Assembly and Activity of Engineered Minicellulosomes

Qi Xu, John Baker, Bill Michener, Roman Brunecky, Bill Adney, Shi-you Ding and Michael E. Himmel

Goals of our project: (1) Elucidate the mechanism of biomass degradation by *C. thermocellum* cellulosomes; (2) construct active engineered minicellulosomes mainly using the recombinant *C. thermocellum* enzymes; (3) modify *C. thermocellum* to degrade biomass more efficiently.

Table 1. Overexpression in *E. coli* of all fifty-four *C. thermocellum* genes that encode cellulosomal enzymes related to biomass degradation

Module structure	Solubility
Cellulases	
CBM3b-GH5-Doc1	Soluble
GH5-CBM6-FN3-Doc1	Soluble
GH5-Doc1	Soluble
GH5-Doc1	Soluble
GH5-Doc1	Soluble
GH8-Doc1	Partially soluble
CBM4-Ig-GH9-2(Fn3)-CBM3b-Doc1	Soluble
CBM4-Ig-GH9-Doc1	Soluble
Ig-GH9-Doc1	Partially soluble
GH9-CBM3c-CBM3b-Doc1	Partially soluble
GH9-CBM3c-CBM3b-Doc1	Partially soluble
GH9-CBM3c-Doc1	Soluble
GH9-CBM3c-Doc1	Soluble
GH9-CBM3c-Doc1	Partially soluble
GH9-CBM3c-Doc1	Soluble
GH9-CBM3c-Doc1	Partially soluble
GH9-CBM3c-Doc1	Partially soluble
GH9-CBM3c-Doc1	Not determined
GH9-Doc1	Partially soluble
GH9-Doc1	Inclusion bodies
GH48-Doc1	Partially soluble
Xylanases	
CBM22-GH10-Doc1	Soluble
CBM22-GH10-Doc1	Soluble
GH11-CBM4-Doc1-CE4	Soluble
Other hemicellulases	
GH16-Doc1	Soluble
GH18-Doc1	Soluble
CBM-GH26-Doc1	Soluble
GH26-Doc1	Soluble
GH30-CBM6-Doc1	Soluble
GH53-Doc1	Partially soluble
GH81-Doc1	Partially soluble
Putative glycosidases	
GH2-CBM6-Doc1	Inclusion bodies
GH39-2(CBM6)-Doc1	Inclusion bodies
GH43-CBM6-Doc1	Soluble
GH43-CBM13-Doc1	Inclusion bodies
GH43-2(CBM6)-Doc1	Soluble
Xyloglucanhydrolase	
GH74-Doc1	Soluble
Putative carbohydrate esterases	
Fn3-CE12-Doc1-CBM6-CE12	Partially soluble
CE3-CE3-Doc1	Partially soluble
Doc1-CE6	Soluble
CE1-CBM6-Doc1	Partially soluble
Putative pectinases	
GH28-Doc1	Partially soluble
PL1-Doc1-CBM6	Soluble
PL1-Doc1-CBM6-PL9	Soluble
PL10-UN-Doc1	Inclusion bodies
Doc1-CBM6-PL11	Inclusion bodies
Multifunctional components	
CBM30-Ig-GH9-GH44-Doc1-UN	Soluble
GH26-GH5-CBM9-Doc1	Soluble
GH30-GH54-GH43-Doc1	Partially soluble
GH54-Doc1-GH43	Inclusion bodies
GH54-GH43-Doc1	Partially soluble
CE1-CBM6-Doc1-GH10	Partially soluble
CBM22-GH10-CBM22-Doc1-CE1	Partially soluble
GH5-Doc1-CE2	Partially soluble

Part A: Optimization of reaction conditions for *C. thermocellum* cellulase

The following combinations of buffer and pH were evaluated for use in assay of *C. thermocellum* cellulase activity on crystalline cellulose (1 mg/mL total protein acting against 1 % Avicel at 40º C, with 300 mM NaCl, 1 mM CaCl2 in all assay mixtures):

- 50 mM Tris, pH 7.0
- 50 mM Tris, pH 7.0, 300 mM imidazole
- 20 mM MES, pH 6.0
- 20 mM acetate

The results (Figure 1) showed that native *C. thermocellum* cellulases were more active at pH 6.0 (20 mM MES) and at pH 5.0 (20 mM acetate). In our further work, cellulosome and minicellulosome activities were assayed at pH 6.0 in 20 mM MES (with NaCl and CaCl2 as noted above), because some recombinant cellulosomal enzymes were not stable at pH 5.0.
(Note: *C. thermocellum* cellulase was prepared from the total protein of cell-free broth of *C. thermocellum*, ATCC 27405, grown on 0.7 % Avicel.)

Part B: Comparison of *C. thermocellum* and fungal cellulases

The following three samples were used for activity assaying against 1 % avicel:

- Native cellulosomes (Cth, see the Part A)
- Total cell-free protein from culture broth of a new species of *Trichoderma* (Tne)
- GC220---commercial enzyme mixture produced from *T. reesei* (Tre)

(Note: reaction conditions as described in Part A)
The following conclusions could be drawn from Figure 2:

- Fungal cellulase activity (Tne: 61.7 % and Tre: 54.8 % conversion) was stronger than that of *C. thermocellum* (31.8 %).
- After a lengthy (155-hour) digestion the major product of digestion by fungal enzymes was glucose, whereas products of *C. thermocellum* cellulase digestion contained slightly more cellobiose than glucose (Table 2).
- Somewhat unexpectedly, higher cellodextrins, such as cellotriose and cellotetraose, were not detected at digestion times ranging from 14 to 155 hours apparently.

