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Clean Fractionation Pretreatment Reduces Enzyme Loadings for Biomass Saccharification and Reveals the Mechanism of Free and Cellulosomal Enzyme Synergy

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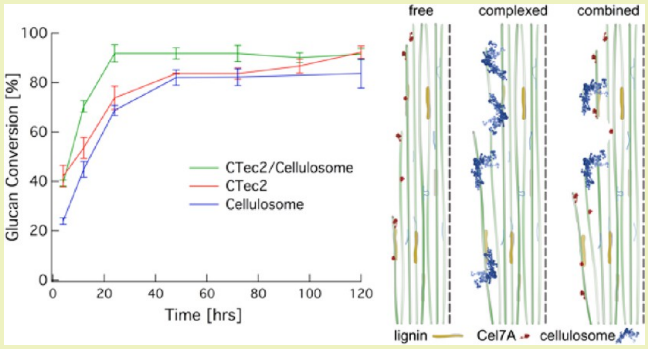
Supporting Information

ABSTRACT: Enzymatic depolymerization of polysaccharides is often a key step in the production of fuels and chemicals from lignocellulosic biomass. Historically, model cellulose substrates have been utilized to reveal insights into enzymatic saccharification mechanisms. However, translating findings from model substrates to realistic biomass substrates is critical for evaluating enzyme performance. Here, we employ a commercial fungal enzyme cocktail, purified cellulosomes, and combinations of these two enzymatic systems to investigate saccharification mechanisms on corn stover deconstructed either via clean fractionation (CF) or deacetylated dilute sulfuric acid pretreatments. CF is an organosolv pretreatment method utilizing water, MIBK, and either acetone or ethanol with catalytic amounts of sulfuric acid to fractionate biomass components. The insoluble cellulose-enriched fraction (CEF) from CF contains mainly cellulose, with minor amounts of residual hemicellulose and lignin. Enzymatic digestions at both low and high solid loadings demonstrate that CF reduces the amount of enzyme required to depolymerize polysaccharides relative to deacetylated dilute acid-pretreated corn stover. Transmission and scanning electron microscopy of the digested biomass provides evidence for the different mechanisms of enzymatic deconstruction between free and cellulosomal enzyme systems and reveals the basis for the synergistic relationship between the two enzyme paradigms on a process-relevant substrate. These results also demonstrate that the presence of lignin is more detrimental to cellulosome action than to free fungal cellulases. As enzyme costs are a major driver for biorefineries, this study provides key inputs for evaluation of CF as a pretreatment method and synergistic mixed enzyme systems as a saccharification strategy for biomass conversion.



KEYWORDS: *Cellulase, Biofuels, Lignin, Cellulose, Organosolv*

INTRODUCTION

Plant biomass represents an immense renewable source of raw material for the production of fuels and chemicals. Cost effective depolymerization of plant cell wall polysaccharides remains a major challenge in processes designed to convert carbohydrates to fuels and chemicals.[1,2] To date, many biochemical conversion processes have been developed that utilize biomass pretreatment coupled to enzymatic saccharification for the production of sugars.[1,3] Pretreatments are generally designed to make biomass more amenable to enzymatic or microbial attack by making the cellulose more accessible, in part, by partially removing or redistributing the hemicellulose and lignin matrix of the cell wall.[1,3] Pretreatment and enzymatic hydrolysis are major economic drivers for biorefineries[4−8] and thus require a highly integrated design approach, as the chemistry and severity of pretreatment directly impacts the susceptibility of the resulting biomass to enzymatic digestion, which in turn dictates the requirements for the enzyme cocktail composition and loading.

In nature, microbes have evolved diverse enzyme systems to deconstruct polysaccharides, and these natural enzyme cocktails provide a starting point for designing industrial enzymes. Most biomass-degrading organisms characterized to date secrete "free" enzymes, which diffuse independently and contain single catalytic domains for deconstructing cellulose, hemicellulose, and in some cases lignin. The soft-rot fungus *Trichoderma reesei* (*Hypocrea jecorina*) is the most well studied model organism that utilizes a free enzyme paradigm.[1,9] The cellulolytic enzyme

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system of *T. reesei* and similar organisms primarily comprises endoglucanases and cellobiohydrolases (CBHs). Endoglucanases are thought to hydrolyze chains in amorphous regions of cellulose to create attachment and detachment points for both reducing and nonreducing end-specific CBHs,[10] which processively hydrolyze cellulose chains into crystalline regions without substrate detachment between hydrolytic events. Oxidative enzymes, now termed lytic polysaccharide monooxygenases, have recently been discovered and characterized,[11−16] which are thought to perform endo-like cleavage of cellulose and hemicellulose chains in crystalline regions, thus complementing the activity of cellulase and hemicellulases. Additional enzymes with specificity to hemicelluloses, pectins, and in some cases lignin are also commonly utilized by biomass-degrading organisms.[17]

In contrast to the free enzyme paradigm, another enzyme system evolved in some anaerobic organisms wherein enzymes are organized into large extracellular macromolecular complexes termed cellulosomes. This paradigm was first discovered in the anaerobic thermophile *Clostridium thermocellum*,[18−20] which has since become a primary candidate for consolidated bioprocessing, a process that utilizes cellulolytic microbes for simultaneous deconstruction and conversion of carbohydrates to fuels.[21] Cellulosomes are composed of lignocellulose-degrading enzymes, noncovalently bound via cohesin−dockerin interactions to a scaffoldin protein either associated with the bacterial cell or free in solution.[22] Once assembled, *C. thermocellum* cellulosomes can contain up to nine catalytic domains selected from 70 different glycoside hydrolases (GHs). The large multimodular complex contains multiple enzymatic specificities in close proximity, which is hypothesized to aid in degrading the complex mixture of polysaccharides found in plant cell walls.[23,24]

Visualization of cellulase and cellulosome action on model cellulose substrates has shed light on the mechanistic differences of these two enzyme paradigms.[25−32] Starting with the work of Chanzy and Henrissat, these discoveries have aided in the interpretation of the mode of free cellulase action.[25] Driven by the presence of reducing end-specific GH Family 7 CBHs, free fungal enzyme cocktails act on cellulose substrates mainly from the reducing end, leading to the sharpening of one end of cellulose.[25,26,28−30] Recently, the differences in the mechanism of free cellulases and cellulosomes have been visualized on larger more complex layered bundles of cellulose microfibrils.[30] In contrast to free cellulase cocktails, cellulosomes splay the microfibrils apart at one end—also presumably the reducing end—given the abundance of reducing end-specific GH Family 48 CBHs in cellulosomes.[30] The splaying effect was hypothesized to increase the available surface area for enzyme action. These observations have greatly informed our collective interpretation of cellulolytic enzyme mechanisms, but a corresponding detail regarding enzymatic mechanisms on pretreated substrates has not yet been elucidated.

