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Surface and ultrastructural characterization of raw and pretreated switchgrass

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ABSTRACT

The US Department of Energy-funded Biomass Refining CAFI (Consortium for Applied Fundamentals and Innovation) project has developed leading pretreatment technologies for application to switchgrass and has evaluated their effectiveness in recovering sugars from the coupled operations of pretreatment and enzymatic hydrolysis. Key chemical and physical characteristics have been determined for pretreated switchgrass samples. Several analytical microscopy approaches utilizing instruments in the Biomass Surface Characterization Laboratory (BSCL) at the National Renewable Energy Laboratory (NREL) have been applied to untreated and CAFI-pretreated switchgrass samples. The results of this work have shown that each of the CAFI pretreatment approaches on switchgrass result in different structural impacts at the plant tissue, cellular, and cell wall levels. Some of these structural changes can be related to changes in chemical composition upon pretreatment. There are also apparently different structural mechanisms that are responsible for achieving the highest enzymatic hydrolysis sugar yields.

1. Introduction

Leading lignocellulosic biomass conversion research teams from Auburn, Michigan State, Purdue, and Texas A&M Universities; Genencor (a Danisco division); Ceres Corp.; the National Renewable Energy Laboratory (NREL); and the University of California at Riverside have collaborated on a US Department of Energy Office of the Biomass Program (OBP)-funded project to investigate pretreatment and enzymatic saccharification of switchgrass. These participants are members of the Biomass Refining Consortium for Applied Fundamentals and Innovation (CAFI), which was originally formed in 2000. Since its inception, the CAFI Team has developed unique comparative performance and economic data on leading pretreatments applied to both corn stover (Eggeman and Elander, 2005; Kim and Holtzapple, 2005; Kim and Lee, 2005; Liu and Wyman, 2005; Lloyd and Wyman, 2005; Mosier et al., 2005b; Teymouri et al., 2005; Wyman et al., 2005a,b) and poplar wood (Balan et al., 2009; Bura et al., 2009; Gupta and Lee, 2009; Kim et al., 2009; Kumar and Wyman, 2009; Lu et al., 2009; Sierra et al., 2009; Wyman et al., 2009) in earlier projects ("CAFI 1" and "CAFI 2" projects). These projects also improved the understanding of these pretreatments and established comparative procedures and methods. More recently, this approach was extended to switchgrass, an important feedstock with different characteristics than either corn stover or poplar wood, in the OBP-funded the "CAFI 3" project. The goal of the CAFI 3 project was to develop leading pretreatment technologies for application to switchgrass and evaluate their effectiveness in recovering sugars from the coupled

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operations of pretreatment and enzymatic hydrolysis. Key chemical and physical characteristics were measured for selected pretreated solids displaying significant variations in performance for the CAFI pretreatment processes.

The CAFI team also seeks to better understand interactions among pretreatment and enzymatic hydrolysis to gain insight that will facilitate process definition and improvements in process yields to facilitate commercialization of cellulosic conversion technologies. One of the key elements of the CAFI 3 project that was performed by NREL involved the use of its Biomass Surface Characterization Laboratory (BSCL) to conduct surface and ultrastructural analysis of various switchgrass feedstock types and pretreated solids generated in the CAFI 3 project.

Over that past few years, several groups have incorporated structural characterization by microscopy into their repertoire of analysis tools used to understand the mechanisms by which pretreatment improves the digestibility of biomass (Chundawat et al., 2011; Donohoe et al., 2008; Kristensen et al., 2008; Kumar et al., 2009; Zeng et al., 2007). Because of its relatively high resolution, and ability to image whole intact biomass particles, most of these studies have utilized scanning electron microscopy (SEM) as their primary imaging tool. These direct observations of changes in cell wall architecture have led to new insights into surface erosion mechanisms and re-localization of cell wall matrix components to increase enzyme accessibility and improve enzymatic digestion. Here, we utilize a correlative microscopy approach including multiple light and electron imaging modes to investigate the impact of pretreatment on disrupting biomass tissue, cellular arrangement, and cell wall architecture. We report the imaging analysis on raw switchgrass and representative pretreated switchgrass samples from each CAFI pretreatment process. Relationships between imaging observations and feedstock composition, pretreated solids composition, and performance of pretreated solids upon subsequent enzymatic hydrolysis are also reported.

2. Methods

2.1. Untreated and pretreated switchgrass

Four unique samples representing three different switchgrass varieties were provided to CAFI research teams by Ceres Corporation (Thousand Oaks, CA), as described in Table 1. Square bales of each switchgrass type were stored in a building after harvest until dried to less than 10% moisture and knife- or ball-milled to 2–6 mm size. Samples of unmilled switchgrass were used to identify anatomical features and to examine gross morphological properties. Compositional analysis of the three switchgrass types is reported in other CAFI 3 project papers published in this same issue.

CAFI teams from Auburn, Michigan State, Purdue, and Texas A&M Universities and the University of California at Riverside provided pretreated Dakotah switchgrass samples to NREL for imaging analysis. The pretreatment conditions for each sample used in imaging analysis are shown in Table 2. Specific descriptions for each CAFI pretreatment methodology is reported in earlier CAFI project publications (Kim and Holtzapple, 2005; Kim and Lee, 2005; Liu and Wyman, 2005; Mosier et al., 2005; Teymouri et al., 2005) and were used in conducting the CAFI 3 pretreatments on switchgrass.

