UNDERSTANDING PLANT CELL WALL PHENOTYPES THAT CONTRIBUTE RECALCITRANCE TO ALKALINE-OXIDATIVE PRETREATMENTS AND ENZYMATIC HYDROLYSIS

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ABSTRACT

UNDERSTANDING PLANT CELL WALL PHENOTYPES
THAT CONTRIBUTE RECALCITRANCE TO ALKALINE-OXIDATIVE
PRETREATMENTS AND ENZYMATIC HYDROLYSIS

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Plant cell walls represent the majority of the terrestrial plants by mass and mainly consist of polysaccharides and lignin. These constituents are considered as an abundant and renewable carbon resource providing great potential for the production of petroleum-displacing biofuels and biochemicals. However, the complex, rigid and heterogeneous plant cell wall structure typically requires a combination of biological and chemical treatments to break down the cell wall and maximize the yield of the products. Pretreatments are thermal or chemical processes that facilitate the subsequent sugar-releasing enzymatic hydrolysis. Alkaline pretreatments and alkaline-oxidative pretreatments are one of the main categories of pretreatments that function well on monocot grasses, which include the cereal stovers and the energy crops. These pretreatments target ester cross-links between lignin and hemicelluloses within the grass cell walls formed by the hydroxycinnamates, as well as the ester linked side chains such as acetyl groups in hemicelluloses, results in significant removal of lignin and hemicelluloses. The removal of these cell wall constituents significantly increases the accessibility of water and enzymes to the cell walls. Structural characteristics including higher order structures within the cell wall, covalent and non-covalent cross-linking, and porosity may all impact the conversion process. Also, plant cell walls can exhibit substantial heterogeneity between plant cell types and tissues. The goal of this work is to employ novel and conventional characterization tools to identify how properties of diverse cell walls are impacted by pretreatments and how this correlates to reduced cell wall “recalcitrance”, which is important

for both plant design with properties suited for deconstruction and the design of deconstruction strategies.

This work consists of several related studies. The roles that glycans play in the plant cell wall recalcitrance was investigated using taxonomically and structurally diverse biomass feedstocks. Their responses to alkaline oxidative pretreatment and how differing features of the cell wall matrix contribute to its recalcitrance was assessed by a set of studies. Different mechanisms of improving hydrolysis yields following alkaline oxidative pretreatment were discovered for monocots and dicots. In monocot grasses, the "loosening" of the glycans due to alkaline oxidative pretreatment indicating the overall weaker associations between cell wall polymers in grasses than in dicots, which results in significantly improved hydrolysis yields after the pretreatment. It was found that the hydrolysis yields of alkali pretreated corn stovers are not necessarily correlated with the original contents of lignin and hydroxycinamates, but they are highly related with the removal of these compounds. Multivariate models were applied to predict the hydrolysis yields based on cell wall properties quantified or predicted by high-throughput pyrolysis molecular beam mass spectrometry (py-MBMS). It was found that the untreated cell wall composition properties including lignin and xylan content, as well as the hydrophilicity, set the initial recalcitrance of the cell walls. However, for the alkaline pretreated corn stover, hydrolysis yields were only predictable by the release of lignin and hydroxycinnamates. These findings indicate that the final yield of a biomass feedstock is not necessarily correlated with the structural features, but is highly pretreatment-oriented, and the properties contributing to a "reduced recalcitrance" phenotype following a specific pretreatment are not necessarily the same properties that contribute to recalcitrance in untreated cell wall.

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KEY TO ABBREVIATIONS

AFEX	ammonia fiber expansion
AG	arabinogalactan
AHP	alkaline hydrogen peroxide
AIC	Akaike information criterion
BIC	Bayesian information criterion
CAD	cinnamyl alcohol dehydrogenase
CBM	carbohydrate binding module
CCRC	complex carbohydrates research center
CDTA	1,2-Cyclohexylenedinitrilotetraacetic acid
CoMPP	comprehensive microarray polymer profiling
COMT	caffeic acid O-methyl transferase
Cp	Cathodic potential
CS	corn stover
Ct	*Clostridium thermocellum*
DI	de-ionized
ELISA	enzyme-linked immuno assay
FA	ferulate/ferulic acid
FTIR	Fourier transform infrared spectroscopy
G	guaiacyl
GAX	glucuronoarabinoxylan
GC	gas chromatography
GFP	green fluorescent protein
GH	glycosyl hydrolase

