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Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass†

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One of the key barriers to low cost biological conversion of cellulosic biomass into renewable fuels and chemicals is the recalcitrance of plants to deconstruction by chemical, enzymatic, and/or microbial routes. A deeper understanding of the source of biomass recalcitrance is sorely needed so that specific cell wall chemical and structural features that limit the release of sugars can be identified in different plants. In this study, biomass from two phylogenetically different plants, the monocot switchgrass (*Panicum virgatum*) and the woody dicot poplar (*Populus trichocarpa*) were studied. Sets of samples that varied in composition and structure were generated from each native biomass *via* defined chemical and enzymatic extractions. The two native biomasses, as well as their extracted residues, were characterized, and the enzymatic digestibility of all samples was tested to shed light on substrate-related features that limit sugar release. Based on the results from this study, lignin and hemicellulose were found to influence the enzymatic digestibility of both poplar and switchgrass, but the degree of influence varied significantly. Xylan removal from switchgrass resulted in materials that achieved nearly 100% glucose yields at high enzyme loading in subsequent enzymatic hydrolysis, whereas chlorite extractions that reduced the lignin content had the most beneficial effect in poplar. While lignin content likely plays an important role in biomass recalcitrance particularly in plants such as poplar that contain higher levels of lignin, this work identified subsets of hemicellulose that were key recalcitrance-causing factors in switchgrass. The findings and research approach presented in this study strongly suggest that different strategies will need to be adopted when trying to engineer poplar and switchgrass for reduced recalcitrance or when designing processing conditions to efficiently convert a specific biomass feedstock into sugars.

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1 Introduction

Cellulosic biomass is currently the only available resource for large-scale production of renewable fuels, chemicals, and biomaterials.[1,2] In order to convert cellulosic biomass into any of these products, sugars that are stored as high molecular weight polymers in the plants' cell walls must first be deconstructed and released into solution as monosaccharides or short oligosaccharides. The efficient release of sugars at high yields is vital to the production of low cost products. To achieve this goal, one of the key barriers that must be overcome is the recalcitrance of biomass, which refers to the resistance of plant cell walls to deconstruction by chemical, enzymatic, and/or microbial routes. Biomass recalcitrance is likely a multi-scale phenomenon that includes plant ultrastructural, molecular, and chemical features,[3,4] and in many cases can largely be overcome by pretreatment processes that often include the use of chemicals and high temperatures and pressures to disrupt the cell wall structure prior to enzymatic hydrolysis.[5] To date, there have been numerous biomass features proposed to contribute to biomass recalcitrance. Unfortunately though, the published literature is often conflicting, and as a result, no clear picture has emerged about what plant features most strongly limit efficient sugar release.

Plant cell wall structures are highly dynamic in nature and vary significantly from biomass to biomass; hence, there is a need to understand plants individually and perhaps apply different processing routes to each for bioenergy applications. This complexity and the highly cross-linked nature of the cell wall itself likely contributes to biomass recalcitrance,[3] particularly the presence and integration of hemicellulose and lignin,

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both of which have been proposed to limit access of enzymes to cellulose. The removal or reduction of these components has been strongly correlated with improved enzymatic digestibility.[6,7] For example, studies have demonstrated that plants genetically modified for lower lignin content exhibited higher susceptibility to sugar release by pretreatment and enzymatic hydrolysis.[8,9] Conversely, it has also been reported that there was no correlation between lignin content and sugar release from pretreatment and enzymatic hydrolysis in a large natural population of poplar.[10] As opposed to lignin content, there is significantly less literature available that has studied the effects of reduced hemicellulose content *via* genetic modification or natural variation. In one study, tobacco lines with genetically reduced xylan content did not result in improved cellulose extractability;[11] however the range of xylan content in the modified lines was somewhat limited (16.8–23.5%), and the extractability was determined by chemical extraction, not by pretreatment and/or enzymatic hydrolysis.

Lignin and hemicellulose content likely cannot tell the whole story; the composition of lignin and hemicellulose may also be equally important. Extensive hemicellulose branching and substitutions are understood to restrict the ability of enzymes to degrade wall polysaccharides.[12] Along these lines, the degree of acetylation on the xylan backbone is one such commonly proposed feature. Chemical removal of acetyl groups,[7,13] as well as genetic modification of plants for reduced *O*-acetylation[14,15] have both been demonstrated to increase enzymatic digestibility. However, there have also been other studies suggesting that the impact of acetylation is minimal.[16] A study by Mortimer *et al.*[17] demonstrated that modified *Arabidopsis* lines lacking almost all xylan substitutions exhibited improved cell wall extractability; and while these results may not be directly applicable to sugar release by pretreatment and enzymatic hydrolysis, they do suggest that reduced xylan branching may result in cell wall material that is less recalcitrant to deconstruction.

The composition and structure of lignin within the cell wall can also vary substantially and may influence biomass recalcitrance. Its integration within the wall and associations with other wall components provides significant strength.[18] As a result, the reduction of lignin–carbohydrate associations has been proposed as a mechanism to increase digestibility.[19–21] Likewise, altered lignin composition is thought to impact a plant's recalcitrance since its structure and ability to crosslink with other subunits can vary with composition. However, while some studies have reported correlations between lignin composition (syringyl to guaiacyl, S/G, ratio) and digestibility,[22–24] others found no influence between the two.[8,25]

Another set of polysaccharides that has been proposed previously to influence biomass recalcitrance is pectins. As such, Lionetti *et al.*[26] reported that the genetic reduction of demethyl-esterified homogalcturonan (HGA) increased enzymatic saccharification efficiency. Unfortunately, there is very little other work that has evaluated the influence of pectins on biomass digestibility, so the effect is unclear.

Based upon the diverse set of plant features that have been proposed to impact biomass recalcitrance, as well as the sometimes conflicting reports that may only be applicable to the specific plant tested, a deeper understanding of biomass recalcitrance is sorely needed so that specific cell wall chemical and structural features that limit the release of sugars can be identified. Only in this way can improved enzymes and pretreatment processes, as well as superior biomass feedstocks be intelligently designed and implemented.

One of the major difficulties in identifying features that contribute to biomass recalcitrance is the complexity of plant cell walls and the lack of high throughput and reliable tools to analyze them. The recent expansion of available cell wall glycan-directed monoclonal antibodies (mAbs) and probes such as carbohydrate binding modules (CBMs) offer new approaches for studying plant cell wall structure and composition.[27–29] Since cell walls account for the majority of plant biomass, these glycan-directed mAbs can be useful for analyzing lignocellulosic feedstocks both *in situ*[30] and *in vitro*.[31] In a previous study,[32] we employed a mAb-based Glycome Profiling technique to track the cell wall composition and structure of untreated and hydrothermally pretreated poplar (*Populus trichocarpa*) biomass. Observed chemical and structural changes were then related to improvements in subsequent enzymatic digestibility to identify features that potentially influence biomass recalcitrance. A multitude of changes were observed to occur simultaneously during hydrothermal pretreatment, making it difficult to relate a specific cell wall characteristic to reduced biomass recalcitrance.[32] However, the study provided hints regarding structures that may or may not contribute to biomass recalcitrance, and provided a foundation for the current study in which more targeted research was carried out to further probe the effect of specific cell wall components on recalcitrance.