Table 1
Ecotype and harvest information of switchgrass feedstocks.

	Alamo 1	Alamo 2	Shawnee	Dacotah
Latitude-of-Origin	29°N		38°N	46°N
Ecotype	Southern lowland		Northern upland	
Morphology	Thick stems		Thin stems	
Harvest Location	Ardmore, OK 34°N (Elev. 870 ft)		Stillwater, OK 36°N (Elev. 960 ft)	Pierre, SD 44°N (Elev. 1420 ft)
Plant date	June 2005	June 2007	June 2005	December 1999
Harvest date	December 2006	November 2007	December,2006	May 2008[a]

[a] Plot was allowed to stand over the winter.

Table 2
Pretreated switchgrass samples (Dacotah variety) used in NREL imaging analysis. AFEX refers to ammonia fiber expansion pretreatment (Michigan State University), lime refers to lime pretreatment (Texas A&M University), SAA refers to soaking in aqueous ammonia pretreatment (Auburn University), LHW refers to liquid hot water pretreatment, and DA refers to dilute sulfuric acid pretreatment (University of California at Riverside).

Pretreatment method	Chemicals loading (per g dry biomass)	Temperature (°C)	Duration	Moisture level (per g dry biomass)
AFEX	1.5 g NH_3	150	30 min	2 g H_2O
Lime	1 g $Ca(OH)_2$	120	4 h	15 g H_2O
SAA	0.15 g NH_4OH	160	1 h	9 g H_2O
LHW	6.7 g H_2O	200	10 min	6.7 g H_2O
DA	0.005 g H_2SO_4	160	10 min	19 g H_2O

2.1.1. Pretreatment sample compositional analysis and enzymatic hydrolysis

The compositional analysis of each CAFI pretreatment sample listed in Table 2 was determined by Purdue University and is reported in other CAFI 3 project papers published in this same issue. Enzymatic hydrolysis results of each CAFI pretreatment sample listed in Table 2 was compiled by Michigan State University and is reported in other CAFI 3 project papers published in this same issue.

2.2. Stereomicroscopy

Whole pieces of the various tissue fractions of untreated switchgrass (Shawnee variety) and pretreated switchgrass (Dacotah variety) were examined without further processing. Images were captured on a Nikon SMZ1500 stereomicroscope and captured with a Nikon DS-Fi1 CCD camera operated by a Nikon Digital Sight system (Nikon Instruments, Melville, NY). Adobe Photoshop was used to rotate, crop, resize, and adjust contrast, brightness and white balance of images.

2.3. Sample preparation

Untreated and pretreated switchgrass tissue was prepared using microwave processing as described previously (Donohoe et al., 2008). Briefly, samples were fixed 2 × 6 min (with variable power) in 2.5% gluteraldehyde buffered in 0.1 M sodium cacodylate buffer (EMS, Hatfield, PA) under vacuum. The samples were dehydrated by treating with increasing concentrations of acetone for 1 min at each dilution (15%, 30%, 60%, 90%, and 3× 100% acetone). After dehydration, the samples were infiltrated with EPO-Nate 812 resin (EMS, Hatfield, PA) by incubating at room temperature (RT) for several hours to overnight in increasing concentrations of resin (15%, 30%, 60%, 90%, 3× 100% resin, diluted in acetone). The samples were transferred to flat-bottomed capsules and the resin polymerized by heating to 70 °C overnight. EPON embedded samples were sectioned to ~2 μm with a glass knife and to ~75 nm with a Diatome diamond knife (Diatome, Hatfield, PA) on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany).

2.4. Bright-field and fluorescence microscopy

Sectioned samples were positioned on glass microscope slides and stained with 0.01% toluidine blue (TBO) or left unstained for epi-fluorescence imaging. Images were captured using a Nikon

C1 Plus microscope (Nikon, Tokyo, Japan), equipped with the Nikon C1 confocal system with Hg lamp and four lasers (403 nm, 561 nm, 643 nm, and Argon tunable 458/477/488/515 nm), and operated via Nikon's EZ-C1 software. Images were captured with a SPOT RTKE CCD camera (Diagnostic Instruments, Sterling Heights, MI). Adobe Photoshop was used to rotate, crop, resize, and adjust contrast, brightness and white balance of images.

2.5. Scanning electron microscopy

Imaging by scanning electron microscopy (SEM) was performed using a FEI Quanta 400 FEG instrument under low vacuum (0.40–0.65 Torr) operating with the gaseous solid-state detector (GAD). Samples were prepared for imaging by freezing in liquid nitrogen followed by lyophilization. Dry samples were mounted on aluminum stubs using carbon tape, and conductive silver paint was applied to the sides of the samples to reduce charging. The samples were not sputter coated. Imaging was performed at beam accelerating voltages from 12.5 to 25 keV.

2.6. Transmission electron microscopy

Thin sections were positioned on 0.5% Formvar coated copper slot grids (SPI Supplies, West Chester, PA). Grids were post-stained for 6 min with 2% aqueous uranyl acetate and 6 min with 1% aqueous $KMnO_4$ to selectively stain for lignins. Images were taken with a 4 mega-pixel Gatan UltraScan 1000 camera (Gatan, Pleasanton, CA) on a FEI Tecnai G2 20 Twin 200 kV LaB6 TEM (FEI, Hilsboro, OR).

