Example 2

## Catalytic Activities of Native CelE, CelEcc and CelEcc_CBM3a with Cellulose I, Xylan and Mannan

It was shown that CelEcc_CBM3a has a much stronger binding ability to insoluble substrates than native CelE and CelEcc. Consequently, it was expected that CelEcc_CBM3a would have a much better catalytic reactivity with the insoluble substrates than native CelE and CelEcc. The experiment regarding catalytic activities of native CelE, CelEcc and CelEcc_CBM3a with cellulose I, xylan and mannan, was thus performed. The catalytic reactions were carried out at 60° C., and the reaction time was 20 hours. The experiment was conducted with an amount of 8 mg of enzyme per gram of glucan. The products obtained in the reaction were detected by DNS assay. FIG. **6** showed that native CelE, CelEcc, and CelEcc_CBM3a had different rates and relative activities for hydrolyzing insoluble substrates. As expected, the engineered construct of CelEcc_CBM3a had a catalytic reactivity with phosphoric acid amorphous cellulose (PASC) of at least 50% higher than that of native CelE. The engineered construct of CelEcc_CBM3a had a catalytic reactivity with phosphoric acid amorphous cellulose (PASC) of at least three times over that of CelEcc. While CelEcc_CBM3a showed a comparable reactivity with xylan and mannan to that of native CelE, the presence of CBM3a increased the catalytic reactivity by about 100% compared with that of CelEcc. The results demonstrated that CelEcc_CBM3a could also hydrolyze SIGMACELL, filter paper, beta-glucan, galactan, mannan, galactomannan, xylan, and lichenan.

## Example 3

## Catalytic Activities of CelEcc, Native CelE, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with Either Cellulose I (SIGMACELL-20) or Cellulose III Prepared from SIGMACELL-20 by Extractive AFEX

Due to the specific binding ability of CBM3a, one would be interested in discovering the catalytic reactivity of the engineered construct of CelEcc_CBM3a with different insoluble substrates. Thus, an experiment regarding catalytic activities of CelEcc, native CelE, CelEcc_CBM3a, CelK, and CelRcc_CBM3a with either cellulose I or cellulose III was performed. Cellulose I was obtained directly as SIGMACELL-20, and cellulose III was prepared from SIGMACELL-20 by extractive AFEX (E-AFEX). CelKcc and CelRcc_CBM3a were produced following the same method and procedure described in U.S. Patent Publication No. 2010/0304405, which is incorporated by reference herein. The results were depicted in FIG. **7**.

As shown in FIG. **7**, in addition to the increased activity with PASC, CelEcc_CBM3a appeared to have significant improvements of catalytic reactivity with crystalline cellulose I, the natural allomorph commonly found in biomass. For example, the catalytic reactivity of CelEcc_CBM3a with cellulose I was found to be at least four times over that of native CelE, and the catalytic reactivity of CelEcc_CBM3a with cellulose I was found to be at least two times over that of CelEcc. Surprisingly, the engineered construct of CelEcc_CBM3a showed even more dramatic improvement in its reaction with cellulose III, a non-natural allomorph formed during extractive AFEX pretreatment. The catalytic reactivity of CelEcc_CBM3a with cellulose III was almost five times over that of native CelE, and the catalytic reactivity of CelEcc_CBM3a with cellulose III was at least three times over that of CelEcc. Further, it was found that CelEcc_CBM3a was more reactive with cellulose III than either CelKcc or CelRcc_CBM3a (FIG. **7**). As a comparison, CelRcc_CBM3a was found to be strongly reactive with cellulose I, but weakly reactive with cellulose III.