In this study, biomass from two phylogenetically different plants [the monocot switchgrass (*Panicum virgatum*) and the woody dicot poplar (*Populus trichocarpa*)] were selected that vary significantly from one another in cell wall structure and composition. To analyze specific cell wall components that contribute to biomass recalcitrance in each, we generated a set of samples *via* targeted chemical and enzymatic extractions that varied in composition and structure. After characterizing the samples that were generated, their enzymatic digestibility was tested at two enzyme loadings, including an industrially relevant low enzyme loading and a significantly higher loading to shed light on purely substrate-related features that limit sugar release. As a result, correlations could be drawn between cell wall chemical and structural features that contribute to the recalcitrance of poplar and switchgrass, as well as those that do not appear to play a large role.

2 Results and discussion

2.1 Characterization of cell wall components in native poplar and switchgrass biomass

2.1.1 Compositional analysis. Poplar and switchgrass are phylogenetically distant and represent two potentially important bioenergy crops in North America that differ widely in terms of their anatomy, composition, and structure. To characterize the overall differences between poplar and switchgrass biomass, the glucan, xylan, and acid insoluble residue (AcIR,

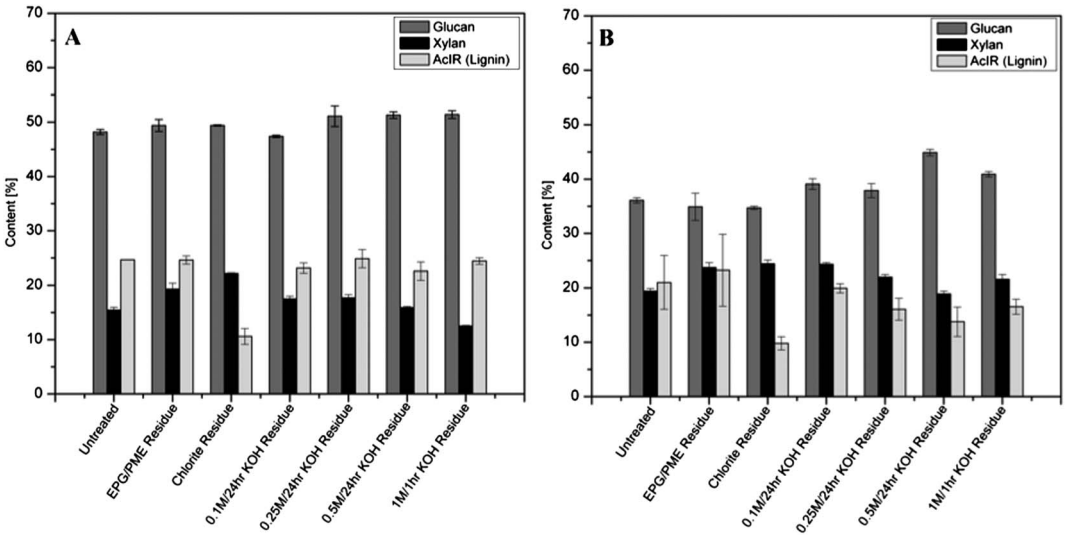

Fig. 1 Composition of poplar (A) and switchgrass (B) biomass samples, including both untreated and extracted materials. Glucan, xylan, and acid insoluble residue (which approximates Klason lignin) contents were determined as described in Materials and methods. Analyses were performed in triplicate, with the error bars representing the corresponding standard deviations.

which provides an estimate of Klason lignin) contents were determined by wet chemistry compositional analysis. As displayed in Fig. 1, switchgrass contained less glucan (36.1%) and AcIR (21.0%) than poplar (48.2% glucan, 24.7% AcIR), but contained a higher proportion of xylan (19.4%) than poplar did (15.5%).

2.1.2 Glycome Profiling. To provide more detailed information on cell wall compositional and structural differences between poplar and switchgrass, Glycome Profiling was applied to both biomass materials. Glycome Profiling employs a set of sequential chemical extractions to solubilize different cell wall components, depending on how tightly these components are bound into the walls. The resulting wall extracts are then screened with a comprehensive mAb toolkit to provide insight as to what glycan components are present in the extracts.[28,31] The Glycome Profiles of poplar and switchgrass shown in Fig. 2 differ substantially, further demonstrating the differences that exist between the cell wall chemistry/composition and structure of the two species. To highlight this, the Glycome Profiling results from poplar and switchgrass will each be summarized, followed by a comparison of the similarities and differences in the composition and structure of the two biomass species as revealed by Glycome Profiling.

2.1.2.1 Poplar. Mild extractions using reagents such as ammonium oxlalate and sodium carbonate resulted in the removal of a wide variety of pectin and pectic-arabinogalactan epitopes in poplar. These included epitopes recognized by the following groups of antibodies: homogalacturonan (HG) backbone-1 and -2, rhamnogalacturonan (RG)-1 backbone, RG-1b and -1c, RG-1/AG, and AG-2 through AG-4. Additionally, ammonium oxalate and sodium carbonate extractions also removed some xylan epitopes from poplar as indicated by the strong binding of xylan-5 and xylan-7 groups of antibodies to these extracts, particularly the carbonate extract. However, the total amount of carbohydrate material recovered during these extractions was low as compared to the subsequent extractions (as denoted by the red bars above profiles).

Harsher alkaline extractions tend to remove the hemicellulose components of the cell wall. In poplar, xylan epitopes recognized by xylan-4 through xylan-7 groups of mAbs were abundantly present in the 1 M KOH extract, whereas very low levels of xylan-3 epitopes were detected. In the 4 M KOH extract, significant amounts of additional xylan (*i.e.*, xylans that were more strongly integrated into the wall) were removed from poplar (as indicated by binding of xylan-4 through -7 groups of mAbs). Both fucosylated and non-fucosylated xyloglucans were also released by the 4 M KOH treatment. Additionally, there were more tightly bound rhamnogalacturonan and arabinogalactan epitopes that were also removed in the KOH extractions that were similar in profile to those released in the previous oxalate and carbonate treatments.