In addition to different modes of action between free cellulases and cellulosomes, there is a disparity in their performance on model cellulose substrates relative to pretreated biomass. Specifically, cellulosomes exhibit superior performance relative to free enzyme cocktails when degrading model cellulose, and the mixture of the two systems has a synergistic effect in performance.[20,30,33] Conversely, on dilute acid-pretreated biomass, which contains significant amounts of lignin, free cellulases exhibit superior performance to cellulosomes, and the combination of the two systems is not synergistic. This behavior limits the utility of cellulosomes for use with dilute acid-pretreated substrates.[30] Informed by work on fungal CBHs from Valjamae et al.[34] and Westh et al.[35] wherein they demonstrate that dissociation rates are rate limiting in cellulose depolymerization with CBH action alone, we proposed that once engaged, the multiple carbohydrate-binding modules and catalytic domains of the cellulosome decrease the probability of disengaging from the substrate for productive binding to other locations, thus limiting the extent of cellulose hydrolysis.[30] We also hypothesized that cellulosome inhibition when acting on biomass substrates may also be due to nonspecific binding to lignin.[30] However, assays using free and cellulosomal enzymes were only conducted on a single type of pretreated biomass, namely, dilute acid-pretreated biomass. Beyond dilute acid pretreatment, there are many other pretreatment options under development.[3,7,36−40] Some of these pretreatments may be particularly able to render biomass more digestible by cellulosomes or by combinations of free cellulases and cellulosomes, especially in the case of enhanced lignin removal.[41−47]

To that end, here we investigate the differences between free cellulases and cellulosomes in the saccharification of pretreated biomass from clean fractionation (CF).[41,44,45] CF employs water, methyl isobutyl ketone (MIBK), and either ethanol or acetone with sulfuric acid to separate biomass into three fractions enriched mainly in cellulose, hemicellulose, or lignin, with the efficiency of fractionation dependent on the severity of the pretreatment.[40,43,44] Following pretreatment and fractionation, an organic fraction enriched in lignin, an aqueous fraction enriched in hemicellulose-derived sugars, and an insoluble cellulose-enriched fraction (CEF) are obtained, the lattermost wherein significant amounts of both lignin and hemicellulose have been removed. CF enables upstream separation of the primary components in biomass, which in turn enables more selective upgrading of individual intermediate streams.[40,43,44] In a companion study,[48] we reported the full details of the CF pretreatment of corn stover and the compositional analysis and mass balance results. We examined six conditions using both an MIBK/ethanol/water system (Ethanol-CF) and an MIBK/acetone/water solvent system (Acetone-CF). For each solvent system, temperatures of 120 and 140 °C and sulfuric acid concentrations of 0.025, 0.05, and 0.1 M were used. Each of these substrates is examined in this study.

Another primary aim of this study is to quantify the extent of enzyme loading reduction possible using CF pretreatment relative to deacetylation and dilute acid pretreatment.[5,49] Partially digested CEF substrates were investigated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to visualize the impact of lignin removal and to examine mechanistic differences between free and complexed enzymes. Finally, we demonstrate that the combination of the two enzyme systems results in a synergistic improvement in enzyme performance on CF biomass. Overall, these results highlight the continued potential for pairing advanced pretreatment technologies with novel enzyme cocktail formulations to further reduce the cost of producing fuels and chemicals from lignocellulosic biomass.

RESULTS AND DISCUSSION

Substrate Digestions by Free and Complexed Enzyme Systems. As described in a companion study, we conducted Acetone-CF and Ethanol-CF on corn stover at 120 and 140 °C with sulfuric acid loadings of 0.025, 0.05, and 0.1 M.[48] Table S1