3. Results and discussion

Compared to the corn stover and poplar wood samples analyzed in the previous CAFI projects, switchgrass plants have narrow stems and leaves and proved to be relatively more easily handled for microscopy sample preparation. The native, un-pretreated switchgrass samples did retain a significant amount of plant cellular material. Remnants of the plant cytoplasm and organelles such as chloroplast could be clearly identified within cell lumen (Fig. 1 arrows). Usually as the cell desiccates and loses volume, these structures collect at intercellular connecting cell pits. These cell remnants appeared throughout the switchgrass samples and are seldom seen in senesced, field-dried agricultural residue samples like corn stover. While the appearance of the plant cell wall material is visually striking, possibly the most important observation is that none of these remnants remain following any of the pretreatments regardless of the pretreatment chemistries or severities.

3.1. Stereomicroscopy shows color changes and inter-particle variability

Stereomicroscope images of untreated switchgrass and the various CAFI pretreatment conditions (as listed in Table 2) are shown in Fig. 2. These images display color changes and clumping of switchgrass particles caused by the various pretreatments. The control material contained a mixture of stem and leaf particles that were dry and brown, or moist and green. In each of the pretreated samples only occasional traces of green coloration were seen and the particles had turned more uniformly brown. Most samples appeared darker after pretreatment, but the lime pretreated material was lighter in color (orange to light brown, Fig. 2E). These color changes correspond to the removal of chlorophyll and plant cell cytoplasmic contents from the particles. Also, in the case of the AFEX, dilute acid, and LHW pretreatments, the uniform brown color likely corresponds to some lignin re-localization to the surfaces of the particles as was seen in the higher resolution imaging analysis.

Another characteristic of the pretreated particles that can be qualitatively observed by stereomicroscopy is aggregation and moisture content. All pretreated samples showed a tendency to clump relative to the control sample. Particles from SAA pretreatment and lime pretreatment showed the strongest tendency to aggregate. The AFEX pretreated samples exhibited the least tendency to clump (Fig. 2B). One factor that contributes to the particles' tendency to aggregate is their moisture content. The AFEX pretreated samples appeared to be the driest material and the lime pretreated material was very wet. The remaining treatments

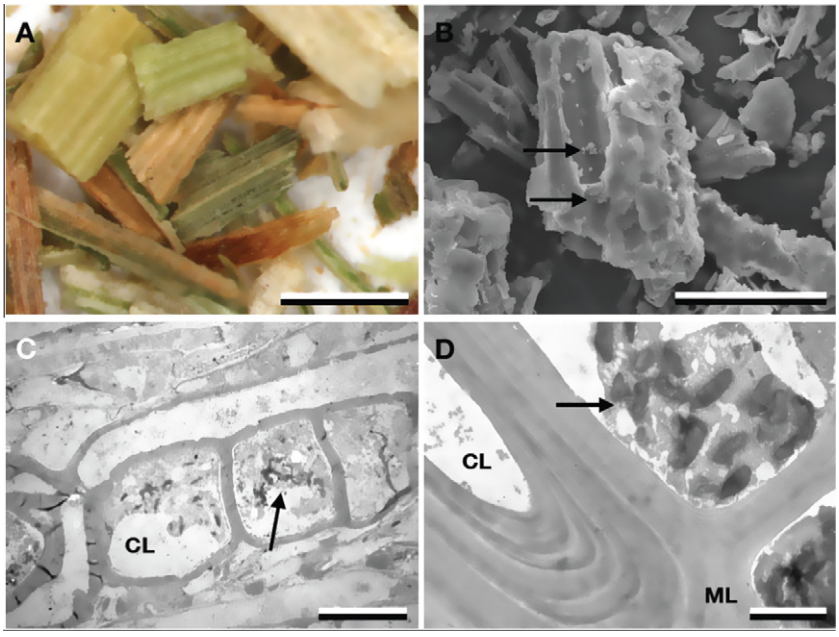

Fig. 1. Stereo (A), SEM (B), and TEM (C, D) micrographs highlighting aspects of green harvested Shawnee switchgrass. Many of the native, milled switchgrass particles were green (A). Residual, partly desiccated plant cells can be visualized within the cell lumen by SEM and TEM (B–D arrows). CL, cell lumen; ML, middle lamella. Scale bars = 1 mm (A), 100 μm (B), 10 μm (C), 2 μm (D). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