The subsequent chlorite extraction fragments and removes lignin and releases associated carbohydrates from the cell wall. As such, xylans were the predominant polysaccharide associated with lignin in poplar (as indicated by the binding of mAbs belonging to groups xylan-5 through -7). While no xyloglucans were present in the chlorite extract, the presence of pectic and arabinogalactan epitopes that were again similar in profile to those released in the previous oxalate and carbonate treatments, suggest that they too were potentially associated with lignin.

Finally, a 4 M KOH post chlorite (PC) extraction was used to solubilize very tightly bound sets of wall polysaccharides that became more extractable after removal of lignin and its associated glycans. This post-chlorite extract contained a wide diversity of polysaccharides in poplar, including xyloglucan, xylan, and pectins/arabinogalactans.

2.1.2.2 Switchgrass. Significant levels of pectin and pectic-arabinogalactan epitopes were removed by the mild oxalate and carbonate extractions in switchgrass. Epitopes released by these treatments were recognized by Linseed Mucilage RG-1, RG-1b,

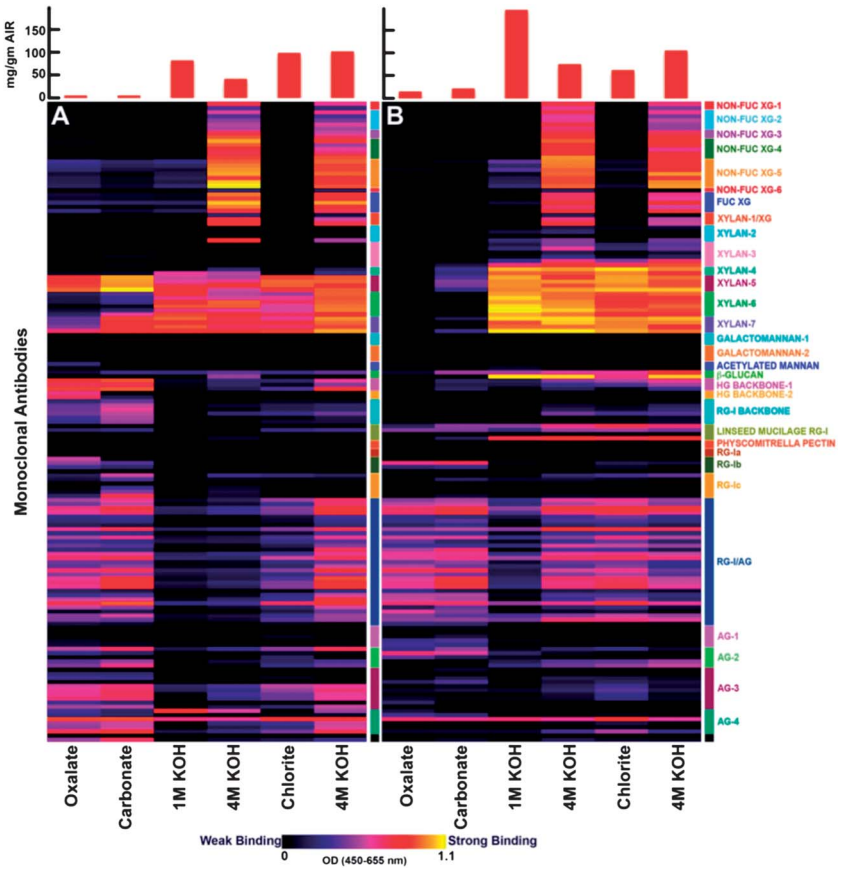

Fig. 2 Glycome Profiling of raw untreated poplar (A) and switchgrass (B) biomass. Sequential extracts of alcohol insoluble residues (AIR) of each biomass were prepared by treatment with various reagents (as labeled at the bottom of each heatmap). Extracts were loaded onto ELISA plates and screened against an array of plant glycan-directed monoclonal antibodies (see ESI, Table S1†). The legend panel on the right of the figure displays the identity of the polysaccharides predominantly recognized by each group of mAbs. Antibody binding is represented as colored heat maps, with black signifying no binding, pink/red representing intermediate binding, and bright yellow representing the strongest binding. The bar graphs at the top indicate the amount of soluble material recovered at each extraction step per gram of alcohol insoluble residue (AIR).

RG-1/AG, and lesser amounts of AG-1 through AG-4 mAb groups. Conversely, no xylan was detected in the oxalate extract and only negligible amounts of xylan mAb groups 4, 5, and 7 were removed from switchgrass by sodium carbonate. Furthermore, binding of a β-glucan-directed antibody, LAMP,[33] was also identified in the carbonate extract, indicating the presence of 1–3 linked β-glucans.

The 1 M KOH extract from switchgrass contained very high levels of xylan epitopes as indicated by the strong binding of xylan-3 through xylan-7 groups of mAbs. There was also a dramatic increase in the amount of carbohydrate material recovered in this fractionation step as indicated by the red bars above the profiles. These results demonstrate the abundance of xylans in switchgrass, of which a significant portion is extractable with 1 M KOH. In the 4 M KOH extract, significant amounts of additional xylan (*i.e.*, xylans that are more strongly integrated into the wall) were removed as indicated by binding of xylan-3 through -7 groups of mAbs. Both fucosylated and non-fucosylated xyloglucans were also released by the 4 M KOH treatment in switchgrass. Additionally, there were more tightly bound epitopes that were removed in subsequent KOH and chlorite extractions that were similar in profile to those released in the previous oxalate and carbonate treatments, including those recognized by the Linseed Mucilage RG-1, RG-1/AG, and to a lesser extent, the AG-2 through AG-4 mAb groups. Furthermore, monoclonal antibody, BG-1 that is specific to mixed linkage glucans (1–3, 1–4 linked β-glucans)[34] exhibited significant binding to all strong base (KOH) extracts.

Based on the chlorite extract, xylans are the predominant polysaccharide associated with lignin in switchgrass (as indicated by the binding of mAbs belonging to groups xylan-3 through -7). Additionally, epitopes recognized by both β-glucan directed mAbs (LAMP and BG-1), as well as a diverse set of pectin and arabinogalactan epitopes were identified in the chlorite extract of switchgrass, including all of those seen in the milder oxalate and carbonate treatments with the exception of AG-1.

In the 4 M KOH post-chlorite treatment, a diverse set of polysaccharides were removed from switchgrass, including xyloglucan, xylan, pectins/arabinogalactans, and β-glucans, which included both 1–3 linked glucans (LAMP) and 1–3,1–4 mixed-linkage glucans (BG-1).

2.1.3 Summary of Glycome Profiling studies. The profiles revealed that while poplar and switchgrass share a number of chemical and structural features, they differ significantly in

terms of cell wall composition, as well as structure and extractability, which agrees well with past reports on the composition of monocot and dicot cell walls.[35] Glycome Profiling is a semi-quantitative method, so although we cannot determine the exact content of each of these classes of polysaccharides, we can make observations about their presence, extractability and relative amounts.

