Energy & Environmental Science

Cite this: DOI: 10.1039/c1ee02112e

www.rsc.org/ees

PAPER

Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production†

Jaclyn D. DeMartini,[abe] Sivakumar Pattathil,[ce] Utku Avci,[ce] Kaitlyn Szekalski,[c] Koushik Mazumder,[ce] Michael G. Hahn[cde] and Charles E. Wyman*[abe]

Received 10th July 2011, Accepted 17th August 2011
DOI: 10.1039/c1ee02112e

To better understand how hydrothermal pretreatment reduces plant cell wall recalcitrance, we applied a high throughput approach ("glycome profiling") using a comprehensive suite of plant glycan-directed monoclonal antibodies to monitor structural/extractability changes in *Populus* biomass. The results of glycome profiling studies were verified by immunolabeling using selected antibodies from the same toolkit. The array of monoclonal antibodies employed in these studies is large enough to monitor changes occurring in most plant cell wall polysaccharides. Results from these techniques demonstrate the sequence of structural changes that occur in plant cell walls during pretreatment-induced deconstruction, namely, the initial disruption of lignin-polysaccharide interactions in concert with a loss of pectins and arabinogalactans; this is followed by significant removal of xylans and xyloglucans. Additionally, this study also suggests that lignin content per se does not affect recalcitrance; instead, the integration of lignin and polysaccharides within cell walls, and their associations with one another, play a larger role.

[a]*Chemical and Environmental Engineering Department, University of California, Riverside, Riverside, CA, 92507, USA. E-mail: Charles.wyman@ucr.edu; Fax: +951-781-5790; Tel: +951-781-5703*
[b]*Center for Environmental Research and Technology, Bourns College of Engineering, University of California, Riverside, 1084 Columbia Ave, Riverside, CA, 92507, USA*
[c]*Complex Carbohydrate Research Center, The University of Georgia, 315 Riverbend Rd., Athens, Georgia, 30602, USA*
[d]*Department of Plant Biology, University of Georgia, Athens, GA, 30602, USA*
[e]*BESC BioEnergy Science Center, Oak Ridge National Laboratory, Oak Ridge, TN, 37831, USA*
† Electronic supplementary information (ESI) available. See DOI: 10.1039/c1ee02112e

Introduction

Lignocellulosic biomass is the only sustainable resource for large-scale production of liquid transportation fuel that has the potential to significantly reduce the world's dependence on petroleum.[1–3] One of the primary barriers to low cost biological conversion of lignocellulosic biomass to renewable fuels is the plants' recalcitrance, which refers to the resistance of cell walls to deconstruction by enzymes or microbes.[4,5] To overcome this obstacle, biomass is subjected to pretreatment prior to enzymatic hydrolysis in order to disrupt the plant cell wall's structure and thereby allow hydrolyzing enzymes better access to the cellulose

Broader context

Currently, the only promising resource for large-scale sustainable production of liquid transportation fuels to reduce dependence on petroleum and associated greenhouse gas emissions is lignocellulosic biomass. However, the key barrier to low cost production of fuels and chemicals is the plants' recalcitrance, which refers to the resistance of cell walls to deconstruction by chemicals, heat, enzymes, or microbes. Lignocellulosic biomass is typically pretreated prior to enzymatic hydrolysis to disrupt cell wall structure and thereby allow hydrolyzing enzymes better access to the cellulose core. However, few details are known about the effects of pretreatment on cell wall structure and composition, making it difficult to intelligently design and optimize biomass deconstruction, including more efficient pretreatment processes and enzyme cocktails. Our study employed a novel glycome profiling technique in which cell wall glycan-directed monoclonal antibodies were applied to monitor structural/extractability changes in untreated and hydrothermally-pretreated *Populus* biomass. We demonstrate that hydrothermal pretreatment causes an initial disruption of lignin-polysaccharide interactions in concert with a loss of pectins and arabinogalactans, followed by significant removal of xylans and xyloglucans. Additionally, this study also suggests that the integration of lignin and polysaccharides within cell walls, and their associations with one another, play a large role in recalcitrance.

core.[6] However, few details are known as to the exact effects of pretreatment on cell wall structure and composition, thus making it difficult to intelligently design and optimize biomass deconstruction.

To gain insight into the effects of pretreatment, laboratories conventionally perform a general wet chemistry compositional analysis to determine basic carbohydrate and lignin content of the untreated and pretreated biomass.[7] More recently, though, investigations into the effects of pretreatment on biomass and cell wall structure have been improved through the application of microscopic techniques. Such studies can provide visual evidence of disruptions to microfibrils,[8] the creation of pits or holes in the cell wall,[9] the fate of lignin,[9,10] and the distribution of xylan throughout the cell walls of pretreated materials.[11] However, detailed information regarding the deconstruction of plant biomass and its polysaccharide components remains far from complete. Such information is essential to rationally design processes that more effectively prepare biomass for the subsequent step of enzymatic hydrolysis while keeping costs low. Optimized pretreatment processes, when combined with improved enzyme mixtures and the use of biomass species with reduced recalcitrance, have the potential to make the production of fuels from lignocellulosic biomass more commercially viable.[2]

