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Cell wall-associated transition metals improve alkaline-oxidative pretreatment in diverse hardwoods

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Abstract

The responses of four diverse hardwoods (hybrid poplar, silver birch, hybrid aspen, and sugar maple) to alkaline hydrogen peroxide (AHP) pretreated at ambient temperature and pressure were analyzed to gain a deeper understanding of the cell wall properties that contribute to differences in enzymatic hydrolysis efficacy following alkaline-oxidative pretreatments. The enzymatic hydrolysis yields of these diverse hardwoods increased significantly with increasing the cell wall-associated, redox-active transition metal content. These increases in hydrolysis yields were directly correlated to improved delignification. Furthermore, we demonstrated that these improvements in hydrolysis yields could be achieved either through elevated levels of naturally-occurring metals, namely Cu, Fe, and Mn, or by the addition of a homogeneous transition metal catalyst (*e.g.* Cu 2,2'-bipyridine complexes) capable of penetrating into the cell wall matrix. Removal of naturally-occurring cell wall-associated transition metals by chelation resulted in substantial decreases in the hydrolysis yields following AHP pretreatment, while re-addition of metals in the form of Cu 2,2'-bipyridine complexes and to a limited extent Fe 2,2'-bipyridine complexes prior to pretreatment restored the improved hydrolysis yields. Glycome profiles showed improved extractability xylan, xyloglucan, and pectic epitopes with increasing hydrolysis yields for the diverse hardwoods subjected to the alkaline-oxidative pretreatments, demonstrating that the strength of association between cell wall matrix polymers decreased as a consequence of improved delignification.

Broad Impacts and Highlights

This work is the first demonstration that cell wall-associated, redox-active transition metals play a critical role in the efficacy of oxidative delignification for cell wall deconstruction. These results suggest that altering the abundance and localization of these metals represents an important strategy that can be used to influence the yield of biomass conversion processes involving lignin-modifying pretreatments. The implications of these results are that this previously unrecognized cell wall property, which impacts recalcitrance, can be optimized for an oxidative deconstruction strategy either through biological means during plant growth (*i.e.* environment and/or plant genotype) or by the addition of a metal catalyst during processing.

Keywords: Alkaline hydrogen peroxide (AHP), biofuels, Cu(bpy), delignification, glycome profiling, oxidation, pretreatment

1. Introduction

Biofuels derived from sustainably-produced, non-food plant biomass have the potential to displace a significant fraction of the petroleum-derived liquid transportation fuel used globally while simultaneously contributing to greenhouse gas reduction targets and economic growth.[1] For biomass-to-biofuels processes, diverse biomass feedstocks and conversion pathways (*e.g.* biochemical, thermochemical, catalytic, hybrid, *etc*.) can be envisioned, and the physical and chemical properties of the biomass will have an important impact on the conversion process.[2]

As a feedstock for biomass-to-biofuels processes, woody biomass exhibits several advantages that facilitate logistics relative to herbaceous feedstocks, including year-round availability and high bulk density.[3, 4] "Hardwoods" (or woody dicotyledons) are industrially significant feedstocks for the forest products industry, with fast-growing species such as hybrid poplar and *Eucalyptus* spp. proposed to play an important role in supplying future cellulosic biorefineries.[5] While typically less recalcitrant than "softwoods" (or gymnosperms), hardwoods are significantly more recalcitrant to cell wall "deconstruction" processes than potential herbaceous feedstocks, often requiring harsh pretreatment approaches to enable the enzymatic release of cellulosic sugars at comparable levels.[6] As a consequence of this higher recalcitrance of woody biomass, it is notable that the vast majority of ongoing biomass-to-biofuels commercialization efforts target thermochemical conversion routes for woody feedstocks, while conversion pathways involving cell wall pretreatment and hydrolysis followed by biological conversion have generally been targeted at herbaceous feedstocks.[7]

Recalcitrance of hardwoods arises due to multiple structural features at different size scales, which cooperatively contribute to decreased accessibility of cell wall polysaccharides to hydrolytic enzymes.[2] At the nanometer scale within the cell wall, these factors can include the composition, such as the lignin content,[8-10] the ratio of the monomers in the lignin (which impact the relative abundance of intra-lignin linkages),[9] and the properties and abundance of non-cellulosic polysaccharides,[11] which together impact the supramolecular organization of the cell wall. At the micron scale, cell wall morphological factors that impact recalcitrance are manifested in properties such as overall density of the xylem,[12] differences in cell wall

thickness,[13] and differences in the relative abundance of certain tissue types such as reaction wood.[14]

In addition to the above-mentioned structural and compositional heterogeneity, plant cell walls also exhibit substantial heterogeneity in both the content and the distribution of metals, silicates, and other inorganic elements. Differences are observed between disparate plant taxa, between related species, within a single species both as a function of its genotype and in response to its environment, as well as between different cell types within a single plant. In addition, temporal differences are also observed over the life of the plant.[15-18] These variations can impact the response of a given biomass feedstock to different pretreatment processes, although there has been limited work on this question.

The pretreatment of lignocellulosic biomass to reduce recalcitrance prior to enzymatic hydrolysis is a critical step required for biochemical conversion of cell wall polysaccharides to biofuels, with the overall conversion efficiency of the process depending heavily on the efficacy of the pretreatment step.[10] Various approaches have been applied to hardwoods including soda pulping,[19] dilute acid,[20] dilute acid sulfite,[21] alkali-buffered sulfate (green liquor),[22] acidic ethanol organosolv pretreatment,[23, 24] and liquid hot water pretreatments,[25] as well as pretreatments that decrystallize cellulose such as ionic liquids.[26] In addition, oxidative pretreatments have been widely used by the pulp and paper industry for bleaching and delignification.[27-29] More recently, these pretreatments have also been examined for cellulosic biofuels applications with lignin solubilization as the route for reducing biomass recalcitrance.[8, 30-32]

Redox-active transition metals have been shown to exhibit substantial variability in woody plants with contents spanning four orders of magnitude in abundance (e.g., commonly reported values for Cu are in the range of 0.1-10 ppm and Fe and Mn are in the range of 10-100 ppm).[33] During oxidative delignification or bleaching, cell wall-associated transition metals can catalyze the formation of reactive oxygen species through Fenton chemistry. These reactive oxygen species, however, have also been shown to contribute to the oxidative scission of polysaccharides.[34, 35] As a result, strong precautions are often taken prior to oxidative pretreatment to remove metals via

chelation and extensive washing, or to complex the metals during the delignification processes through the addition of Mg salts and silicates.[36] This demonstrates that intrinsic metals present in biomass can, in fact, play an important and active role in affecting the pretreatment processes. Furthermore, given the importance of lytic polysaccharide monooxygenases in enzyme cocktails used to hydrolyze lignocellulosic biomass,[37] it is quite possible that limited oxidative polysaccharide scission may be beneficial to cellulosic biofuels processes.