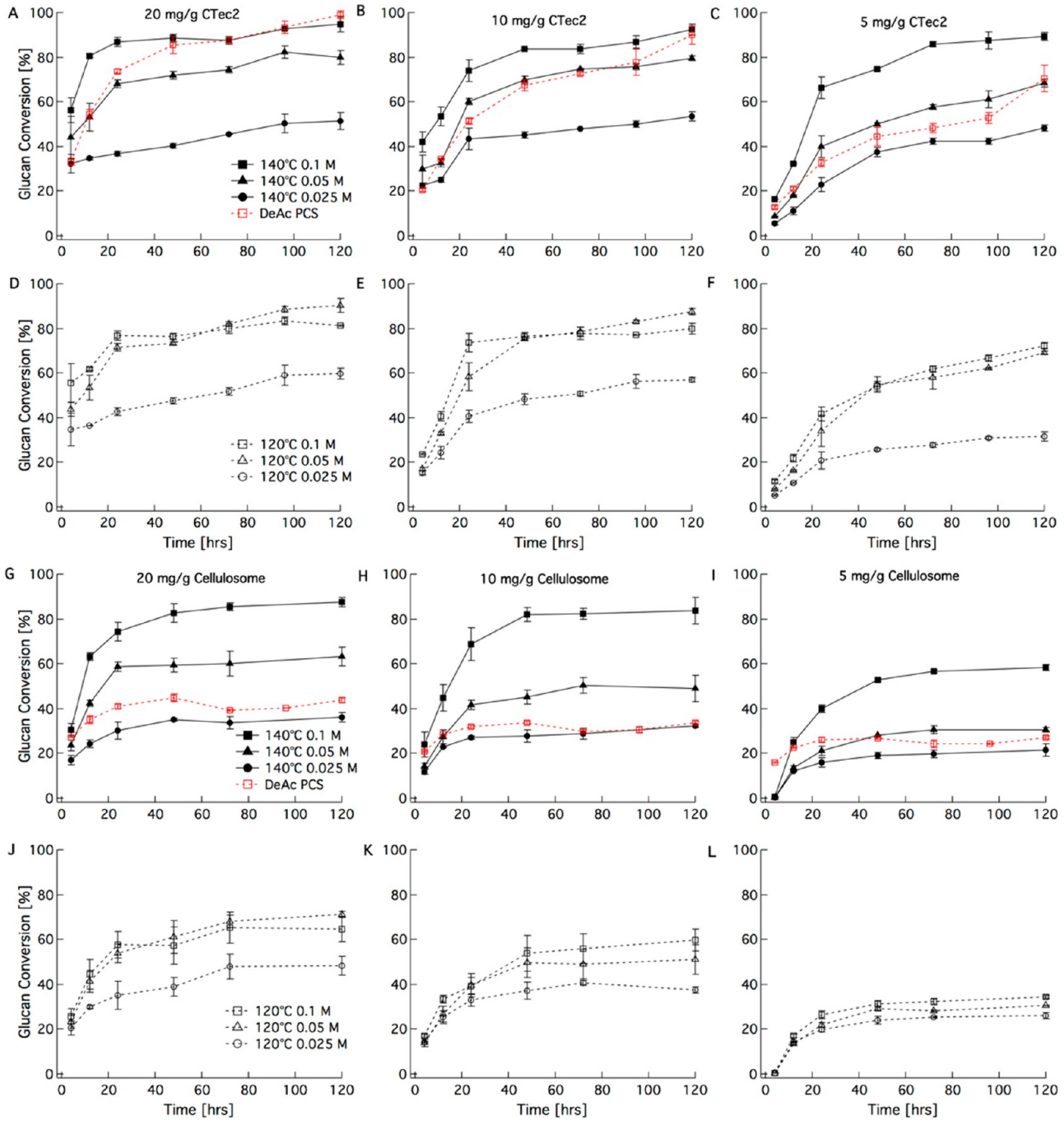

Figure 1. Glucan conversion in the cellulose-enriched fraction from Acetone-CF. The isolated cellulose-enriched fractions (CEF) from Acetone-CF pretreatment at 0.1, 0.05, and 0.025 M sulfuric acid at 140 °C (A–C, G–I) and 120 °C (D–F, J–L) were assayed for the extent of enzyme saccharification by the free fungal enzyme preparation CTec2 (A–F) or *C. thermocellum* cellulosomes (G–L) loaded at 20 mg (A, D, G, and J), 10 mg (B, E, H, and K), and 5 mg (C, F, I, and L) of protein per gram of glucan. CTec2 reactions contained 30 mM NaAc pH 5.0 and were incubated at 50 °C. The cellulosome reactions contained 30 mM NaAc pH 5.5, 100 mM NaCl, 1 mM $CaCl_2$, and 10 mM cysteine and were incubated at 60 °C.[30] Digestions of pretreated biomass contained 1% (w/v) solid slurries in 1.4 mL reactions and were mixed by continuous rotation at 10–12 rpm. Glucan conversion was measured by quantifying the amount of glucose and cellobiose released using HPLC.

in the Supporting Information provides the compositional analysis of the CEF, which exhibits increased crystallinity (50% relative to 37% for the starting material) and low amounts of residual hemicellulose and lignin.[48] Additionally, we compare the enzymatic digestibility of CF-pretreated corn stover with that of corn stover pretreated in mild alkaline conditions (deacetylation) as described in ref 49 followed by 0.5 wt % sulfuric acid at 150 °C for 20 min in a steam gun reactor. The deacetylated dilute acid-pretreated corn stover is referred to as DeAc PCS.

In this study, the time to reach 80% glucan conversion to soluble sugars is used as a metric for comparing enzyme performance. We incubated enzymes with 1% solid slurries of Acetone-CF and Ethanol-CF CEFs and DeAc PCS for 5 days. The results for both CF pretreatments are generally similar, so the Acetone-CF data are shown in the main text with

corresponding results for the Ethanol-CF substrates provided in the Supporting Information. Digestions were conducted in 1.4 mL volumes with enzymes loaded at 5, 10, 15, and 20 mg of protein per gram of glucan. Aliquots were sampled over 120 h to measure cellobiose, glucose, and xylose release by HPLC analysis as the percentage of glucan (Figure 1 and Figure S1, Supporting Information) and xylan (Figure 2 and Figure S2, Supporting Information) hydrolyzed to soluble sugars.

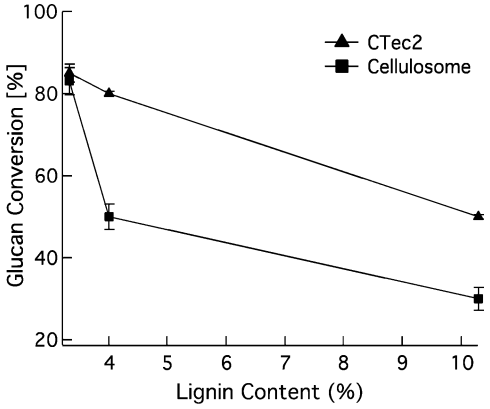

Figure 2. Enzymatic digestibility of the CEF from Acetone-CF pretreatment as a function of lignin content, plotted as glucan conversion at 72 h using 10 mg/g enzyme loading at three different CF severities.

The enzyme preparation CTec2 (Novozymes) was used as the fungal free enzyme cocktail. At the highest severity of Acetone-CF pretreatment, 140 °C and 0.1 M H_2SO_4, CTec2 achieves 80% glucan conversion in less than 12 h (Figure 1A and Figure S1A, Supporting Information) at a 20 mg/g loading, compared to 48 h with DeAc PCS. At an enzyme loading of 10 mg/g, CTec2 was able to convert 80% of the CEF glucan in 2 days compared to 5 days on DeAc PCS (Figure 1B and Figure S1B, Supporting Information). An enzyme loading of 5 mg/g converted 80% of the glucan within 72 h (Figure 1C). The improvement of the CEF glucan conversion compared to DeAc PCS was more apparent at the lower enzyme loadings of 10 and 5 mg/g. Substrates pretreated at the lowest CF severity, 120 °C and 0.025 M sulfuric acid, were able to achieve only 50% glucan conversion at an enzyme loading of 20 mg/g (Figure 1D).

Cellulosomes performed well on CF substrates, achieving 80% conversion within 48 h at 20 mg/g loading (Figure 1G and Figure S1C, Supporting Information). A glucan conversion of 80% was observed using cellulosome loadings as low as 10 mg/g on substrates pretreated at 140 °C and 0.1 M sulfuric acid (Figure 1H and Figure S1D, Supporting Information). At a 20 mg/g loading (Figure 1G-I, red line), cellulosomes were only able to hydrolyze 40% of the DeAc PCS glucan. The benefit of the CF over dilute acid pretreatment for enzymatic saccharification is clearly more dramatic for cellulosomes compared to free enzymes.

High xylan conversion was also observed where the glucan conversion reached 80% using both enzyme systems. CTec2 was able to hydrolyze 80% of the xylan from the CEF treated at 140 °C at enzyme loadings of 20 mg/g (Figures S2A and S3A, Supporting Information), 10 mg/g (Figures S2B and S3B, Supporting Information), and 5 mg/g (Figure S2C, Supporting Information) but was unable to achieve 80% xylan conversion with the CEF treated at 120 °C (Figure S2D−F, Supporting Information). In similar pretreatment conditions, where the glucan conversion reached 80% (Figure 1F−H and Figure S1C,D, Supporting Information), cellulosomes also converted 80% of the xylan (Figures S2 F−H and S3C,D, Supporting Information) from CEF. It is noteworthy that the highest severity CF pretreatments removed most of the hemicellulose, resulting in less than 5% xylan dry weight in the initial CEF substrate (Table S1, Supporting Information).

