Electronic Supporting Information

Fungal Cellulases and Complexed Cellulosomal Enzymes Exhibit Synergistic Mechanisms in Cellulose Deconstruction

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Cellulosome Activity Enrichment by Purification

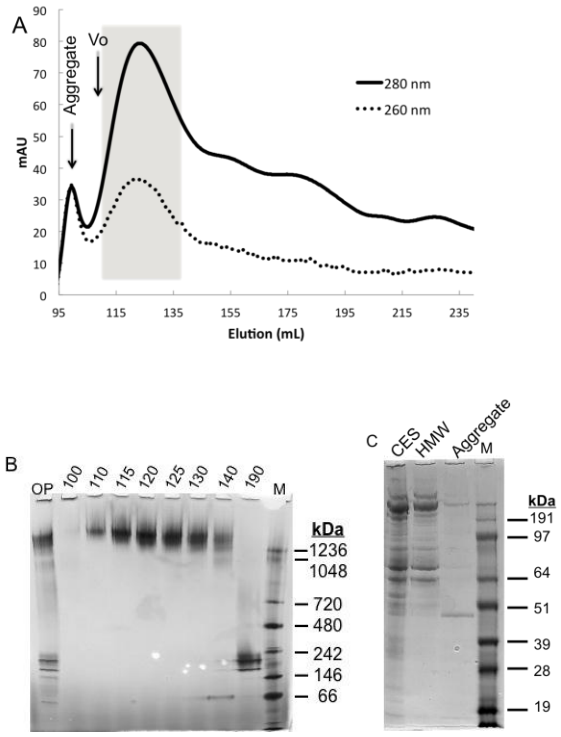


Fig. S1 Size Exclusion Chromatography (SEC) purification of High Molecular Weight (HMW) cellulosomes. Concentrated cellulosome-enriched sample (CES) was applied to a HiPrep Sephacryl HR S400 26/60 SEC column (GE) to separate HMW cellulosomes (> 1 MDa) (110-135 mL elution volume) from aggregates (95-110 mL) and free enzymes (> 140 mL). The (A) SEC chromatogram of UV absorbance at (solid lines) 280 nm and (dotted line) 260 nm. CES on put (OP) and eluted fractions were analyzed by (B) native PAGE on a Novex 3-12% Bis-Tris Gel (Invitrogen) along with (B, Lane M) NativeMark™ Unstained Protein Marker. HMW cellulosome fractions in the elution volumes between 112-135 mL (highlighted) were pooled. The CES, pooled HMW, and aggregate compositions were also compared on (C) 3-12% Novex (Invitrogen) SDS-PAGE and stained with Colloidal blue protein stain kit (Life Technologies) then scanned with HP image scanner.

The cellulosome preparation used in this study was produced from the culture-filtrate of *C. thermocellum* by successive affinity-selection (binding to microcrystalline cellulose), elution from the cellulose with 1% triethylamine, and subsequent concentration over a 300 kDa nominal-pore-size ultrafiltration membrane to produce a cellulosome-enriched secretome (CES), which was then further fractionated by size-exclusion chromatography, according to the general procedure outlined in Lamed *et al*. [1]

Free cellulases, non-cellulolytic proteins, and aggregated proteins are present along with the cellulosomes in the extracellular growth medium. We used size exclusion chromatography (SEC) to separate the high molecular weight (HMW > 1MDa) cellulosomes from the non-cellulosomal (smaller) and aggregated (much larger) material. Fig. S1A illustrates separation and pooling of fractions containing the HMW cellulosomes. We used

native PAGE to identify the fractions that contained the HMW cellulosomes (Fig. S1). The fractions that eluted between 112 and 135 mL contained a discrete HMW cellulosome band and were pooled and concentrated to 1 mg/mL for enzymatic characterization. The peak eluted before the void volume (Vo, Fig. S1A) contained a combination of aggregated proteins and potentially nucleic acid indicated by the absorbance peak at 260 nm depicted in the SEC chromatogram. The two prominent bands found in the aggregated peak were identified by trypsin digestion followed by MS/MS and identified as CipA (197 kDa) and ABC transport protein CbpB (50 kDa) (Fig S1C).