## Example 4

## Multifunctional Catalytic Activities of CelEcc_CBM3a

FIG. **10** is a set of diagrams and graphs showing multifunctional catalytic activity of CelEcc_CBM3a. FIG. **10**A is diagram showing the three-dimensional structure of CelEcc_CBM3a and indicating that CelEcc_CBM3a can be individually reacted with biomass, e.g., AFEX-switchgrass (AFEX-SG). FIG. **10**B is a graph showing NIMS analysis (see Reindl, et al., 2011 and Reindl et al., 2012) of the products from the catalytic reaction of CelEcc_CBM3a (Cthe_0797; GH5) with AFEX-switchgrass. In the NIMS analysis, reducing sugars released by enzyme action from the biomass react with a mass-spectral probe containing a hydroxylamino functional group. Upon mixing and reaction, the hydroxylamino group condenses with the reducing sugar to form a stable, covalent oxime bond. After labeling, the sugars can be identified by using mass spectrometry. The individual mass spectral peaks in FIG. **10**B have unique masses corresponding to the chemical structures identified. The multifunctional enzyme of CelEcc_CBM3a produces products derived from both cellulose and hemicellulose after its reaction with biomass, in this case AFEX-SG. FIG. **10**C is a graph showing NIMS analysis (of the products from the catalytic reaction of BglA (Cthe_0212; GH1) with AFEX-SG as a control experiment. The reaction of BglA with biomass was carried out in 100 μL of 50 mM phosphate and at pH 6.0. The biomass contained 10 mg AFEX-SG. The volume of enzyme (BglA) was 5 μL and the concentration of stock solution was 1 mg/mL. The reaction was carried out at 60° C. for up to 96 hours and samples were taken at various time points. At each of these time points, a 2 μL aliquot of the reaction mixture was transferred into a vial containing 6 μL of 100 mM glycine acetate at pH 1.2, 0.5 μL of a 50 mM aqueous solution of [U]-$^{13}$C glucose, 2 μL of $CH_3CN$, 2 μL of MeOH, 1 μL of NIMS probe [100 mM in 1:1 (v/v) $H_2O$:MeOH], and 0.1 μL of aniline. The mixture was incubated at room temperature for 16 hours. The mixture was then used in the NIMS assay.

FIG. **11** is a set of graphs showing the time-dependent catalytic reactions and kinetics of enzymes with biomass, in this case ionic-liquid treated switchgrass (IL-SG). FIG. **11**A is a graph showing the time-dependent catalytic reactions and kinetics of CelB (Cthe_0536) with IL-SG. The reaction of CelB with biomass was carried out in 100 μL of 50 mM phosphate at pH 6.0. The biomass contained 10 mg IL-SG. The volume of enzyme was 5 μL of CelB and the concentration of stock solution was 1 mg/mL. The reaction was carried out at 60° C. for up to 96 hours and samples were taken at various time points. At each of these time points, a 2 μL aliquot of the reaction mixture was transferred into a vial containing 6 μL of 100 mM glycine acetate at pH 1.2, 0.5 μL of a 50 mM aqueous solution of [U]-$^{13}$C glucose, 2 μL of $CH_3CN$, 2 μL of MeOH, 1 μL of NIMS probe [100 mM in 1:1 (v/v) $H_2O$:MeOH], and 0.1 μL of aniline. The mixture was incubated at room temperature for 16 hours. The mixture was then used in the NIMS assay. In the reaction of CelB, glucose (g1), cellobiose (g2), and cellotriose (g3) were observed, with cellobiose accumulating as the majority product. In total, the three hexose products accounted for only ~15% conversion of the cellulose present in IL-SG. The low yield of solubilized sugar is consistent with the known function of CelB as a non-processive endoglucanase.

FIG. **11**B is a graph showing the time-dependent catalytic reactions and kinetics of CelEcc_CBM3a (Cthe_0797) with IL-SG. The reaction of CelEcc_CBM3a with biomass were carried out in 100 μL of 50 mM phosphate at pH 6.0. The biomass contained 10 mg IL-SG. The volume of enzyme was 5 μL of CelEcc_CBM3a and the concentration of stock solution was 2 mg/mL. The reaction was carried out at 60° C. for up to 96 hours and samples were taken at various time points. At each of these time points, a 2 μL aliquot of the reaction mixture was transferred into a vial containing 6 μL of 100 mM glycine acetate at pH 1.2, 0.5 μL of a 50 mM aqueous solution of [U]-$^{13}$C glucose, 2 μL of $CH_3CN$, 2 μL of MeOH, 1 μL of NIMS probe [100 mM in 1:1 (v/v) $H_2O$:MeOH], and 0.1 μL of aniline. The mixture was incubated at room temperature for 16 hours. The mixture was then used in the NIMS assay. CelEcc_CBM3a (2.2% enzyme/biomass loading w/w) simultaneously hydrolyzed cellulose and hemicellulose in the biomass to give a ~50% yield of the expected solubilized products including both hexoses and pentoses.

Table 1 shows the numerical values of individual rate constants ($h^{-1}$) for the reactions with IL-SG according to the kinetic schemes shown in FIGS. **12** and **13**. Rate constants were normalized to the nmol of enzyme active sites. Rate constants of $k_6$ to $k_{11}$ are not included in the kinetic scheme for cellulose reaction.