The presence of lignin has long been shown to be detrimental to enzymatic saccharification of pretreated biomass via mechanisms such as small molecule inhibition,[50,51] nonspecific binding,[52,53] and restriction of cellulose accessibility.[54,55] As a comparison of the effect of lignin on free and complexed enzyme systems, Figure 2 shows the glucan conversion at 72 h with a 10 mg/g loading at three CF pretreatment severities. The decrease in conversion as a function of increasing lignin content is significantly more detrimental to cellulosomes than CTec2. Cellulosomes were able to convert 80% of the CEF glucan at two enzyme loadings (10 and 20 mg/g) on the highest severity CF material. The CEF obtained using lower severity CF pretreatment conditions contains more lignin and more hemicellulose, resulting in a dramatic decrease in conversion. Because xylan conversion was high in most of the conditions tested, we do not think that the

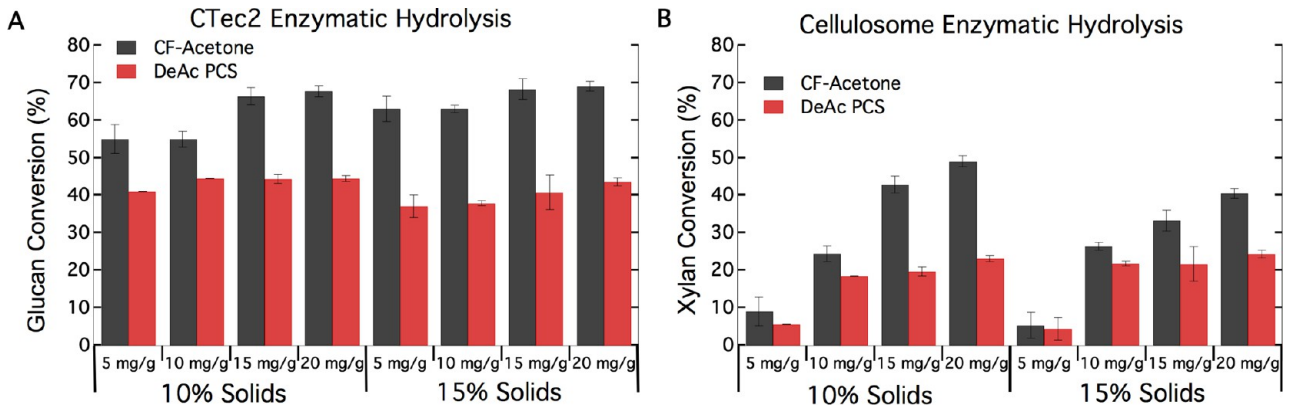

Figure 3. Glucan conversion in high solid biomass enzymatic saccharification. The CEF obtained/isolated from Acetone-CF pretreatment at 140 °C with 0.1 M H_2SO_4 was digested at solids loading of 10% and 15% by incubating with 5, 10, 15, and 20 mg of protein per gram of glucan using CTec2 (A) and cellulosomes (B) in 100 μL reaction vessels. The mixture was harvested at 120 h, and the glucose and cellobiose were quantified by HPLC. Each condition was replicated in triplicate, and error bars represent ± standard deviations.

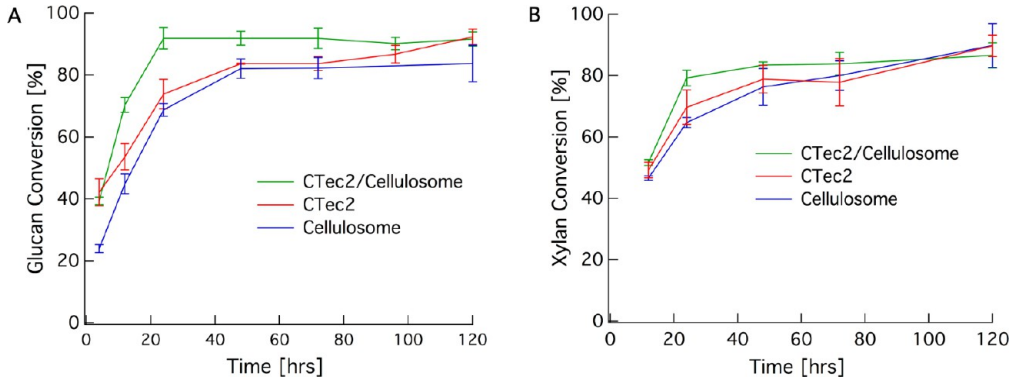

Figure 4. CF-CEF synergistic hydrolysis by free and complexed enzyme systems. Acetone-CF produced CEF (0.1 M sulfuric acid, 140 °C) was assayed to compare the extent for CTec2 (red), cellulosomes (blue), and an equal mixture by mass of CTec2 and cellulosomes (green). All enzyme systems were assayed at an equivalent 10 mg/g. Conditions are described in the Methods section and described in detail in ref 30. Digestions of biomass contained 1% (w/v) solid slurries in 1.4 mL reactions and were mixed by continuous rotation at 10−12 rpm. The percentage of glucan (A) and xylan (B) conversion was measured by the amount of glucose, cellobiose, and xylose released, determined using HPLC to derive the percent conversion of glucan or xylan.

resulting conversion is due to the presence of recalcitrant hemicellulose. Also, the decrease in cellulosome activity may be the result of the nonproductive binding of cellulosomes to lignin and/or enzyme accessibility to the cellulose. We note that the correlation of enzymatic activity to lignin content likely contains elements of cellulose accessibility, small molecule inhibition, and changes to the three-dimensional cell wall architecture. The changes in the cell wall architecture are shown below.

The high conversions of the CEF achieved at low enzyme loadings are promising, as cellulase enzymes are a key cost driver identified in techno-economic analyses.[6] To examine enzymatic conversion in more industrially relevant conditions, we increased the solids loadings to 10% and 15% (100 and 150 mg biomass per mL). It is noted that the conversion of DeAc PCS glucan and xylan is significantly higher using pilot-scale reactors than we observed using small-scale high solid reactors (100 μL).[49] The effect of scale and reaction vessel mixing has been previously reported,[56] and we acknowledge that further studies in optimized reactor conditions are necessary. However, one of the intentions of this study was to compare the enzymatic hydrolysis of corn stover pretreated by deacetylation and dilute acid pretreatment to CF, which is most readily accomplished by running small-scale (100 μL) enzymatic reactions with DeAc PCS and CEF in 10% and 15% solid slurries.

After incubating the high solid reactions for 120 h, CTec2 converted 30% more of the CEF glucan compared to DeAc PCS (65% vs 43%) (Figure 3A). The trend for improved digestibility of the Acetone-CF CEF was consistent at all enzyme and solids loadings. The enzymatic digestions of CEF using complexed enzymes showed a dramatic improvement of glucan conversion at high solid loadings compared to the DeAc PCS substrate (Figure 3B). High solid digestions of DeAc PCS were only able to convert ∼13% of the glucan. However, the cellulosome digestion of CEF converted ∼50% of the glucan. Thus, the conversion improvement between DeAc PCS and CF-CEF was ∼5 fold. We attribute the trend of conversion improvement to lignin removal and increased cellulose accessibility achieved by the CF pretreatment. We also observe a dramatic improvement of the xylan conversion in the CF-pretreated samples compared to DeAc PSC (Figure S4, Supporting Information).