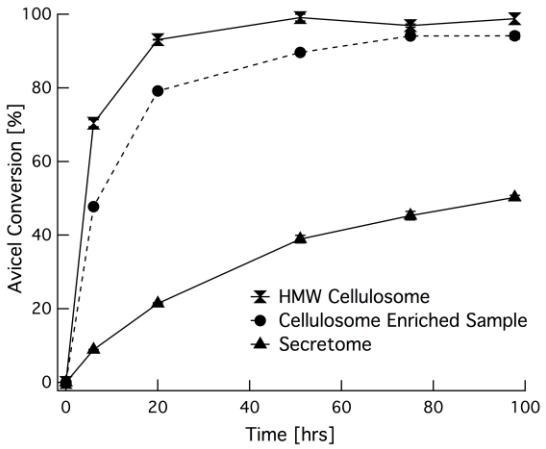


Fig. S2. Enhanced activity of chromatographically selected cellulosome fraction. Enzymatic digestion of Avicel PH-101 by SEC-purified cellulosomes (—), cellulosome-enriched secretome (---), and *C. thermocellum* secretome (···). 10 mg of protein was loaded per g of Avicel in a 1% solids loading.

The *C. thermocellum* secretome, cellulosome-enriched sample (CES), and the HMW cellulosomes were compared for their enzymatic activity on Avicel. The enzymes mixtures were loaded equally at 10 mg/g. Fig. S2 shows that purification of the HMW cellulosome increases the specific activity. Twenty hours into the digestion cellulosome purification improved the activity by 14% compared to the cellulosome-enriched secretome and 4-fold with respect to the secretome.

Optimization of Cellulosome Enzymatic Activity Conditions

Fig. S3 shows the overall results for optimization of cellulosome enzymatic activity as a function of the presence of sulfhydryl protectants, β-glucosidase, and $CaCl_2$. Some crucial enzymes of the *C. thermocellum* cellulosome are oxygen-sensitive.[2, 3] Thus, we used cysteine as a reducing agent to remove oxygen from the enzymatic assays.[4] Fig. S3 also shows the sccarification of Avicel by the HMW cellulosomes assayed with or without cysteine protectant. In the absence of cysteine, we observe low Avicel conversion, which is consistent with the observation that certain cellulosomal enzymes are subject to oxygen-induced inactivation.[2]

Additionally, calcium stabilizes cohesin-dockerin interactions and other cellulosomal domains,[4-7] and it is necessary for catalysis in some cellulosomal enzymes.[4] Ten mM $CaCl_2$ was maintained in all assays, and EDTA was added (with Ca^{2+} kept in molar excess over the EDTA) to scavenge trace amounts of other transition metal ions that may promote oxidation of sulfhydryl groups.[4] Lastly, because many cellulases, including important members of the cellulosomal array, are known to be strongly inhibited by cellobiose, we added a β-glucosidase that had been chromatographically purified from a commercial *Aspergillus niger* preparation (Novozyme 188, Novozymes USA) to mitigate product inhibition.[4, 7, 8]

The addition of both sulfhydryl-protectants and β-glucosidase results in a dramatic increase in activity in comparison with the activity observed in the absence of either of these two adjuvants. In addition to the effects of adding protectants and a β-glucosidase, slightly higher sustained activities are attained when the digestions are conducted under anaerobic conditions (Fig. S4). The differences in activity observed between the anaerobic and aerobic assays, however, are small compared to the differences between the reactions with and without protectants and β-glucosidase.

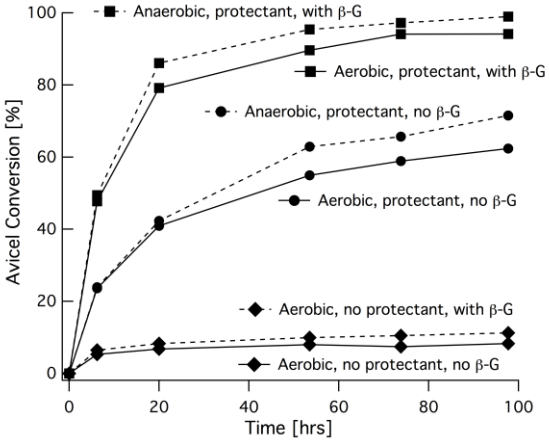


Fig. S3 Optimization of cellulosome activity on Avicel as a function of aerobic or anaerobic conditions, β-glucosidase presence, and the presence of chemical protectants. The optimized conditions, described in the text, were used in the remainder of experiments in this study for activity assay containing cellulosomes.