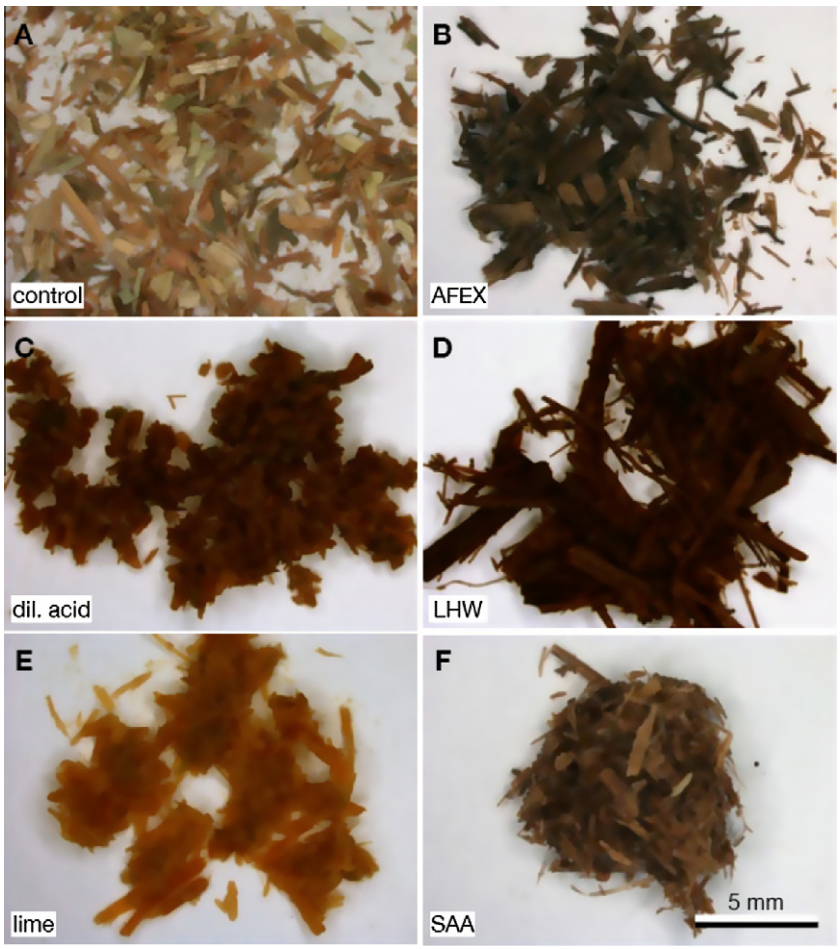

Fig. 2. Stereomicroscope images taken at ~1× magnification of untreated switchgrass and the various CAFI pretreatment conditions (as listed in Table 2). This view displays changes in coloration and clumping of switchgrass particles caused by the pretreatments. Most samples have lost any green coloration and have turned a darker brown. The lime treated material is noticeably lighter in color. SAA and lime treated samples displayed the strongest tendency to aggregate. Scale bar = 5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

appear similarly moist. A final observation made by stereomicroscopy was that the dilute acid pretreated sample had a noticeably smaller average particle size.

3.2. Light microscopy reveals changes in morphology and re-localization of cell wall components

With light microscopy analysis, differences in the way that the various pretreatments impact switchgrass particles at the tissue and cellular-scale structure can be observed. Light microscopy images of untreated and pretreated switchgrass (at pretreatment conditions as listed in Table 2) are shown by epi-fluorescence imaging in Fig. 3 (Row A) and after staining with toluidine blue (TBO) in Fig. 3 (Row B). Additional light microscopy images with detailed annotation are provided as Supplementary Data.

The images confirm that while the various pretreatments can dramatically change the chemical composition of the biomass (upper chart in Fig. 3), the general cellular and tissue structure remains largely intact. Vascular bundles, surrounded by cells with thickened cell walls appeared expanded or swollen in some samples, but even the smaller, thinner-walled cells that appeared deformed at the resolution of light microscopy were usually still present in the samples. Nonetheless, there were several interesting and consistent patterns among the samples.

The AFEX samples displayed the most obvious signs of mechanical disruption of general cell morphology. The thinner-walled parenchyma cells and even some of the thicker-walled fiber cells structure show deformation and collapse of the cell lumen along with broken cell walls. The AFEX pretreated samples also show some regions of lignin concentration, with dark staining, as sometimes irregularly shaped globules lying on lumen surfaces and within delaminated cell walls.

The second pattern revealed by light microscopy is that in the dilute acid and especially the LHW pretreated samples, both the staining and fluorescence pattern show an increased concentration of signal in the middle lamella and cell corners. This pattern is most evident in the TBO stained images where, compared to the control, the pretreated cell walls appear to have lighter staining overall, but the middle lamella and cell corners are distinctly dark. The dark staining regions are areas of higher lignin concentration.

Finally, the SAA and lime pretreated samples both show loss of lignin as evidenced by decreased TBO staining and auto-fluorescence. However, the pattern of loss is slightly different. In the lime pretreated samples, the thicker-walled cells still appear to retain most of their lignin with the signal becoming lower and more diffuse predominately in the thinner-walled cells. Interestingly though, the lime pretreatment appears to have extensively removed material from the middle lamella between cells leaving