GHG	greenhouse gas
Glc	glucose/glucan
GM	glucomannan
GP	glycome profiling
GX	glucuxylan
H	p-hydroxyphenyl
HCA	hydroxycinnamate/hydroxycinnamic acid
HG/HGA	homogalacturonan
HPLC	High-performance liquid chromatography
HSQC	heteronuclear single quantum coherence
mAb	monoclonal antibody
MBMS	molecular beam mass spectrometry
MLG	mixed-linked glycan
MLR	multiple linear regression
MS	mass spectroscopy
NIR	Near-infrared spectroscopy
NMR	nuclear magnetic resonance
NREL	national renewable energy laboratory
PC	post-chlorite
*p*CA	para-coumarate/para-coumaric acid
PCA	principal component analysis
PLS	partial least square
py	pyrolysis
RG-I	rhamnogalacturonan I
S	syringyl

SG	switchgrass
WRV	water retention value
XyG	xyloglucan

1. INTRODUCTION

1.1 BACKGROUND

The consumption of fossil fuels from ancient plants buried under the ground for approximately 300 million years is the primary contributor to GHGs emissions. Lignocellulosic biomass, as the most abundant terrestrial carbon resource, can be utilized as feedstocks for biofuels via diverse routes. These routes are beneficial to society due to the potential of reducing GHGs emission and the promotion of regional energy independency/diversification. These feedstocks include agricultural and forestry residues, and energy grasses [1, 2]. Structural polymers within plant cell walls (*i.e.* cellulose, hemicelluloses, and lignins) offer potential for long-term sustainable production of renewable fuels, chemicals, polymers, and materials that are currently produced from petrochemicals. Many of the promising conversion pathways for these products are based on a series of biochemical and/or catalytic reactions starting with the sugars derived from cellulose (glucose) and hemicelluloses (primarily xylose in angiosperms).

The recalcitrance of plant cell walls to deconstruction and conversion is considered to be the most crucial factor to overcome in order to develop successful bioprocessing technologies for lignocellulose conversion to renewable fuels and chemicals [3]. As such, in order to generate high sugar yields from the cellulose and hemicellulose within plant cell walls, a pretreatment is required in combination with the subsequent polysaccharide hydrolysis by either enzymes or an acid catalyst [4]. The unique ultrastructure of biomass, heterogeneous and layered plant cell wall structure, results in the difficulties in assessing the changes during the biomass conversion processes. Traditional compositional analysis can only provide limited scope of information on the cell wall structures such as the composition of the structural carbohydrates

and total content of lignin. Plant cell wall characteristics including the ultrastructure of the cell walls, the covalent bonds and side-chains, the porosity and charges within the cell wall polymers and the variation in different plant parts and phenotypes of plants may all impact the deconstruction but are not receiving enough attention for detailed assessment or accurate quantification. Novel and conventional characterization tools are desired to identify how properties of diverse cell walls are impacted by type of conversion processes and how this correlates to reduced cell wall recalcitrance [3], which is important for both plant design with properties suited for deconstruction and for design deconstruction processes.

1.2 PLANT CELL WALLS

1.2.1 Plant cell wall organization

Plant cell walls comprises the majority of the lignocellulosic biomass materials [5]. In typical plant cell walls, secondary cell wall exists around the cell membrane and lumen, surrounded by the primary cell wall. Primary cell wall is first grown and appears early than secondary cell wall. In the primary cell wall, cellulose exhibits as the crystalline microfibrils, and hemicelluloses coalesce with the surface of cellulose microfibrils [6]. The hydrogen bonded matrix structure comprising of cellulose microfibrils and hemicelluloses contribute to the strength of plant cell walls [7]. Lignin mostly exist in the secondary cell wall and also in the middle lamella layer between cells to resist water and enhance rigidity [8]. Cell wall cellulose is mostly crystalline and composed of 36 chains of β-1-4 linked D-glucosyl unit [9]. In nature, cellulose crystalline structure has distinct but coexisting crystallite forms, I_{α} and I_{β}[10]. Hemicelluloses are branched polysaccharides which consist of different monomeric sugars including glucose, xylose, arabinose, mannose and galactose [11]. The composition of hemicelluloses vary by species.