TABLE 1

Apparent rates $(h^{-1})^a$ in reactions with IL-SG according to the kinetic schemes of FIG. 12 and 13.

| Rate | cellulose CelB | cellulose CelEcc_CBM3a | hemicellulose CelB | hemicellulose CelEcc_CBM3a |
|---|---|---|---|---|
| $k_1$ | 0.002 | 0.022 | 0.000 | 0.020 |
| $k_2$ | 0.027 | 0.089 | 0.000 | 0.054 |
| $k_3$ | 0.004 | 0.006 | 0.000 | 0.073 |
| $k_4$ | 0.000 | 0.002 | 0.000 | 0.059 |
| $k_5$ | 0.000 | 0.005 | 0.000 | 0.026 |
| $k_6$ | — | — | — | 0.000 |
| $k_7$ | — | — | — | 0.000 |
| $k_8$ | — | — | — | 0.000 |
| $k_9$ | — | — | — | 0.000 |
| $k_{10}$ | — | — | — | 0.000 |
| $k_{11}$ | — | — | — | 0.013 |

[a] rates were normalized to the nmol of enzyme active sites; Rates $k_6$-$k_{11}$ are not included in the kinetic scheme for cellulose reaction.

FIG. **12** is a set of graphs and diagrams showing time-dependent catalytic reactions and kinetics of CelEcc_CBM3a (Cthe_0797) for each of the individual products from cellulose contributing to the overall reaction profile summarized in FIG. **11**B. FIG. **12**A shows a numerical simulation of the product evolution curves for each of the individual products detected from the reaction of CelEcc_CBM3a (Cthe_0797) with the cellulose component in IL-SG. The solid lines show the graphic results of the differential equations corresponding to the kinetic scheme shown in FIG. **12**B. The differential equations for the kinetic model of FIG. **12**B are provided in FIG. **12**C. Table 1 shows the numerical values for the individual rate constants shown in FIG. **12**B. The individual rate constants were calculated by using the differential equations shown in FIG. **12**C. According to this analysis, CelEcc_CBM3a hydrolyzed 51% of the cellulose in IL-SG to a mixture of glucose (15%, relative to total cellulose in IL-SG), cellobiose (35%), and cellotriose (1%). The simulations show that CelEcc_CBM3a has a ~4.5-fold preference for releasing cellobiose from IL-SG over other products. CelEcc_CBM3a has a small propensity to release cellotriose and to hydrolyze solubilized cellotriose and cellobiose to produce other minor products. These properties correspond to reactions of purified CelEcc_CBM3a with purified cellotetraose, suggesting that the fundamental properties of the engineered enzyme are the same in reactions with biomass and the purified or artificial substrates.

FIG. **13** is a set of graphs and diagrams showing time-dependent catalytic reactions and kinetics of CelEcc_CBM3a (Cthe_0797) for each of the individual products from hemicellulose contributing to the overall reaction profile summarized in FIG. **11**B. FIGS. **13**A and **3**B show a numerical simulations of the product evolution curves for each of the individual products detected from the reaction of CelEcc_CBM3a (Cthe_0797) with the hemicellulose component in IL-SG. The solid lines show the graphic results of the differential equations corresponding to the kinetic schemes shown in FIG. **13**C. The differential equations for the kinetic model of FIG. **13**C are provided in FIG. **13**D. Table 1 shows the numerical values for the individual rate constants shown in FIG. **12**B. The individual rate constants were calculated by using the differential equations of FIG. **13**D. Since the method of NIMS cannot distinguish between the masses of various pentose isomers such as xylose, arabinose and others, generic names for pentose products were used. After 24 hours of reaction, the product formed from the reactions of CelEcc_CBM3a consists of pentose (p1, 6%, relative to total hemicellulose in IL-SG, FIG. **13**A), pentobiose (p2, 16%, FIG. **13**A) and pentotriose (p3, 17%, FIG. **13**A), and pentotetraose (p4, 8%, FIG. **13**B) and pentopentaose (p5, 3%, FIG. **13**B). Table 1 shows that the apparent rates for releasing the three major products were overall similar (within ~25%).

Pentotriose accumulated at the fastest initial rate. In order to successfully model the hemicellulose product cascade, it was necessary to include slow, secondary hydrolysis of pentopentaose to pentotriose and pentobiose, and also hydrolysis of pentotetraose to 2 mol of pentobiose. Similar products were detected in the reactions of purified CelEcc_CBM3a with purified xylohexaose, indicating that CelEcc_CBM3a can react with both the insoluble hemicellulose fraction and some of the soluble pentose oligomers released from biomass.