We previously demonstrated that copper-catalyzed alkaline hydrogen peroxide (Cu-AHP) pretreatment of hybrid poplar resulted in the substantial improvement of sugar yields following enzymatic hydrolysis.[31, 38] In this manuscript, we focus on relating biomass properties to oxidative pretreatment efficacy to uncover factors that affect recalcitrance and the effectiveness of alkaline hydrogen peroxide (AHP) and Cu-AHP pretreatments. We compared the enzymatic hydrolysis yields following these alkaline-oxidative pretreatments of four different hardwoods (silver birch, a hybrid aspen, a hybrid poplar, and sugar maple) and correlated these results to diverse cell wall properties. Herein we demonstrate that there is a strong positive relationship between the quantity of redox-active metals in the cell wall and enzymatic hydrolysis yields following AHP pretreatment. These results are consistent with our hypothesis that the delivery of copper to the cell wall during Cu-AHP pretreatment is a major factor contributing to the increased efficacy of the pretreatment.[39]

2. Experimental

2.1 Biomass and cell wall characterization

The biomass used in this work included debarked 18-year old hybrid poplar (*Populus nigra* L. var.*charkoviensis x caudina* cv. NE-19) that was grown at the University of Wisconsin Arlington Agricultural Research Station and provided through the Great Lakes Bioenergy Research Center (GLBRC). Bark-free sugar maple (*Acer saccharum* Marsh.) wood chips were obtained from Todd Smith (Devereux Sawmill, Inc., Pewamo, MI). Silver birch (*Betula pendula* Roth.) that was grown in northern Sweden, debarked, and chipped was acquired from Curt Lindström (Smurfit-Kappa Kraftliner AB, Piteå, Sweden). Hybrid aspen (*P. tremula* L. *x P. tremuloides* Michx.) chips were obtained from Dr. Raymond Miller (Michigan State University Forest Biomass Innovation Center) and are from debarked, 10-year-old trees grown near Escanaba,

Michigan. All biomass was milled using a Wiley MiniMill (Thomas Scientific, Swedesboro, NJ) to pass through a 20-mesh size screen, air dried, and stored in airtight bags prior to pretreatment studies.

Quantification of glucan, xylan, acetate, acid-insoluble lignin (Klason lignin), and ash present in untreated biomass were determined using the NREL two-stage sulfuric acid hydrolysis method,[40] with sugar and acetate quantification by HPLC using an Aminex HPX-87H (Bio-Rad, Hercules, CA) column, and uronic acids quantified enzymatically using the K-Uronic assay (Megazyme, Wicklow, Ireland). Minor cell wall polysaccharides, non-crystalline glucan, and deoxy sugars were determined by quantification of their alditol acetate derivatives following polysaccharide hydrolysis by trifluoroacetic acid (TFA) as described elsewhere.[41, 42] Xylan content determined by the two-stage sulfuric acid hydrolysis method was corrected by subtracting mannan and galactan contents as determined by the TFA hydrolysis method which used a different analytical approach because xylose, mannose, and galactose were not resolved in the HPLC system used following the two-stage method. The extractives content was determined gravimetrically following sequential solvent extraction as reported in our previous work.[43] Total solids solubilization used in determining lignin removal during pretreatment was estimated as initial glucan divided by final glucan based on the assumption that glucan content was relatively unaffected by pretreatment. The lignin S/G ratios were determined by the microscale thioacidolysis method as described previously.[42] It should be noted that this method only quantifies those units linked via β–O-4 bonds, and the total lignin S/G ratio may vary somewhat from this measured value. The identity and quantity of the redox-active metals in the hardwood samples were determined by inductively coupled plasma mass spectrometry (ICP-MS) performed at A&L Great Lakes Laboratories (Fort Wayne, Indiana).

2.2 Pretreatment

Three different pretreatment strategies were tested on each of the biomass samples: alkali only, alkaline hydrogen peroxide (AHP), and copper-catalyzed AHP (Cu-AHP). In all cases, 0.51 g of biomass (~0.50 g dry basis; 3-5% moisture content) were pretreated in a total of 5.0 mL aqueous solution (10% w/v solids loading). For alkali-only pretreatment, the solution contained 50 mg NaOH (100 mg NaOH/g biomass), while AHP pretreatment also contained 150 μL of 30% H_2O_2

(*i.e.* 100 mg H_2O_2/g biomass). The Cu-AHP pretreatment solution was prepared as described above for AHP except that 125 µL of a solution containing 40 mM $CuSO_4$ as well as 125 µL of a solution containing both 40 mM $CuSO_4$ and 160 mM 2,2'-bipyridine (bpy) were added to the biomass slurry after the addition of NaOH (2 mM Cu^{2+} and 4 mM bpy final concentration) but prior to the addition of H_2O_2. The final pH for the alkali-only pretreatment was 13.2, while it was approximately 11.5 for AHP and Cu-AHP due to the addition of H_2O_2. For all three pretreatments, the reactants were vortexed and the slurry incubated with orbital shaking at 180 rpm and 30 °C for 24 hours. Solutions containing only biomass and deionized water acted as controls.

2.3 Enzymatic hydrolysis

Following pretreatment, 0.5 mL of 1 M citric acid buffer (pH 4.8) was added to the pretreated slurry, and the slurry was slowly titrated with 72% (w/w) H_2SO_4 to adjust the pH to 5.0 prior to enzymatic hydrolysis. An enzyme cocktail consisting of Cellic CTec3 and HTec3 (gift from Novozymes A/S, Bagsværd, DK) at a loading of 34 mg protein/g glucan for CTec3 and 38 mg protein/g glucan for HTec3 was added to the hydrolysis reaction (protein content was provided by Novozymes). The total volume of the pretreated biomass slurry was adjusted to 10 mL by the addition of deionized water, and the samples were incubated at 50 °C for 72 hours with orbital shaking at 210 rpm. Following enzymatic hydrolysis, the solid and liquid phases were separated by centrifugation, and the amount of glucose and xylose released into the aqueous phase was quantified by HPLC (Agilent 1260 Series equipped with an Aminex HPX-87H column operating at 65 °C, a mobile phase of 0.05 M H_2SO_4, a flow rate of 0.6 mL/min, and detection using an Agilent 1260 infinity refractive index detector). The yield of glucose and xylose released was defined as the amount of solubilized monosaccharide divided by the total sugar content of the biomass prior to pretreatment as determined by chemical composition analysis. Prior to each analysis, standard curves were generated using pure solutions of glucose and xylose to convert peak area to concentration of monomeric sugar. The error bars in the figures represent the standard deviation from three or more biological replicates (i.e. multiple experiments using the same biomass).