1.2.2 Plant cell wall lignins

Lignin is the most complicated and hydrophobic compound in plant cell wall materials. It is polymer composed primarily of three *p*-hydroxyphenyl alcohols (monolignols) including coniferyl, sinapyl, and a lesser extent *p*-coumaryl alcohols which form the guaicyl (G), sinapyl (S), and *p*-hydroxyphenyl (H) substituents in lignin [12]. Other "non-canonical" aromatic monomers taken from other points of the monolignol biosynthesis grid are known to be incorporated into the lignin polymer as well [13, 14]. The linkages between lignin units are primarily β-O-4 and α-O-4 ether bonds and a fraction of condensed bonds. The "C-C" bonds including β–5, β–β, and 5–5 and the biphenyl ethers such as 4-O-5 and 5-O-4 bonds are called condensed structure [15]. The total content and relative abundance of monolignols, their linkages, and degree of crosslinking with polysaccharides varies by plant species, plant developmental stage, and plant tissues [16-18].

Lignin composition and cell wall structural organization in grasses is significantly different from herbaceous and woody dicots (forbs and hardwoods, respectively) or gymnosperm lignins. One distinguishing feature of the monocot lignins is the considerable incorporation of the *p*-hydroxycinnamic acids including ferulic and *p*-coumaric acids [19-21]. Ferulate monomers and dimers are known to be ester-linked to glucuronoarabinoxylan and involved in ether and C-C linkages to the lignin polymer that act as crosslinks between polymer chains [19]. Monomers of *p*-coumaric acid are proposed to be esterified to the lignin polymer at the γ-carbon of the side chain region of β-O-4 linked syringyl moieties [22] and to a lesser degree esterified to glucuronoarabinoxylan [18]. Grass lignins are significantly more condensed and have higher phenolic hydroxyl content than the lignins of dicots [21, 23]. An important implication of this is that more than 50% of grass lignins can be solubilized by treatment with alkali [24] due to the destruction of alkali-labile ester linkages along with the high free phenolic content improving lignin solubility in alkali [25].

1.2.3 Plant cell wall polysaccharides

The major classes of matrix polysaccharides include the xylans including glucuronoarabinoxylans (GAXs) and glucuronoxylans (GXs), glucomannans (GMs), xyloglucans (XyGs), mixed-linkage glucans (MLGs), pectins, and cell wall protein glycosylations [26-28]. The abundance, composition, and substitution patterns of these glycans vary temporally during plant growth and cell wall expansion, spatially within cell walls and between plant tissues, and taxonomically across diverse plants. In dicots, pectins, XyGs and GAXs are the predominant non-cellulosic glycans in the primary cell walls with GXs the predominant hemicellulose in dicot the secondary cell walls [29, 30]. In grasses, GAXs have occupy a significant fraction of the interstitial space between cellulose microfibrils in the primary cell walls in addition to mixed-linkage MLGs and grass-specific XyGs and GMs [31, 32]. Pectins are complex and can comprise up to 30% of the primary cell walls of dicots and significantly less in grasses [30] where some of this function is thought to be performed by other glycans including MLGs and GAXs [32]. Structural proteins may comprise up to 10% of the cell wall in some plant tissues and can be significantly glycosylated, for example with arabinogalactans (AGs) [26].

1.3 DECONSTRUCTION OF PLANT CELL WALLS

1.3.1 Cell wall deconstruction to fuels and chemicals

Plant cell walls conversion to fuels and chemicals are generally through biochemical or thermochemical methods [33]. Thermochemical conversion including pyrolysis and gasification require high temperature to convert the biomass to bio-oils and synthesis gas, respectively. Biochemical conversion to fuels and chemicals via fermentation or catalytic routes usually require a low-severity thermochemical pretreatment which partially breaks down the cell wall, then an enzymatic hydrolysis step to convert the pretreated biomass to

fermentable sugars. Compared with thermochemical conversion, which requires low residence time but is not selective, biochemical conversion provides high selectivity in converting biomass to desired end products.