As such, significant levels of pectin and pectic-arabinogalactan epitopes were removed by the mild oxalate and carbonate extractions of both poplar and switchgrass, demonstrating that they are loosely held within the cell walls of both plants. Additionally, there are also more tightly bound pectic-arabinogalactan epitopes that were removed in subsequent KOH and chlorite extractions that were similar in profile to those released in the previous oxalate and carbonate treatments. The presence of pectic and arabinogalactan epitopes in the chlorite extract suggests that they too are potentially associated with lignin in both plant types. Overall, subtle differences exist between the presence and structure of pectins and arabinogalactans in poplar *versus* switchgrass; as outlined above, a wider variety of these epitopes were identified in poplar than in switchgrass, most notably those recognizing the homogalacturonan and rhamnogalacturonan backbones of pectin. Additionally, 1-3-β-glucan and 1,3-1-4 mixed linkage β-glucan epitopes are present at high levels in switchgrass, as denoted by binding of the BG1 and LAMP antibodies, whereas only faint binding with these antibodies was observed in poplar.

As compared to the pectin and arabinogalactan portion of the cell walls, more striking differences were observed in the hemicellulose fractions of poplar and switchgrass. For example, distinct subsets of xylan were identified in poplar that are bound very loosely (and hence extractable with oxalate and carbonate) within the cell wall, whereas significant levels of xylan could only be removed by stronger treatments (various KOH concentrations and chlorite) of switchgrass. The relative abundance of xylans also differ significantly between the two biomass species. The binding intensity of xylan-3 through xylan-7 groups of mAbs in the 1 M and 4 M KOH extracts of switchgrass, as well as the higher amount of cell wall material recovered from these treatments, hint at a greater presence of xylans in switchgrass as opposed to poplar. Additionally, a significantly large portion of this xylan is extractable with 1 M KOH in switchgrass. Conversely, although xyloglucan is present in both poplar and switchgrass, it appears to comprise a relatively larger portion of poplar than it does in switchgrass.

Glycome Profiling also demonstrated that two subsets of polysaccharides are directly associated with lignin in both poplar and switchgrass: hemicellulose and pectins/arabinogalactans. Different levels of cell wall carbohydrate were removed from the two species, suggesting that the degree of lignin–carbohydrate associations likely vary between the two plants. As such, more cell wall material was removed in the chlorite extract of poplar, which hints that there may be a higher level of association between lignin and carbohydrates in this plant due perhaps to its higher lignin content (Fig. 1). Furthermore, the hemicellulose–lignin associations in poplar included just xylans, while in switchgrass, both xylans and β-glucans are associated with lignin. The composition of xylan–lignin interactions also differ slightly between poplar and switchgrass, as demonstrated by the presence of epitopes recognized by the xylan 3–7 groups of antibodies in the chlorite extract of switchgrass, whereas xylan epitopes recognized by only the xylan 5–7 groups of antibodies were identified in the chlorite extract of poplar. No xyloglucans were present in the chlorite extract of either poplar or switchgrass, suggesting that this hemicellulose is not associated with lignin in either plant.

2.2 Development and characterization of poplar and switchgrass samples produced by chemical and enzymatic extractions

Based on the chemical, structural, and extractability differences observed between poplar and switchgrass, we hypothesized that the causes of biomass recalcitrance likely varied between the two species, and that the targeted removal of different fractions of the cell wall would impact the recalcitrance of the residual biomass differently. We observed differences in the pectin/arabinogalactan, hemicellulose, and lignin fractions of poplar and switchgrass, as well as in the interactions between the individual components. Furthermore, as discussed in the introduction, all of these cell wall components have been studied as potential recalcitrant features, although the influence of each is unclear. As a result, a total of 12 samples were produced from native poplar and switchgrass biomass in this study *via* single-step enzymatic or chemical extractions to create a set of cell wall samples that varied in composition and structure. In particular, we targeted the extractions toward specific wall components in order to compare the effects of pectin/arabinogalactan, hemicellulose, and lignin on recalcitrance in both poplar and switchgrass. The composition of the resulting samples was analyzed by wet chemistry compositional analysis. Additionally, Glycome Profiling was applied to the materials solubilized by each extract to determine all detectable polysaccharides that were removed from the cell walls of poplar and switchgrass by each treatment/extraction.

As such, endopolygalacturonase and pectin-methylesterase (EPG/PME) were applied to native poplar and switchgrass to release enzyme-accessible pectins and arabinogalactans, whereas a sodium chlorite treatment was applied to remove a significant portion of the lignin. Finally, a set of single-step alkali extractions was also conducted with various concentrations of KOH to remove primarily xylans. The mechanism of each of the extractions is summarized in Table 1, as well as the primary effects of each on the biomass as observed through Fig. 2 and 3.

The EPG/PME treatment was successful in removing a portion of pectins and arabinogalactans (Fig. 3), including those recognized by mAbs within groups RG-1b, RG-1/AG and AG groupings, as well as Linseed Mucilage RG-1 in poplar. Furthermore, subsets of xylan (as recognized by the binding of mAbs belonging to groups xylan-4 through -7) were also removed in concert with pectins and arabinogalactans in poplar, whereas in switchgrass, no other epitopes recognized by the mAbs utilized were observed. It is also important to note

Table 1 Description of chemical and enzymatic extractions performed on poplar and switchgrass biomass in this study

Extraction	Type of reaction	Primary reactions taking place	Primary effects observed in Fig. 2 and 3
EPG/PME	Enzymatic	Hydrolyzes methyl esters in pectin (PME). Hydrolyzes 1,4-linked α-D-GalA residues (EPG)	Removal of pectins and associated arabinogalactans
Sodium chlorite	Oxidation	Oxidation and fragmentation of aromatic residues in lignin. May hydrolyze Ara*f* residues	Removal of lignin (~50%) and associated carbohydrates
0.1 M KOH[a,b]/24 h	Strong base	Hydrolyzes ester (methyl, acetyl and feruloyl) bonds. May disrupt H-bonds between cellulose and xylans	Removal of xylans and pectic arabinogalactans
0.25 M KOH[a,b]/24 h	Strong base	Hydrolyzes ester (methyl, acetyl and feruloyl) bonds. May disrupt H-bonds between cellulose and xylans	Removal of xylans, xyloglucans, and pectic arabinogalactans
0.5 M KOH[a,b]/24 h	Strong base	Hydrolyzes ester (methyl, acetyl and feruloyl) bonds. May disrupt H-bonds between cellulose and xylans	Removal of xylans, xyloglucans, and pectic arabinogalactans
1 M KOH[a,b]/1 h	Strong base	Hydrolyzes ester (methyl, acetyl and feruloyl) bonds. May disrupt H-bonds between cellulose and xylans	Removal of xylans, xyloglucans, and pectic arabinogalactans

[a] The KOH solutions contain 1% (w/v) sodium borohydride to prevent β-elimination reactions from occuring at the reducing ends of the polysaccharides. [b] All base extracts were immediately adjusted to pH 5 with glacial acetic acid.