2.4 Chelation of metals from biomass

Biomass (3 g) was mixed with 30 mL of a solution containing 0.2% (w/v) of the chelator diethylenetriaminepentaacetic acid (DTPA). The pH of the slurry was adjusted to 7.0 with 5 M NaOH, and the solution was incubated for 24 hours at 30 °C. The biomass was then washed thoroughly with 10 volumes of distilled H_2O to remove the DTPA, dried at room temperature for 2 days, and stored in airtight bags. Biomass incubated for 24 hours at 30 °C with only distilled water was used as a control.

Pretreatment reactions as described above were performed either in the presence of 2,2'-bipyridine (bpy) plus Cu^{2+}, Mn^{2+}, or Fe^{2+} ([metal]:[bpy] = 2 mM:4 mM) or in the absence of added bpy and metal to ascertain the effect these metal complexes have on the pretreatment of chelated woody biomass. The error bars represent the standard deviation from three or more biological replicates.

2.5 Glycome profiling

Glycome profiling was performed on either untreated or pretreated biomass as described previously.[43-45] Briefly, this involves subjecting AIR (alcohol insoluble residues) prepared from the plant biomass samples to six sequential extractions using increasingly harsh reagents to selectively solubilize cell wall matrix polysaccharides on the basis of the relative tightness with which they are integrated into the plant cell walls. These extracts were then screened against a panel of 155 plant cell wall glycan-specific monoclonal antibodies (mAbs) that were obtained either from laboratory stocks (CCRC, JIM, and MAC series) at the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or from BioSupplies (Australia) (BG1, LAMP).[45, 46] A complete list of the mAbs employed in this study is provided in the Supplemental Information, **Table S1.** Hierarchical clustering of binding data for these mAbs against 54 structurally known plant polysaccharides allowed classification of these mAbs into the categories used in this work, with the data on the binding specificity and cross-reactivity for each mAb accessible in a publicly-available web database (Wall*Mab*DB; http://www.wallmabdb.net). The mAb binding data were normalized to a "per mass original biomass" basis to facilitate comparison as described in our previous work.[43]

3. Results and Discussion

Plant cell walls are known to exhibit differing responses to alkaline and alkaline-oxidative deconstruction approaches as a consequence of differences in cell wall chemistry and ultrastructure.[2, 47, 48] To ascertain the key traits associated with differences in the plant cell walls in hardwoods that contribute to these variations, we analyzed the enzymatic hydrolysis yields of four hardwoods (hybrid poplar, hybrid aspen, birch, and maple) following alkali-only, AHP, or Cu-AHP pretreatment. Each of these biomass samples exhibited differences in composition (polysaccharides, lignin, extractives, and ash) as well as redox active metal content. These characteristics were compared with their enzymatic hydrolysis yields following pretreatment. Glycome profiling studies were also performed to acquire a deeper understanding of how pretreatment alters cell wall structure and integrity among these biomass types. Together, these data provide a comprehensive analysis of key aspects that contribute to the recalcitrance of hardwoods and also suggest strategies for improved deconstruction or design of bioenergy feedstocks with improved traits.

3.1 Cell wall composition

The four diverse hardwoods investigated in this study represent industrially promising feedstocks and provide substantial phylogenetic diversity within the rosid clade of the core eudicots. Sugar maple is one of the most abundant hardwoods in the forests of the eastern U.S.,[49] while silver birch is the most abundant and industrially important hardwood in northern Europe. In addition, hybrid poplars such as the poplar and aspen used in this work have been proposed as important bioenergy feedstocks to supply future biorefineries.[3, 5] Of note, the hardwoods used in this study are all "diffuse-porous", indicating that they do not exhibit distinct differences in the wood density and pore structure between earlywood and latewood as in other hardwoods.[12]

Cell wall composition can be an important determinant of recalcitrance, and not surprisingly, the four hardwoods exhibited a relatively wide range of composition (**Table 1**). Differences include lignin contents that ranged from 18% (birch) to approximately 24% (maple), as well as non-cellulosic polysaccharide contents with xylosyl residue contents ranging from 16% (hybrid poplar) to more than 22% (birch). Other notable differences include higher content of certain glycosyl residues that are characteristic of non-cellulosic cell wall glycans in the aspen (such as

fucosyl, rhamnosyl, uronosyl, glucosyl, and arabinosyl residues) relative to the other hardwoods. The higher proportions of these glycosyl residues in aspen may indicate a higher content of fucosylated xyloglucan (*i.e*. elevated contents of fucosyl and non-cellulosic glucosyl residues), pectic polysaccharides (*i.e.* elevated contents of uronosyl, arabinosyl, rhamnosyl, and fucosyl residues), and/or more highly substituted glucuronoarabinoxylan (*i.e.* elevated contents of uronosyl and arabinosyl residues).[50]

3.2 Response to alkaline-oxidative pretreatments

To ascertain the susceptibility of the four hardwoods to different alkaline pretreatments, each biomass was subjected to pretreatment by alkali-only, AHP, or Cu-AHP followed by enzymatic hydrolysis (**Fig. 1, Table S2**). While the birch and aspen both responded well to alkali-only pretreatment, the enzymatic hydrolysis yields of hybrid poplar and maple rose much more modestly when only NaOH was used for pretreatment. Intriguingly, while three of the four woody biomass feedstocks tested had high glucose hydrolysis yields following AHP pretreatment and exhibited only a slight increase in hydrolysis yields when AHP was performed in the presence of copper 2,2'-bipyridine complexes (Cu-AHP), the hybrid poplar behaved quite differently. In the case of hybrid poplar, hydrolysis yields following alkali-only pretreatment and AHP pretreatment were nearly identical, while glucose yields more than doubled following Cu-AHP pretreatment (**Fig. 1**). Hemicellulose (Xyl+Man+Gal) yields demonstrated similar results (**Table S2**). Overall, birch exhibited the highest sugar yields following Cu-AHP pretreatment and enzymatic hydrolysis, followed by aspen, hybrid poplar, and sugar maple.