1.3.2 Pretreatments

Pretreatments utilize hydrothermal or chemical treatment to physically solubilize or chemically alter the biomass components to facilitate the enzymatic hydrolysis [34]. The best-studied pretreatment is acidic hydrothermal pretreatment, which utilizes the diluted acid in varied reaction temperature and pressure to hydrolyze and solubilize xylan, melt and redistribute lignin and increase the enzymatic accessible surface area of the cell walls to improve digestibility [35, 36]. Steam explosion pretreatment is also a hydrothermal pretreatment to break apart the cell wall components, but using high pressure steam penetrates into heated lignocellulosic material to let the material expands, which leads to size reduction and increasing porosity of the substrate [37]. The reaction variables of steam explosion pretreatment depend on the particle size and moisture content of the samples [38]. Steam explosion pretreatment and dilute acid pretreatment both result in lignin melting at its glass transition point and generating small lignin globules which limit the enzyme penetration to cellulose microfibrils [39, 40]. Ammonia fiber expansion (AFEX) pretreatment is a thermochemical pretreatment that aims to overcome biomass recalcitrance by effectively redistributing but without removing hemicelluloses and lignin [41-44]. During AFEX pretreatment, lignocellulosic materials are treated by liquid ammonia under high temperature and pressure for a certain time then followed by pressure release, and the treated materials have the same composition as the untreated material but significantly increased digestibility [34, 45]. Besides the above mentioned pretreatments, many other thermochemical pretreatments also have been shown to be effective for improving biomass saccharification

such as lime pretreatments [46], organic solvent pretreatments [47, 48], ionic liquid pretreatments [49] and sulfite/ SO_2 pretreatments [50], etc.

Biological pretreatments usually function as biological delignification, which is another category of approaches to improve the digestibility of the lignocellulosic biomass. Compared with thermal chemical pretreatments, biological delignification requires lower energy input and is more environmental friendly in terms of the formation of less toxic byproducts than the thermochemical pretreatments [51]. Lignin-degrading microorganisms can be found in forest leaf litter/composts, and the microorganisms applied for biological pretreatment such as *Phanerochaete chrysosporium* have been reviewed recently [52]. A best known wood-decay fungi is white rot fungi, which produces extracellular lignin-modifying enzymes, including laccase, lignin peroxidases and manganese peroxidases, and leave the lighter-colored and bleached cellulose [53]. In white-rot fungi, redox-active metals such as copper, iron and manganese act as cofactors or are thought to participate in the reaction cycle of the active enzymes and directly participate in the process of lignin degradation [54]. Employing the effective features of biological delignification, oxidative and catalytic pretreatment approaches have been developed, which offer chemical pretreatments at mild temperatures with minimal chemical input [55, 56].

Alkaline hydrogen peroxide (AHP) pretreatment is one type of alkaline oxidative pretreatment has been studied as a chemical pretreatment [57-60] and as a delignifying post-treatment [61, 62] and is based on treatment of biomass with hydrogen peroxide at alkaline pH (optimally at pH 11.5) at ambient or near-ambient temperatures and pressures. AHP pretreatment is originally a bleaching process widely applied in the paper industry for brightening, which is based on hydrogen peroxide oxidation of lignin under alkaline condition, while alkali results in saponification reactions with hemicellulose esters and lipids. The solubilization and cleavage of lignin and hemicelluloses can effectively increase the

hydrolysis conversion of residual plant cell walls [58, 59, 61]. Alkaline pretreatments are particularly effective for grasses, and it is known that AHP is less effective on herbaceous dicots [60] and woody dicots [56]. We hypothesize that the digestibility improvement resulting from AHP pretreatment may be attributable to the destruction of ferulate crosslinks as well as mild oxidation and solubilization of lignin, and the metals in the plant cell walls act as redox-active reactants and participate in the lignin degradation by hydrogen peroxide. These outcomes have the net effect of improving the overall hydrophilicity of the cell wall matrix which can allow for water and hydrolytic enzyme penetration.