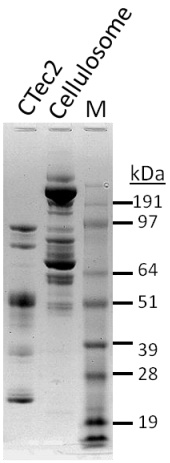


Fig. S4 SDS-PAGE of CTec2 and purified HMW cellulosomes.

Free and Complexed Enzymes Have Similar Ability to Digest Untreated Switchgrass

To test the digestion of unpretreated biomass (switchgrass), we used high loadings (50 mg/g) of cellulosomes and CTec2. We observe that the long-term conversion was identical using the two different enzymatic systems (Fig. S5). This shows that the two enzyme systems have similar ability to degrade the accessible cellulose.

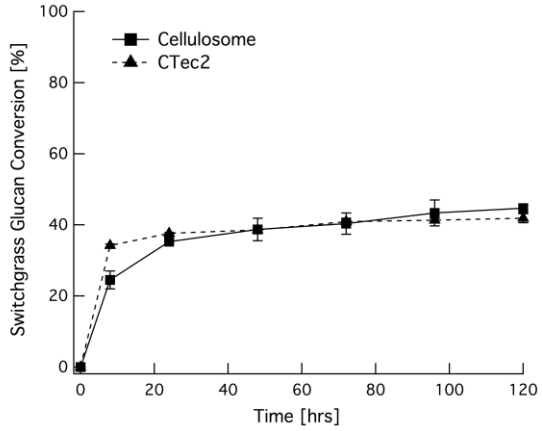


Fig. S5 Digestions of untreated switchgrass by the HMW cellulosome and CTec2 enzyme systems. 2% solids slurry was mixed with enzymes loaded at 50 mg/g.

Low Biomass Conversion by Cellulosomes is Not Due to the Lack of Hemicellulase Enzymes

Cellulosomes are known to contain hemicellulase enzymes.[9] To explore the hypothesis that hemicellulase enzymes associated with the cellulosome are insufficient to enable effective degradation of complex cell wall carbohydrates, we added purified hemicellulases to the cellulosome to assay the scarification of pretreated switchgrass. We used the following hemicellulase enzymes: acetylxylan esterase (Axe), arabinofuranosidase (AbfB), furilic acid esterase (Fae), β-1,4 xylanase (XynA), and xylobiase (XylD)[10]. The total amount of protein loaded was 10 mg of protein per g of cellulose. The "cellulosome only" reaction contained 10 mg/g of purified cellulosome. The "hemicellulase-augmented" reaction contained 5 mg/g of the HMW cellulosome plus five hemicellulases loaded at 1 mg/g of each hemicellulase for a total of 10 mg of protein per g cellulose. We found that substituting hemicellulases for part of the cellulosome loading actually reduced the overall conversion (Fig. S6). This result confirms that lowering the cellulosome loading reduces the conversion, and indicates that the poor conversion by the cellulosome is not due to a deficiency of hemicellulases.

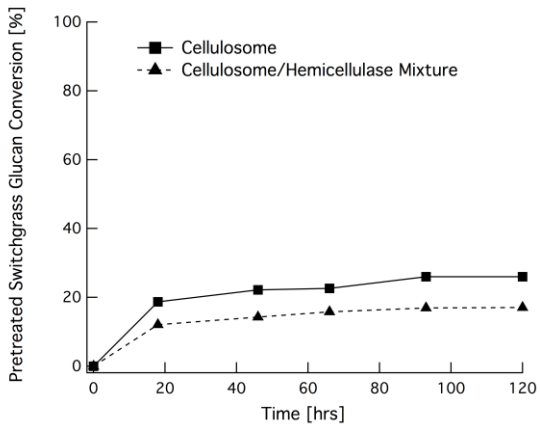
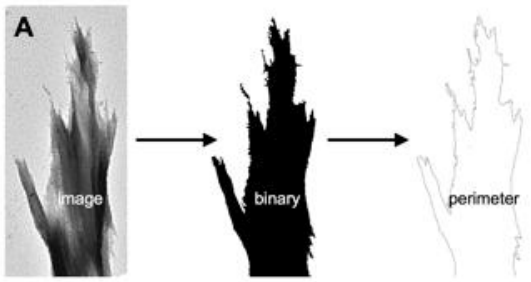


