Electronic Supplementary Material: Saccharification of thermochemically pretreated cellulosic biomass using native and engineered cellulosomal enzyme systems

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1. Biomass composition

The composition of untreated, ammonia fiber expansion (AFEX) and dilute-acid treated switchgrass (SG), on a dry weight basis (dwb), was estimated using standard NREL LAP Protocols (http://www.nrel.gov/biomass/analytical_procedures.html) and is listed in the table below (dry weight basis or dwb). The composition of Avicel used in this study was predominantly enriched in cellulose (~98-99%; dwb).

Sample Description	Glucan	Xylan	Galactan	Arabinan	Mannan	Ash	Water Extractives	Ethanol Extractives	Extractive-free Ash	Acid-Soluble Lignin	Acid-insoluble Lignin
Untreated/AFEX treated SG	31.0	19.2	1.8	3.3	0	5.2	14.5	3.7	3.7	6.1	17.8
Dilute Acid Pretreated SG	48.2	6.8	0.0	0	0	4.8	3.9	8.9	5.9	2.1	24.9

2. Production and characterization of the rosettazyme complexes

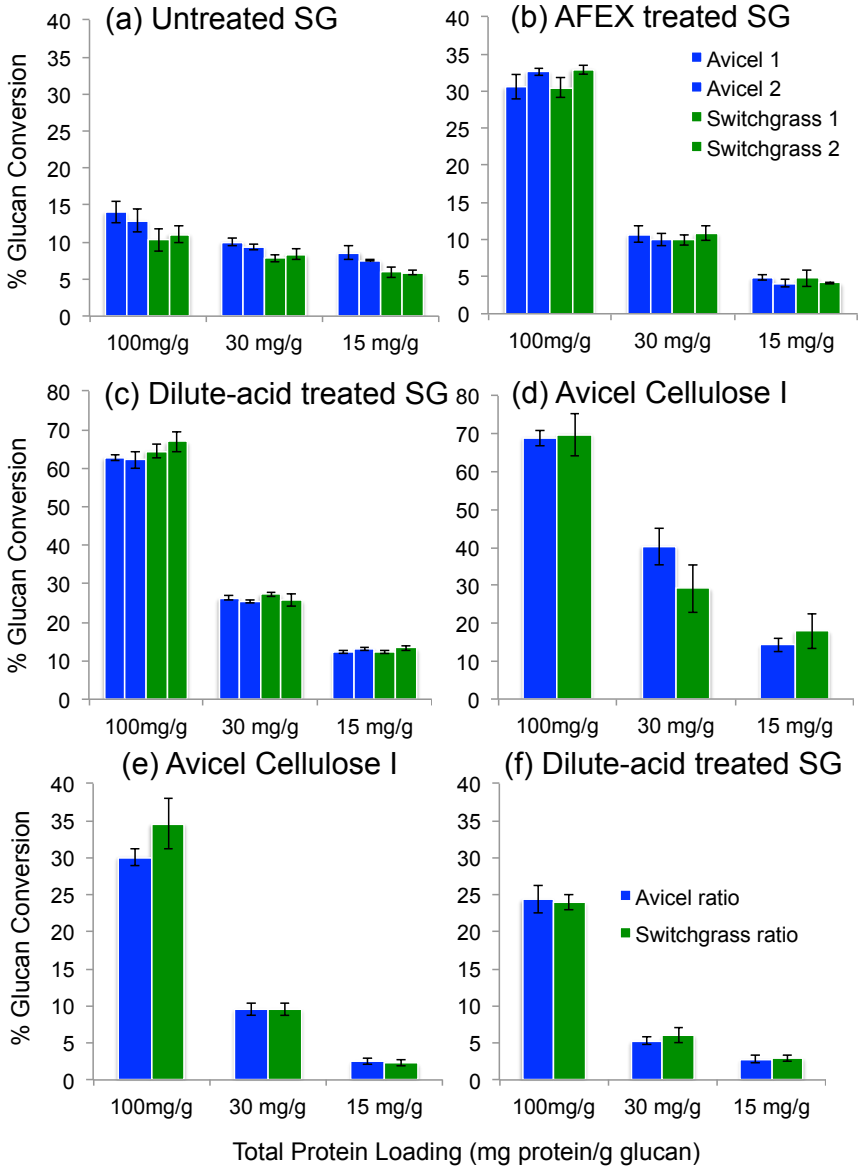
For purification of the soluble enzyme (Cel9F, Cel9K, Cel8A, Cbh9A, Cel5B, Xyn11A, Xyn10C, Xyn10Z, Man5A and CtXynGH30) fraction, frozen cells (-80°C) were thawed (0.25 mL/g wet weight) in a protease inhibitor (PI) solution (Sigma-Aldrich, St. Louis, MO), the cell suspension was sonicated (Branson Sonifier 450, 60% duty cycle, output 6) 3x for 2 min each with a one-minute pause between cycles and centrifuged (17,000xg, 4°C, 30 min). Heat-labile *E. coli* proteins were precipitated from the supernatant by heating in a water bath (65°C, 30 min) cooling on ice for 10 min and centrifugation (17,000xG, 4°C, 30 min). The supernatant was filtered (0.45 µm, PES membrane, Millipore) diluted in 25 mM HEPES, 1 mM EDTA pH 7.5 and the enzymes were purified chromatographically using a MonoQ column (Amersham Biosciences) and an elution gradient of 1 to 400 mM NaCl in 25 mM HEPES, 1 mM EDTA pH 7.5 for all enzymes except Cel9K, which used 20 mM Tris-HCl, pH 8.0. The proteins were concentrated using Vivaspin 30,000 MWCO centrifugal concentrators (Sartorius). Buffer was exchanged using a Bio-Rad EconoPac 10DG-desalting column equilibrated with 20 mM Tris-HCl at pH 8.0. All proteins were purified to electrophoretic homogeneity.

