

# Construction of a Bacterial Cellulase Cocktail for Saccharification of Regenerated Cellulose and Pretreated Corn Stover

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To apply bacterial cellulases for efficient saccharification of biomass, three *Clostridium thermocellum* cellulases and a *Thermoanaerobacter brockii*  $\beta$ -1,4-glucosidase were synthesized in *Escherichia coli*, and the proportions among them were optimized. When the activities of CelD, CBHA, CBH48Y, and CglT were set at 554, 0.91, 0.91, and 856 mU per assay, respectively, the percent conversion of regenerated cellulose (0.92 g/L) reached 80.9% within 24 h at 60 °C without shaking. Meanwhile, the percent conversion of pretreated corn stover (0.62 g/L) reached 70.1%. Gradually raising the loads of regenerated cellulose from 0.92 to 4.58 g/L resulted in a linear increase in glucose production from 870 to 3208  $\mu$ g ( $R^2=0.997$ ), as well as a decrease in the percent conversion from 80.9% to 59.6%. These findings suggested that the cellulase cocktail is efficient in saccharification of regenerated cellulose, as well as pretreated corn stover, and has potential applications in the biofuels industry.

*Keywords:* Cellulases; Saccharification; *Clostridium thermocellum*; *Thermoanaerobacter brockii*; Biomass

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## INTRODUCTION

Currently, cellulosic biofuel technologies are confronted with two primary problems: the high cost of cellulases and the lack of an efficient pretreatment technology for biomass (Berlin *et al.* 2007; Taherzadeh and Karimi 2007; Agbor *et al.* 2011). To cut down on the cost of cellulases, efficient cellulases have been recruited to improve the performance of cellulase systems. Cumulative studies have suggested that cellulases from both filamentous fungi and bacteria hold great potential for future industry applications, although the latter is currently less widely applied in the cellulosic biofuel industry than the former (Maki *et al.* 2009). Most importantly, many bacteria thrive in extreme environmental niches and produce stress-resistant cellulases, which is of great value for industrial applications.

Meyer *et al.* (2009) found that a minimal enzyme cocktail had a better performance than native *Trichoderma reesei* enzymes for hydrolysis of pretreated barley straw samples and pointed out the necessity of constructing a minimal cellulase cocktail for efficient biomass processing that could also be applicable to bacteria. *Clostridium thermocellum* is one of the most famous anaerobic cellulolytic bacteria because of its efficient cellulase system, which consists of both cellulosomal and non-cellulosomal cellulases (Gold and Martin 2007). Many *C. thermocellum* cellulases have been characterized, and most of them have functioned well, with good thermostability. Thus, it is necessary to construct a basic cellulase cocktail, mainly from *C. thermocellum*, for the gradual establishment of an efficient cellulase system for biomass saccharification. A core cellulase system consists of

the following: (1) an endo-1,4- $\beta$ -D-glucanase, which scissors the long cellulose chains; (2) two exo-1,4- $\beta$ -D-glucanases, one of which acts on the reducing side of cellulose chains, and the other on the non-reducing side, to produce cellobiose; and (3) a  $\beta$ -1,4-glucosidase, which acts on cellobiose to produce glucose. Fortunately, all of these enzymes could be found in *C. thermocellum*.

The final step of saccharification is accomplished by  $\beta$ -1,4-glucosidase, which will decrease the inhibitory effect of cellobiose on both endo-1,4- $\beta$ -D-glucanases and exo-1,4- $\beta$ -D-glucanases, which would have resulted in decreases in yield and reaction rate (Shen *et al.* 2008). However, *C. thermocellum* has not yet evolved an efficient  $\beta$ -1,4-glucosidase because there is no selection pressure, *i.e.*, it can directly assimilate cellodextrins (Zhang and Lynd 2005). Recently, Prawitwong *et al.* (2013) found that the percent conversion of cellulose greatly increased from about 30% to more than 90% by supplementing the crude enzymes of *C. thermocellum* S14 with a thermostable  $\beta$ -1,4-glucosidase from *Thermoanaerobacter brockii* at 60 °C for 7 days. This enzyme could possibly be employed in the core cellulase system mentioned above.