Fig. S6 The effect of hemicellulase addition on cellulosome scarification of pretreated switchgrass. Cellulosomes were loaded either alone at 10 mg/g (−) or at 5 mg/g of cellulosome in combination with 1 mg/g of each hemicellulose (Axe, AbfB, XynA, XylD, and Fae) (---). The reactions were loaded with 2% biomass solids and incubated at 60°C in 25 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 10 mM L-cysteine, 10 mM CaCl2, 2 mM EDTA and 2 mg/g β-glucosidase.

TEM Analysis



B

	Particles	Perimeter [µm]	Std Dev
Ctec2	12	8.39	2.65
C'some	16	16.00	3.33
Combo	8	17.10	2.49

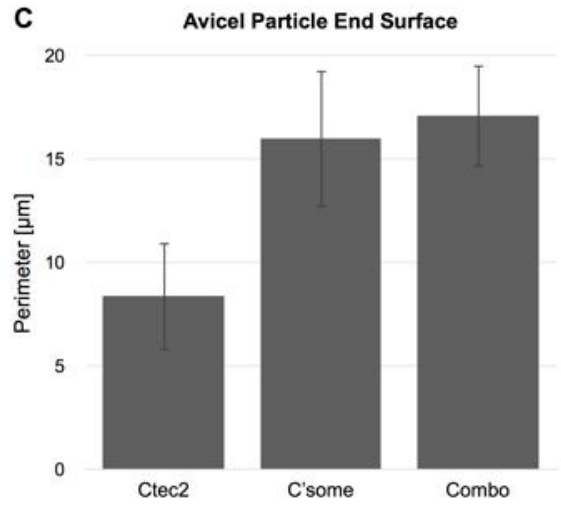


Fig. S7 Digested Avicel particle image analysis. TEM micrographs were processed using ImageJ software to generate a (A) binary image of the tapered, splayed or combination morphology particle ends and the *analyze particles* tool was used to calculate the perimeter of the binary objects. (B) The number of Avicel particles analyzed, perimeter measured, and standard deviation is presented in table and (C) graphical form.

Representative TEM micrographs are presented in Fig. S8. The principal observation from the imaging of these digested biomass samples was that there is extreme variability within each sample and that it is not straightforward to determine consistent morphological properties that distinguish the CTec2 digested samples from the cellulosome-digested samples (Fig. S8A' and S8B'). However, the immuno-localization of enzyme in the pretreated biomass particles does give some insight into enzyme penetration into the particles (Fig. 3 in the main text).

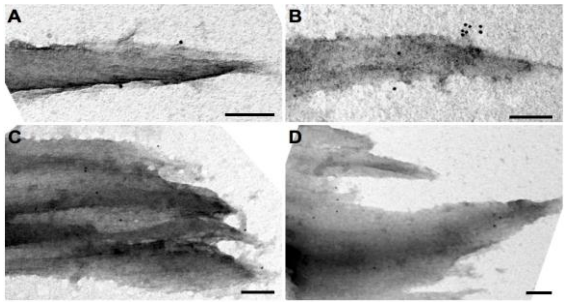


Fig. S8 TEM micrographs of immuno-labeled Avicel PH-101 digested with CTec2 for 120 hours (A, B) or HMW cellulosomes for 24 hours (C, D) to achieve a cellulose conversion of ~65% in each case. Samples were immuno-labeled with 15 nm gold conjugated antibodies that appear as black spots on the micrographs to localize Cel7A enzymes (A,B) or the cellulosome scaffoldin protein (C,D) on or within the cellulose microfibril bundles. Scale bars = 200 nm.