This disclosure further incorporates the concurrently submitted sequence listing in computer readable form. The above description, attached figures and their descriptions are intended to be illustrative and not limiting of this invention, which is defined by the appended claims. Furthermore, all cited publications are incorporated by reference herein in their entirety.

## REFERENCES CITED

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6. Miller, G. L. *Anal. Chem.* 1959, 31, 426-428.
7. Reindl, W., K. Deng, J. M. Gladden, G. Cheng, A. Wong, S. W. Singer, S. Singh, J. C. Lee, C. H yao, T. C. Hazen, A. K. Singh, B. A. Simmons, P. D. Adams, and T. R. Nothen. Colloid-based multiplexed screening for plant biomass-degrading glycoside hydrolase activities in microbial communities. *Energy Environ. Sci.* 2011, 4, 2884-2893.
8. Reindl, W., K. Deng, X. Cheng, A. K. Singh, B. A. Simmons, P. D. Adams, and T. R. Nothen. Nanostructure-initiator mass spectrometry (NIMS) for the analysis of enzyme activities. *Current Protocols in Chemical Biology* 2012, Jun. 1, 123-142.

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SEQUENCE LISTING

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cacatcggac ctgctccgga ctataaaatt gacgaagcat ggctgaacag agttgaggaa    240

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Asn Pro Arg Thr Thr Lys Ala Met Ile Glu Lys Val Arg Glu Met Gly
        35                  40                  45

Phe Asn Ala Val Arg Val Pro Val Thr Trp Asp Thr His Ile Gly Pro
    50                  55                  60

Ala Pro Asp Tyr Lys Ile Asp Glu Ala Trp Leu Asn Arg Val Glu Glu
65                  70                  75                  80

Val Val Asn Tyr Val Leu Asp Cys Gly Met Tyr Ala Ile Ile Asn Leu
                85                  90                  95

His His Asp Asn Thr Trp Ile Ile Pro Thr Tyr Ala Asn Glu Gln Arg
            100                 105                 110

Ser Lys Glu Lys Leu Val Lys Val Trp Glu Gln Ile Ala Thr Arg Phe
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Lys Asp Tyr Asp Asp His Leu Leu Phe Glu Thr Met Asn Glu Pro Arg
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        195                 200                 205

Asp Ser Arg Val Ile Val Ser Ile His Ala Tyr Ser Pro Tyr Phe Phe
    210                 215                 220

Ala Met Asp Val Asn Gly Thr Ser Tyr Trp Gly Ser Asp Tyr Asp Lys
225                 230                 235                 240

Ala Ser Leu Thr Ser Glu Leu Asp Ala Ile Tyr Asn Arg Phe Val Lys
                245                 250                 255

Asn Gly Arg Ala Val Ile Ile Gly Glu Phe Gly Thr Ile Asp Lys Asn
            260                 265                 270

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        275                 280                 285

Val Ser Arg Gly Ile Ala Val Phe Trp Trp Asp Asn Gly Tyr Tyr Asn
    290                 295                 300

Pro Gly Asp Ala Glu Thr Tyr Ala Leu Leu Asn Arg Lys Thr Leu Ser
305                 310                 315                 320

Trp Tyr Tyr Pro Glu Ile Val Gln Ala Leu Met Arg Gly Ala Gly Val
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Glu Pro Leu Val Ser Pro Thr Pro Thr Pro Thr Leu Met Pro Thr Pro
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Ser Pro Thr Val Thr Ala Asn Ile Leu Tyr Gly Asp Val Asn Gly Asp
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Gly Lys Ile Asn Ser Thr Asp Cys Thr Met Leu Lys Arg Tyr Ile Leu
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Arg Gly Ile Glu Glu Phe Pro Ser Pro Ser Gly Ile Ile Ala Ala Asp
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Leu Ile Ser Glu Val Arg Arg Asn Tyr Pro Asp Ala His Ile Phe Cys
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Cys Val Gly Pro Met Leu Trp Gly Thr Gly Leu Asp Leu Cys Arg Ser
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Tyr Val Thr Glu Val Val Asn Asp Cys Asn Arg Ser Gly Asp Leu Lys
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Val Tyr Phe Val Glu Phe Pro Gln Gln Asp Gly Ser Thr Gly Tyr Gly
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gaagtggtaa actatgttct tgactgcggt atgtacgcga tcataaatgt tcaccatgac     300