Beyond the chemical and microscopic techniques mentioned above, tools that can analyze plant cell wall composition and structure remain limited.[12] To address this concern, this study employed a novel glycome profiling technique in which cell wall glycan-directed monoclonal antibodies (mAbs) were used to monitor structural/extractability changes in untreated and hydrothermally-pretreated *Populus* biomass. The worldwide collection of plant cell wall glycan-directed mAbs is now sufficiently large that they can be used to monitor cell wall structural changes involving most major classes of plant polysaccharides.[13] The combination of glycome profiling, immunolocalization, along with sugar release data from the enzymatic hydrolysis of untreated and hydrothermally-pretreated *Populus* biomass made it possible to draw correlations between plant cell wall structure and digestibility.

Results and discussion

Cell wall changes in hydrothermally-pretreated biomass revealed by glycome profiling

We used three approaches to investigate how poplar cell walls are deconstructed during hydrothermal pretreatments of different lengths (11, 28, and 70 min): 1) wet chemistry compositional analysis of the untreated and pretreated poplar solids for glucan, xylan, and acid insoluble residue contents; 2) glycome profiling of sequential chemical extracts of the untreated and pretreated biomass samples; and 3) immunolabeling to study the *in situ* spatial distribution of carbohydrate epitopes in intact untreated and pretreated biomass materials. All three approaches showed dramatic changes in the composition and structure of the biomass during deconstruction by hydrothermal pretreatment, with increasingly long pretreatments yielding greater changes. The wet chemistry compositional analyses provided the most limited information regarding changes, demonstrating that the xylan content decreased with increasing pretreatment time, ranging from 17% in the untreated material to 5% in the 70 min pretreated material (Fig. 1). Furthermore, the analyses also demonstrated that there was a corresponding increase in glucan content from 42 to 62%, as well as a slight increase in acid-insoluble residue content (which closely approximates Klason lignin in poplar) from 25 to 33% for the untreated and 70 min pretreated materials, respectively, reflecting the preferential solubilization of non-glucan biomass components by hydrothermal pretreatment. These results are consistent with typical results under similar conditions.[9]

Significantly more information on the deconstruction of poplar cell walls by hydrothermal pretreatment was revealed by the glycome profiling and immunolocalization studies. Glycome profiling employs a set of increasingly harsh sequential extractions to solubilize different carbohydrate components from the cell walls, depending on how tightly these components are bound into the walls. For example, extracts released by mild reagents such as oxalate and carbonate tend to be enriched in arabinogalactans and pectic components, while the extracts resulting from the harsher alkaline extractions tend to be enriched in hemicellulosic polysaccharides (xylans and xyloglucans). More tightly bound lignin components of the cell wall materials are removed using a chlorite treatment, and a post chlorite 4M KOH treatment solubilizes additional carbohydrates after removal of lignin. These wall extracts can then be screened with a comprehensive mAb toolkit to define what glycan components are solubilized in each extraction step.

The results (Fig. 2) demonstrate that the glycome profiles of the hydrothermally-pretreated biomass samples were entirely different from that of the unpretreated biomass, even for the mildest pretreatment condition of 11 min at 180 °C. Specifically, there were three distinct differences in the glycome profiles between the untreated and mildly pretreated material: 1) a loss of almost all lignin-bound arabinogalactan and xylan epitopes in the chlorite extract, suggesting that these lignin-carbohydrate associations in the wall were particularly labile; 2) a significant

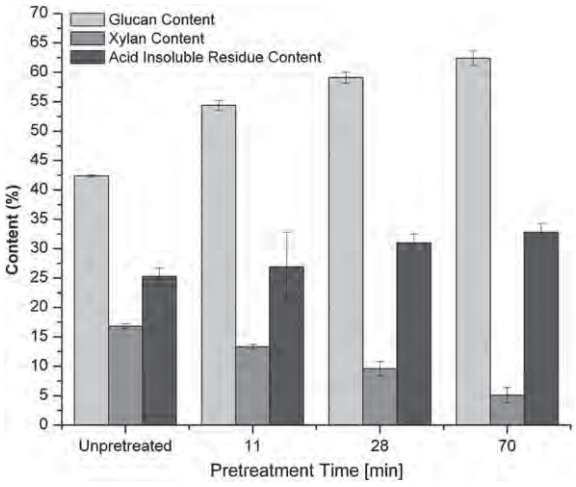

Fig. 1 Composition of untreated and hydrothermally-pretreated *Populus trichocarpa* biomass. Glucan, xylan, and acid insoluble residue (Klason lignin) contents were determined as described in Materials and Methods. Tests were performed in triplicate, with the error bars representing the corresponding standard deviations.