Table 2. Ratio of cellobiose and glucose converted by cellulases

	Cellobiose (%)	Glucose (%)
Cth	53.1	46.9
Tne	5.0	95.0
Tre	5.7	94.3

Part C: Library of cellulosomal enzymes for assembly of engineered minicellulosomes:

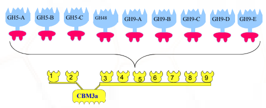
- All **fifty-four** of the *C. thermocellum* cellulosomal genes identified as being related to biomass degradation, including cellulases, hemicellulases, pectinases and multifunctional modules, have been cloned and overexpressed in *E. coli* (Table 1). **Forty-six** of these were soluble or partially soluble, and we are working on making all 54 enzymes soluble.
- The activities of 23 purified recombinant cellulases have been assayed, and 16 of them have apparent activity on MUC (4-methylumbelliferyl-*beta*-D-cellobioside)(Table 3).

Table 3. All *C. thermocellum* cellulosomal cellulases and their activities on MUC

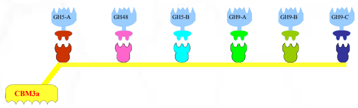
	GH5	GH8	GH9	GH48
Number in genome	7	1	16	1
Number soluble	7	1	14	1
Number active on MUC	7	0	8	1

Part D. Assembly of engineered cellulosomes

Our first goal is to construct active, engineered ("mini-") cellulosomes for efficient degradation of crystalline cellulose.
Two methods have been designed and employed for the assembly of engineered minicellulosomes:
(1) In order to do activity-screening of recombinations of various fusion enzymes for new engineered minicellulosomes from our cellulosomal enzyme library, a truncated *cipA* with 9 cohesins of the same type (without X domain and dockerin II) and 9 recombinant enzymes with that type of dockerin, applied in equal molar loadings, were used for undirected (by the experimenter) assembly of engineered minicellulosomes. Assay of their activity is in progress.



(2) In a more directed, engineered exercise, a chimeric scaffoldin with 6 cohesins taken from six different species was used for the specifically-ordered assembly of 6 *C. thermocellum* enzymes, each of which had been fused to a dockerin corresponding to one of the cohesins.



Part E. Activities of engineered minicellulosomes -- Effects of *beta*-glucosidase

Avicelase activity of the engineered minicellulosome from Part D(1) was determined, either alone (MC) or in the presence (MC+Beta-G) of added fungal beta-glucosidase (20 µg/ml). Reaction conditions: Please see Part C. The results demonstrated the feasibility of engineered minicellulosomes.

- The engineered minicellulosome showed some activity, but its activity was half of that of *C. thermocellum* cell-free total protein (Cth)(Figure 3 and Table 4).
- Beta-glucosidase helped to increase the glucose production, but had little effect on total conversion (Figure 3 and Table 4).

Table 4. Ratio of cellobiose and glucose converted by native and mini-cellulolosomes

	Total Conversion of avicel (%)	Percentage of cellobiose (%)	Percentage of glucose (%)
Cth	31.8	53.1	46.9
MC	13.5	77.3	22.7
MC+Beta-G	13.9	15.3	84.7

Figure 1. The Effect of Reaction Conditions on Activity of Native Cellulosome

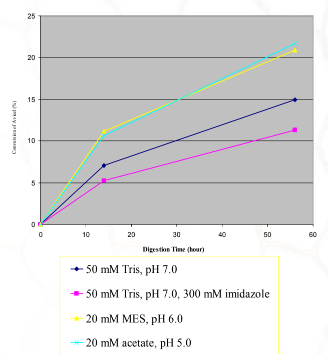


Figure 3. Saccharification of Avicel by Minicellulosomes

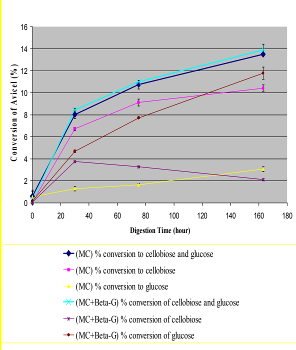
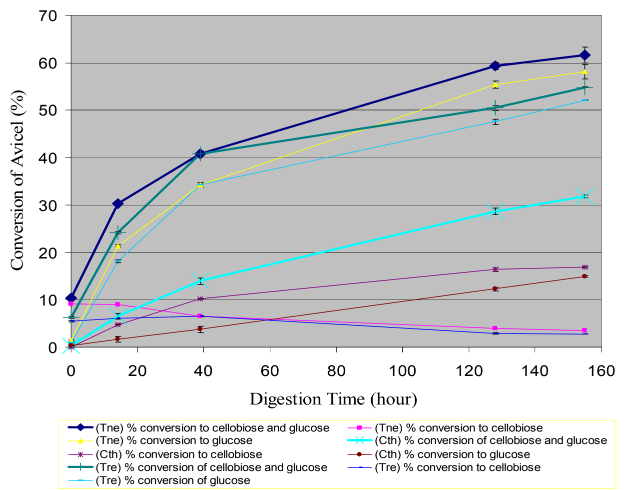


Figure 2. Comparison of cellulase activity among three species



Presented at the BEST Retreat Meeting, 21-24 June 2009, Asheville, North Carolina

The BioEnergy Science Center (BESC) is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science

NREL is a national laboratory of the U.S. Department of Energy, Office of Energy Efficiency and Renewable Energy, operated by the Alliance for Sustainable Energy, LLC. • NREL/PO-270-46338