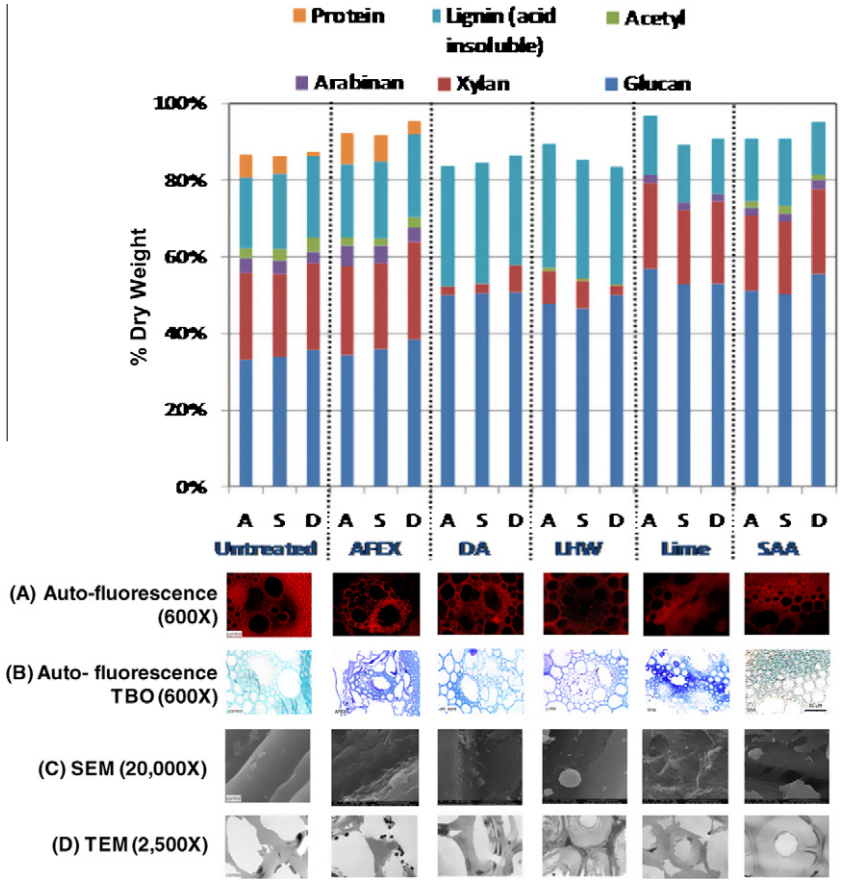

Fig. 3. Compositional analysis of native switchgrass and of solids resulting from each CAFI pretreatment (compositional analysis performed by Purdue University). A refers to Alamo variety, S refers to Shawnee variety, D refers to Dacotah variety. In lower portion, auto-fluorescence light microscope (epi-fluorescense (Row A) and toluidine blue (TBO) staining (row B)), scanning electron microscope (Row C), and transmission electron microscope (Row D) images of each corresponding CAFI sample are displayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detached cells with newly accessible surfaces. In the SAA samples, the staining and fluorescence signal loss and diffusiveness appeared more uniform across the width of the cell wall and more uniform across the different cell types with the middle lamella remaining intact.

3.3. Scanning electron microscopy (SEM) displays cell wall erosion and microfibril unsheathing

Scanning electron microscopy images of untreated switchgrass and the various CAFI pretreatment conditions (as listed in Table 2) are shown in Fig. 3 (Row C). Additional SEM images with detailed annotation are provided as Supplementary Data. These images show significant surface disruption in AFEX pretreated samples. The relatively smooth cell wall surfaces displayed in control samples have become extremely irregular from apparent deposition of re-localized cell wall matrix material. The dilute acid and LHW pretreated samples show evidence for lignin re-localization into lignin-rich globules that are especially apparent at high magnification (20,000×).

One of the most striking visual confirmations of the mode of action for a pretreatment approach was revealed by SEM surface analysis of the lime pretreatment sample. Here, the surface appears to be deeply etched with widespread and nearly complete removal of the cell wall matrix, leaving behind a thin layer exposed cellulose microfibrils on the cell wall surface. It appears that this extensive erosion does not penetrate very far into the cell wall, but it likely creates a highly accessible surface for initial cellulase binding. There appears to be a more homogeneous surface texture in the SAA samples, which is indicative of a more uniform pattern of lignin relocation and removal.

3.4. Transmission electron microscopy (TEM) reveals delamination and changes in cell wall porosity

Transmission electron microscopy images of untreated switchgrass and the various CAFI pretreatment conditions (as listed in Table 2) are shown in Fig. 3 (Row D). Additional TEM images with detailed annotation are provided as Supplementary Data. These images show differences among the pretreatment methods at the cell wall ultrastructure scale. Un-pretreated cell walls display a relatively uniform staining pattern across the cell wall layers, indicative of finely distributed lignin and no major gaps in the lamellar structure. Plant cell material was attached to the cell wall lumen surfaces in control samples, but is not observed in any of the pretreated samples.

The cell walls from the AFEX pretreatment show the most dramatic evidence for lignin re-localization. Globules (up to hundreds of nanometers in diameter) of coalesced lignin-rich material were seen on cell wall surfaces and within newly formed delamination/pore zones. The dilute acid and LHW pretreated samples also displayed some lignin globules on cell wall surfaces. In addition, the dilute acid samples displayed delamination of cell wall lamella and some coalescence of lignin in the middle lamella, cell corners, and delamination gaps. The LHW samples, however, revealed a widespread increase in porosity across the width of even the

Table 3
Summary of the general observations across switchgrass tissue structures, cells, and cell walls as determined by various analytical microscopy imaging techniques on untreated and variously pretreated switchgrass samples.