In this study, a core cellulase cocktail, consisting of an endo-1,4- $\beta$ -D-glucanase (CelD) (Joliff *et al.* 1986), a reducing-end-acting exo-1,4- $\beta$ -D-glucanase (CBH48Y) (Liu *et al.* 2012), a non-reducing-end-acting exo-1,4- $\beta$ -D-glucanase (CBHA) (Kataeva *et al.* 2002) from *C. thermocellum* DSM 1237, and a  $\beta$ -1,4-glucosidase (CglT) from *T. brockii* (Breves *et al.* 1997), was established for the hydrolysis of regenerated cellulose and pretreated corn stover. By using the cellulase cocktail, more efficient members of cellulases and hemicellulases could be introduced for better biomass saccharification in the future.

## EXPERIMENTAL

### Materials

Enzymes for molecular cloning were purchased from Takara, Dalian, China. pEasy E2 expression vector and *Escherichia coli* Transetta ( $F^- ompT hsdS_B (r_B^+ m_B^-) gal dcm$  (DE3) pRARE (argU, argW, ileX, glyT, leuW, proL) (CamR)) were both from TransGen, Beijing, China. Avicel PH101 (Sigma-Aldrich, St. Louis, USA) was used to produce regenerated cellulose. The glucose quantification kit was from Senbeijia, Nanjing, China. *p*-Nitrophenyl- $\beta$ -D-glucoside (pNPG) was from Sangon Biotech, Shanghai, China. Other chemical reagents were analytically pure and obtained from Sinopharm Chemical, Shanghai, China.

### Methods

#### Gene clone

The *celd*, *cbh48y*, and truncated *cbha* gene were PCR cloned from the genomic DNA of *C. thermocellum* DSM 1237 (corresponding to ATCC 27405, DSMZ, Germany) using LA taq and primers, as listed in Table 1. The *cglT* gene was PCR cloned from an artificial synthetic gene with codons optimized (GenBank accession: KP401766), which was synthesized by the BGI company (Shenzhen, China). The gene fragments of *celd* and *cglT* were double digested by *EcoRV* and *XhoI*, and *cbha* was double digested by *SmaI* and *XhoI*, which were introduced to the *EcoRV/XhoI* sites of expression vector pET 22r (Geng *et al.* 2012) separately. The PCR product, *cbh48y*, was directly T-cloned into a pEasy E2 expression vector. The resulting plasmids carrying *celd* or *cbha* were transformed in *E. coli* BL21 separately. The resulted plasmids carrying *cbh48y* or *cglT* were transformed into *E.*

*coli* Transetta for better gene expression. All the resulting plasmids were verified by sequencing.

**Table 1.** Gene Information

Gene names	Accession No.	Gene sizes (kb)	Primers (5'→3')*	Domains of the corresponding proteins	Vector	Expression host ( <i>E. coli</i> )
<i>celd</i>	4810443	1.9	f: <u>TATGATATC</u> GCAAAAATA ACGGAGAAT r: TATCTCGAG TATTGGTAAT TTCTCGAT	Glycoside hydrolase family 9; dockerin type I	pET 22r	BL21 (DE3)
<i>cbha</i> **	4808416	2.8	f: <u>ATTCCCGGG</u> TATATACTTC CGCAGCCT r: <u>ATTCTCGAG</u> ACCGCCCGG CGGCGTTC	Glycoside hydrolase family 9; Carbohydrate-binding domain type 3a	pET 22r	BL21 (DE3)
<i>cbh48y</i>	4808766	2.7	f: <u>AGCTTTAAA</u> CCTTCCAG r: CGGTTCGTT TCCCGAT	Glycoside hydrolase family 48; Carbohydrate-binding domain type 3a	pEasy E2	Transetta (DE3)
<i>cgl</i>	KP40176 6	1.3	f: <u>TATGATATC</u> GCAAAATTT CCAAGAGAT r: <u>ATACTCGAG</u> ATCTTCGATA CCATCATC	Glycosyl hydrolase family 1	pET 22r	Transetta (DE3)
*Restriction sites are underlined; **A truncated gene						

#### *Gene expression and protein purification*

Each transformant carrying one of the four genes was inoculated into 4 mL of Luria–Bertani (LB) media supplemented with a proper antibiotic and grown at 37 °C overnight. Then, the overnight culture was inoculated into 500 mL of LB, grown to OD 1.2 at 37 °C, and induced at 50 to 100 µM isopropyl-β-D-thio-galactoside (IPTG) at 25 °C to 30 °C, 120 rpm, for 6 to 12 h.