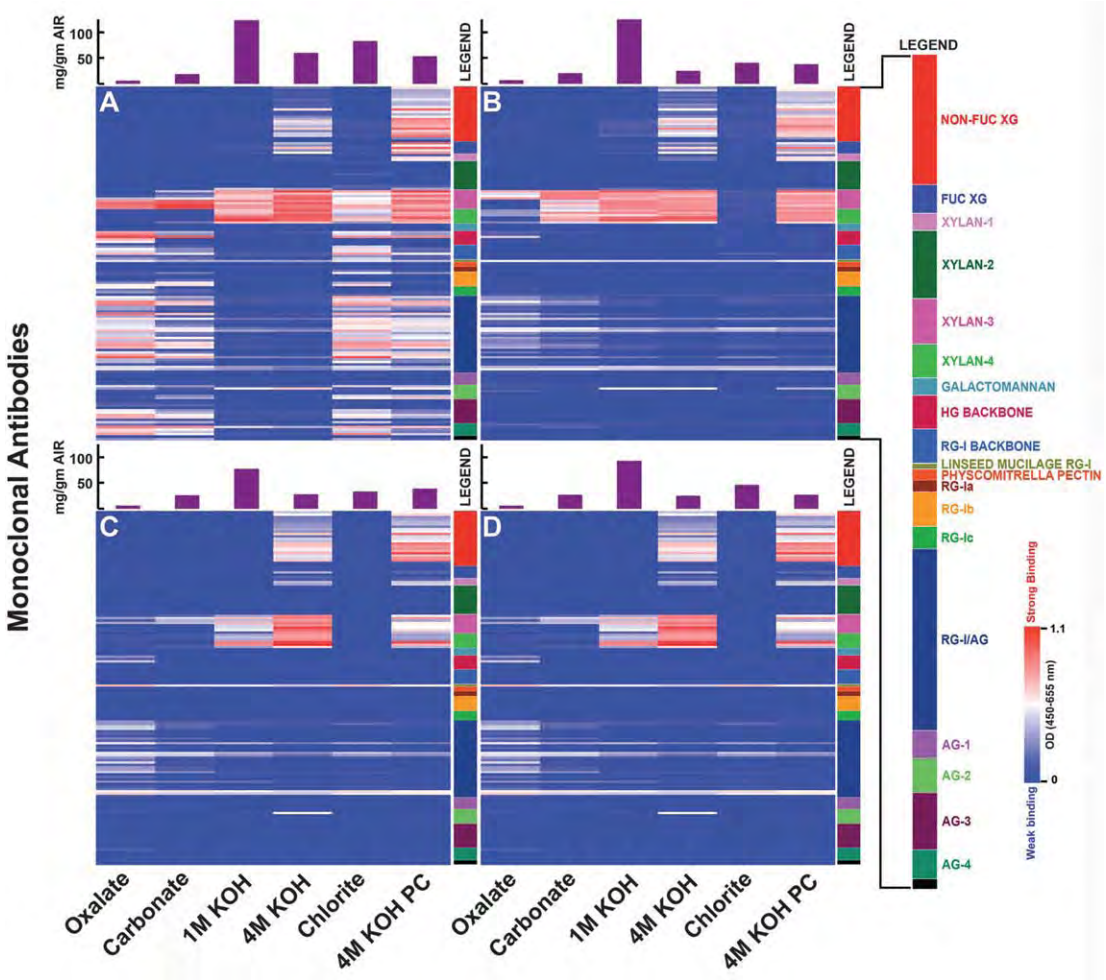

Fig. 2 Glycome profiling of untreated and hydrothermally-pretreated *Populus trichocarpa* biomass. Sequential extracts were prepared from untreated (A), 11 min pretreated (B), 28 min pretreated (C), and 70 min pretreated (D) biomass. The extracted materials released from each biomass sample by various reagents (as labeled at the bottom of each map) were loaded onto the ELISA plates and were screened against an array of plant glycan-directed monoclonal antibodies. The legend panel on the right of the figure displays the nature of the polysaccharides predominantly recognized by these mAbs. Antibody binding is represented as colored heat maps, with dark blue signifying no binding, white representing intermediate binding, and bright red representing the strongest binding. The bar graphs at the top indicate the amount of material recovered at each extraction step per gram of alcohol insoluble residue (AIR).

reduction in pectic and arabinogalactan epitopes (those recognized by the AG, RG-I/AG and pectic backbone antibody groups); and 3) a slight shift of xylan components from harsher extracts (1 M KOH through 4 M KOH PC) to milder extracts (oxalate and carbonate). However, the amounts of xylan epitopes recognized by the xylan-3 and -4 groups of mAbs remained largely unchanged after the 11 min pretreatment. The levels of xyloglucan (both fucosylated and non-fucosylated) epitopes also appeared largely unaffected by the mild pretreatment.

Poplar biomass subjected to longer hydrothermal pretreatment times (28 and 70 min) showed a further reduction in wall polysaccharide epitopes recognized by the entire mAbs toolkit. In particular, pectic and arabinogalactan epitopes disappeared more completely under the longer pretreatments, suggesting that these epitopes were significantly more sensitive to deconstruction than were hemicellulose epitopes. Furthermore, an increasing loss of xylan epitopes, as well as fucosylated and non-fucosylated xyloglucan epitopes, was also observed in poplar biomass pretreated for 28 and 70 min. Nonetheless, significant amounts of xylans and non-fucosylated xyloglucan epitopes remained strongly bound in the cell wall after hydrothermal pretreatment for 28 min, as seen in the 4 M KOH and 4 M KOH PC extracts of this pretreated biomass. For the 70 min pretreated material, while most of the wall polysaccharide epitopes recognized by the antibodies in the toolkit were gone, some xylan and xyloglucan epitopes remained, particularly in the 4M KOH extract.