3.3 Cell wall properties impacting efficacy of alkaline-oxidative pretreatments

Intrigued by these distinct trends, we sought to identify the factors that govern the efficacy of the three alkaline pretreatments on the different hardwoods. Both lignin and hemicelluloses were removed from the cell walls during pretreatment (**Fig. 2a** and **2b**). Lignin and hemicelluloses form a physical barrier,[10, 51] hindering the ability of enzymes to access and hydrolyze the cellulose, and therefore an important objective of essentially all pretreatment strategies is to overcome this barrier by removing, relocalizing, and/or modifying the lignin and hemicelluloses.[52, 53] For delignifying pretreatments, including alkaline and alkaline-oxidative procedures, lignin removal has been shown to act as a strong predictor of hydrolysis yields,[8, 48]

and likewise, in the present work lignin removal is strongly correlated to glucose hydrolysis yields across all pretreatments and feedstocks (**Fig. 2c** and **3a**). For dilute acid and liquid hot water pretreatments, lignin relocalization and acid-catalyzed hydrolysis of xylan are major outcomes of the pretreatment. Consequently, for these pretreatments, xylan hydrolysis, which may be an indirect indicator of lignin redistribution due to xylan's intimate association with lignin, is a well-known predictor of glucose hydrolysis yields.[21, 25] In the present work, xylan removal can be correlated to both lignin removal and glucose hydrolysis yields for all the feedstocks except birch, in which xylan was largely retained (**Fig. 2b**). Our previous work found higher degrees of polymerization of alkali-extracted silver birch xylans relative to sugar maple and hybrid poplar xylans, which may explain the distinct behavior of birch biomass, whereby the larger birch xylans may be less soluble in the pretreatment liquors used in the current work and more likely to remain sorbed to the cell wall.[54]

Because lignin removal is one of the primary outcomes of the alkaline and alkaline-oxidative pretreatments contributing to improved hydrolysis yields, the cell wall properties contributing to improved delignification during uncatalyzed AHP pretreatment were investigated. The lignin S/G ratio can vary substantially from plant to plant, with values ranging from 1.7 to 3.9 in *Populus trichocarpa*[55] and from 2.7 to 7.3 in silver birch.[56] Increasing S/G ratios in diverse hardwoods lead to increasing rates of delignification during alkaline pulping,[57] presumably due to decreased crosslinking because of the extra methoxyl group in S monomers.[58] Indeed, increasing the S/G ratio in transgenic hybrid poplar has been shown to increase alkaline delignification efficacy.[59] The impact of S/G ratio is less obvious, however, in studies utilizing acidic pretreatments, although work has demonstrated improved enzymatic hydrolysis with increasing S/G ratios for dilute acid[60] and liquid hot water pretreated[9] *P. trichocarpa* as well as liquid hot water pretreated transgenic *Arabidopsis thaliana*[61] and alfalfa (*Medicago sativa*).[62]

In the present study, four different hardwoods were compared, and a positive correlation was found between the S/G ratio and both lignin removal (**Fig. S1a**) and hydrolysis yields (**Fig. 3b**). Silver birch, with the lowest lignin content (18%) and the highest S/G ratio (2.7), showed the highest glucose hydrolysis yields for all pretreatments (**Fig. 1**). However, the glucose yields for silver birch were only slightly higher than those for aspen even though the S/G ratio in the aspen

was significantly lower (1.3). In addition, sugar maple had the second highest S/G ratio (1.7) and yet resulted in lower glucose yields following enzymatic digestion compared to both birch and aspen for all pretreatment tested. Together, these results highlight the fact that while lignin content and composition are clearly important, other factors also impact how these four hardwoods respond to alkaline and alkaline-oxidative pretreatments.

3.4 Importance of metal ions during alkaline-oxidative pretreatment

One particularly interesting observation was that the addition of copper 2,2'-bipyridine [Cu(bpy)] complexes during AHP pretreatment (Cu-AHP) substantially increased the hydrolysis yields of hybrid poplar (and substantially increased the delignification, **Fig. 2a**) but only slightly increased the hydrolysis yields of the other hardwoods as identified in our previous work.[31] We hypothesized that AHP requires the presence of metal ions to be an effective pretreatment. We further hypothesized that while the birch, aspen, and maple samples already contained sufficient redox-active metal ions in their cell wall (thereby obviating the need for the additional copper ions during pretreatment), the hybrid poplar samples contained a relatively low level of redox-active metal ions. In this scenario, the addition of Cu(bpy) complexes during AHP pretreatment would compensate for the low natural levels of metal ions in our hybrid poplar samples.

To test our hypotheses, ICP-MS was performed to quantify cell wall redox-active metal ions in the four different hardwood samples (**Table 2**). The values ranged from only 7 ppm found in the hybrid poplar to over 111 ppm in the silver birch. As predicted, a strong positive correlation was discovered between the redox-active metal content of the woody biomass and both lignin removal (**Fig. S1b**) and enzymatic hydrolysis yields (**Fig. 3c**) following AHP pretreatment. Importantly, this same correlation was not observed following Cu-AHP pretreatment. Not surprisingly, analysis via ICP-MS of the cell wall metal ion content in Cu-AHP pretreated biomass demonstrated that all samples exhibited a large increase in the amount of copper relative to the untreated samples, with copper essentially dominating the metal ratio (**Table S3**). These results are consistent with our previously published data indicating that during Cu-AHP pretreatment, the copper catalyst penetrates into the cell wall matrix.[39]

To corroborate this important relationship between intracellular metal content and efficacy of AHP pretreatment, each of the different hard woods was incubated with the metal chelator DTPA prior to pretreatment. DTPA, an octadentate ligand with high affinity for metal cations, has been employed previously to study the effects of metals on pulp bleaching[36] and is often used to stabilize H_2O_2 or in a separate chelation state to remove metal ions during the oxidative pulp bleaching.[35] ICP-MS analysis of the chelated biomass revealed a substantial decrease in metals, with DTPA treatment removing approximately 96% of cell wall-associated redox metals (**Table 2).**

The effect of chelation was dramatic for hardwoods that initially contained a large amount of redox-active metal ions. For example, the glucose hydrolysis yields of chelated silver birch were significantly diminished following uncatalyzed AHP pretreatment relative to biomass that had not been chelated, with glucose yields reduced from 70% to only 50% (**Fig. 4a**). Likewise, chelated aspen and sugar maple also exhibited lower enzymatic hydrolysis yields following uncatalyzed AHP pretreatment relative to unchelated samples. Conversely, the hydrolysis yields of hybrid poplar, which naturally had very low cell wall metal content, were not affected by incubation with DTPA.

To verify that the decreased efficacy of uncatalyzed AHP following incubation with DTPA was due to the loss of the metal ions, chelated biomass was subjected to metal-catalyzed AHP pretreatment (**Fig. 4b**). As expected, chelation of the hardwoods with DTPA prior to pretreatment had only minimal influence on the efficacy of Cu-AHP, presumably because the addition of Cu(bpy) complexes obviated the need for naturally occurring intracellular metal ions. Interestingly, the addition of Cu(bpy) complexes led to significantly higher enzymatic hydrolysis yields than the addition of either Mn(bpy) or Fe(bpy) complexes (*i.e.* Mn-AHP and Fe-AHP) (**Fig. S2**). Whether this difference is due to the superior reactivity of Cu(bpy) complexes, the result of better penetration of Cu(bpy) complexes into the plant cell wall (**Table S3**), or some other property cannot be determined from these data.