Synergistic Enzyme Combinations. Previously, we have shown that the combination of free and complexed enzyme systems improves the conversion of crystalline cellulose.[30] Here, we demonstrate for the first time that combining free and complexed enzyme systems can synergistically degrade pretreated plant cell wall polysaccharides. By combining equal amounts of the two enzyme systems on a mass basis, 80% conversion of the CEF glucan was achieved in less than 20 h of digestion period compared to 48 h using either free or complexed enzymes alone (Figure 4A). Xylan conversion also improved using the enzyme combination (Figure 4B). Furthermore, combining the two enzyme systems using a protein loading of 10 mg/g (5 mg/g of each) achieved similar conversion levels as loading 20 mg/g of CTec2 alone (Figure 1). Combining these two systems decreased both the time and amount of enzyme needed to reach 80% glucan and xylan conversion (Figure 4). The degree of synergistic enhancement to glucan conversion was similar to what was observed previously using model cellulose substrates.[30]

Biomass Surface Morphology Investigation by SEM. On the basis of the results from Figures 1−4, we employed electron microscopy to investigate morphological differences of the digested materials and to elucidate differences in the degradation mechanisms of these two enzymes systems. At the cellular scale, CF-pretreated biomass revealed various degrees of cell separation, which trended with pretreatment severity and the extent of lignin removal.[48] The CF pretreatment with lower acid concentrations left more intact clusters of cells and vascular bundles, while increasing acid concentration and temperature produced CEF tissue with decreased intercell adhesion. Complete disjoining of cells, evidenced as separated individual cells, was observed in samples treated at intermediate acid concentrations, as reported in the companion study.[48] Cell separation was extensive in the samples treated with 0.1 M sulfuric acid in either acetone or ethanol.[48]

The cellular-scale particle morphology is similar to that produced by other pulping technologies and is also observed in biomass treated with dilute acid in a steam explosion reactor.[37] This cell separation primarily results from the removal of the lignin-rich middle lamellar region of the cell walls, dramatically reducing intercell adhesion of the remaining biomass and facilitates disassembly of cellular bundles. In the context of

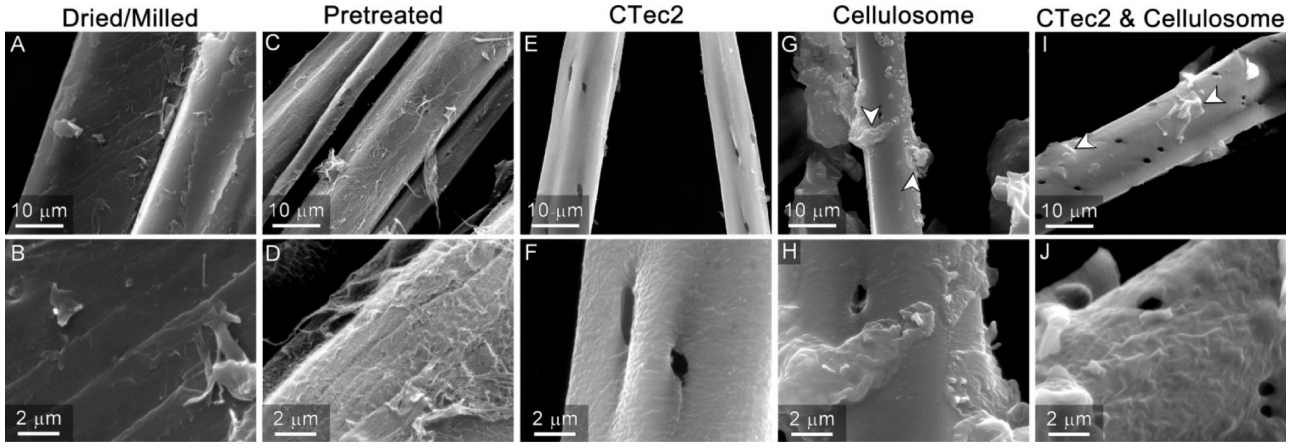

Figure 5. Scanning electron microscopy (SEM) of pretreated and digested corn stover fiber cells. (A,B) SEM was used to image the surface of corn stover fiber cells after the drying and milling process. (C,D) CF pretreatment caused separation of cellular clusters and extensive nanofibrillation of cellulose on the surface of the cell walls. (E,F) Digestion by a commercial cocktail of fungal enzymes removed the fibrillated cellulose at the surface and produced a wrinkled texture. (G,H) Digestion by purified cellulosomes produced delamination pockets (arrowheads) and abrasions on the surfaces of the fiber cells. (I,J) The combination of the two enzyme systems resulted in a combination of the two surface morphologies. The enzymatically digested biomass were sampled at a time point where 50% of the glucan was hydrolyzed.

enzymatic conversion, this process reduces the average biomass particle size, increasing surface area and accessibility.

The surface textures of CEF corn stover fiber cells were compared by SEM and are shown in Figure 5. Fiber cells were chosen for image analysis because they represent an extensively lignified and recalcitrant tissue type and remained identifiable in each sample following digestion. After drying and milling, fiber cells are largely present in clusters of vascular tissue (Figure 5A,B). The surface texture of these cells was relatively smooth compared to the pretreated cells (Figure 5C,D). CF pretreatment caused disjoining of individual fiber cells from the clusters of vascular bundles (Figure 5C) due to removal of lignin from the compound middle lamellae. Evidence of nanofibrillation was observed on the surface of fiber cells following CF pretreatment (Figure 5D), which improves the accessibility of microfibrils to enzymes and contributes to the enhanced digestibility of CF-CEF.

Digestion of the CEF material by free fungal enzymes (CTec2) ablated virtually all of the fibrillated cellulose from the exterior surface of the fiber cells (Figure 5E). Additional changes in texture with respect to the pretreated material were revealed by higher-magnification surface imaging (Figure 5F), wherein the surface of these cell walls contained a subtle wrinkled topology that may be attributed to exposure of underlying bundles of cellulose microfibrils deeper within the cell wall. While digestion of the pretreated material by cellulosomes removed fibrillated cellulose from the exterior surface, it also produced a surface morphology that was completely distinct from that produced by the fungal enzyme cocktail (Figure 5G,H). Evidence of cellulosome-induced delamination that formed discrete raised pockets (Figure 5G,H, arrowheads) and surface abrasion was present in many locations on the exterior of the fiber cells. Combination of the two enzyme systems resulted in a notable combination of the two surface morphologies: roughened wrinkled surface texture and delaminated pockets. Synergy between these two deconstruction mechanisms can arise when a pocket of cell wall lamella is peeled up by cellulosomes, exposing new and otherwise inaccessible surface area to the free enzymes.