Molecular sieve chromatography of wall extracts and immunolabeling of biomass

The ELISA assays carried out for glycome profiling were performed with loading of equal amounts of carbohydrate of each extract onto the plates. However, for many of the extracts from hydrothermally-pretreated biomass, there was far lower total signal for many antibodies than was observed for the equivalent extracts from untreated poplar biomass. For example, the

chlorite extract of the mildly pretreated poplar contained considerable carbohydrate material, but almost no antibody binding (Fig. 2). This is in contrast to the untreated material in which the chlorite extract contained significant amounts of both pectic and hemicellulosic epitopes. This observed loss of signal in the ELISAs for pretreated samples could arise for two reasons: 1) a mass removal of epitope structures (resulting from the breaking of bonds during the hydrothermal pretreatment process), or 2) the inability of smaller-sized components that may be produced by hydrothermal pretreatments to adsorb onto the ELISA plate since it is known that small polysaccharides, such as rhamnogalacturonan II (which has a molecular mass of 5–10 kDa[14]), do not adhere to ELISA plates.[13] The size of the extracted materials (except for the oxalate fractions, where the amounts of material were too low to permit analysis) was examined by molecular sieve chromatography, and the results demonstrated that extracts from the hydrothermally-pretreated materials contained shorter polysaccharide chains compared to the equivalent extracts from unpretreated samples. These results suggest that the absence of antibody binding to the extracts from pretreated biomass could largely be explained by the cleavage of the polysaccharides to small fragments. However, they do not exclude the possibility that the epitopes themselves have been removed or destroyed by the pretreatments.

To test for epitope destruction *in situ* that might be correlated with the glycome profiling results described in the previous section, imunolabeling of the untreated and pretreated poplar was performed (Fig. 3) to reveal information on the *in situ* spatial distribution of carbohydrate epitopes in intact biomass before and after deconstruction. Accordingly, antibodies selected from diverse antibody groups based on the glycome profiling results, in addition to the carbohydrate-binding module, CBM2a, which binds to crystalline cellulose,[15] were used for the glycan epitope localizations. With the exception of crystalline cellulose (CBM2a binding) and the xylan epitope recognized by CCRC-M149 (of the xylan-3 group), which showed no appreciable decline as a result of hydrothermal pretreatment, a general decline in wall polysaccharide epitopes recognized by the selected mAbs was observed with increasing pretreatment time (Fig. 3). Thus, immunolabeling of many pectic (HG and RG-I) and arabinogalactan (AG) related epitopes declined or disappeared even after the shortest hydrothermal pretreatment, consistent with the results of glycome profiling. Immunolabeling of the fucosylated xyloglucan epitope by CCRC-M1 also declined appreciably after mild pretreatment, while labeling of a non-fucosylated xyloglucan epitope by CCRC-M88 was still evident even after the 70 min pretreatment. Each of the xylan epitopes examined showed different immunolabeling patterns in the biomass samples. CCRC-M150 (xylan-2 group) exhibited no binding to any of the samples, whereas CCRC-M108 (xylan-1 group) showed a low level of labeling in untreated biomass that disappeared after mild pretreatment. CCRC-M138 (xylan-4 group) labeled untreated and mildly pretreated biomass strongly, but labeling declined dramatically after 28 and 70 min pretreatments. Thus, immunolabeling clearly showed that epitopes were lost in the pretreatment process, further supporting the conclusion that the significant changes observed in glycome profiling largely reflected the removal of epitopes during deconstruction by pretreatment.

Past approaches and new insights

Detailed studies of cell wall deconstruction caused by pretreatment have heretofore been limited by the availability of tools capable of analyzing plant cell walls. Chemical analyses provided basic compositional data (Fig. 1), but in general did not supply more detailed compositional information or structural characteristics. However, a substantially increased collection of plant cell wall glycan-directed monoclonal antibodies is now available that is sufficiently large and diverse to monitor changes occurring in most major plant cell wall polysaccharides.[13] A previous study employed four cell wall glycan-directed probes in a Comprehensive Microarray Polymer Profiling (CoMPP) technique[16] to examine the effects of hydrothermal pretreatment on wheat straw.[17] The results of the previous study and the current study reported here are largely in concurrence, despite the fact that our study employed different pretreatment conditions with a woody dicot, which has a significantly different structural makeup than wheat straw. To wit, significant reductions in hemicellose contents were observed in both studies only under the harshest pretreatment conditions. Although the work by Alonso-Simón and coworkers[17] was an important step toward providing more detailed analysis of cell walls following deconstruction by pretreatment, the study only monitored four glycan epitopes covering three wall polymers. The current study utilized 155 antibodies against a broad diversity of wall glycan structures, with each major wall polymer class being monitored by antibodies against multiple epitopes on each of those polymers.[13] This larger antibody toolkit yielded a more complete picture of changes that occurred during hydrothermal pretreatment and provided greater insight into the polymers that were attacked most quickly by hydrothermal pretreatment. In the case of the poplar biomass examined here, these were the arabinogalactans and lignin-associated glycans. In addition, we were able to document that not all polysaccharides of a given class were equally affected by the pretreatment. Thus, the changes in the poplar biomass resulting from pretreatment were complex, in keeping with the known complexity of cell walls.