Cells were harvested and disrupted using a supersonic-wave disrupting method described by Yan *et al.* (2013) and centrifuged at 20,000 g for 15 min. Subsequently, the supernatant was heat treated at 60 °C for 25 min and centrifuged at 8,000 g for 10 min. The supernatant was loaded onto a nickel column (HisTrap FF column, GE Healthcare, Piscataway, NJ), washed with 40 mM imidazole, and eluted with 500 mM imidazole. Finally, the purified enzymes were buffer-exchanged into a solution (5 mM Na<sub>3</sub>PO<sub>4</sub> and

50 mM NaCl, pH 7.4) using a HiTrap desalting column (GE Healthcare) and stored at -20 °C for use. The purity of proteins was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Shanghai, China), followed by Coomassie Brilliant Blue R-250 staining.

#### *Enzymatic activity assay*

The enzymatic activities of CelD, CBHA, and CBH48Y were assayed using the DNS method (Miller 1959). A standard assay contains 10 µL of properly diluted enzymes, 200 µL of regenerated amorphous cellulose (5.38 g/L glucose equivalent) (Zhang *et al.* 2006), 100 µL of buffer (100 mM NaAc-HAc, 20 mM CaCl<sub>2</sub>, pH 6.0), and 90 µL of water. The enzymatic activity of CelD was determined at 60 °C for 1 h, while the activities of CBHA and CBH48Y were determined at 60 °C for 12 h. The activity of CglT was determined by employing the above reaction system, except using pNPG as a substrate at 60 °C for 25 min, as described by Magalhães *et al.* (2006). A unit of enzymatic activity was defined as the number of micromoles of reducing sugar or *p*-nitrophenol released per minute per milligram of protein. For thermostability tests, suitable amounts of enzymes were subjected to the standard enzymatic assay after having been incubated at 60 °C for 24 h.

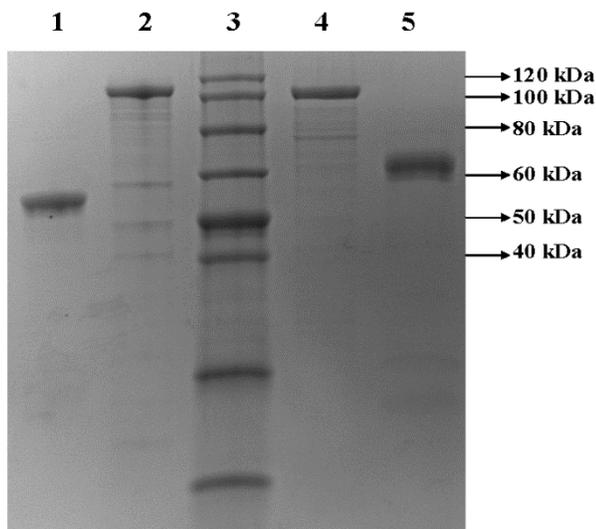
#### *Cellulose saccharification*

Regenerated cellulose and corn stalk pretreated by cellulose-solvent-based lignocellulose fractionation (Zhu *et al.* 2009), were used as substrate to investigate the effects of various compositions of the four enzymes on the saccharification efficiency. Unless otherwise stated, the saccharification was carried out at 60 °C for 24 h without shaking in a total volume of 1170 µL, with a proper content of cellulose and cellulases, as well as 200 µL of acetate buffer. Glucose content was determined using a glucose oxidase kit (Senbeijia, Nanjing, China). The content of cellulose fraction in the pretreated corn stalk was calculated as described by Zhu *et al.* (2009). Unless otherwise stated, the percentage production of total reducing sugar divided by total cellulose (in glucose equivalents) was used to represent saccharification efficiency. All results are the average of three replicates, and Student's *t*-test was performed with Excel 2007 (Microsoft Corp., Redmond, WA) to analyze the significance of the differences.