Investigation of Cell Wall Cross Sections by Transmission Electron Microscopy (TEM). TEM was used to investigate the influence of CF pretreatment on cell wall ultrastructure and its accessibility to enzymatic deconstruction. The CF-pretreated cell walls displayed some delamination and a reduction in staining density that would be expected from removal of lignin caused by CF pretreatment (Figure 6A–F). TEM was also used to determine if the surface deconstruction phenomena observed by SEM were observable deeper into the cell wall interior. While SEM revealed extensive nanofibrillation on exterior cell wall surfaces following CF pretreatment, examination of these cell walls in cross section revealed that this phenomena was highly localized to the cell wall surface and that the underlying cellulose was not fibrillated (Figure 6D–F). This morphology differs significantly from the pretreated substrates that are produced by pretreatments that employ explosive decompression and cause extensive delamination and nanofibrillation throughout the cell wall interior.[57] Subsequent digestion of the CF-pretreated material by fungal free enzymes produced a tapered cellulose bundle morphology on cell wall fragments that were likely delaminated during pretreatment (Figure 6G,H). However, the perimeter of regions of the wall that were not delaminated appeared relatively uniform (Figure 6I). In contrast, digestion with cellulosomes showed evidence of nonuniform surface disruption that penetrated well into the cell wall interior (Figure 6J–L). Cell walls treated with the combination of free and complexed enzymes displayed a combination of the uniform surface-oriented dissolution of the free enzymes and the abrasive penetrating disruption of the cellulosomes (Figure 6M–O). Extensive channels penetrating well beyond the surface are evident. These structural observations of the digested substrate further support the explanation of synergistic mechanisms between free and complexed enzyme systems, wherein the larger-scale disruption of the cellulosomes provides the free enzymes additional access to cellulose surfaces and ends deeper into the cell wall interior. In turn, localized deconstruction of the cell wall interior by the infiltrated free enzymes likely weakens the wall in localized regions and facilitates additional disruption by the cellulosomes.

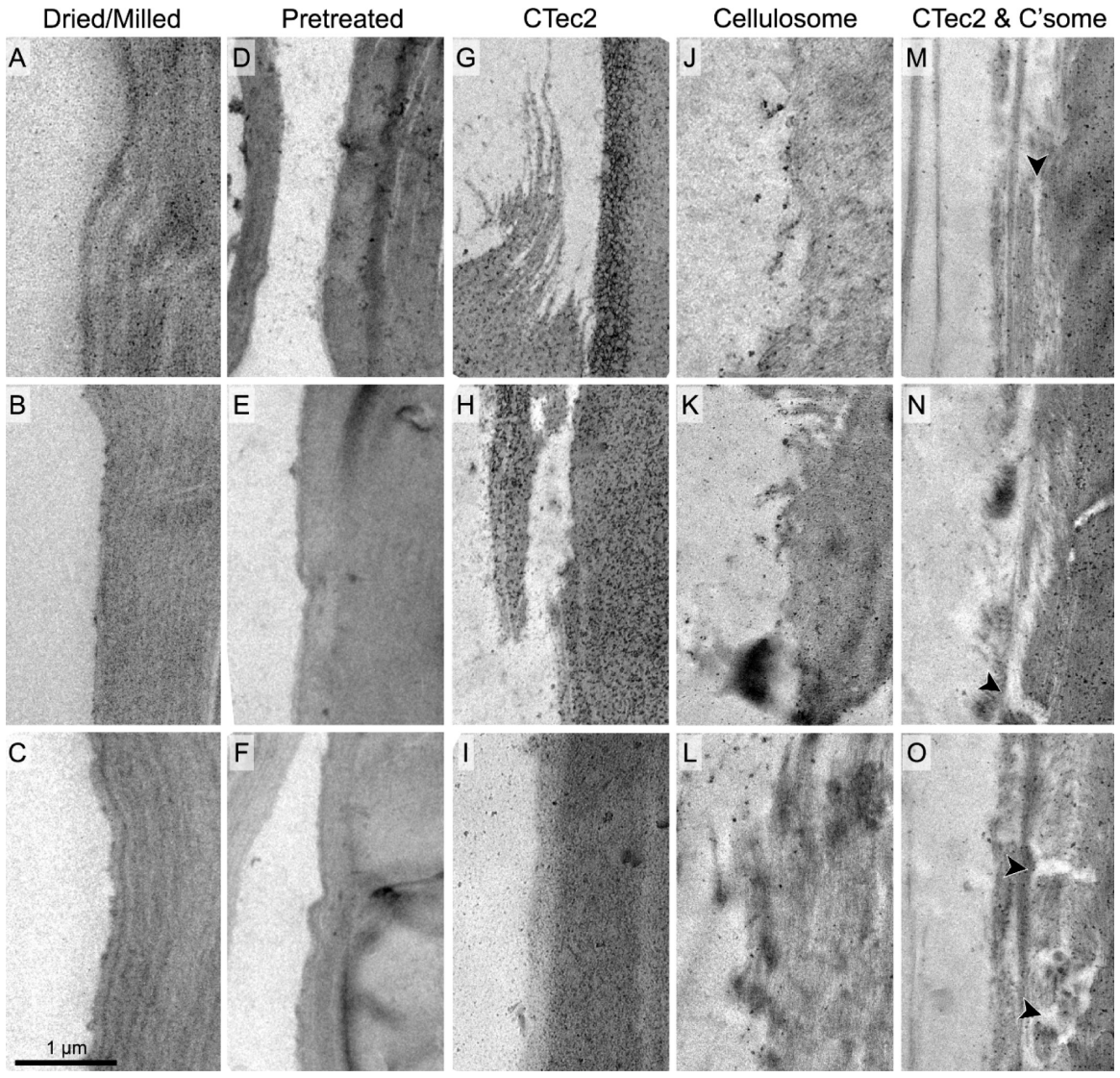

Figure 6. Transmission electron microscopy of pretreated and digested corn stover fiber cells. (A–C) TEM was used to image the internal structure of corn stover fiber cell walls after the drying and milling process. (D–F) CF pretreatment caused some delamination and other evidence of structural change within the lamella of secondary cell walls. (G–I) Digestion by the CTec2 cocktail of fungal enzymes produced more regular cell wall surfaces and modified cell wall fragments. (J–L) Digestion by purified cellulosomes revealed irregular cell wall surfaces. (M–O) Digestion by a combination of CTec2 and cellulosomes resulted in walls that displayed highly irregular wall surfaces and a pattern of extensive fissure or channel-like voids (arrowheads) into the cell wall. The enzymatically digested biomass were sampled at a time point where 50% of the glucan was hydrolyzed.