Sample	Tissues			Cells		Cell wall architecture		
	Color	Aggregation	Moisture	Morphology	Lignin	Thickness	Delamination	Lignin
Control	Green/brown	Loose	Dry	Control	Diffuse	Control	None	Control
SAA (Auburn)	Brown/green	Clumped	Moist	Deformed	Diffuse	Swollen	Minimal	Decreased
AFEX (MSU)	Brown	Loose	Dry	Collapsed	Concentrated	Thinned	Extensive	Re-localized
LHW (Purdue)	Brown	Clumped	Moist	Altered	Concentrated	Expanded	Extensive	Re-localized
Lime (Texas A&M)	Tan/orange	Clumped	Wet	Deformed	Diffuse	Thinned	Minimal	Decreased
Dilute acid (UC Riverside)	Brown	Clumped	Moist	Deformed	Concentrated	Expanded	Moderate	Re-localized

thickest cell walls. In addition, the LHW samples showed a striking and extensive delamination in the cell walls. This level of delamination and porosity increase should have a dramatic impact on increasing enzyme accessibility.

Cell walls in the lime pretreatment sample showed a slight decrease in staining that correlates to a loss of lignin and cell wall that often appeared thinned, with an irregular scalloped surface. Another feature of the lime pretreatment was that the spaces in the cell corners were enlarged and at higher magnification some removal of material from the middle lamella was revealed. The SAA pretreated samples displayed swollen cell walls and an uniform decrease in lignin staining across the layers of the cell wall. The extensive swelling may be an indication of a general loosening of the cell wall structure, but no extensive delamination was seen in the SAA treated cell walls.

3.5. Summary of imaging observations

A summary of the general qualitative observations across switchgrass tissue structures, cells, and cell walls is presented in Table 3. This table compiles some of the major observed features from the different imaging techniques discussed above.

3.6. Relating imaging observations to compositional changes and enzymatic hydrolysis

When comparing the pretreated solids compositional data in Fig. 3 to the various images generated for each of the pretreated samples, it is clear that observed structural changes upon pretreatment cannot be easily related to the composition data. At first glance, pretreated samples look similar to controls at all scales and it is not obvious that nearly 40% of the mass has been solubilized by several of pretreatment conditions, although dramatic differences in individual component solubilization is seen across the various pretreatment conditions. In addition, even in the relatively small and simple switchgrass stems, there can be cell-to-cell variability in how the pretreatments impact cell wall structure. It is not possible to directly visualize the compositional and chemical changes in pretreated samples, but instead the microscopic analysis reveals the architectural changes in cell walls that may result from and facilitate those compositional changes taking place.

This phenomenon is especially evident for the AFEX pretreated switchgrass, which shows virtually no gross change in composition upon pretreatment, but does display significant structural changes at the cellular and cell wall ultrastructural level. Some of the observed structural changes appear to be consistent with the AFEX compositional data, as there is strong evidence of lignin rearrangement into lignin globules that accumulate in cell pits, cell corners, and delamination zones (Fig. 4A, arrowhead), but not necessarily gross lignin removal. For the pretreatments that achieve significant hemicellulose removal (dilute acid and LHW pretreatment), there is significant cell wall delamination evident (Fig. 4B arrow), along with areas of lignin re-localization and globule formation that are typical of these pretreatment approaches on other feedstocks, such as corn stover. Imaging results on the pretreatments that result in substantial lignin removal (lime and SAA pretreatment) do show evidence of lower lignin intensity in the staining-based imaging, along with removal of the lignin-rich middle lamella and some cell wall swelling (Fig. 4C and D). Delamination and increased porosity is one of the major themes for how pretreatment changes cell wall architecture to improve digestibility. Fig. 4 shows a comparison of delamination caused by pretreatment.

When comparing pretreated solids enzymatic digestibility data to the various images generated for each of the pretreated samples, it is clear that different types of structural changes to cell walls can enable improved digestibility. As reported in other CAFI 3 project papers published in this same issue, overall glucose and xylose yields (including soluble oligomers) are highest for the lime, LHW and SO_2 pretreatments (SO_2 pretreated switchgrass sample was not available for imaging analysis), with somewhat lower overall yields for the other pretreatments. In all pretreatments, glucose production occurred primarily in the enzymatic hydrolysis step, while xylose production occurred to a greater extent in the pretreatment step for the acidic pretreatments and the LHW pretreatment (washed sample).

The pretreated solids that achieved the highest overall sugar yields for samples available for imaging analysis (LHW-washed and lime pretreatments) showed very different structural effects upon pretreatment. The LHW pretreatment achieved high enzymatic digestibility by, in addition to hemicellulose removal and lignin re-localization, a massive amount of cell wall delamination and increase in porosity. These impacts were clearly revealed by TEM analysis (Fig. 4B). The lime pretreatment achieved high enzymatic digestibility by, in addition to partial hemicellulose removal and by removing and re-arranging lignin, also creating extensive new surface area by etching away cell wall matrix and leaving microfibrils exposed on cell wall structures. This phenomenon was revealed by SEM analysis (Fig. 5C). Surface erosion or etching has emerged as another of the major themes in how pretreatment changes cell wall architecture to improve digestibility. Fig. 5 shows a comparison of surface erosion caused by pretreatment. The other sample that highlights the impact of structural changes to cell wall architecture on enzyme accessibility is the AFEX pretreatment. Although the AFEX pretreatment had somewhat lower enzymatic hydrolysis sugar yields, there were still significant structural impacts at the cellular and cell wall level even without significant changes in composition upon pretreatment. AFEX samples displayed extensive lignin re-localization and globule formation along with evidence of delamination and increased porosity.