## RESULTS AND DISCUSSION

### **Expression and Purification of the Four Cellulases**

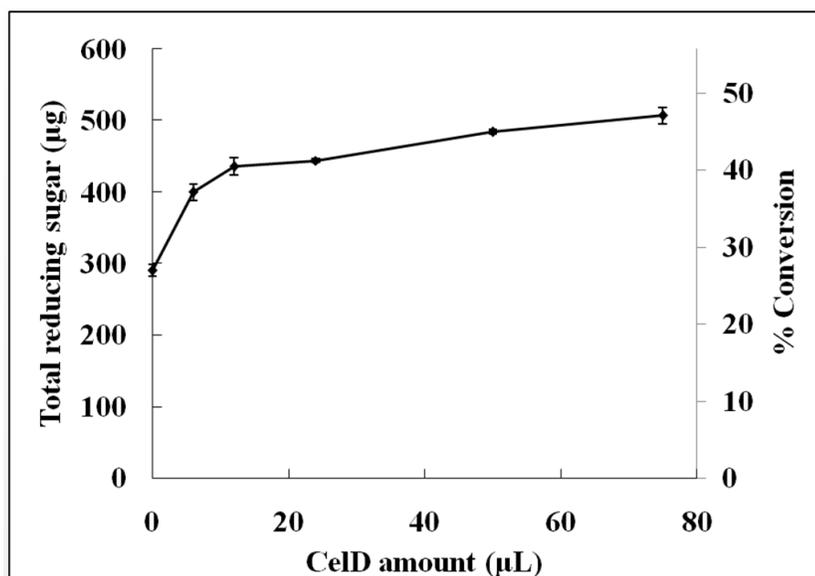
The four purified cellulases each presented a strong band, as analyzed by SDS-PAGE (Fig. 1). The sizes of CelD, CBHA, CBH48Y, and CglT were all as expected (72.4, 104.9, 105.7, and 51.7 kDa, respectively). Although some proteins partially formed inclusion bodies during the expression process, the soluble fractions of the four cellulases were successfully purified using the four steps of centrifuging, heat treatment, nickel-affinity purification, and desalting, as described above. The productivities of these cellulases were currently not considered in this study. Of course, some efficient strategies could be employed to decrease the inclusion bodies and increase the protein production (Esposito and Chatterjee 2006).



**Fig. 1.** Purified cellulases on a 10% SDS-PAGE gel stained with Coomassie Brilliant Blue R-250: Lane 1, CglT; Lane 2, CBH48Y; Lane 3, protein marker; Lane 4, CBHA; Lane 5, CelD

### Effect of CelD Load on Saccharification

The exoglucanase activities (CBHA plus CBH48Y at a 1:1 ratio) and  $\beta$ -glucosidase activities were set at 0.28 and 11.6 U/g cellulose, respectively, which were situated at medium levels as were found in our preliminary tests (data not shown). As the load of the endoglucanase, CelD, increased from 0 to 12  $\mu$ L (85.9 U/g cellulose), the production of total reducing sugar remarkably increased from 290 to 436  $\mu$ g (percent conversion from 27.0% to 40.5%). However, as the CelD load further increased 6.5 times (from 12 to 75  $\mu$ L), the production of total reducing sugar only slightly increased, to 47.1% (Fig. 2).

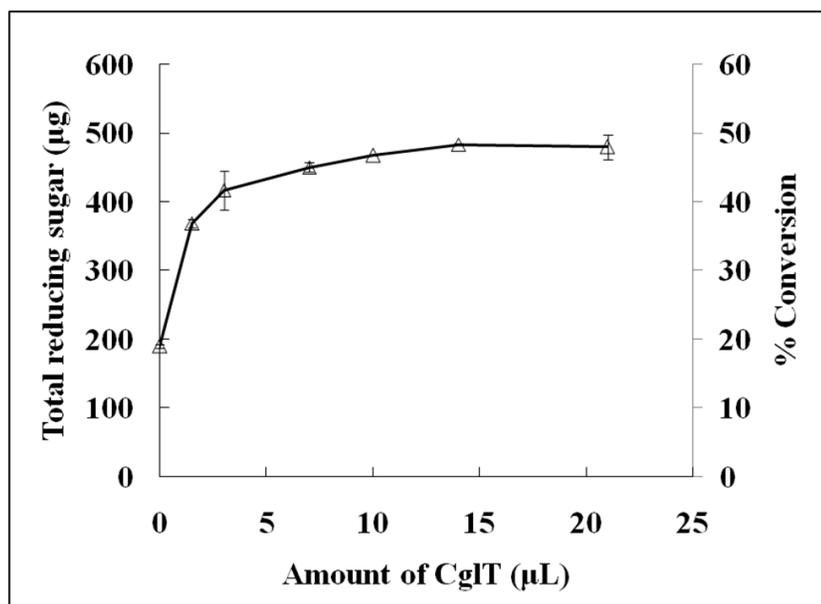


**Fig. 2.** Effect of CelD load on saccharification. The activity of CelD was 7.7 mU/ $\mu$ L. The exoglucanase activities (CBHA plus CBH48Y at a 1:1 ratio) and  $\beta$ -glucosidase activities were set at 0.28 and 11.6 U/g cellulose, respectively. The load of regenerated cellulose was 2.69 g/L, and the total reaction volume was 400  $\mu$ L. Saccharification levels are presented in the form of total reducing sugar and percent conversion.