The importance of redox-active metal ions to improve delignification and hydrolysis during pretreatment processes is not unprecedented. For example, Wei *et al*. reported that the addition

of Fe during dilute acid pretreatment improved both glucose and xylose yields, presumably by acting as a Lewis acid to improve xylan hydrolysis, and they further suggested that other transition metal ions such as Mn and Cu might have similar effects.[63] Manganese complexes in the presence of hydrogen peroxide have been reported to catalyze the oxidation of lignin model compounds[64] as well as the delignification of poplar[65] and spruce.[66] In addition, copper–phenanthroline complexes have been employed to catalyze the oxidation of loblolly pine[67] and other softwoods,[68, 69] while oxidation with alkaline cupric oxide has been utilized to characterize lignin structure in a variety of woody species.[70, 71] And finally, aqueous formic acid induced depolymerization of woods has been demonstrated with a variety of reducing metals, including zinc, manganese, and iron, to generate valuable aromatic products.[72] Together, these data highlight the importance of considering the availability of transition metals when designing pretreatment processes for woody biomass.

3.5 Glycome profiling

Glycome profiling was next employed to gain insight into the differences in the composition and distribution of non-cellulose cell wall glycans, the variations in the strength of association between these glycans and other cell wall matrix polymers, and how these composition/distributions and association strengths are impacted by various pretreatments. As a tool for mapping xylan structures, recent work employing synthetic xylooligomers demonstrated that select xylan-binding mAbs from this panel are capable of distinguishing glycan features that include differences in length, substitution type, frequency, and pattern.[73] Previous work employed glycome profiling as a tool to identify differences in the abundance and extractability of cell wall glycans in taxonomically-diverse plants including poplar subjected to AFEX™ pretreatment,[44] AHP pretreatment,[43] and hydrothermal pretreatment.[74]

In the current work, the four hardwoods subjected to either no pretreatment, alkali-only pretreatment, AHP pretreatment, or Cu-AHP pretreatment were subjected to glycome profiling with the complete glycome profile data presented as Supplemental Information (**Table S1**). A subset of the complete glycome profile data (oxalate, 1 M KOH, and 4M KOH post-chlorite extracts) that highlight major differences between the four hardwoods and their responses to pretreatment is presented in **Fig. 5**. Note that the mAb binding results are normalized to epitope

abundance per mass of original biomass so that results from different extracts could be compared on the same basis.[43, 44] Overall, glycome profile analyses delineated differences in the both the relative abundance and extractability of non-cellulose cell wall glycans among different species and between pretreatments (**Fig. 5**; **Table S1**).

Several notable trends were observed between the abundance of different classes of glycan epitopes in cell wall extracts as a consequence of the pretreatments. The first observation is that increasing hydrolysis yields associated with (in ascending order) alkali-only, AHP, and Cu-AHP can be linked to increasing extractabilities of xylan and pectic polysaccharide epitopes as indicated by their relatively enhanced abundance in the oxalate extracts from pretreated poplar, aspen, and birch (**Fig. 5a**). Conversely, the inverse of this trend was observed in the 4 M KOH post-chlorite extracts (**Fig. 5c**) and, to a lesser extent, in the 1 M KOH extracts (**Fig. 5b**). This indicates that following alkali-oxidative pretreatments, the glycans were shifted from the harshest extract (implying intimate initial association with lignin) to the mildest extract (indicating weak, easily disrupted associations with other cell wall matrix polymers). One such pretreatment-induced cell wall modification may be linked to lignin removal (**Fig. 2a**), which disrupts lignin-xylan association and results in enhanced xylan extractability. These observations are consistent with the previous studies where alkaline pretreatments such as AHP and AFEX™ were demonstrated to induce enhanced extractability of non-cellulosic matrix polysaccharides such as xylan and pectin in diverse phylogenies of plants.[43, 44] A second observation is that the maple responded poorly to all of the treatments, and this was reflected in the glycome profiling results which show the least changes in the epitope abundances for all extracts and pretreatment conditions. In contrast, Cu-AHP pretreatment of aspen resulted in the near complete depletion of xyloglucan epitopes in the 1 M KOH extract (**Fig. 5b**) and of the xyloglucan and xylan epitopes in the 4 M KOH post-chlorite extracts (**Fig. 5c**). These epitopes were largely depleted in the harshest extracts of Cu-AHP pretreated aspen, but were enriched in the oxalate extract (**Fig. 5a**), indicating that the extractability of these glycans was substantially increased by the pretreatment-induced cell wall modifications. Notably, the Cu-AHP pretreated aspen exhibited the highest lignin removal (**Fig. 2a**), which is consistent with the shift of glycan epitopes observed in the glycome profiles.

A final notable observation is that the largest difference in hydrolysis yields between pretreatment conditions for a single feedstock is for Cu-AHP pretreated poplar (**Fig. 1**), which also correlated with a substantial increase in lignin removal (**Fig. 2b**). The most obvious difference in the glycome profile for this pretreatment is that the 4 M post-chlorite extract from Cu-AHP pretreated poplar contained substantially less xylan and xyloglucan epitopes than did the extracts from poplar subjected to other pretreatment conditions (**Fig. 5c**). These results indicate that lignin-associated xylan and xyloglucans were liberated as a consequence of the improved delignification imparted by addition of the Cu catalyst. Overall, the results of the glycome profiling provide indirect support of the mechanism proposed for the role of cell wall-associated metals in alkaline-oxidative pretreatment efficacy, whereby increasing metal content increased delignification resulting in improved hydrolysis yields. Specifically, the results showed that increasing delignification efficacy could be linked to differences in the extractability of non-cellulosic glycans.