For purification of enzymes (Cel48S, Cel9R) in the insoluble fraction, cell pellets were sonicated as above, centrifuged (17,000xg, 4°C, for 30 min), and the pellet was suspended in 20 mM Tris-HCl, 1 mM EDTA, 0.5% Triton X-100 at pH 8.0 and PI Cocktail from Sigma (500 µL PI cocktail per 10 mL Tris/EDTA/Triton X-100 solution). After centrifugation (as described above), pellets were rinsed in 20 mM Tris-HCl pH 8.0 with PI Cocktail (500 µL PI Cocktail per 20 ml Tris solution). Proteins were unfolded in 5M urea, 0.1M Tris (pH 8.0) (10 mL/g original cell pellet) for 60 min at room temperature. The solution was centrifuged as before and proteins were refolded by 20-fold dilution into 20 mM Tris-HCl, 1.5 mM cellobiose, 0.1 mM EDTA, pH 8.0, with stirring. The protein solution was concentrated to 25 mL in a stirred cell apparatus with 30K NMWL PES membrane filter (Millipore), diluted to 50 ml with 20 mM Tris-HCl (pH 8.0), and filtered (0.22 µm PES syringe filter). The filtered Cel9R protein solution was separated chromatographically (MonoQ) with a linear elution gradient (1 to 400 mM NaCl in 20 mM Tris-HCl pH 8.0), and concentrated using Vivaspin 15 30,000 MWCO centrifugal concentrators (Sartorius). For Cel48S, the protein solution was diluted with 20 mM Tris-HCl (pH 8.0) and concentrated, using Vivaspin 15 30,000 MWCO centrifugal concentrators (Sartorius), until the urea concentration was below 50 µM. Both refolded proteins were purified to electrophoretic homogeneity. The refolded enzyme, Cel48S, had measurable but low activity on crystalline cellulose that was comparable to previous reports.[1] This result is not surprising considering that

Cel48S has been reported in the literature to give very low activity on cellulose unlike other clostridial cellulases.[2] Our approach to prepare recombinant Cel48S has been used previously to solve the crystal structure of Cel48S[3] and characterize the activity of recombinant Cel48S[1] on crystalline cellulose.