The production of reducing sugar at a CelD load of 50  $\mu\text{L}$  was not significantly different from that at 75  $\mu\text{L}$  ( $p=0.057$ ). Apparently, endo-1,4- $\beta$ -D-glucanase can provide new ends for exo-1,4- $\beta$ -D-glucanase and thus is important for saccharification.

### Effect of CglT Load on Saccharification

The endoglucanase and exoglucanase activities (CBHA plus CBH48Y at a 1:1 ratio) were set at 85.9 and 0.28 U/g cellulose, respectively. As the load of the  $\beta$ -glucosidase, CglT, was increased from 0 to 10  $\mu\text{L}$  (16.6 U/g cellulose), the production of total reducing sugar greatly increased, from 190 to 468  $\mu\text{g}$  (percent conversion from 17.6% to 43.5%). As the load of CglT was further increased 2.1 times (from 10 to 21  $\mu\text{L}$ ), the production of total reducing sugar only slightly increased, to 44.6% (Fig. 3). The production of reducing sugar at a CglT load of 10  $\mu\text{L}$  was not significantly different from that at 21  $\mu\text{L}$  ( $p=0.22$ ). Usually, the middle product, cellobiose, has an inhibitory effect on both endo-1,4- $\beta$ -D-glucanases and exo-1,4- $\beta$ -D-glucanases (Shen *et al.* 2008); therefore, increasing the load of CglT greatly increased the production of reducing sugar.



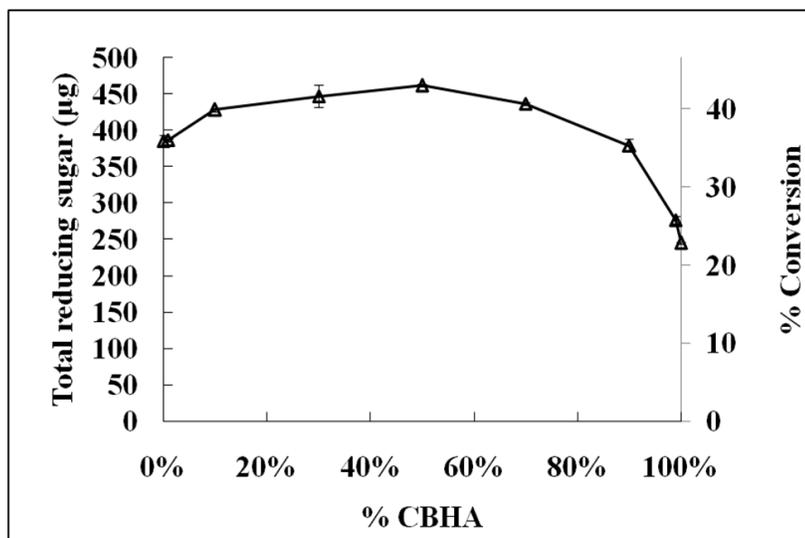
**Fig. 3.** Effect of CglT load on saccharification. The activity of CglT was 1.79 mU/ $\mu\text{L}$ . The endoglucanase activity and exoglucanase activities (CBHA plus CBH48Y at a 1:1 ratio) were set at 85.9 and 0.28 U/g cellulose, respectively. The load of regenerated cellulose was 2.69 g/L, and the total reaction volume was 400  $\mu\text{L}$ . Saccharification levels are presented in the form of total reducing sugar and percent conversion.

### Effect of CBHA and CBH48Y Proportion on Saccharification

The endoglucanase activity, exoglucanase activities (CBHA plus CBH48Y), and  $\beta$ -glucosidase activities were set at 85.9, 0.28, and 16.6 U/g cellulose, respectively. As the fraction of CBHA increased from 0% to 100% (*i.e.*, CBH48Y decreased from 100% to 0%), the production of total reducing sugar underwent an inverse-U fluctuation (Fig. 4). The highest production of reducing sugar was 462  $\mu\text{g}$  (42.9% conversion) at 50% CBHA, in contrast to only 385 and 245  $\mu\text{g}$  reducing sugar at 100% CBH48Y and 100% CBHA, respectively. Hence, the optimal proportion of CBHA:CBH48Y was 1:1.