CONCLUSIONS

In this study, we compared the biomass deconstruction mechanisms of free fungal enzymes and isolated cellulosomes on CF-pretreated corn stover. The free enzyme cocktail consists of separate proteins with individual catalytic specificities and CBMs. In contrast, cellulosomal complexes comprise many enzymes with different substrate specificities bound together on a scaffoldin. Examples from each enzyme system are among the leading candidates for industrial-scale biochemical deconstruction of lignocellulosic feedstocks. Key differences in the deconstruction mechanisms between these two enzyme systems have been previously demonstrated on model cellulose substrates.[30] These purified cellulose particles are discrete bundles of microfibrils with readily accessible ends on which free enzymes preferably taper one end of the particle and cellulosomes separate individual cellulose microfibrils increasing available surface area. In contrast, plant cell walls are chemically and structurally more complex. Microfibrils in the cell wall are longer, their ends are not readily accessible to enzymes, and the cell wall matrix polymers make the microfibrils markedly less accessible. Here, we have shown the first evidence that the mechanistic differences between these two enzyme systems hold for CF-pretreated cell walls as well. Utilizing CF to isolate a CEF, where the majority of the lignin and hemicellulose were removed, enabled the visualization of morphological differences in the plant cell walls treated with free or complexed enzymes by electron microscopy. We observed dramatic differences in the plant cell wall surface even under relatively low magnification using SEM (Figure 5). When free enzymes are combined with CEF, they are able to gain access to cell wall surfaces and easily digest loose exposed microfibrils. In contrast, when CEF was incubated with cellulosomes, the surface of the cell walls displayed raised pockets of delamination. These structural changes are also observed in thin sections of cryopreserved

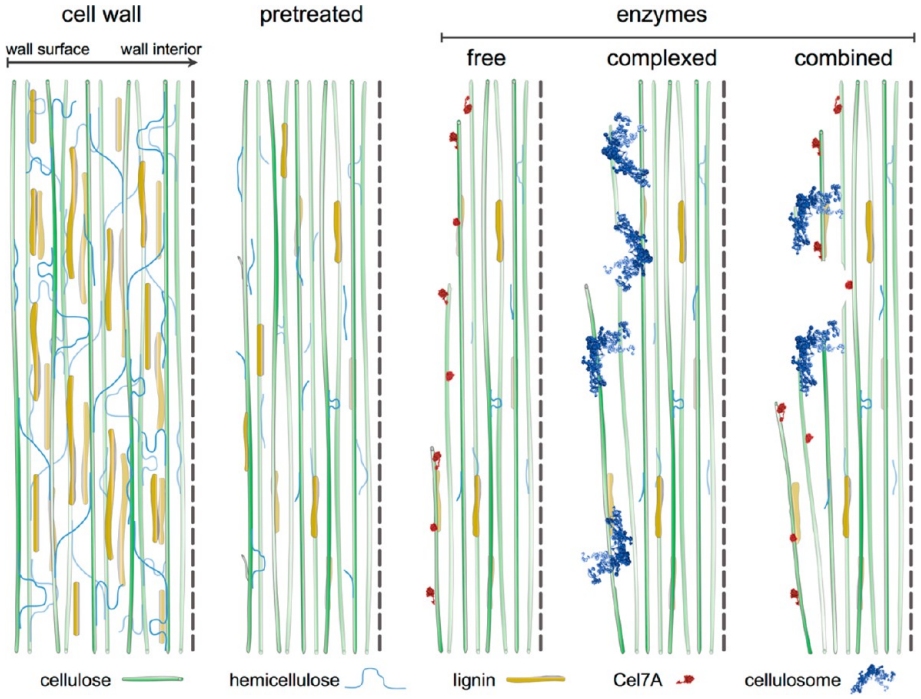

Figure 7. Illustration of plant cell walls before and after CF pretreatment and models of hydrolysis by free (red) and complexed (blue) enzyme systems. Free enzymes with single CBMs and catalytic units hydrolyze cell wall polysaccharides by utilizing endoglucanases and CBHs to react with accessible cellulose surfaces. Complexed enzymes with multiple catalytic and binding specificities likely have lower off-rates and once bound at multiple points of contact disrupt the biomass surface resulting in an increase in surface area. Combining these two enzyme paradigms on fractionated biomass synergistically deconstructs the cell walls by increasing the reactive surface area allowing free enzymes to better diffuse and processively hydrolyze the substrate. Also, by removing the majority of the lignin and hemicellulose from the cellulose fraction, CF enhances the cellulosome activity enabling the benefits of combining these two deconstruction mechanisms.

digested cell walls using TEM, where cellulosomes were found to delaminate pockets of the cell wall surface lamina (Figure 6). A summary interpretation of the structural analysis of digested CF-pretreated biomass is illustrated in Figure 7.

Combining the two enzyme systems was shown to be synergistic at degrading model cellulose substrates.[30] Interestingly, combining these two deconstruction mechanisms on CF-CEF also synergistically enhances the glucan and xylan hydrolysis and results in a combination of ablated cell wall surfaces and pocket-type delaminations, with channels penetrating into the cell wall. (Figure 6M–O). Presumably, these mutually beneficial mechanisms aid in physical and chemical deconstruction, where large complexed enzymes expose more reactive surface area to allow for the processive hydrolysis action by free fungal enzymes (illustrated in Figure 7).

In conclusion, this study demonstrates the differences in enzyme deconstruction mechanisms between two of the predominantly studied cellulase enzyme paradigms. The results from enzymatic digestions, SEM, TEM, and synergy studies suggest that free enzymes readily hydrolyze the exposed surface microfibrils and penetrate into walls where the accessibility allows. Contrarily, complexed enzymes physically deconstruct the biomass by generating peeled pockets of cell wall layers that increases the accessibility of the deeper cell wall lamella. We show that combining these two systems results in an enhancement of cell wall polysaccharide hydrolysis due to the synergistic enzyme mechanisms. Using CF combined with all three enzyme cocktail formulations, an 80% glucan conversion can be achieved using a lower enzyme loading than traditional dilute acid pretreatment. Using CF substrates also enabled the visualization of the different enzymatic mechanisms in deconstruction of plant cell walls. These results indicate that combining pretreatment methods and tailoring the enzyme cocktail composition may lead to a reduction in enzyme cost in the growing biofuels industry.

METHODS

Clean Fractionated Cellulose-Enriched Fraction Isolation. Ten grams of corn stover, knife milled to pass a 20 mesh (~0.85 mm) screen, was mixed in a single-phase mixture of methyl isobutyl ketone (MIBK)/ethanol (EtOH)/H_2O (16/34/50 g/g/g, 100 mL)[45] or MIBK/acetone/H_2O (11/44/44, g/g/g, 100 mL) with sulfuric acid (0.025, 0.05, or 0.1 M) and was loaded into a 316 stainless steel pressure reactor, as described previously.[48] The reactor was sealed and heated in an electric heating block at 120 or 140 °C for 56 min. After the reaction, the reactor was cooled in ice water. The reaction mixture was separated into a solid fraction and an aqueous fraction via filtration. The solid fraction was thoroughly washed first with the same solvent (200 mL) followed by deionized H_2O (650 mL) to obtain the CEF. The CEF was washed three times in ddH_2O and stored in 30 mM sodium acetate (NaAc) pH 5.0 at 4 °C prior to enzymatic hydrolysis. Compositional analysis of each CEF was conducted according to the National Renewable Energy Laboratory's Laboratory Analytical Procedure[58] and is summarized in Table S1 of the Supporting Information.