3. Varying enzyme composition of native cellulosomes and rosettazymes has limited impact on their substrate-specific hydrolysis yields

Cellulosomes isolated from *C. thermocellum* grown on microcrystalline cellulose (Avicel 1 or 2) or pretreated switchgrass (Switchgrass 1 or 2) were tested on switchgrass (untreated; a, AFEX treated; b, dilute-acid treated; c) and Avicel (cellulose I; d) with total protein loadings ranging from 15 to 100 mg/g glucan. All assays were carried out for 24 hours. Note that 1 and 2 depict two replicate cell cultures used to isolate the cellulosomes (see figure and details in previous work[4]). As discussed in the paper, there were no significant differences in the activity of the two distinct cellulosomes composition on various substrates tested. Similarly no significant difference was observed in the activity (on Avicel Cellulose I; e, or Dilute-acid treated switchgrass; f) of the twelve-enzyme rosettazyme complex with enzyme composition mimicking the composition of cellulosomes isolated from *C. thermocellum* grown on Avicel (Avicel ratio) or dilute-acid pretreated switchgrass (Switchgrass ratio).



Similar results were also seen for xylose yields for both cellulosomes and rosettazymes of distinct cellulase composition (data not shown).

4. Additional enzymatic hydrolysis data for purified *T. reesei* cellulases on crystalline cellulose I and cellulose III allomorphs

Enzymatic assays were carried out using combinations of purified fungal cellulases Cel7A (CBH I), Cel6A (CBH II), and Cel7B (EG I) on cellulose I and III. All fungal cellulases were purified from a Trichoderma derived cellulase cocktail as described elsewhere.[5] Enzymatic hydrolysis assays were carried out similarly to as described in experimental section. To minimize end-product inhibition and simultaneously hydrolyze sugar oligomers into monosaccharides, a fungal-derived β-glucosidase[5] was added at 5% of the total mg of cellulase loaded in each assay. Assays were done in triplicates for a combination of single, binary or ternary mixtures of CBH I, CBH II and EG I (as shown in table below) at total enzyme loadings ranging from 5-30 mg/g glucan. All samples were incubated in a shaker at 200 rpm, with a bead in each well to facilitate mixing, for 24 hours. Commercial Trichoderma cellulase cocktail included as a control for these experiments was Spezyme CP (supplemented with Novo 188 at 15 mg/g glucan each). Composition of Spezyme CP is similar to Accellerase 1500 as described elsewhere.[6] Data shown below for all assays using purified fungal cellulases has been reproduced from our previous study[7] and similar findings have been reported in another study[5] as well. The two key findings and interpretations from these experimental results were;

(i) Synergistic combinations of purified fungal exo- and endo-cellulases gave preferentially higher hydrolysis yield on pretreated crystalline cellulose III versus native cellulose I.

(ii) Combination of the major cellulase components of a Trichoderma cocktail (Cel7A, Cel6A and Cel7B) can reproduce the cellulosic substrate preferences seen for a crude commercial cellulase cocktail. Analogously, a twelve-enzyme rosettazyme complex also partly reproduces the patterns of cellulosic substrate preferences seen for the native cellulosome complex.

					Cellulose I		**Cellulose III**	
	Enzyme loading (mg/g glucan)			**Total Cellulase**	**Glucose Yield** *Mean*	*Std Dev*	**Glucose Yield** *Mean*	*Std Dev*
Expt #	**CBH I**	**CBH II**	**EG I**	**Loading**				
1	5	0	0	5	5.7	1.4	3.3	0.5
2	0	5	0	5	5.8	0.3	3.1	3.1
3	0	0	5	5	2.0	0.1	5.1	1.1
4	5	5	0	10	19.0	0.2	19.6	1.0
5	0	5	5	10	14.9	1.4	14.3	2.4
6	5	0	5	10	15.5	0.2	26.1	1.2
7	5	5	5	15	39.9	0.6	78.3	6.0
8	10	0	0	10	7.0	0.3	4.5	1.3
9	0	10	0	10	6.8	0.9	3.7	0.2
10	0	0	10	10	2.7	0.3	4.8	0.3
11	10	10	0	20	22.9	4.6	28.5	0.2
12	0	10	10	20	17.3	1.0	22.3	2.0
13	10	0	10	20	25.9	1.2	47.8	8.6
14	10	10	10	30	52.0	4.6	90.2	0.5
Commercial Trichoderma cellulase cocktail				30	52.7	2.8	82.3	4.6

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