The reducing-end-acting and non-reducing-end-acting exo-1,4- $\beta$ -D-glucanases are two oppositely functioning enzymes and usually function synergistically in cellulose

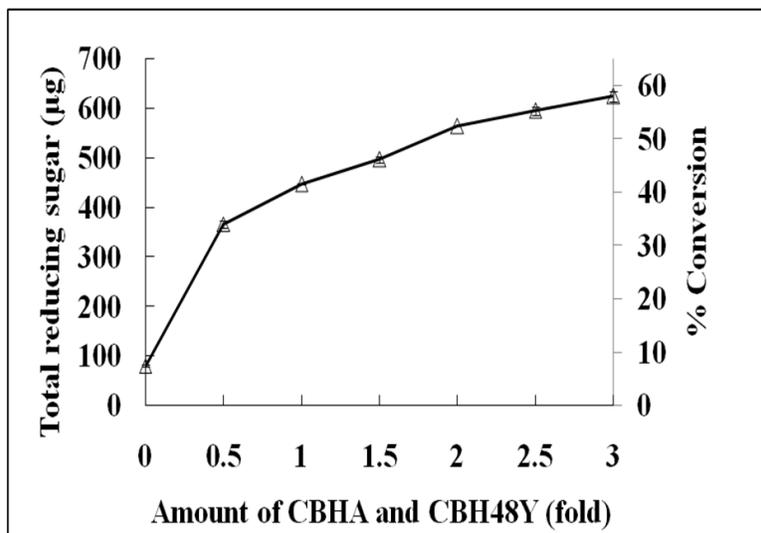
hydrolysis. Hence, the cooperation of CBHA and CBH48Y maximally produced 120% reducing sugar compared with CBH48Y alone, and produced 188% reducing sugar compared with CBHA alone. Similarly, Liu *et al* (2012) reported 134% enhancement in reducing sugar using a combination of recombinant CBHA and CBH48Y (expressed in *Bacillus subtilis*) compared with that of the sum of each single enzyme. Barr *et al.* (1996) also found synergistic effects between these two classes of exocellulases from *Thermomonospora fusca* and *T. reesei*. These two types of exoglucanase worked simultaneously from the two ends of a cellulose chain without competition on the substrate, showing increased efficiency in cellulose decomposition.



**Fig. 4.** Effect of CBHA and CBH48Y proportion on saccharification. The endoglucanase activity, exoglucanase activities (CBHA plus CBH48Y), and  $\beta$ -glucosidase activities were set at 85.9, 0.28, and 16.6 U/g cellulose, respectively. The load of regenerated cellulose was 2.69 g/L, and the total reaction volume was 400  $\mu$ L. Saccharification levels are presented in the form of total reducing sugar and percent conversion.

### Effect of Exoglucanase Load on Saccharification

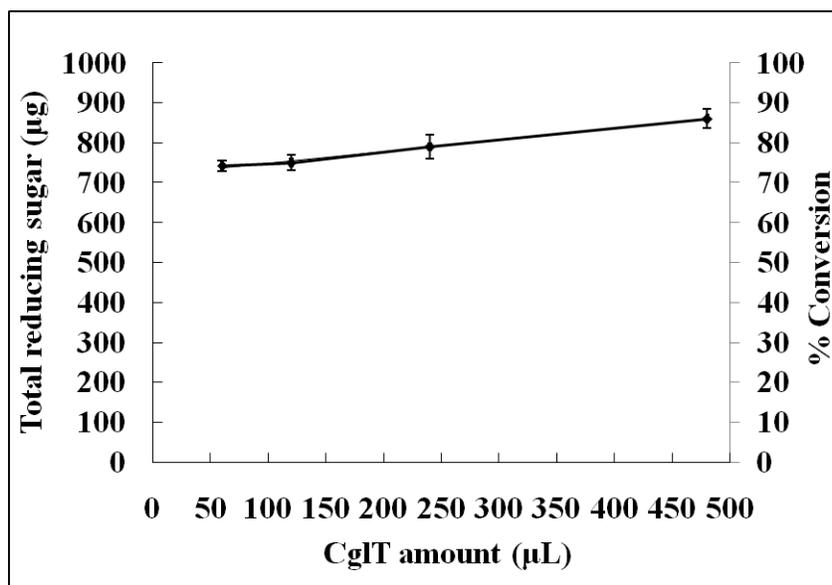
The endoglucanase activity and  $\beta$ -glucosidase activities were still set at 85.9 and 16.6 U/g cellulose. The exoglucanase activities of 0.28 U/g cellulose were set as one-fold, and the proportion of CBHA:CBH48Y was fixed at 1:1 (Fig. 5). As the load of exoglucanases was increased from 0 to 3.0 fold, *i.e.*, from 0 to 0.84 U/g cellulose, the production of total reducing sugar remarkably increased from 80 to 624  $\mu$ g (7.4% to 58% conversion), suggesting a great influence of exoglucanases on the saccharification of regenerated cellulose. In addition, the exoglucanase activities were much less than that of the endoglucanase and  $\beta$ -glucosidase, which was consistent with other reports. Gao *et al.* (2010) recently optimized purified commercial fungal cellulases and found that the optimal activities of endo-1,4- $\beta$ -D-glucanase, exo-1,4- $\beta$ -D-glucanase, and  $\beta$ -D-glucosidase activities were 60.3, 0.32, and 372 U/g glucan, respectively (or 9.0, 14.6, and 3 mg/g glucan, respectively). Obviously, the optimization of both fungal and bacterial cellulases suggests the usage of a heavy  $\beta$ -1,4-glucosidase load (to effectively decompose cellobiose), followed by endo-1,4- $\beta$ -D-glucanase (to provide new ends for exo-1,4- $\beta$ -D-glucanase) and exo-1,4- $\beta$ -D-glucanase, the last to produce cellobiose.