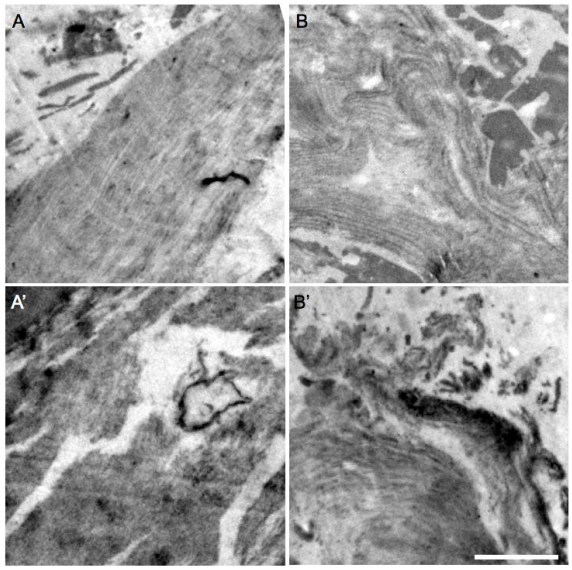


Fig. S9 TEM micrographs of (A, B) dilute acid pretreated switchgrass samples and (A', B') enzymatic digestions of pretreated switchgrass. The dilute acid pretreated biomass particles (A, B) display extensive fracturing and delamination within the cell walls from the milling and pretreatment process. The pretreated particles digested for 24 hours with (A') CTec2 or with (B') cellulosomes for 24 hours displayed extensive variability in cell wall morphology and patterns of deconstruction. There were not

obvious differences in the morphological properties of the biomass cell walls that would explain the difference in the performance of free and complexed enzymes. Scale bar = 2 µm.

Cellulosome pH and Temperature Activity Optima

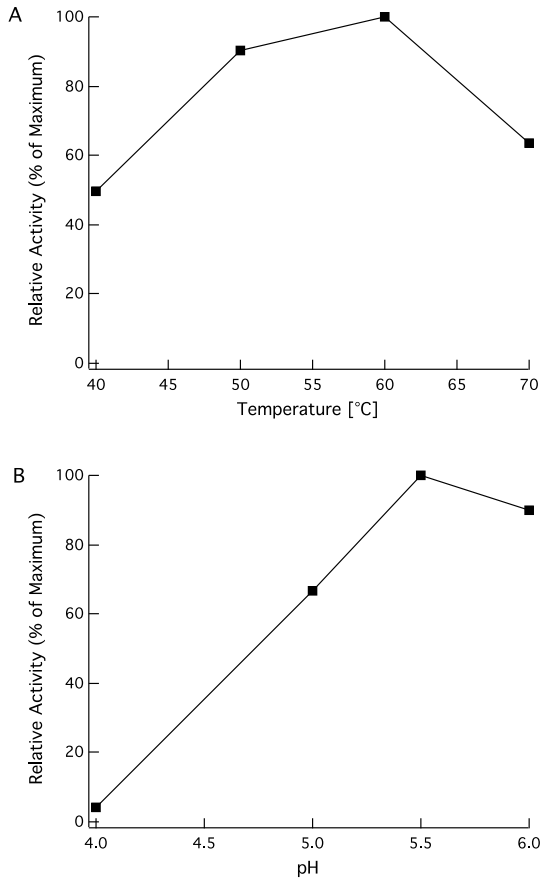


Fig. S10 Effect of (A) temperature and (B) pH on the enzymatic activity of purified HMW *C. thermocellum* cellulosomes on Avicel. Cellulosomes were loaded at 5 mg/g in a 1% Avicel slurry. Temperature digestions were conducted in buffer containing 30 mM Na-Ac pH 5.5, 100 mM NaCl, 10 mM $CaCl_2$, 2 mM EDTA and incubated at the temperatures indicated. The effects of pH were measured in buffer containing 100 mM NaCl, 10 mM $CaCl_2$, 2 mM EDTA and Na-Ac pH 4.0-6.0. pH was measured after the digestion mixture was combined to confirm a stable pH. Digestions were conducted in 0.7 mL volumes and agitated by continuous mixing at 10 rpm. After 24 hours, 100 µL of each reaction was sampled and the cellobiose and glucose was quantified by HPLC. The percentage of maximum was calculated by dividing the percent conversion by the maximum conversion obtained at the same 24-hour time point.