Furthermore, although hydrothermal pretreatment has been previously reported to remove a significant portion of hemicellulose,[18,19] the resolution and scope of biomass monitoring was improved in the current study. Here we found that arabinogalactans of various types were the first wall components that were lost upon hydrothermal pretreatment of poplar, a result that has not been reported previously. On the other hand, hemicelluloses such as xylans and xyloglucans required harsher pretreatments to be removed. Both glycome profiling and immunolabeling further suggested that the fucosyl-containing epitope present on xyloglucans is removed more easily than were non-fucosylated xyloglucan epitopes. Furthermore, some xylan epitopes showed remarkable resistance to the pretreatment.

This study also sheds new light on lignin-carbohydrate associations in poplar, and their fate during deconstruction by hydrothermal pretreatment. Although the lignin-carbohydrate associations observed in the chlorite extractions of this study likely do not represent all such associations that exist in the cell walls of poplar, the results suggest that there are at least two major classes of polymers that are associated with lignin, the

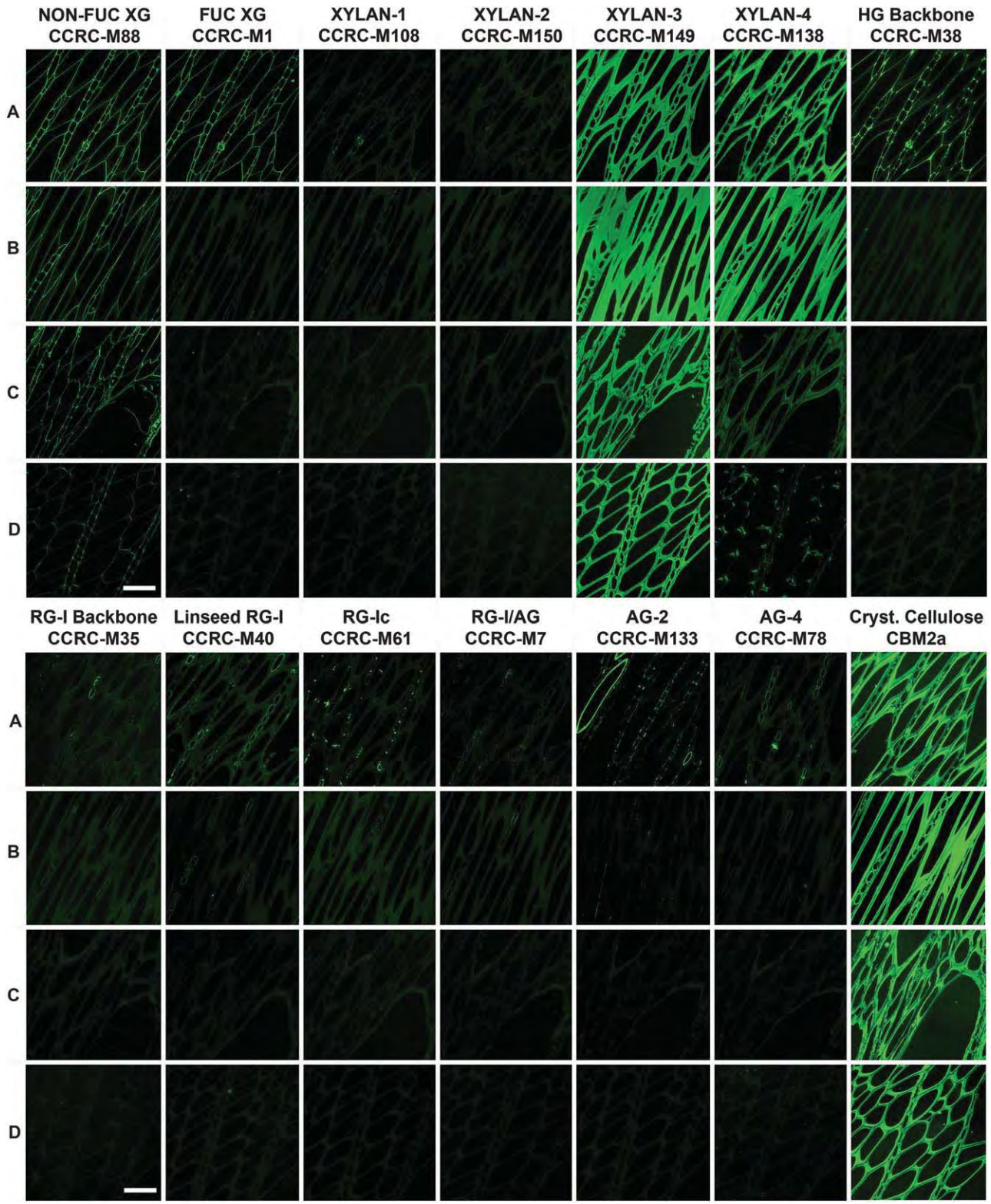

Fig. 3 Immunofluorescent labeling of untreated (A), 11 min pretreated (B), 28 min pretreated (C), and 70 min pretreated (D) *Populus trichocarpa* biomass with selected mAbs representative of different groups of antibodies that recognize distinct epitopes present on various plant cell wall glycans, as indicated at the tops of the images. Sections (250 nm) were taken from biomass samples, and (immuno)labeling was carried out as described in Materials and Methods. Scale bars = 50 μm.

xylans and pectins/arabinogalactans. In fact, arabinogalactans have been previously found to be removed concurrently with lignin during delignification of lupin by chemical treatments,[20,21] although the nature of these lignin-carbohydrate complexes still remains largely a mystery. The fate of lignin and its associations with carbohydrates during pretreatment is equally unclear. Although it has been previously reported that the morphology of lignin changes as a result of pretreatment, including an increase in the degree of condensation[22] and a re-localization,[10] there is no clear-cut consensus on its fate. Some data[10] suggest that in dilute acid pretreatment above the melting temperature of lignin, lignin coalesces within the cell wall, migrates out, and then re-deposits as droplets on the biomass surface. It remains less clear whether the lignin droplets stay complexed with carbohydrates during removal and re-deposition. Glycome profiling results reported here suggest that the lignin-carbohydrate associations that we were able to monitor, including lignin-pectic/arabinogalactan and some lignin-xylan associations, were disrupted by even the mildest hydrothermal pretreatment.