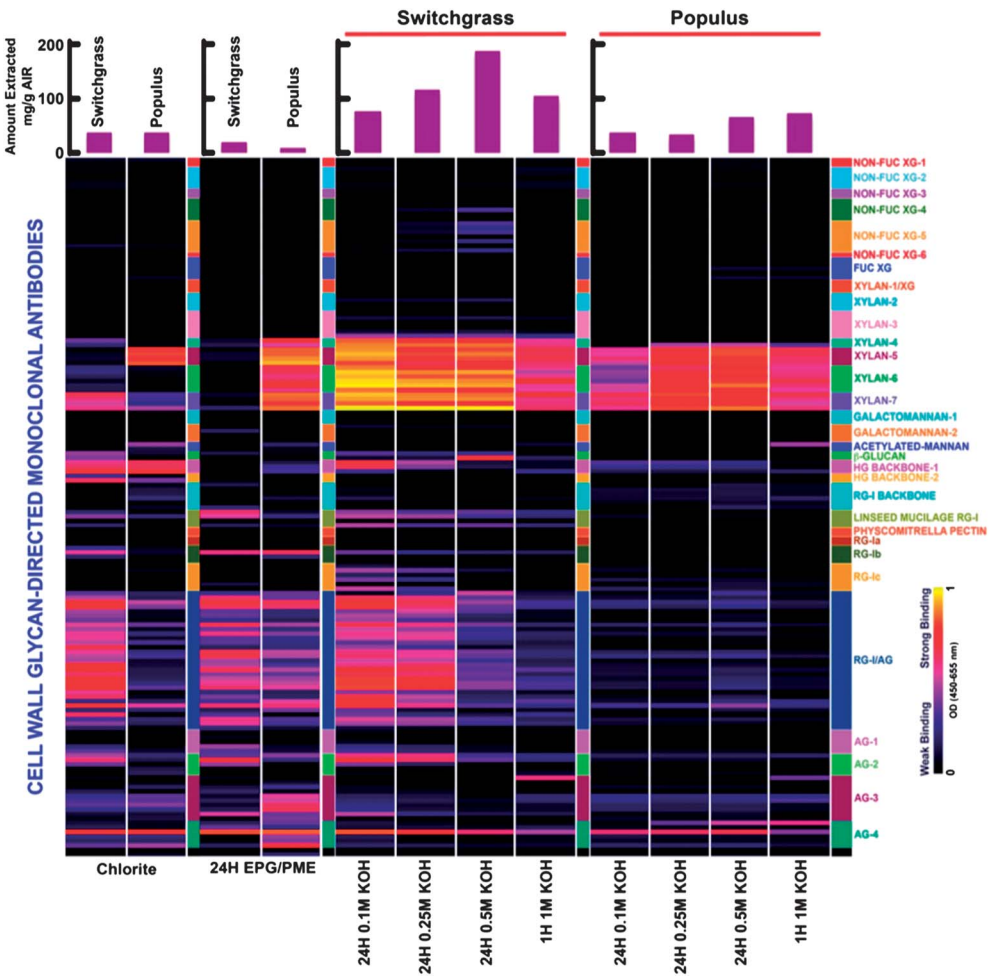

Fig. 3 Glycome Profiling of single step extracts of poplar and switchgrass cell wall samples. Individual chemical (chlorite, 0.1 M KOH/24 h, 0.25 M KOH/24 h, 0.5 M KOH/24 h, and 1 M KOH/1 h) and enzymatic (EPG/PME) extractions were carried out on AIR from both biomass samples as described in Materials and methods. In order to characterize which cell wall glycans were released from the cell walls by each of these extractions, the solubilized extracts were loaded onto the ELISA plates and screened against an array of plant glycan-directed monoclonal antibodies (ESI, Table S1†). Antibody binding is represented as colored heat maps, with black signifying no binding, pink/red representing intermediate binding, and bright yellow representing the strongest binding. The bar graphs at the top indicate the amount of material recovered in the solubilized extracts per gram of AIR.

that the EPG/PME treatment likely also released rhamnogalacturonan (RG-II) and smaller fragments of homogalacturonan (HG) that are not detected by Glycome Profiling due to their small sizes and thus their inability to adhere to the ELISA plate.

After chlorite extraction, wet chemistry compositional analyses of the resultant insoluble residues demonstrated that a large portion of AcIR was removed in poplar (57% lower AcIR content) and switchgrass (53% lower AcIR content) (Fig. 1). Glycome Profiling of the solubilized materials further showed that various classes of polysaccharides were removed upon lignin fragmentation in the chlorite treatment. In particular, pectins and arabinogalactans were removed from both poplar and switchgrass in concert with lignin, including those recognized by the mAb groups HG backbone-1 and -2, RG-1/AG, AG, as well as RG-1b in poplar. Additionally, xylan epitopes (those recognized predominantly by xylan-5 mAbs in poplar and xylan-7 mAbs in switchgrass) were also removed from the cell walls of both biomass materials by the chlorite treatment.

The last set of extractions involved KOH treatments to remove large portions of xylans in both poplar and switchgrass. Lesser amounts of pectins and arabinogalactans were also released by the KOH treatments, as well as β-glucans from switchgrass (Fig. 3).

Although the extractions performed in this study resulted in the removal of diverse cell wall components from the biomass, the use of Glycome Profiling and wet chemistry compositional analysis allowed us to track each of these changes. Furthermore, as discussed in the Materials and methods, the extractions used in this study were conducted at room temperature (except the chlorite extraction performed at 70 °C) and atmospheric pressure. The use of milder reaction conditions likely reduced large-scale changes in the composition and ultrastructure of the biomass, as compared to the hydrothermal pretreatment study carried out previously, which employed high temperature and pressure.[32] Thus, the chemical composition of the extracted insoluble residues was more similar to that of the corresponding untreated biomass (Fig. 1), than the compositions of the untreated *versus* pretreated materials after hydrothermal pretreatment.[32] Furthermore, the milder nature of the targeted extractions carried out in the present study was also reflected in the physical appearances of the resulting insoluble materials, which were very similar to that of the untreated materials. The only exception was the chlorite residue that turned white in color for both plant biomass samples. In hydrothermal pretreatments, the resulting materials were substantially darker in color and slightly smaller in particle size.