1.3.2 Enzymatic hydrolysis

Fungi and bacteria utilizing cellulose as a carbon source have evolved a complex array of enzymes that hydrolyze cellulose to glucose [63]. The lignocellulose-degrading enzymes are classified as families with different three-dimensional structures and catalytic mechanisms [64]. A vast natural diversity of cellulases are belonging to GH (glycosyl hydrolases) families. In order to deconstruct the crystalline cellulose, the enzymatic hydrolysis needs the synergy of three types of cellulolytic enzymes [65]. Cellulases including endoglucanase and cellobiohydrolase adsorb onto the substrate via binding domains. Catalytic domain of endoglucanases splits the bond in glucan chain within the cellulose microfribrils and produce the free chain ends as well as increase accessibility of the substrate by the other cellulases. Cellobiohydrolases cut the end of cellulose chain to produce cellobiose by threading the chain end into the catalytic tunnel to initiate the hydrolysis of the β-glycosidic bond and simultaneous slide forward the enzyme along the cellulose chain. The process is repeated until cellulases desorb from the cellulose. Meanwhile, β-glucosidase hydrolyzes cellubiose to two units of glucose. The enzyme cocktail secreted by the filamentous fungi *Trichoderma reesei* represents a most widely studied noncomplexed cellulase system [66]. Many microorganisms employ a “cellulosome”, a large extracellular multiprotein complex

comprising enzyme modules and scaffold protein, and bacteria *Clostridium thermocellum* represents the most widely studied complexed cellulase systems [67]. In addition, accessory enzymes such as hemicellulases [68] and lytic polysaccharide monooxygenase (AA9) [69] can expedite the catalysis of cellulases. Currently, development of synthetic enzyme cocktails with feedstock-specific activities and protein engineering to alter the catalytic properties of enzymes are the two main routes to improve the biomass saccharification [70, 71].

1.4 PLANT CELL WALL RECALCITRANCE

1.4.1 Cell wall lignin

Lignin is a hydrophobic component in the cell wall, which can prevent water penetration and provides chemical resistance to degradation [3, 72]. Lignin impacts cell wall saccharification yields primarily as a physical obstacle and by non-specific binding to cellulases [3], or the hydrophobic nature of lignin retards the solubilization of xylan associated with lignin [73]. Studies have shown strong negative correlations between lignin content and enzymatic hydrolysis yields for different types of biomass [74, 75]. The impact of lignin content and composition on the digestibility of biomass vary by the types of feedstock and pretreatment chemistry. Yang et al. suggested that the cellulose digestibility was strongly correlated to lignin removal for flowthrough operated pretreatment with or without acid addition [75]. Another study showed xylan content decreasing in the center of the cell wall while remaining in the lumen and middle lamella layer during dilute acid pretreatment, which can be explained by the hydrophobic nature of lignin preventing the solubilization of xylan associated to lignin [73]. During dilute acid hydrolysis of *Populus*, both lignin content and the ratio between syringyl and guaiacyl monomer (S/G ratio) in lignin have been shown to impact the sugar yield, with slightly lower S/G ratio yielding significantly higher extent of hydrolysis [76]. Conversely, for hot water pretreatment, sugar release was higher for *Populus*

with higher S/G ratios [74]. For *Arabidopsis* tissue after hot water pretreatment, cell walls with higher S/G ratio also gave a higher glucose yield [77]. For alfalfa following dilute acid pretreatment, significant differences in hydrolysis yields between various lines with different lignin content and S/G ratio were observed. And for those lines having high H lignin content, S/G ratio alone doesn't impact sugar yield [78]. The correlations between the digestibility and *p*-coumaric acid and ferulic acid do not follow the same mechanism and depend on their connection to other cell wall components (lignin and xylan). Since the majority of *p*-coumaric acid is esterified to lignin, the amount of *p*-coumaric acid esters are more of an indicator of the degree of lignification in a forage plant than a direct predictor of digestibility [79].