**Fig. 5.** Effect of exoglucanase load on saccharification. One-fold activity of CBHA and CBH48Y was set at 0.30 mU (0.28 U/g cellulose). The endoglucanase activity and  $\beta$ -glucosidase activities were still set at 85.9 and 16.6 U/g cellulose. The load of regenerated cellulose was 2.69 g/L, and the total reaction volume was 400  $\mu$ L. Saccharification levels are presented in the form of total reducing sugar and percent conversion.

### Effect of $\beta$ -glucosidase in the High Enzyme-Load Situation

When all four enzymes were set at the optimized proportion and at double activities (CelD at 515 U/g cellulose, the two exoglucanases together at 1.68 U/g cellulose at a 1:1 ratio, and CglT at 99.6 U/g cellulose), the production of total reducing sugar reached 742  $\mu$ g (68.9% conversion) (Fig. 6).

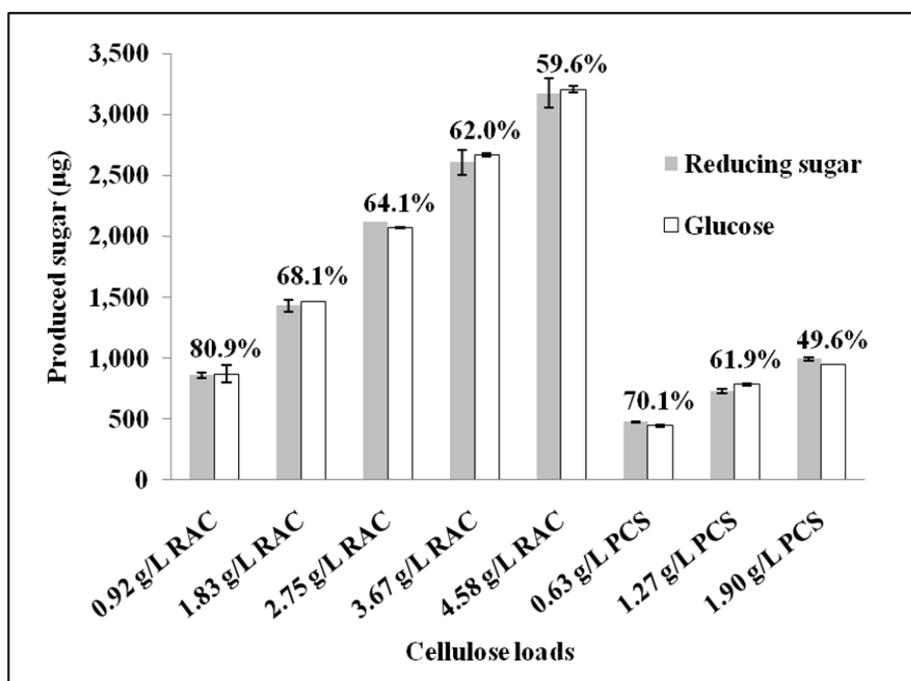


**Fig. 6.** Effect of  $\beta$ -glucosidase in the high enzyme-load situation. The activity of CglT was 1.79 mU/ $\mu$ L. The activity of CelD was 515 U/g cellulose, and the activity of the two exoglucanases together was 1.68 U/g cellulose at a 1:1 ratio. The load of regenerated cellulose was 0.92 g/L, and the total reaction volume was 1170  $\mu$ L. Saccharification levels are presented in the form of total reducing sugar and percent conversion.