2.3 Digestibility of poplar and switchgrass samples generated by chemical and enzymatic extraction

The enzymatic digestibility of the native poplar and switchgrass biomasses, as well as that of all insoluble residues remaining after the diverse single step extractions, was tested at two enzyme loadings, including an industrially relevant low enzyme loading and a significantly higher loading to shed light on purely substrate-related features that limit sugar release. Given the characterization of all of these samples by both chemical and immunological methods, some conclusions could be drawn about specific cell wall chemical and structural features that contribute to the recalcitrance of poplar and switchgrass, as well as those that do not appear to play a large role.

Figure 4 plots the glucose yield in enzymatic hydrolysis as a function of incubation time for the switchgrass (Fig. 4A and B)

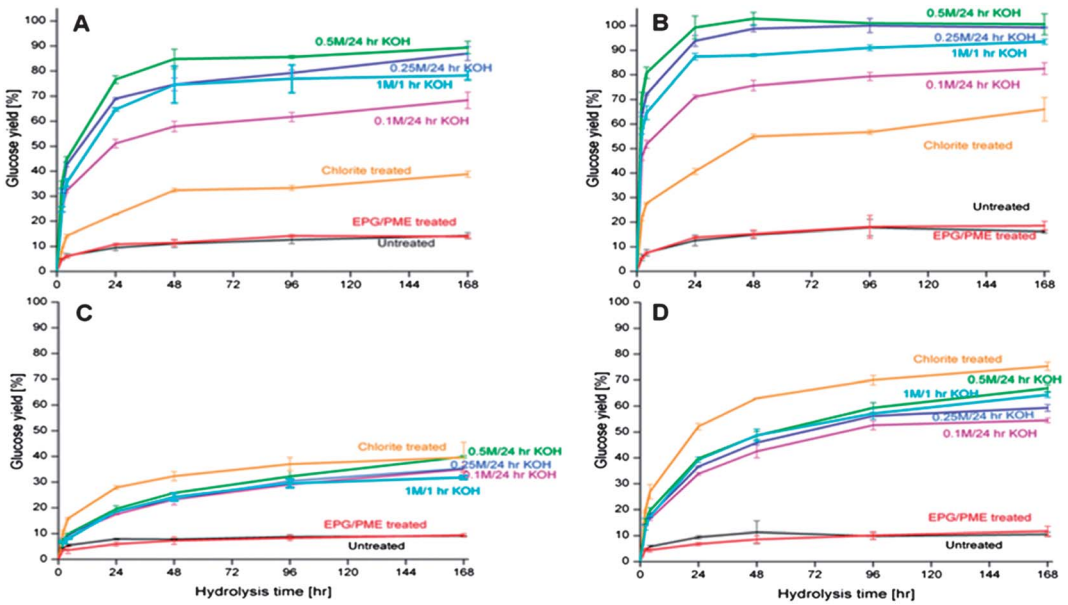

Fig. 4 Enzymatic hydrolysis time profiles of switchgrass (A and B) and poplar cell wall residues (C and D), at low enzyme loading (A and C) and high enzyme loading (B and D). The low and high enzyme loadings corresponded to 15 + 3.2 mg cellulase + β-glucosidase, and 120 + 25 mg cellulase + β-glucosidase per g glucan in the raw biomass, respectively. The different color data sets represent the various samples tested that were produced by single step extractions of poplar and switchgrass cell wall samples. Individual chemical (chlorite, 0.1 M KOH/24 h, 0.25 M KOH/24 h, 0.5 M KOH/24 h, and 1 M KOH/1 h) and enzymatic (EPG/PME) extractions were carried out on both biomass samples as described in Materials and methods.

and poplar (Fig. 4C and D) samples. Yields are shown for hydrolyses conducted at both a low enzyme loading (Fig. 4A and C) and a significantly higher loading (Fig. 4B and D). The glucose yields for the untreated materials were low for both poplar (<11%) and switchgrass (<17%) at both enzyme loadings. Equally low were the glucose yields exhibited by the EPG/PME-treated residues for both poplar and switchgrass. They performed almost identically to the untreated materials. However, other than these samples, the digestibility of all other materials varied greatly, particularly for switchgrass. Significantly higher glucose yields were obtained for the chlorite and KOH-treated residues than for the corresponding untreated material. Thus, at low enzyme loading, all of the KOH-treated switchgrass residues achieved high yields, particularly the 0.25 M and 0.5 M KOH samples that exhibited final glucose yields of 87 and 89%, respectively. In contrast, the KOH-treated poplar samples only achieved a maximum glucose yield of 40% at low enzyme loading. At high enzyme loading, the glucose yields did increase in poplar; final glucose yields of between 55 and 67% were achieved. However, these yields were still lower than those of the KOH-treated switchgrass samples at both low and high enzyme loadings. In switchgrass, the KOH-treated materials reached glucose yields of theoretical 100% yields with high enzyme loading.

The chlorite-treated switchgrass residues did not perform as well as the KOH treated switchgrass biomass. At low and high enzyme loadings, the 168 h glucose yields of the chlorite-extracted switchgrass were 39 and 66%, respectively. In the chlorite-treated poplar, the final glucose yield was 40% at low enzyme loading and 75% at high enzyme loading.

2.4 Evaluating the influence of cell wall components on the recalcitrance of poplar and switchgrass: past findings and new insights

Significant differences were observed in the cell wall structures of poplar and switchgrass, translating into variable causes of biomass recalcitrance in the two species. The most influential cell wall feature resulting in the largest improvement in glucan digestibility when removed from the cell wall of one plant did not have the same effect in the other, pointing to the complexity of biomass recalcitrance and the difficulty in developing strategies to decrease recalcitrance for a wide range of biomass feedstocks. No single and specific cell wall component was found to be the key determinant of digestibility for both plant species tested in this study. In switchgrass, the removal of hemicellulose by KOH treatments resulted in the greatest improvement in glucose yields, whereas the removal of lignin by a chlorite treatment was more important for improved glucose yields in poplar.

The removal of hemicellulose from switchgrass, specifically xylan (including methyl glucuronoxylan, arabinoxylan, and unsubstituted straight chain xylan), resulted in an increase in the final glucose yield of between 54 and 75% at low enzyme loading and 66 to 84% at high enzyme loading, as compared to the untreated switchgrass. While the removal of xylan also improved the digestibility of poplar, the effects were not as substantial. Thus, the final glucose yields increased by a maximum of 31% at low enzyme loading and 57% at high enzyme loading.

For poplar on the other hand, we found that the most significant improvement in the glucose yields came after the removal of a fraction of lignin by the chlorite treatment. The chlorite-treated residues of both poplar and switchgrass had AcIR contents that were about 50% lower than those of the corresponding untreated materials (Fig. 1). In switchgrass, the removal of this fraction of lignin improved glucose yields by about 24 and 50% for low and high enzyme loading, respectively. In poplar, glucose yields increased more substantially, by 31 and 65% over the untreated biomass for low and high enzyme loadings, respectively. The amounts of cell wall material removed in the chlorite extractions were similar in both poplar and switchgrass; however, the removal improved the digestibility of poplar more so than it did in switchgrass, and also had a greater impact on final yields than did the removal of hemicellulose. The exact reasons for this remain unknown, but we believe that differences in the cell wall composition and structure in poplar and switchgrass resulted in the variable effects that were observed for the single-step extractions between the two species.