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Gly Phe Asn Ala Val Arg Val Pro Val Thr Trp Asp Thr His Ile Gly
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Pro Ala Pro Asp Tyr Lys Ile Asp Glu Ala Trp Leu Asn Arg Val Glu
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                85                  90                  95

Val His His Asp Asn Thr Trp Ile Ile Pro Thr Tyr Ala Asn Glu Gln
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Arg Ser Lys Glu Lys Leu Val Lys Val Trp Glu Gln Ile Ala Thr Arg
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Phe Lys Asp Tyr Asp Asp His Leu Leu Phe Glu Thr Met Asn Glu Pro
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            180                 185                 190

Ala Ala Thr Gly Leu Asp Val Ala Leu Asn Asp Leu Val Ile Pro Asn
        195                 200                 205

Asn Asp Ser Arg Val Ile Val Ser Ile His Ala Tyr Ser Pro Tyr Phe
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225                 230                 235                 240

Lys Ala Ser Leu Thr Ser Glu Leu Asp Ala Ile Tyr Asn Arg Phe Val
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Gly Phe Asn Ala Val Arg Val Pro Val Thr Trp Asp Thr His Ile Gly
65                  70                  75                  80

Pro Ala Pro Asp Tyr Lys Ile Asp Glu Ala Trp Leu Asn Arg Val Glu
                85                  90                  95

Glu Val Val Asn Tyr Val Leu Asp Cys Gly Met Tyr Ala Ile Ile Asn
            100                 105                 110

Leu His His Asp Asn Thr Trp Ile Ile Pro Thr Tyr Ala Asn Glu Gln
        115                 120                 125

Arg Ser Lys Glu Lys Leu Val Lys Val Trp Glu Gln Ile Ala Thr Arg
    130                 135                 140

Phe Lys Asp Tyr Asp Asp His Leu Leu Phe Glu Thr Met Asn Glu Pro
145                 150                 155                 160

Arg Glu Val Gly Ser Pro Met Glu Trp Met Gly Gly Thr Tyr Glu Asn
                165                 170                 175

Arg Asp Val Ile Asn Arg Phe Asn Leu Ala Val Val Asn Thr Ile Arg
            180                 185                 190

Ala Ser Gly Gly Asn Asn Asp Lys Arg Phe Ile Leu Val Pro Thr Asn
        195                 200                 205

Ala Ala Thr Gly Leu Asp Val Ala Leu Asn Asp Leu Val Ile Pro Asn
    210                 215                 220

Asn Asp Ser Arg Val Ile Val Ser Ile His Ala Tyr Ser Pro Tyr Phe
225                 230                 235                 240

Phe Ala Met Asp Val Asn Gly Thr Ser Tyr Trp Gly Ser Asp Tyr Asp
                245                 250                 255

Lys Ala Ser Leu Thr Ser Glu Leu Asp Ala Ile Tyr Asn Arg Phe Val
            260                 265                 270

Lys Asn Gly Arg Ala Val Ile Ile Gly Glu Phe Gly Thr Ile Asp Lys
        275                 280                 285

Asn Asn Leu Ser Ser Arg Val Ala His Ala Glu His Tyr Ala Arg Glu
    290                 295                 300

Ala Val Ser Arg Gly Ile Ala Val Phe Trp Trp Asp Asn Gly Tyr Tyr
305                 310                 315                 320

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                325                 330                 335

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            340                 345                 350

Val Glu Ser Leu Asn Ala Thr Pro Thr Lys Gly Ala Thr Pro Thr Asn
        355                 360                 365

Thr Ala Thr Pro Thr Lys Ser Ala Thr Ala Thr Pro Thr Arg Pro Ser
    370                 375                 380

Val Pro Thr Asn Thr Pro Thr Asn Thr Pro Ala Asn Thr Pro Val Ser
385                 390                 395                 400

Gly Asn Leu Lys Val Glu Phe Tyr Asn Ser Asn Pro Ser Asp Thr Thr
                405                 410                 415

Asn Ser Ile Asn Pro Gln Phe Lys Val Thr Asn Thr Gly Ser Ser Ala
            420                 425                 430

Ile Asp Leu Ser Lys Leu Thr Leu Arg Tyr Tyr Tyr Thr Val Asp Gly
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Gln Lys Asp Gln Thr Phe Trp Cys Asp His Ala Ala Ile Ile Gly Ser
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Asn Gly Ser Tyr Asn Gly Ile Thr Ser Asn Val Lys Gly Thr Phe Val
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Lys Met Ser Ser Ser Thr Asn Asn Ala Asp Thr Tyr Leu Glu Ile Ser
                485                 490                 495