Structural disruption of cell wall architecture to improve enzyme accessibility is clearly one of the key effects of pretreatment. The pore space that needs to be created for an enzyme to gain new access into a cell wall is on the order of tens of nanometers. Therefore, further structural analysis of these types of samples should

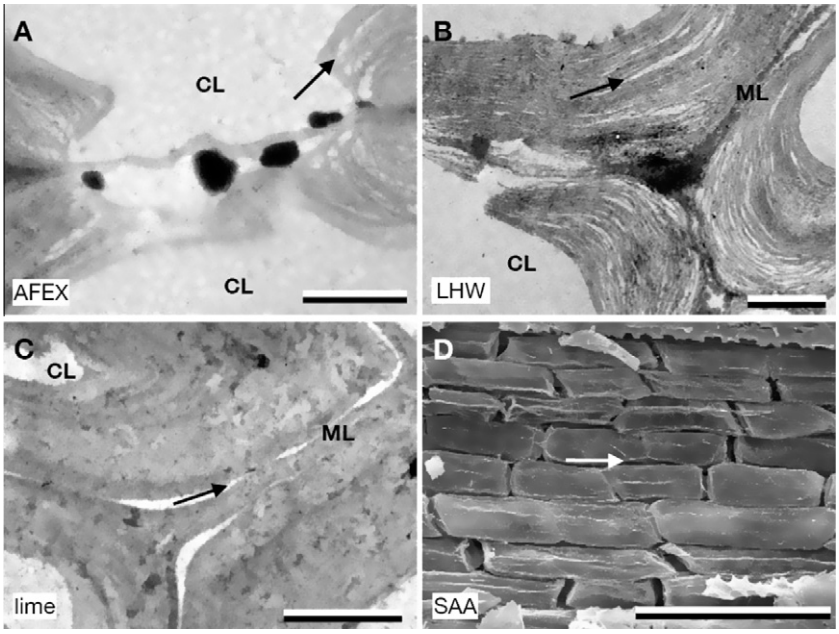

Fig. 4. TEM (A–C) and SEM (D) micrographs highlighting the impact of various pretreatments on delaminating cell walls and increasing porosity. The AFEX pretreatment delaminated secondary cell wall particularly near cell pits (arrow). The cell pits also accumulated coalesced lignin globules (arrowhead). LHW samples showed extensive delamination throughout the cell walls (arrow). Lime and SAA pretreatment separated adjacent cells by disrupting and dissolving the middle lamella (arrows). CL, cell lumen; ML, middle lamella. Scale bars = 1 μm (A–C), 100 μm (D).

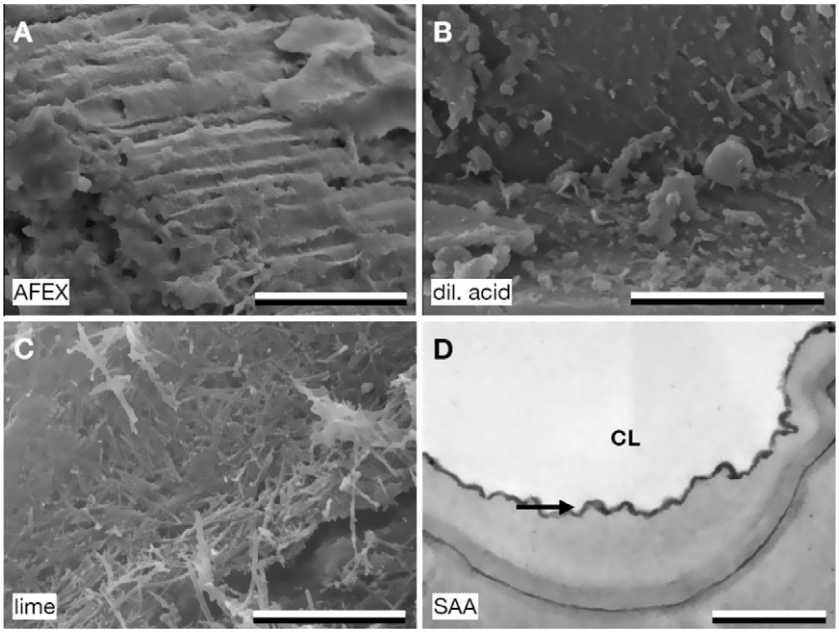

Fig. 5. SEM (A–C) and TEM (D) micrographs highlighting the impact of various pretreatments on eroding the cell lumen surface of cell walls. The AFEX and dilute acid samples show a combination of surface erosion and re-deposition of material on the surface (A, B). The lime pretreatment partially unsheathed layers of microfibrils (C). The SAA sample displays an irregularly eroded surface (D arrow). CL, cell lumen. Scale bars = 100 μm (A), 5 μm (B, C), 1 μm (D).

focus on the highest resolution imaging technologies to reveal changes at the nano-scale. Another route for further analysis is to create a better chemical overlay on the structural data provided by microscopy. This could include Raman and IR imaging spectroscopy. This work has illustrated the value of correlative microscopic approaches to structural analysis of biomass to understand both surface and sub-surface changes in biomass particle architecture by different pretreatment methods across a range of scales.