Additionally, labeling of the biomass with CBM2a showed that the distribution of crystalline cellulose did not appear to change between the untreated and pretreated materials. Some past studies measured slight increases in cellulose crystallinity following dilute acid pretreatment of biomass,[8,22] while others[9] reported that hydrothermal pretreatment did not result in an increase in the degree of cellulose crystallinity. Our immunolabeling results, although not quantitative, support the latter claim. It is interesting that despite the significant changes observed in the hemicellulose, pectin, and arabinogalactan structures during deconstruction by hydrothermal pretreatment, there was no apparent disruption to the overall crystalline cellulose structure of the cell wall at any of the pretreatment times tested in this study.

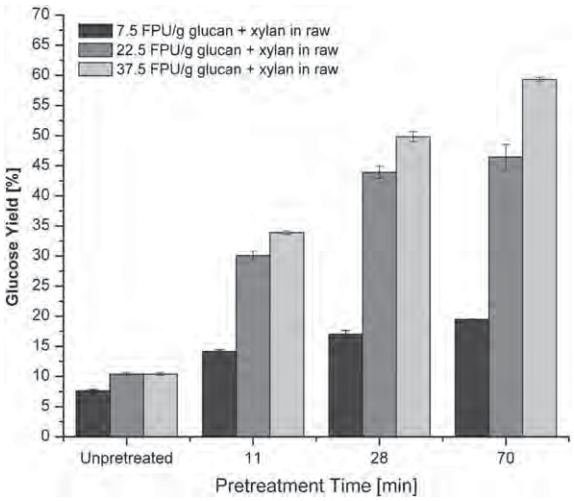

Fig. 4 Sugar release data from enzymatic hydrolysis of untreated and hydrothermally-pretreated *Populus trichocarpa* biomass. Poplar biomass samples were digested with cellulase supplemented with xylanase at three enzyme loadings as described in Materials and Methods. Results are expressed as glucose yields for tests performed in triplicate, with error bars representing the corresponding standard deviations.

Relating cell wall changes to digestibility

Considerable differences were observed in the glucan digestibility of *Populus trichocarpa* biomass pretreated for various times (Fig. 4). Overall, glucose yields were lower than expected, which we suspect may be due in part to the drying of pretreated materials prior to enzymatic hydrolysis, since this has been previously reported to negatively affect enzymatic digestibility.[23] Despite this, enzymatic hydrolysis of all of the pretreated materials resulted in increased glucose release for all enzyme loadings when compared to the untreated biomass. The largest increase in glucose yield between temporally adjacent materials (*e.g.*, untreated *vs.* 11 min pretreated, 11 min *vs.* 28 min pretreated, or 28 min *vs.* 70 min pretreated) occurred between the untreated and 11 min pretreated materials. These results can then be related to the hierarchy of changes that were observed to take place in the cell wall, namely the initial disruption of lignin-arabinogalactan/pectin and some lignin-xylan interactions, which occurred in concert with the loss of arabinogalactans in the 11 min pretreatment. These changes were associated with an increase in digestibility of up to 24% as compared to the untreated material, depending on enzyme loading. These changes were then followed by the increasing loss of some, but not all, xylans and most xyloglucans in the 28 and 70 min pretreatments, resulting in further increases in glucose yields upon subsequent enzymatic digestion.