1.4.2 Cell wall polysaccharides

The complex matrix of non-cellulosic glycans within the cell wall is another major contributor of the cell wall recalcitrance besides lignin. The content, diversity, interactions, and distribution of polysaccharides play roles in recalcitrance by setting the cellulose accessibility and the cell wall porosity [30, 80, 81]. Non-covalent bonds including H-bonding, van der Waals, and hydrophobic forces between and within polysaccharides are responsible for stable crystalline regions of cellulose and the ability of unsubstituted regions of glycans containing β-1,4 backbones (i.e. MLGs, xylans, GMs, and XyGs) to form junction zones with each other and to "sheath" the surface of cellulose microfibrils [82, 83]. This capacity of hemicelluloses to sheath the surface of cellulose microfibrils has been identified as one potential means through which non-cellulosic polysaccharides contribute to cell wall recalcitrance [84]. Ionic bonds such Ca^{2+} crosslinks in homogalacturonan (HGA) are important in controlling cell wall porosity (and penetration of enzymes) and potentially aiding cell-cell adhesion [30] while electrostatic repulsion of charged uronic acid groups in GAXs are hypothesized to play a role in ordering cellulose microfibrils, preventing aggregation, and patterning lignin deposition [85]. Covalent cross-links within the cell wall

include ferulate and diferulate bridges between and within xylans, lignin, and pectins [27], isodityrosine cross-links in structural proteins, and ether and aryl linkages between structural proteins and ferulates esterified to polysaccharides [29, 86]. Taken together, this complex composite structure presents a challenge for characterization and a number of approaches have been developed recently with a focus on relating structural features of the cell wall to its recalcitrance as reviewed recently by Foston et al. [87].

1.4.3 Cell wall ultrastructure

Besides composition, many studies showed that the enzymatic digestibility is affected by the higher order structure of biomass. A study using a fluorescence-labeled cellulase to probe enzyme binding with the substrates showed that the amorphous forms of the celluloses are more accessible by enzyme than crystalline cellulose microfibrils [88]. Zhang et al. demonstrated that an organic solvent-based pretreatment disrupts the hydrogen bonds in cellulose to break down the biomass and the pretreated biomass with increased surface area but unchanged lignin content has dramatically higher cellulose accessibility [89]. Undoubtedly the ultrastructure of biomass including the porosity and polysaccharides accessibility are more important than composition on impacting the cell wall digestibility. However, previous research on characterization of biomass porosity are mostly focused on hardwoods for chemical pulping and papermaking, and the nano-scale porosity properties of lignocellulosic biomass as a bioenergy feedstock still lack adequate attention.

1.4.4 Grass cell wall recalcitrance

The degree of lignification and the composition of cell wall polysaccharides are diverse among grasses, woody dicots and gymnosperm biomass. Outcomes of pretreatment are obviously dependent on the combination of pretreatment chemistry and the plant cell wall properties. A number of promising bioenergy feedstocks are commelinoid monocots, specifically the *Poaceae* or grasses, which include agricultural residues [90] such as corn

stover, wheat straw, rice straw, and sugar cane bagasse, and dedicated perennial energy crops such as switchgrass and *Miscanthus* spp. Many of these commelinoid monocots such as corn stover [91], sugarcane bagasse [92], and sorghum [93] have stems that consist of high-lignin vascular bundles distributed throughout a stem consisting of primarily low-lignin pith parenchyma tissue [94]. As a consequence of the alkali-labile ferulate ester crosslinks within the hemicellulose and lignin [21, 95, 96] in graminaceous monocots, as well as high phenolic hydroxyl contents in their lignins which resulting in increased alkali solubility [25], mild alkali pretreatment of grasses such as maize has shown substantial promise as these can be employed for both fractionating biomass and generating a pretreated biomass that is highly amenable to enzymatic hydrolysis [97, 98]. For the grass cell walls, alkaline saponification partially solubilizes the hemicelluloses by removing the side chains from xylans such as acetate and partially removes lignin based on the degree of lignification and ferulates crosslinks, generates degradation byproducts including organic acids, aldehydes and phenols [99]. Grass cell walls swell after alkaline treatment [100] with higher glycan extractability and is more accessible to either enzyme or water, and alkaline or alkaline oxidative treated monocots yields much higher digestibility compared to dicots.

1.5 PLANT CELL WALL CHARACTERIZATION AND MODELING

1.5.1 Characterization of cell wall structural carbohydrates

Accurate modeling and direct assessment of changes in plant cell wall structure is required in order to fundamentally understand the mechanisms of cell wall alteration by chemical and biological treatments. The monomer composition of biomass can be determined by either NREL two-stage sulfuric acid hydrolysis method [101], solution-state ^{1}H NMR [102, 103], NIR/PLS (near infrared reflectance spectroscopy) [104] and pyrolysis GC/MS [105]. The physical structural characteristics of biomass such as the porosity and morphology can be

profiled by modern microscopes such as scanning electron microscopy [106] and atomic force microscopy [9]. Nuclear magnetic resonance (NMR) is a powerful tool applied to determine the chemical bonds and linkages within the cell wall components such as crystallinity index of cellulose by ^{13}C solid-state NMR [107-109] and the structural fingerprint of the polysaccharide and lignin by 2D HSQC NMR [110, 111].