4. Conclusions

In the present study, cell wall properties were identified in four diverse hardwoods that contributed to improved cell wall deconstruction by an ambient temperature and pressure alkaline-oxidative pretreatment process. The primary outcome of the pretreatments was solubilization and removal of cell wall lignin and xylan. Lignin removal was correlated to increases in enzymatic hydrolysis yields, presumably due the increased accessibility of cell wall polysaccharides to hydrolytic enzymes. Although the initial lignin content did not correlate to delignification efficacy, high S/G ratios were generally associated with higher enzymatic hydrolysis yields following AHP pretreatment. Notably, this study demonstrated the important role of cell wall-associated redox-active transition metals in impacting the efficacy of AHP pretreatment. Specifically, cell wall-associated transition metals intrinsically present in the biomass correlated with increasing hydrolysis yields and delignification. Addition of Cu 2,2'-bipyridine [Cu(bpy)] complexes to transition metal-deficient poplar (total of 7 ppm transition metals) resulted in substantial improvement of hydrolysis yields while providing only minimal improvement for the three hardwoods having transition metal contents ranging from 45 to 111 ppm. This positive contribution of redox-active transition metals to AHP pretreatment was validated by demonstrating that hydrolysis yields in the transition metal-rich hardwoods could be

substantially decreased by removal of the metals via chelation prior to AHP pretreatment, a decrease that could be reversed by re-addition of Cu(bpy) complexes. Additionally, glycome profiling of the diverse pretreated hardwoods revealed that increased delignification during the pretreatments resulted in an increase in the extractability of epitopes for xylan, xyloglucan, and pectin epitopes. Overall, the implications of this work are that cell wall-associated transition metals can play a positive role in oxidative cell wall deconstruction strategies and that this property can be altered to optimize the outcome of the pretreatment.

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Abbreviations

AIR: alcohol insoluble residue; AHP: alkaline hydrogen peroxide; bpy: 2,2'-bipyridine; Cu-AHP: copper-catalyzed alkaline hydrogen peroxide; DTPA: diethylenetriaminepentaacetic acid; HG: homogalacturonan; HPLC: high performance liquid chromatography; ICP-MS: inductively coupled plasma mass spectrometry; mAb: monoclonal antibody; RG: rhamnogalacturonan; S/G: syringyl to guaiacyl; TFA: trifluoroacetic acid; XG: xyloglucan.

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FIGURES

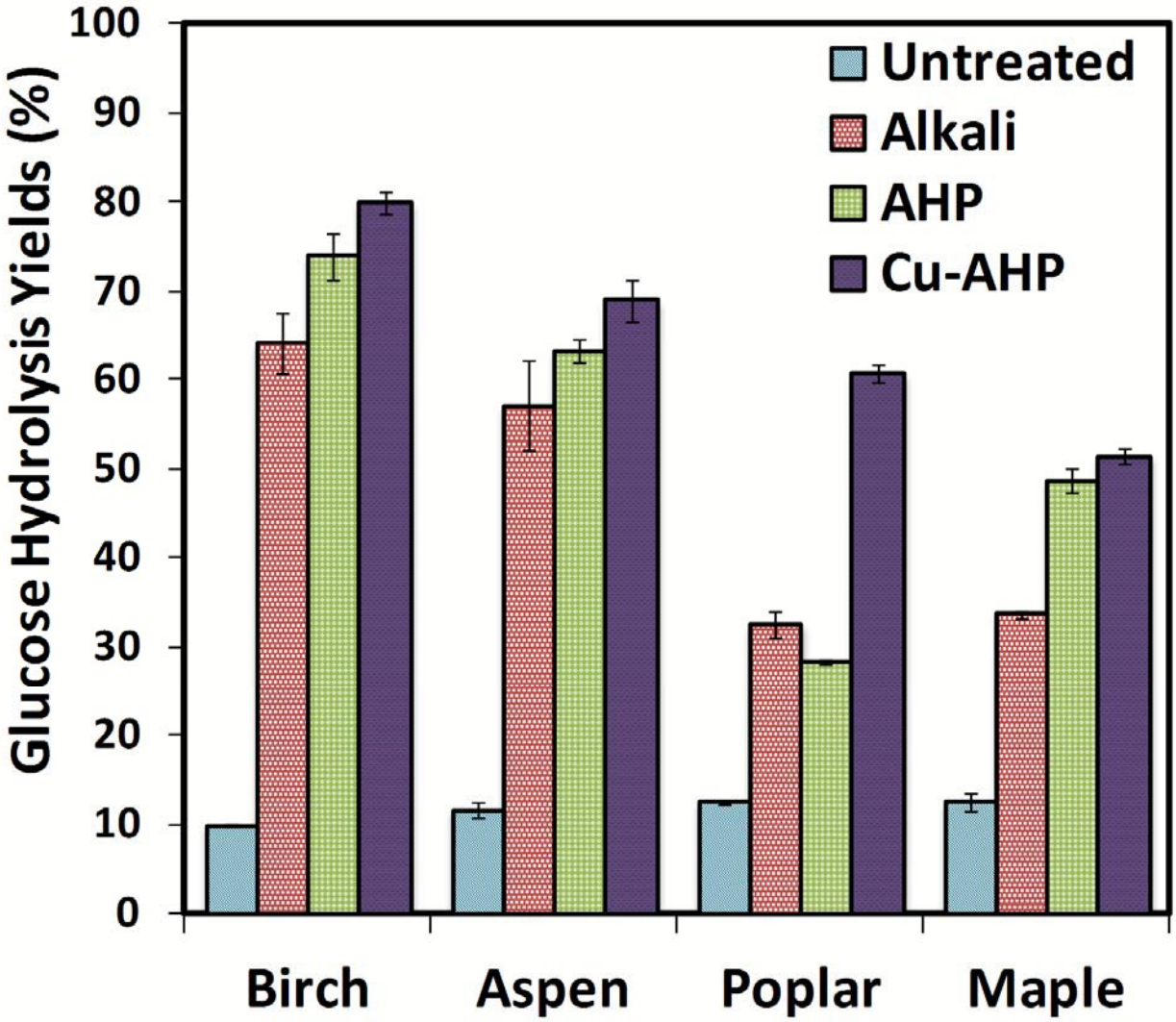

Fig. 1 Glucose yields following enzymatic hydrolysis of various untreated, alkali pretreated, alkaline hydrogen peroxide (AHP) pretreated, and copper-catalyzed AHP (Cu-AHP) pretreated hardwoods.