Besides carbohydrate composition and structure, lignin is also known to play an important role in enzymatic hydrolysis of biomass because it appears to increase nonproductive binding of hydrolyzing enzymes and restrict access of enzymes to cellulose.[18,24–26] Our results show that glucose yields improved even though lignin removal during hydrothermal pretreatment was minimal (Fig. 1), in agreement with previous findings.[9] However, glycome profiling demonstrated that pretreatment altered lignin's role in the cell wall in terms of its association with pectins, arabinogalactans, and some xylans. Thus, these data support the concept that it is not lignin content *per se* that affects recalcitrance. Rather, the integration of lignin and polysaccharides within the cell wall, and their associations with one-another and with other wall components, appear to play a larger role.

Conclusions

A diverse collection of cell wall glycan-directed monoclonal antibodies can monitor structural/extractability changes in pretreated biomass at greater resolution and scope than was previously possible. Results using these antibodies demonstrated that significant changes occur to the lignin and polysaccharide composition, structure, and integration within the *Populus* cell wall even under mild hydrothermal pretreatment conditions. Interestingly though, not all polysaccharides of a given class respond in the same manner to hydrothermal pretreatment, with some carbohydrate structures being more recalcitrant than others. For the first time, more detailed information is available on cell wall changes that occur during hydrothermal pretreatment which result in improved enzymatic digestibility. Although it is difficult to relate a specific cell wall characteristic to reduced biomass recalcitrance due to the multitude of changes that were observed to occur simultaneously during hydrothermal

pretreatment, this information hints as to what structures may or may not contribute to recalcitrance. This not only provides a platform from which more targeted studies can be undertaken to further test the effect of specific cell wall components, it can also aid in the optimization of future pretreatment strategies for improved biofuels production.

Material and methods

Plant material

A single genotype of *Populus trichocarpa* grown at Oak Ridge National Laboratory (ORNL) was used in this study. The logs were debarked, split, and then chipped (Yard Machines 10HP, MTD Products Inc., Cleveland, OH). Two forms of the material were tested: 1) knife milled (Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) biomass containing a 20 mesh (<0.85 mm) to 80 mesh (>0.18 mm) particle size fraction and 2) chips taken from a cross section of the same tree's lateral branch. The chips were approximately 200 × 200 × 40 mm in width, length, and thickness, respectively. Both materials were air dried to a moisture content of around 5%.

Pretreatment

Both forms of *Populus trichocarpa* biomass were subjected to hydrothermal pretreatment as follows. Half-inch (12.7 mm) outer diameter stainless steel tube reactors that were 12.5 inches (317.5 mm) in length were loaded at 5% (w/v) solids concentration with a total reaction mass of 25 g. Both ends of the tubes were closed by stainless steel tube fittings and caps (Swagelok, San Diego Fluids System Technologies, CA)[27] and heated with condensing steam by placing them horizontally in a custom-built steam chamber.[28] Pretreatment was performed on both materials at a temperature of 180 °C for reaction times of 11, 28, and 70 min, based on previous work performed in the UC Riverside laboratory which showed that xylan removal during pretreatment peaked between 28 and 70 min for the same *Populus trichocarpa* biomass.

Following pretreatment, the tube reactors were opened, the contents filtered, and the filtrate collected. The resulting solids were washed with 50 mL of DI water and the liquids were frozen.

Compositional analysis

Glucan, xylan, and acid-insoluble residue (which closely approximates Klason lignin) contents were determined for both the untreated and pretreated solids using a downscaled compositional analysis described elsewhere.[29] This analysis is based on conventional wet chemistry techniques to determine biomass composition[7] but is scaled down by a factor of 100.

Enzymatic hydrolysis

Enzymatic hydrolysis was performed in 1.5 mL high performance liquid chromatography (HPLC) vials at 2% (w/v) solids concentration. Air-dried biomass (20 mg), DI water, and a mixture of 1M citric acid buffer (pH 4.95), sodium azide solution (1 g L^{-1}) was dispensed into vials in triplicate to bring the total reaction mass to 1000 mg. Total protein loadings of 20, 60, and 100 mg of enzyme protein per gram of total glucan plus xylan in the untreated poplar (raw BESC poplar had composition of 42% glucan, 17% xylan, and 25% Klason lignin) were applied at a 3 : 1 ratio of cellulase (Spezyme CP, Lot-Nr. 3016295230) to xylanase (Multifec, Genencore, Palo Alto, CA, Lot-Nr. 4900667792). The cellulase loadings corresponded to 7.5, 22.5, and 37.5 Filter Paper Units (FPU) per gram of total glucan plus xylan in the raw biomass. The vials within the reactor block were then sealed in the same manner as described elsewhere,[29] and the assembly was placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, MD) at 50 °C for 72 h with shaking at 150 rpm.