Our results also suggest that the differences identified in the comparison of switchgrass and poplar cell walls underlie the causes of biomass recalcitrance for these two plant species. For example, one of the possible reasons for the differing effects of the KOH treatments in poplar and switchgrass is the more easily alkali extractable cell wall material in the latter. In general, a KOH extraction of a certain molarity removed significantly more cell wall material from switchgrass than it did from poplar. As a result, the available surface area and porosity in the remaining insoluble residues may be higher, resulting in improved enzyme accessibility. However, when comparing KOH extractions that had similar amounts of mass removal (*e.g.*, 0.1 M KOH/24 h extraction in switchgrass and 1 M KOH/1 h extraction in poplar), the switchgrass residue still exhibited higher glucose yields than did the poplar residue. This suggests that differences in the structural composition of xylans and their integration within the cell walls of poplar and switchgrass may affect their removal and result in differing effects on subsequent enzymatic digestibility of glucan in the biomass of the two plant species.

It is difficult to relate our observations to the structural makeup of the cell walls of monocots *versus* woody dicots because there is no well-defined model for secondary walls. However, based on models of primary cell walls, it is well known that different plant species contain different proportions of the various types of hemicelluloses in their walls.[36–38] For example, xyloglucan is the main hemicellulose in the primary wall of dicots and is thought to interact with and coat cellulose, whereas in monocot grasses, xylans (primarily glucurono-arabinoxylan) play this role.[18] Although hemicellulose contents and proportions vary between primary and secondary cell walls,[35] our data suggests that differences in hemicellulose contents may not be limited to just primary walls, but extend to secondary walls. Furthermore, our data suggest that these differences in hemicellulose class and their manner of

integration into the overall secondary wall structure in different plant species may play varying roles in recalcitrance from one plant to another. For example, our data point to the idea that xylans are the primary hemicelluloses present in the walls of monocot switchgrass, whereas other classes, such as xyloglucans, may play a more pronounced role in poplar. If this were true, the removal of xylans from switchgrass would do more to improve enzyme accessibility and resulting glucose yields than it would from poplar, as we observed in this study. This would also explain why the removal of xyloglucan from switchgrass was not necessary to achieve 90% and 100% yields at low and high enzyme loading, respectively. In poplar on the other hand, the removal of xyloglucan and/or a greater proportion of lignin could be necessary to achieve equally high glucose yields if these components play larger roles in the recalcitrance of poplar than they do in switchgrass. The effects of selective removal of xyloglucan on recalcitrance was not tested in this study.

Another possible reason for the larger improvement observed in the digestibility of the KOH-treated switchgrass over KOH-treated poplar is the fate of lignin–carbohydrate associations in each. Ferulic acid is present at high levels in grasses and is often esterified or linked through ether bonds to carbohydrates. Furthermore, dehydroferulic acid that is esterified to carbohydrates can be esterified to lignin.[18] Thus, the removal of hemicellulose by KOH treatment may also reduce the degree of lignin–xylan associations through de-esterification reactions (Table 1), which would provide another route towards reducing the strength and recalcitrance of the switchgrass cell wall.

One finding of the current study that is common to both plants is that the removal of enzyme-accessible pectins and pectic arabinogalactans by EPG/PME treatment had no effect on enzymatic digestibility of the resulting insoluble wall residues at either enzyme loading. The EPG/PME residue of poplar also had lost a small portion of xylan-5 epitopes; however, this did not result in reduced recalcitrance. The amount of cell wall material removed by this extraction was fairly minimal (less than 20 mg per g biomass), which matches with the low pectin content in poplar wood that is rich in secondary cell walls; pectin content is also low in the cell walls of monocots such as switchgrass.[35] Pectin has also been reported to have properties that contribute to limiting the porosity of cell walls, which may be important with respect to enzyme and solvent accessibility.[18] However, our results do not support a major role for enzyme accessible pectins in negatively influencing enzymatic deconstruction of either poplar or switchgrass biomass by cellulases.

3 Conclusions

A set of biomass samples varying in composition and structure was generated *via* targeted chemical and enzymatic extractions applied to two phylogenetically different plants, the monocot switchgrass and the woody dicot poplar. Wet chemistry compositional analysis and immunological screening of the resulting cell wall extracts using glycan-directed monoclonal antibodies (Glycome Profiling) allowed us to characterize the resulting residual biomass samples in an attempt to relate cell wall chemical and structural changes to limitations in sugar release. The results from this study demonstrate that the differences in cell wall structure and composition between poplar and switchgrass appear to influence which wall components in each plant have the largest impact on recalcitrance to sugar release from the two biomasses. Although both lignin and hemicellulose influenced the enzymatic digestibility of poplar and switchgrass, the degree of influence varied significantly between them. Xylan removal from switchgrass resulted in materials that achieved nearly 100% glucose yields in subsequent enzymatic hydrolysis, suggesting that subsets of hemicellulose are key recalcitrance-causing factors in switchgrass. In contrast, reduction in lignin content and possibly also its associated polysaccharide components by chlorite treatment had the most beneficial effect in reducing the recalcitrance of poplar biomass, with its overall higher lignin content.

The data presented in this study strongly suggest that different strategies will likely be necessary to engineer poplar and switchgrass for reduced recalcitrance. Furthermore, it may prove necessary to design different processing conditions to efficiently convert each specific biomass feedstock into sugars. Studies such as the one reported here can aid in the design of pretreatments to remove specific cell wall components that most severely inhibit enzymatic digestibility for a given biomass, as well as aid in the development of improved enzymes that contain the proper activities to break down the recalcitrant structures remaining after pretreatment of a specific biomass. Both of these routes are important to attain the long-term goal of overcoming biomass recalcitrance and harnessing the biomass for sustainable biofuel production.

4 Materials and methods

4.1 Plant material

Poplar (*Populus trichocarpa*) and switchgrass (*Panicum virgatum*) provided through the BioEnergy Science Center were used in this study. A single genotype of *Populus trichocarpa* and a lowland cultivar switchgrass were grown at Oak Ridge National Laboratory (ORNL) and harvested between 2007 and 2008. After harvest, the materials were sent to the National Renewable Energy Laboratory (NREL), where the poplar logs were debarked, split, and then chipped. Both poplar and switchgrass samples were knife milled (Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) and sieved to produce materials having a 20 mesh (<0.85 mm) to 80 mesh (>0.18 mm) particle size. Materials were dried to approximately 6% moisture content and stored in closed containers at room temperature until use.