Phe Thr Gly Gly Thr Leu Glu Pro Gly Ala His Val Gln Ile Gln Gly
            500                 505                 510

Arg Phe Ala Lys Asn Asp Trp Ser Asn Tyr Thr Gln Ser Asn Asp Tyr
        515                 520                 525

Ser Phe Lys Ser Ala Ser Gln Phe Val Glu Trp Asp Gln Val Thr Ala
    530                 535                 540

Tyr Leu Asn Gly Val Leu Val Trp Gly Lys Glu Pro Gly
545                 550                 555


<210> SEQ ID NO 7
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Clostridium thermocellum

<400> SEQUENCE: 7

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1               5                   10                  15
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We claim:

**1**. A multifunctional polypeptide capable of hydrolyzing a cellulosic material, xylan, and mannan, comprising

the catalytic core (cc) of *Clostridium thermocellum* Cthe_0797 (CelE),

a cellulose-specific carbohydrate-binding module (CBM), wherein the CBM is CBM3 of the cellulosome anchoring protein cohesion region (CipA) of *Clostridium thermocellum* (CBM3a), and

a linker region interposed between the catalytic domain and the cellulose-specific carbohydrate binding module.

**2**. The polypeptide of claim **1**, wherein the linker region is a 5-150 amino acid sequence.

**3**. The polypeptide of claim **1**, wherein the linker region is a 15-40 amino acid sequence.

**4**. The polypeptide of claim **1**, wherein the polypeptide comprises the amino acid sequence of SEQ ID NO:6.

**5**. A polypeptide composition for increasing the rate and the extent of fiber digestion for a mammal comprising the polypeptide of claim **1**.

**6**. A method of hydrolyzing a substrate comprising a cellulosic material, xylan, and mannan, comprising the step of contacting a substrate comprising a cellulosic material, xylan, and mannan with an effective amount of the multifunctional polypeptide of claim **1**, whereby the cellulosic material, xylan, and mannan in the substrate are at least partially hydrolyzed.

**7**. The method of claim **6**, wherein the cellulosic material is selected from the group consisting of filter paper, crystalline cellulose allomorph I, and amorphous cellulose.

**8**. The method of claim **6**, wherein the cellulosic material is cellulose III.

**9**. The method of claim **6**, further comprising the step of pretreating the cellulosic material is under acidic, basic, or oxidative conditions.

**10**. The method of claim **8**, wherein the cellulose III is a non-natural cellulose produced by extractive ammonia fiber expansion treatment of biomass.

**11**. The method of claim **6**, wherein the substrate further comprises SIGMACELL, beta-glucan, galactan, galactomannan, or lichenan.

**12**. The method of claim **6**, wherein the catalytic reactivity exhibited by the multifunctional polypeptide is at least 50% higher than the catalytic reactivity exhibited by native CelE.

**13**. The method of claim **6**, wherein the catalytic reactivity exhibited by the multifunctional polypeptide is at least 1.5 times higher than the catalytic reactivity exhibited by the catalytic core (cc) of native CelE.

**14**. The method of claim **6**, wherein the catalytic reactivity exhibited by the multifunctional polypeptide is at least 2 times higher than the catalytic reactivity exhibited by the catalytic core (cc) of native CelE.

**15**. The method of claim **6**, wherein the method is performed at a temperature of about 60° C.

**16**. The method of claim **6**, wherein the method is performed at a pH of about 6.0.

**17**. The method of claim **6**, wherein the effective amount of multifunctional polypeptide is in the range of 1-100 mg/g (enzyme/glucan).

**18**. The method of claim **6**, wherein the effective amount of multifunctional polypeptide is in the range of 1-10 mg/g (enzyme/glucan).

**19**. A method of increasing the rate and the extent of fiber digestion in a mammal comprising the step of administering to a mammal an effective amount of the multifunctional polypeptide of claim **1**, whereby the cellulosic materials, xylan, and mannan in the fiber consumed by the mammal are at least partially hydrolyzed.

**20**. A method of making a multifunctional polypeptide comprises the step of linking the catalytic core (cc) of *Clostridium thermocellum* Cthe_0797 (CelE) with a cellulose-specific carbohydrate-binding module (CBM) by using a linker region, wherein the linker region is interposed between the catalytic domain and the cellulose-specific carbohydrate binding module, and wherein the CBM is CBM3 of the cellulosome anchoring protein cohesion region (CipA) of *Clostridium thermocellum* (CBM3a).

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