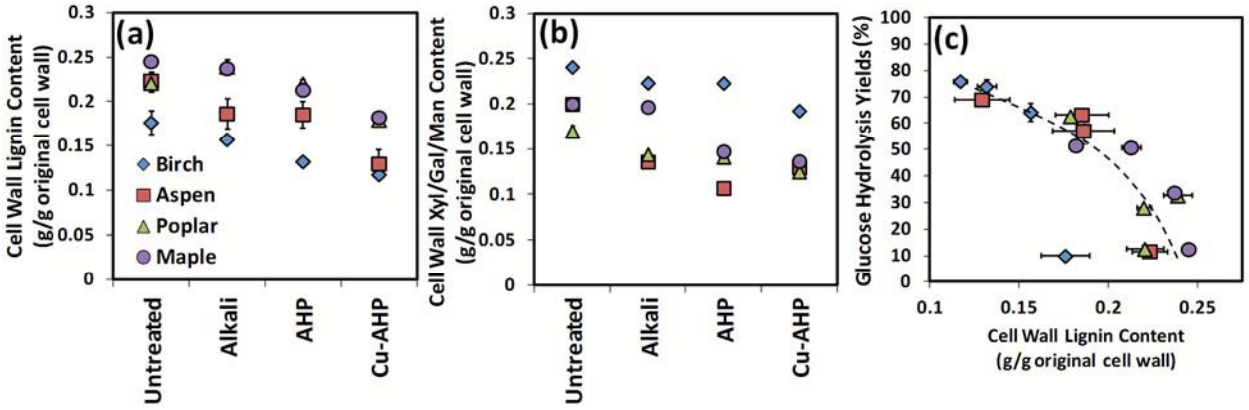

Fig. 2 Correlation between cell wall composition and pretreatment and/or hydrolysis yields. Changes in **(a)** cell wall lignin content and **(b)** cell wall xylan content as a function of pretreatment strategy; **(c)** correlation between lignin content and hydrolysis yields.

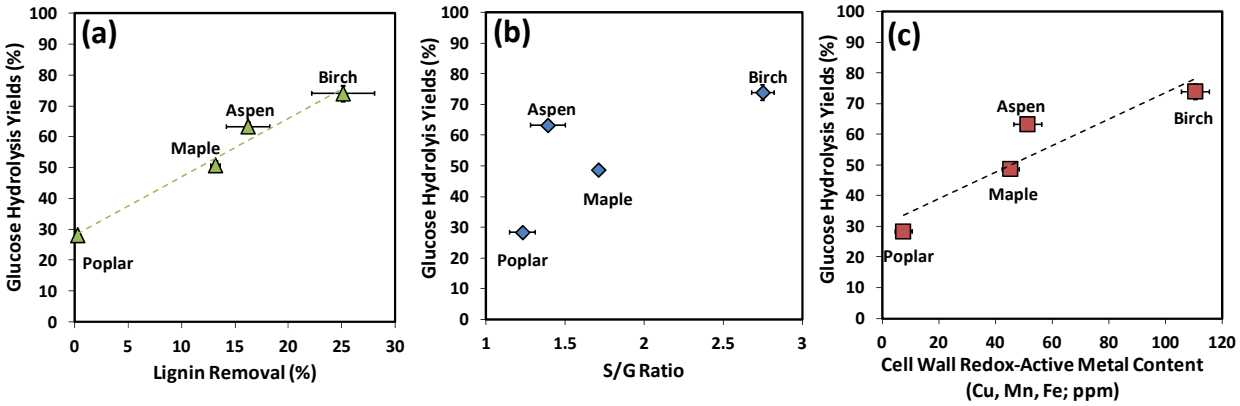

Fig. 3 Factors influencing lignin removal during alkaline hydrogen peroxide-only pretreatment (i.e. without the addition of a supplemental metal catalyst) of diverse hardwoods. Results show the correlation between glucose yield following enzymatic hydrolysis and **(a)** extent of delignification, **(b)** syringyl/guaiacyl (S/G) ratio, and **(c)** cell wall-associated transition metal content.

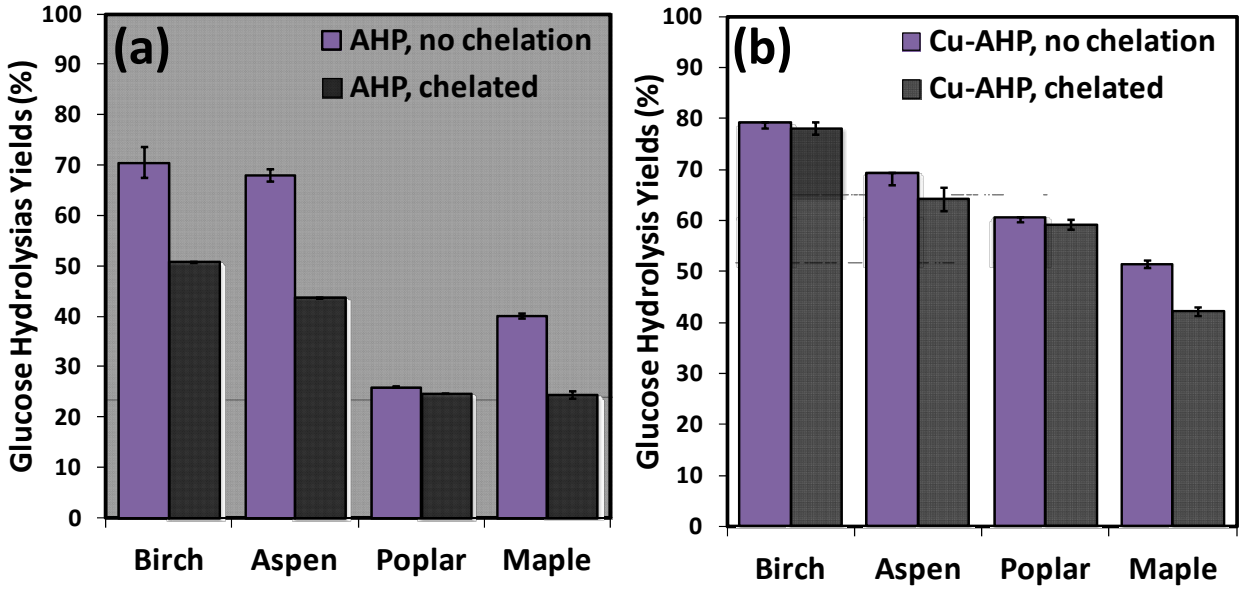

Fig. 4 Effect of chelation on the glucose hydrolysis yields of the diverse hardwoods following **(a)** alkaline hydrogen peroxide (AHP) pretreatment and **(b)** Cu-catalyzed alkaline hydrogen peroxide (Cu-AHP) pretreated.