To further reduce the middle product inhibition by cellobiose, additional CgIT was added. As the CgIT increased from 60 to 480  $\mu\text{L}$  (from 99.6 to 796 U/g cellulose), the production of total reducing sugar kept increasing, from 742 to 860  $\mu\text{g}$  (percent conversion from 68.9% to 80.0%). Subsequently doubling the activities of all the enzymes, however, the production of total reducing sugar only slightly increased, from 80.0% to 81.5%. Obviously, higher  $\beta$ -1,4-glucosidase load further decreased the inhibitory effect of cellobiose and increased the production of glucose.

### Saccharification under Various Substrate Loads

According to the optimized composition, the activities of CelD, CBHA, CBH48Y, and CgIT were fixed at 554, 0.91, 0.91, and 856 mU per assay, respectively (*i.e.*, for 0.92 g/L cellulose, the CelD, CBHA, CBH48Y, and CgIT were set at 515, 0.84, 0.84, and 796 U/g cellulose, respectively). As the load of regenerated cellulose was gradually increased from 0.92 to 4.58 g/L, the glucose production increased linearly ( $R^2=0.997$ ) from 870 to 3208  $\mu\text{g}$ , each approximate to the amount of production of total reducing sugar, respectively (Fig. 7). However, the percent conversion gradually decreased, from 80.9% to 59.6%. For the saccharification of pretreated corn stover, as the cellulose fraction increased from 0.63 to 1.90 g/L, the glucose production gradually increased, from 444 to 944  $\mu\text{g}$ . However, the percent conversion gradually decreased, from 70.1% to 49.6%.



**Fig. 7.** Saccharification under various substrate loads. The activity of CelD, CBHA, CBH48Y, and CgIT was fixed to 554, 0.91, 0.91, and 856 mU per assay, respectively, and the total reaction volume was 1170  $\mu\text{L}$ . The total reducing sugar production and glucose production are shown. The percent values above the bars indicate the percent conversions, which were calculated based on the glucose production. RAC: regenerated amorphous cellulose. PCS: pretreated corn stover.

The yield of saccharification is affected by several factors, for example, enzyme activities, substrate accessibility and concentration, and product inhibition. All four cellulases remained active throughout the saccharification; however, after incubation at

60 °C for 24 h, CelD remained at about 10% activity, and both CBHA and CBH48Y remained at about 60% activity. The highly thermostable CglT was reported to remain at 97% activity under the same situation (Prawitwong *et al.* 2013). The gradual decrease in enzyme activity might have affected the saccharification. Although the corn stover had been pretreated, with its structure destroyed, the residual hemi-cellulose chains might still have interfered with the cellulose accessibility to cellulases or might have directly blocked the hydrolysis (Viikari *et al.* 2012). Therefore, obviously, the percent conversion of pretreated corn stover was lower than that of the regenerated cellulose (Fig. 7). In addition, the continuous decrease in substrate might also have limited the enzymatic activities; that is why prolonging the saccharification to 48 h did not substantially increase glucose production (data not shown). The strong linear relationship ( $R^2$  was 0.997) between the glucose production and the substrate load suggested that a higher substrate load would stimulate higher enzyme efficiency. Hence, it could be better to maintain a high substrate load and allow periodic supplementation of substrate and enzymes, as well as the removal of products, for higher saccharification efficiency.

The relationship between cellulases and biomass is subtle. Different kinds of biomass are composed of various components and structures and therefore require various cellulolytic enzymes; in other words, specific biomass might also require specific components of cellulolytic enzymes. Fortunately, there are many highly evolved cellulolytic microbes, whose genomes have been sequenced (Rubin 2008), from which different types of efficient enzymes could be recruited into the enzyme cocktails, which, in turn, could possibly enhance the level of biomass saccharification.

## CONCLUSIONS

1. Four cellulases were successfully synthesized in *E. coli* and purified. The composition of a cellulase system using these cellulases was optimized for efficient saccharification of both regenerated cellulose and pretreated corn stover.
2. All four cellulases were essential for saccharification, although the proportion among them was very different.
3. Glucose production was positively correlated with the concentration of regenerated cellulose, implying the importance of a high substrate load in saccharification.

## ACKNOWLEDGMENTS

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