4. Conclusions

Each of the CAFI pretreatments on switchgrass results in different structural impacts at the tissue, cellular, and cell wall levels. These impacts can cause changes in chemical composition upon pretreatment, as there is evidence of hemicellulose removal in dilute acid and LHW pretreatments, and evidence of lignin removal/re-arrangement by lime and SAA pretreatments. AFEX pretreat-

ment results in no gross change in composition, but does cause structural changes. When comparing the pretreated solids enzymatic digestibility data, it is not possible to draw a direct correlation to specific structural features that lead to improved digestibility. There are clearly different structural mechanisms that cause enhancement of enzymatic hydrolysis sugar yields. This is most evident when comparing the LHW to the lime pretreatment, as similar enzymatic hydrolysis yields are reported, although substantial differences in pretreated solids composition and structural changes at the cellular and cell wall levels are evident.

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Appendix A. Supplementary data

Additional light microscopy, SEM, and TEM images of untreated and pretreated switchgrass with detailed annotation are available. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.03.092.

References

Balan, V., Sousa, L., Chundawat, S., Marshall, D., Sharma, L.N., Chambliss, K., Dale, B.E., 2009. Enzymatic digestibility and pretreatment degradation products for AFEX-treated hardwoods (*Populus* nigra). Biotechnology Progress 25 (2), 365–375.

Bura, R., Chandra, R., Saddler, J., 2009. Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar. Biotechnology Progress 25 (2), 315–322.

Chundawat, S.P., Donohoe, B.S., da Costa Sousa, L., Elder, T., Agarwal, U.P., Fachuang, L., Ralph, J., Himmel, M.E., Balan, V., Dale, B.E., 2011. Multi-scale visualization and characterization of lignocellulosic plant cell wall deconstruction during thermochemical pretreatment. Energy and Environmental Science. doi:10.1039/c0ee00547f.

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B., 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. Biotechnology and Bioengineering 101 (5), 913–925.

Eggeman, T., Elander, R., 2005. Process and economic analysis of pretreatment technologies. Bioresource Technology 96 (18), 2019–2025.

Gupta, R., Lee, Y.Y., 2009. Pretreatment of hybrid poplar by aqueous ammonia. Biotechnology Progress 25 (2), 357–364.

Kim, S., Holtzapple, M.T., 2005. Lime pretreatment and enzymatic hydrolysis of corn stover. Bioresource Technology 96 (18), 1994–2006.

Kim, T., Lee, Y.Y., 2005. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. Bioresource Technology 96 (18), 2007–2013.

Kim, Y., Mosier, N.S., Ladisch, M.R., 2009. Enzymatic digestion of liquid hot water pretreated hybrid poplar. Biotechnology Progress 25 (2), 340–348.

Kristensen, J., Thygesen, L., Felby, C., Jorgensen, H., Elder, T., 2008. Cell wall structural changes in wheat straw pretreated for bioethanol production. Biotechnology for Biofuels 1 (1), 5.

Kumar, R., Mago, G., Balan, V., Wyman, C.E., 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. Bioresource Technology 100 (17), 3948–3962.

Kumar, R., Wyman, C.E., 2009. Effects of cellulase and xylanase enzymes of the deconstruction of solids from pretreatment of poplar by leading technologies. Biotechnology Progress 25 (2), 301–314.

Liu, C., Wyman, C.E., 2005. Partial flow of compressed-hot water through corn stover to enhance hemicellulose sugar recovery and enzymatic digestibility of cellulose. Bioresource Technology 96 (18), 1978–1985.

Lloyd, T.A., Wyman, C.E., 2005. Total sugar yields for pretreatment by hemicellulose hydrolysis coupled with enzymatic hydrolysis of the remaining solids. Bioresource Technology 96 (18), 1967–1977.

Lu, Y., Warner, R., Sedlak, M., Ho, N., Mosier, N.S., 2009. Comparison of glucose/xylose cofermentation of poplar hydrolysates processed by different pretreatment technologies. Biotechnology Progress 25 (2), 349–356.

Mosier, N., Hendrickson, R., Ho, N., Sedlak, M., Ladisch, M., 2005. Optimization of pH controlled liquid hot water pretreatment of corn stover. Bioresource Technology 96 (18), 1986–1993.

Sierra, R., Granda, C., Holtzapple, M.T., 2009. Short-term lime pretreatment of poplar wood. Biotechnology Progress 25 (2), 323–332.

Teymouri, F., Laureano-Perez, L., Alizadeh, H., Dale, B.E., 2005. Optimization of the ammonia fiber explosion (AFEX) treatment parameters for enzymatic hydrolysis of corn stover. Bioresource Technology 96 (18), 2014–2018.

Wyman, C., Dale, B., Elander, R., Holtzapple, M., Ladisch, M., Lee, Y.Y., 2005a. Coordinated development of leading biomass pretreatment technologies. Bioresource Technology 96 (18), 1959–1966.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005b. Comparative sugar recovery data from laboratory scale application of leading pretreatment technologies to corn stover. Bioresource Technology 96 (18), 2026–2032.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., Mitchinson, C., Saddler, J.N., 2009. Comparative sugar recovery and fermentation data following pretreatment of poplar wood by leading technologies. Biotechnology Progress 25 (2), 333–339.

Zeng, M.J., Mosier, N.S., Huang, C.P., Sherman, D.M., Ladisch, M.R., 2007. Microscopic examination of changes of plant cell structure in corn stover due to hot water pretreatment and enzymatic hydrolysis. Biotechnology and Bioengineering 97 (2), 265–278.