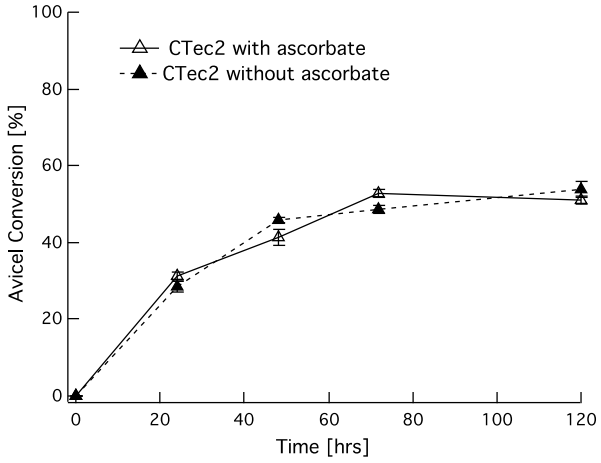


Fig. S11 The effect of ascorbic acid on the digestion of Avicel PH-101 by CTec2.

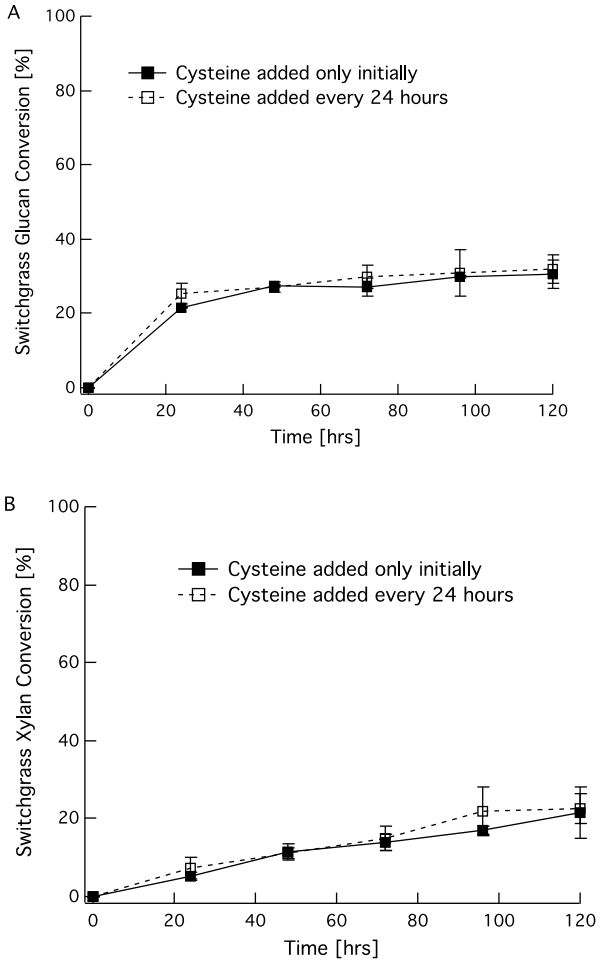


Fig. S12 Enzymatic digestion of pretreated switchgrass with the addition of 10 mM cysteine every 24 hours (❑) or added initially (■). The cellulosome reactions were loaded with 1% biomass solids and incubated at 60°C in 25 mM sodium acetate buffer, pH 5.5, containing 100 mM NaCl, 10 mM L-cysteine, 10 mM $CaCl_2$, 2 mM EDTA and 2 mg/g β-glucosidase. Samples were measured every 24 hours for (A) glucose, cellobiose, and (B) xylose release.