Isolation of HMW Cellulosomes. *C. thermocellum* 27405 was grown on 5 g/L of Avicel and 1191 media in a 5 L Sartorius B+ reactor. The inoculum was cultured in 100 mL bottles on 5 g/L of cellobiose until midlate log phase and then was transferred anaerobically into a bioreactor. The 60 °C culture was continuously sparged with 20 sccm N_2, agitated at 150 rpm, and maintained at pH 7.0 by automatic addition of 1 M NaOH. The supernatant was harvested when the culture reached stationary phase, as indicated by cessation of gas (H_2 and CO_2) production and complete utilization of the substrate. The solids were separated from the secretome by

centrifugation at 5000 rpm for 10 min, and the supernatant was filtered through 0.2 μm nylon disposable filters. The secretome was concentrated using Amicon hollow glass fiber concentrators with a 10 kDa cutoff. To isolate the high molecular weight cellulosomes, concentrated secretome was applied to a GE FPLC equipped with a Sephacryl S-400 size exclusion column in buffer containing 30 mM Bis-Tris pH 6.8, 100 mM NaCl, and 1 mM $CaCl_2$. Fractions containing the cellulosome were separated from aggregates, free enzymes, and noncellulosomal material and were then pooled, concentrated, and stored at 4 °C. Protein concentration was measured by BCA (Pierce).

Fungal Cellulases. CTec2 preparation number NS-22086 PPC 30604 was obtained from Novozymes. The concentrated enzyme mixture was applied to an AKTA FPLC (GE) using a HiPrep 26/10 Sephadex (GE) desalting column to remove stabilizers and other additives that interfere with BCA protein assay and HPLC sugar quantification. Protein concentration was measured by BCA (Pierce).

Activity Assays. All enzyme loadings were loaded as milligrams of protein per gram of glucan. The enzyme activity of the cellulosomes alone was assayed at 60 °C in 30 mM sodium acetate pH 5.5 buffer containing 10 mM $CaCl_2$, 100 mM NaCl, 10 mM cysteine and 2 mg/g of β-glucosidase (*Aspergillus niger*). Fungal cellulase (CTec2) activity alone was measured at 50 °C in 20 mM sodium acetate, pH 5.0. Mixtures of cellulosomes and CTec2 were assayed at 55 °C in 30 mM sodium acetate (NaAc) pH 5.5 buffer containing 1 mM $CaCl_2$, 100 mM NaCl, and 10 mM cysteine. Digestions of 1% solids were conducted in sealed 2 mL vials with continuous mixing by inversion at 10−12 rpm. Unless otherwise noted, substrates were loaded at 10 mg dry biomass per mL in 1.4 mL reaction volumes. Representative (with respect to both solid and liquid phases of the digestion slurry) were withdrawn from well-mixed digestion mixtures at selected time points during the digestions.

High solid digestions were conducted in 500 μL PCR tubes in reaction volumes of 100 μL. High solid digestions were incubated for 120 h, and the entire reaction mixture was used for dilution and sugar conversion measurements.

High and low solid samples were diluted 10-fold with deionized H_2O into 1.5 mL vials then sealed and immersed in a boiling water bath for 10 min to inactivate the enzymes and terminate the reaction. The diluted and terminated digestion aliquots were then filtered through 0.2 μm nominal pore size nylon syringe filters (Pall/Gelman Acrodisc-13) to remove residual substrate and, presumably, most of the denatured enzyme. Released cellobiose, glucose, and xylose in the diluted samples were then determined by HPLC analysis on an Aminex HPX-87H column (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) operated at 55 °C with 0.01 N sulfuric acid as mobile phase at 0.6 mL/min in an Agilent 1100 HPLC system with refractive index detection. The resulting glucose, cellobiose, and xylose concentrations calculated (mg/mL) in each digestion mixture was converted to anhydro-glucose and anhydro-cellobiose concentrations, respectively, by subtracting out the proportional weight added to each molecule by the water of hydrolysis. The sum of the concentrations of anhydro-glucose and anhydro-cellobiose, which sum is equivalent to the weight concentration of the glucan chain that was hydrolyzed to produce the soluble sugars, was then divided by the initial weight concentration of cellulose or xylan in the digestion mixture and multiplied by 100% to yield activity results as percent conversion of cellulose.

Scanning Electron Microscopy. Imaging by scanning electron microscopy (SEM) was performed using a FEI Quanta 400 FEG instrument. Untreated samples, pretreated samples, and samples enzymatically digested to 50% glucan conversion were spun at 13k RPM for 1 min in a desktop microfuge and washed with ddH_2O and freeze-dried prior to imaging. Samples were then mounted on aluminum stubs using conductive carbon tape and sputter coated with 10 nm of iridium. Imaging was performed using beam-accelerating voltages ranging from 15 to 25 keV.

Transmission Electron Microscopy. A total of 1.5 μL of biomass slurries either untreated, pretreated, or enzymatically digested were placed into the No. 707899 type Leica planchets and cryo-preserved in a Leica EMPact2 high-pressure freezer. Freeze substitution was carried out in a Leica AFS2 (Leica, Wetzlar, Germany) unit in 1% OsO_4 in dry acetone with the following temperature regime: −90 °C for 72 h, ramp to −30 °C over 3 h, hold at −30 °C for 21 h, ramp to 3 °C over 3 h, hold at 3 °C for 21 h, ramp to 24 °C over 3 h, hold at 24 °C for ∼1 h, and finally rinse three times in dry acetone at RT. Samples were removed from the planchets using fine-tipped forceps and minimal agitation before proceeding with infiltration. Samples were infiltrated in a graded series of Eponate 812 (EMS, Hatfield, PA) resin over 3 d. Samples were transferred and oriented into Easy Molds (EMS, Hatfield, PA) for polymerization. Resin was polymerized for 48 h in a vacuum oven at 60 °C. Resin-embedded samples were sectioned to ∼75 nm with a Diatome diamond knife on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany). Sections were collected on 0.5% Formvar coated slot grids (SPI Supplies, West Chester, PA). Grids were post-stained for 6 min with 2% aqueous uranyl acetate and 3 min with Reynolds lead citrate. Images were taken with a 4 megapixel Gatan UltraScan 1000 camera (Gatan, Pleasanton, CA) on a FEI Tecnai G2 20 Twin 200 kV LaB6 TEM (FEI, Hilsboro, OR).

■ ASSOCIATED CONTENT

Supporting Information

Compositional analysis data for biomass examined in this study and xylan conversion by free and cellulosomal enzyme systems at high and low solids loadings. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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