4.2 Generation of poplar and switchgrass cell wall extracts and residues

Poplar and switchgrass biomass samples were sequentially washed first with absolute ethanol and then with 100% acetone. The washed residues were then vacuum-dried overnight to produce the alcohol insoluble residue (AIR). All subsequent extractions were performed on distinct AIR samples in 10 mg mL^{-1} suspensions based on the starting dry biomass weight used.

4.2.1 Endopolygalacturonase/pectin methylesterase extraction. Poplar and switchgrass AIR were incubated with a mixture of purified type II *Aspergillus niger* endopolygalacturonase (EPG)-1 and 2 (~1 units per 100 mg AIR; from Complex Carbohydrate Research Center, University of Georgia)[39] and purified *A. niger* pectin methylesterase (PME) (~1 units per 100 mg AIR; obtained from Dr Carl Bergmann, Complex Carbohydrate Research Center, University of Georgia) in 50 mM sodium acetate, pH 5.0, for 48 h at 25 °C with mixing. The pellet was recovered by centrifugation at 3660*g* for 20 min and washed three times with sterile water. The supernatants were stored as the EPG/PME extract and the washed pellet was recovered as the EPG/PME-treated residue.

4.2.2 Chlorite extraction. Poplar (0.5 g) and switchgrass (1.0 g) AIR were each treated with three additions of 0.25 g of sodium chlorite and 100 μL of glacial acetic acid at 70 °C for a total of 180 min. A control run was also performed for the chlorite extraction in which both poplar and switchgrass AIR samples were treated with three additions of 100 μL of glacial acetic acid at 70 °C for a total of 180 min, but with no added chlorite. Glucose yields resulting from the subsequent digestion of these control samples (see below) were similarly low compared to yields from the corresponding untreated biomass materials (data not shown), demonstrating that any effects on sugar release yields were due to the action of the chlorite, and not due to the presence of the glacial acetic acid in the extraction.

4.2.3 KOH extractions. Three samples each of poplar (1.25 g) and switchgrass (1.5 g) AIR were each incubated separately with 0.1 M, 0.25 M, or 0.5 M KOH for 24 hours at room temperature. An additional KOH extraction involved the incubation of 6.0 g poplar and 8.0 g switchgrass AIR in 1 M KOH for 1 hour at room temperature. All alkali extractions contained 1% (w/v) sodium borohydride to prevent β-elimination at the reducing end of released glycans. At the end of the reactions, all extracts were immediately neutralized with glacial acetic acid to prevent further modification of either extracts or cell wall residues. The resulting extracts and residues from the KOH treatments were recovered as described above. Some precipitation was observed to occur when the neutralized extracts were dialyzed to remove salts; these precipitates, if present, were removed by centrifugation at 24 °C at 3000 g for 10 min.

4.3 Glycome Profiling

Glycome Profiling is an ELISA-based method to screen plant cell wall extracts with glycan-directed monoclonal antibodies (mAbs), and was carried out as described previously.[28,31,32] Plant glycan-directed monoclonal antibodies were from laboratory stocks (CCRC, JIM and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or were obtained from BioSupplies (Australia) (BG1, LAMP). A description of the mAbs used in this study can be found in the ESI, Table S1,† which includes links to a web database, Wall*MAb*DB (http://www.wallmabdb.net) that provides detailed information about each antibody.

4.4 Compositional analysis

The composition of all poplar and switchgrass samples was determined using a scaled-down wet chemistry method described in detail elsewhere.[40] Glucan, xylan, and acid insoluble residue (AcIR) contents were measured. In poplar, AcIR closely approximates the Klason lignin content due to its low ash content (0.6%), whereas in switchgrass, the AcIR content is slightly higher than the Klason lignin content due to the higher ash content (5.2%) of this biomass. All compositional analyses were performed in triplicate.

4.5 Enzymatic hydrolysis

Enzymatic hydrolyses of all poplar and switchgrass samples were performed in a downscaled and high throughput 96-well plate reactor system[41,42] at 2% (w/v) solids concentration with a total reaction mass of approximately 440 mg prior to the addition of enzyme. As such, 8.80 mg of dry biomass was added to each well by an automated solid and liquid dispensing robotics platform (Core Module, Freeslate Inc., Sunnyvale, CA, USA). Afterwards, 430.6 μL of deionized (DI) water was transferred into all wells in two additions using an eight-channel pipettor (30–300 μL; Eppendorf, Hamburg, Germany). After allowing the biomass to soak overnight, a mixture of 1 M citric acid buffer (pH 4.95), sodium azide solution (1 g L^{-1}), and diluted enzyme was added to each well (10–100 μL, Eppendorf). Two enzyme loadings were applied, both using a combination of cellulase (Spezyme CP, lot no: 3016295230, Genencor, Palo Alto, CA, USA) and β-glucosidase (Novozymes 188, lot no: 037K0968, Novozymes, Franklinton, NC, USA), with the resulting enzyme mixtures diluted with DI water to a volume ratio of 2 : 1. The high enzyme loading corresponded to approximately 120 mg cellulase protein + 25 mg β-glucosidase protein per gram glucan in the biomass, while the low loading corresponded to approximately 15 mg cellulase protein + 3.2 mg β-glucosidase protein per gram glucan in the biomass. Depending on the enzyme loading and biomass weight used, the volume of enzyme/buffer/sodium azide solution added to each well varied from 28.2 μL to 43.9 μL. After enzyme addition, the wells were sealed as described elsewhere, 40 and the reactor was placed on its side in a temperature-controlled incubation shaker (Multitron Infors-HT, ATR Biotech, MD) set to 50 °C and 150 rpm. Replicate plates were prepared for sampling at different hydrolysis time points, including at 2, 4, 24, 48, 96, and 168 h. At the desired sampling time, the corresponding well plate was removed from the shaker, and the slurry from each individual well was transferred to 2.0 mL polypropylene centrifuge tubes (Safe-Lock 2.0 mL test tubes, Eppendorf). Tubes were centrifuged at 18 200*g* for 5 minutes (5415 D; Eppendorf), after which 300 μL of hydrolyzate was transferred to HPLC vials for analysis. All enzymatic hydrolysis experiments were performed in triplicate.

4.6 Sugar quantitation

Sugar concentrations from compositional analysis and enzymatic hydrolysis testing were measured by refractive index using high performance liquid chromatography (HPLC)

(Alliance 2695 equipped with 2414 RI detector; Waters, Milford, MA, USA). An Aminex HPX-87H column (BioRad, Hercules, CA, USA) heated to 65 °C was used with 5 mM sulfuric acid as the eluent at a flow rate of 0.6 mL min^{-1}.

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