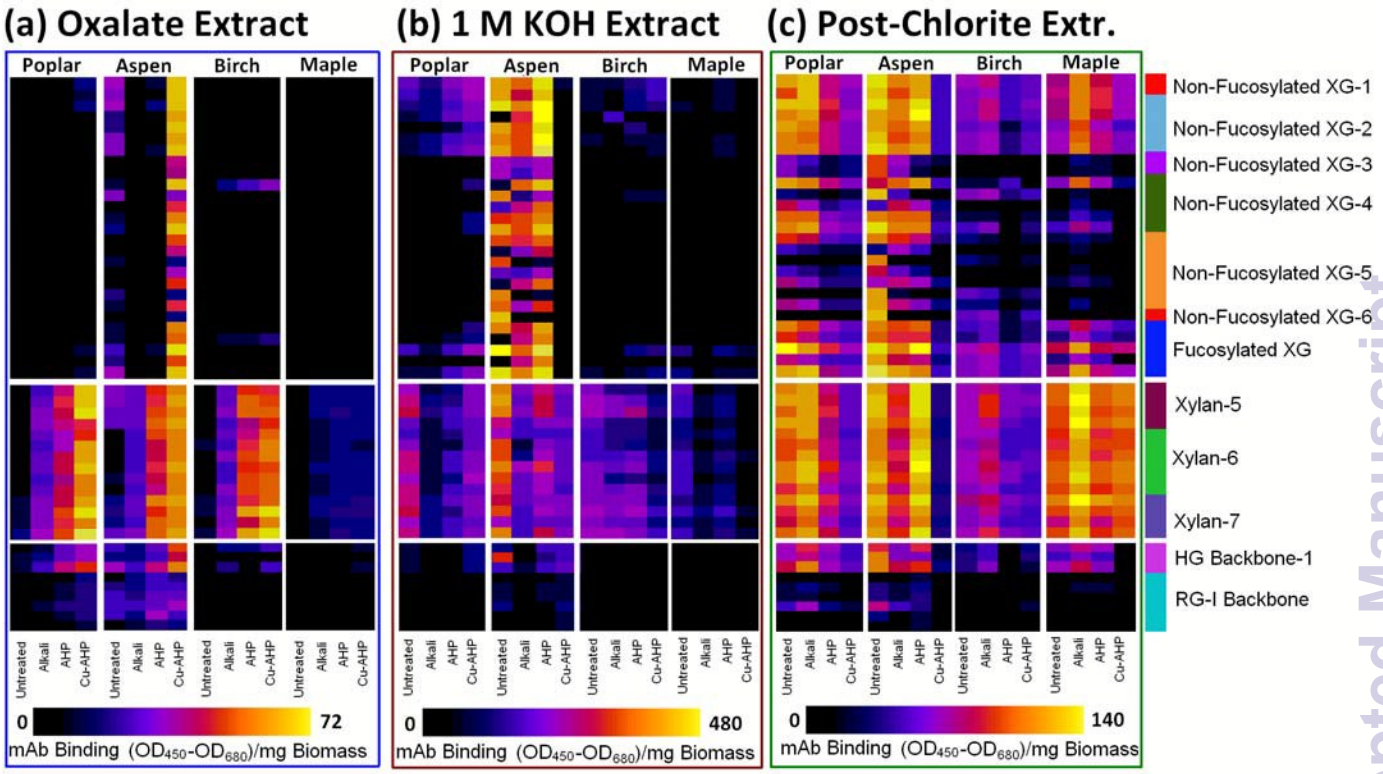

Fig. 5 Glycome profiling results for selected epitopes (xyloglucan, xylan, and pectic backbones) and selected extracts where major differences are observed: **(a)** oxalate, **(b)** 1 M KOH, and **(c)** post-chlorite 4 M KOH. The scales for the heat maps in each subplot are normalized to the maximum value to better visualize differences within each dataset. XG, xyloglucan; HG homogalacturonan; RG rhamnogalacturonan. The complete glycome profiles are provided in **Table S1**.

Table 1 Compositional analysis of raw hardwoods used in this study.[a] Composition is reported as a mass percent on a dry basis.

		Silver Birch	Hybrid Aspen	Hybrid Poplar	Sugar Maple
Glycosyl Residues	**Glc total**	39.0 ± 1.9	32.0 ± 1.7	44.0 ± 1.2	42.0 ± 2.0
	Glc (non-Cel)	2.55 ± 0.08	3.4 ± 0.2	2.60 ± 0.07	2.6 ± 0.1
	Xyl	22.5 ± 0.1	18.2 ± 1.0	15.9 ± 0.2	19.4 ± 0.8
	Ara	0.45 ± 0.02	1.35 ± 0.04	0.41 ± 0.09	0.63 ± 0.01
	Man	1.08 ± 0.02	1.23 ± 0.03	0.8 ± 0.1	0.15 ± 0.08
	Gal	0.63 ± 0.02	0.69 ± 0.04	0.42 ± 0.04	0.44 ± 0.04
	Fuc	0.03 ± 0.02	0.09 ± 0.03	0.02 ± 0.01	0.03 ± 0.01
	Rha	0.44 ± 0.01	0.54 ± 0.03	0.33 ± 0.04	0.36 ± 0.01
	Uronic Acids	1.5 ± 0.3	2.5 ± 0.7	1.36 ± 0.06	0.62 ± 0.04
Acetyl		4.60 ± 1.8	4.8 ± 0.3	4.14 ± 0.09	3.4 ± 0.2
Klason Lignin		17.6 ± 0.8	22.1 ± 0.9	22.1 ± 1.0	24.5 ± 2.5
Ash		0.20 ± 0.03	2.9 ± 0.1	1.9 ± 0.5	0.50 ± 0.03
Extractives		2.9 ± 0.1	12.3 ± 0.5	4.54 ± 0.08	3.7 ± 0.3
Total		**93.48 ± 3.8**	**102.1 ± 5.57**	**98.52 ±3.48**	**98.33 ± 6.12**

[a]Errors represent standard deviations from 3-6 biological replicates except for Extractives which is the standard deviation from 2 biological replicates.

Table 2 ICP-MS analysis for total redox-active metals present in cell wall

Biomass	**Cell Wall Redox-Active Metals (ppm)**			
	Manganese	**Iron**	**Copper**	**Total**[a]
Silver birch (control)[b]	100	10	1	111±5
Silver birch (non chelated)[c]	98	16	1	115±3
Silver birch (chelated)[d]	4	5	1	11±2
Aspen (control)	19	26	6	51±5
Aspen (non chelated)	16	23	6	45±2
Aspen (chelated)	2	11	4	17±3
Hybrid poplar (control)	1	5	1	7±3
Hybrid poplar (non chelated)	1	5	1	7±2
Hybrid poplar (chelated)	1	2	1	4±4
Sugar maple (control)	34	10	1	45±3
Sugar maple (non chelated)	34	6	1	41±4
Sugar maple (chelated)	3	2	1	6±3

[a] Errors represent the standard deviation from 3 biological replicates

[b] Control samples are untreated biomass.

[c] Non-chelated samples were incubated in pure deionized water for 24 hours.

[d] Chelated samples were treated with the chelator DTPA for 24 hours as described in the experimental section.

Table of Contents Entry

Cell wall-associated, redox-active transition metals play a critical role in the efficacy of oxidative delignification