Methods

Isolation of the Secretome and the Cellulosome Enriched Sample from C. thermocellum

C. thermocellum was grown on Avicel PH-101 according to reference 12. The secretome was separated from the cellular debris by centrifuging the cells at 12,000 x g for 30 min at 4°C. The cellulosome-enriched sample was isolated by ammonia sulfate precipitation. After the ammonium sulfate dissolved completely, a precipitate slowly formed and was collected by centrifugation (~8°C, 7000 RPM, 15 min). The supernatant fluids were discarded and the pellet fraction was dissolved in PBS (~300 mL). The clarified supernatant, enriched with cellulosomal and non-cellulosomal components, was filtered via a 0.2 micron filter. The filtrate was then applied to an ultrafiltration device with a nominal molecular-weight cut-off of 300 kDa (Millipore) at 4°C. After reduction of the solution to about 200 mL, the concentrate was analyzed by SDS-PAGE. The cellulosome-enriched secretome was then dialyzed against Tris-buffered saline (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.2) overnight at 4°C (3-L volume x 4 buffer changes). For enzymatic activity assays protein concentration was measured using the BCA protein determination kit (Pierce).

Fractionation of HMW Cellulosomes

5 mL of the cellulosome enriched sample was loaded on a Sephacryl S-400 26/60 SEC column (GE) to purify the high molecular weight (HMW) cellulosomes based on the method described in reference.[1] Separation was run at 1.5 mL/min and 0.5 mL fractions were collected. Elution was monitored by the absorption at 280 nm. Fractions were collected and analyzed using denaturing and native poly-acrylamide gel electrophoresis (PAGE) to identify the cellulosome-containing fractions (Fig. S1). For enzymatic activity assays protein concentration was measured using the BCA protein determination kit (Pierce).

Poly-Acrylamide Gel Electrophoresis

Twenty µL of each sample were mixed with 4x Native Page loading buffer (Life Technologies) and loaded on a Native-PAGE Novex Bis-Tris 3-12% gel (Life Technologies), which utilizes the G-250 compound to eliminate the protein charge effect on electrophoretic migration. The Native gels were run at 4°C at 150V for 120 min.

For denaturing gel electrophoresis protein mixtures were mixed with 4x LDS sample buffer (Life Technologies) and run on a NuPAGE Novex Bis-Tris 4-12% gel (Life Technologies). Proteins were visualized by staining with Colloidal Blue Protein Stain (Invitrogen) and imaged on an HP image scanner (Hewlett Packard).

Hemicellulase Enzyme Purification

Hemicellulase enzyme genes from *Aspergillus niger*, AbfB, XynA, and XlnD, were transformed separately into *Aspergillus nidulans*, as were the genes for *Penicilium funiculosum* FaeA and *Hypocrea jecorina* FaeA. All of the hemicellulase genes were then expressed and purified chromatographically and have been shown to have activity at pH 5.0 and 60°C.[10, 13, 14]

Biomass samples

P. trichocarpa x *P. deltoides* F1 hybrid were grown in Oak Ridge, TN and received no cultural treatment. The Switchgrass was the lowland cultivar Alamo, and the sampling location was Ardmore, Oklahoma. Both were air dried in Colorado for approximately one month to a moisture content of ~15-20%. The poplar logs were debarked, split with an axe, and chipped using a Yard Machines 10HP chipper. The chips were then milled in a Thomas Scientific Wiley Mill (Model 4) using a 1mm screen size. Prior to pretreatment the material was sieved to less than 20 mesh and greater than 80 mesh using a W.S. Tyler Sieve (Model: RX-29 Type: Rotap).

Mass Spectrometry Analysis

To identify proteins, we excised the SDS-PAGE protein gel bands and sent them to the Colorado State Proteomics facility for Trypsin Digestion and Peptide identification. Peptides were purified and concentrated using an on-line enrichment column (Agilent Zorbax C18, 5□m, 5 x 0.3mm). Subsequent chromatographic separation was performed on a reverse phase nanospray column (Agilent 1100 nanoHPLC, Zorbax C18, 5□m, 75 □m ID x 150mm column) using a 42 minute linear gradient from 25%- 55% buffer B (90% ACN, 0.1% formic acid) at a flow rate of 300 nanoliters/min. Peptides were eluted directly into the mass spectrometer (Thermo Scientific LTQ linear ion trap) and spectra were collected over a m/z range of 200-2000 Da using a dynamic exclusion limit of 3 MS/MS spectra of a given peptide mass for 30 s (exclusion duration of 90 s). Compound lists of the resulting spectra were generated using Xcalibur 2.2 software (Thermo Scientific) with an intensity threshold of 5,000 and 1 scan/group.

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