ELISA sample preparation and analysis

Approximately 250 mg (dry weight) each of untreated and pretreated milled *Populus* solids (20–80 mesh size) were sequentially washed with absolute ethanol and 100% acetone. The washed residues were then vacuum-dried overnight. The dried biomass samples were subjected to sequential extraction with increasingly harsh reagents in order to isolate fractions enriched in various cell wall components. All extractions were done in 10 mg mL^{-1} suspensions based on the starting dry biomass weight used. First, the biomass was suspended in 50 mM ammonium oxalate (pH 5.0) and incubated overnight with constant mixing at room temperature. After incubation, the mixture was centrifuged at 3400 g for 15 min at room temperature. The resulting supernatant was decanted and saved as the ammonium oxalate fraction, and the pellet was subsequently washed by re-suspension in the same volume of deionized water and centrifuged again as previously described except that the subsequent supernatant was decanted and discarded. Following the same protocol, the pellet was then subjected to additional sequential extractions using in turn 50 mM sodium carbonate (pH 10) containing 0.5% (w/v) sodium borohydride, and 1 M KOH and 4 M KOH, each containing 1% (w/v) sodium borohydride. The pellet remaining after the final KOH extraction was then treated with sodium chlorite (100 mM)[30] in order to breakdown lignin polymers into smaller components. Lastly, the pellet left following the sodium chlorite treatment was subjected to a final extraction with 4 M KOH containing 1% (w/v) sodium borohydride to isolate material that had previously been secured within the walls by lignin (4 M KOH PC). The resulting residual pellet was not analyzed any further. The 1M KOH, 4M KOH, and 4M KOH PC extracts were neutralized with glacial acetic acid. All extracts were dialyzed against four changes of DI water (with an approximate sample to water ratio of 1 : 60) for 48 h at room temperature and subsequently lyophilized.

After estimating the total sugar contents of the cell wall extracts using the phenol-sulfuric acid method,[31,32] the extracts were dissolved in DI water to a concentration of 0.2 mg mL^{-1}. Next, all extracts were diluted to the same sugar concentration of 60 μg sugar mL^{-1} for loading onto ELISA plates (Costar 3598). Diluted extract (50 μL) was added to each well and allowed to evaporate overnight at 37 °C until dry. The ELISAs were conducted as described[13] using an array of 150 monoclonal antibodies specific to epitopes from most major groups of plant cell wall polysaccharides. Negative controls consisting of water blanks without antigen were included in all assays and their

absorbance subtracted from all samples. None of the monoclonal antibodies that were used show backgrounds in the ELISA assays. ELISA data are presented as heat maps in which antibodies are grouped based on a hierarchical clustering analysis of their binding specificities against a diverse set of plant glycans.[13]

Monoclonal antibodies and CBM

CCRC, JIM, and MAC series of monoclonal antibodies used in this study were obtained as hybridoma cell culture supernatants from the Complex Carbohydrate Research Center collection (available through CarboSource Services; http://www.carbosource.net). The xylan-3 and xylan-4 antibody groupings recognize distinct xylan epitopes and will in general be referred to as xylan-directed antibodies throughout the manuscript. Please note that links to detailed descriptions of all antibodies can be found in Supporting Information S1.† The carbohydrate-binding module, CBM2a, which binds to crystalline cellulose,[15] was obtained from Dr Harry Gilbert (University of Newcastle, Newcastle upon Tyne, United Kingdom).

Microscopy

Treated and untreated samples were fixed in 1.6% (v/v) paraformaldehyde plus 0.2% (v/v) glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.1) for 2 h. Samples were washed with the same buffer (3 times, 15 min each) and water (2 times, 10 min each). Samples were dehydrated through a 35%, 50%, 70%, 95% (v/v), and 100% ethanol series for 25 min each and gradually infiltrated with LR White resin (1 : 3 resin:100% ethanol; 1 : 1 resin:100% ethanol; 3 : 1 resin:100% ethanol; 3 times resin; each step 24 h). Samples were placed into gelatin capsules with fresh LR White and polymerized under ultraviolet light at 4 °C for 48 h. Sectioning and immunolocalization with plant glycan-directed monoclonal antibodies were carried out as previously described.[13] The immunolocalization of bound CBM2a required an additional anti-polyhistidine antibody (H-1029; Sigma) and wash step before applying Alexa Fluor 488-conjugated secondary antibody (goat anti-mouse, A11001, Invitrogen).

Molecular sieve chromatography

The extracted polysaccharides were dissolved in 50 mM ammonium formate (pH 5.0) at a concentration of 5 mg mL^{-1}. A 200 μL aliquot of this solution was run on a Superdex-75 column (GE Healthcare USA) at a flow rate of 0.5 mL min^{-1} using 50 mM ammonium formate (pH 5.0) buffer as eluent on a Dionex HPLC system (Dionex Ultimate 3000). Various glycan peaks were monitored using a refractive index detector (Shodex RI-101).

Acknowledgements

We gratefully acknowledge support by the BioEnergy Science Center, which is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science. We also recognize the Ford Motor Company for their support of the Chair in Environmental Engineering at the University of California Riverside (UCR). The generation of CCRC series of plant cell wall glycan-directed monoclonal antibodies used in this work was supported by the NSF Plant Genome Program (DBI-0421683).

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