1.5.2 Characterization on cell wall lignins

There are generally three categories of characterization methods for quantifying lignin content, including the measurements of the total mass of lignin, the total aromatics of lignin, and the total number of monolignols. To be specific, insoluble solids in two-stage NREL composition analysis is called Klason lignin, which is approximately quantifying the total mass of lignin in the sample. Acetyl bromide soluble lignin (ABSL) has been employed to determine lignin concentration based on the UV absorbance of the aromatics of the extractive-free samples [112]. Thioacidolysis is a method to determine the ratio of lignin monomers by cleaving the β-O-4 inter-unit linkage after lignin isolation from polysaccharides by dioxane [113]. Analytical pyrolysis-GC/MS is another indirect method for quantify lignin content and composition by profiling the pyrolysis products in GC/MS [114-116]. The functional groups and crosslinkings of lignin have been studied by ^{13}C, HSQC, and ^{31}P NMR spectroscopies [117].

1.5.3 Novel characterization tools using specific proteins

Tools utilizing specific proteins such as monoclonal antibodies (mAbs) or carbohydrate binding modules (CBMs) to bind with specific site of biomass have been developed in order to characterize the cell wall structures. Cell wall directed monoclonal antibodies (mAbs) as well as probes assembled by carbohydrate-binding modules (CBMs) from microbial cell wall glycoside hydrolases [118] have been applied to discover cell wall microstructures by locating cell wall epitope *in situ*. A technique called comprehensive microarray polymer

profiling (CoMPP) using microarray with probes of mAbs or CBMs for mapping cell wall composition [119] was able to detect reduction in xylan, arabinoxylans, xyloglucan, and mixed-linked glucan epitopes in hydrothermal pretreated wheat straw [120]. Pattathil et al. employed ~200 glycan-directed antibodies in a ELISA-based screen approach called glycome profiling, which can identify the abundance and extractability of 19 groups of non-cellulosic glycans within the cell walls including mixed-linked glucans, xyloglucans, xylans and pectins, etc.[121]. It has been applied to evaluate the structural changes in poplar under dilute acid pretreatments and discovered that the dilute acid pretreatment primarily removes arabinogalactans, and the fucosyl-containing xyloglucan, then the non-fucosylated xyloglucan [122]. The CBM-GFP has been applied to quantify the accessible area of cellulose [123] and to estimate the initiation sites of hydrolysis and saccharification yields [124].

1.5.4 Data mining and models

Data mining and model development are frequently used in the analytical field of biomass characterization. Some of the research applied multivariate approaches to manipulate the data and build up models to predict biomass component properties. Pyrolysis molecular beam mass spectrometry (py-MBMS) and Fourier transform infrared spectroscopy (FTIR) have been applied using chemometric models to predict the lignin content of switchgrass under different growth conditions [125, 126]. Near-infrared spectroscopy (NIR) has also been used to analyze the nitrogen and carbohydrates in pine needle [127] and the weight loss in spruce wood by brown-rot fungi [128]. Principal component analysis has been utilized to simplify the spectra and correlate the principal components to easily identifiable cell wall features [128]. Other work have been done using chemometric models to relate the cell wall properties including cellulose crystallinity [129], lignin content, *p*-coumaric and ferulic acids content [130], to hydrolysis yields by either neural network [131] or regression models [130].

In terms of the cell wall recalcitrance, the physical properties of biomass including porosity and accessible surface area have impact on enzyme access to cellulose microfibrils. In addition, product inhibition and changes in the substrate properties affect the enzymatic hydrolysis extent and yield. There are many empirical models that incorporate the effects of various substrate and enzyme properties on hydrolysis rates. Studies showed increasing degree of polymerization decreases the availability of chain ends, and the crystallinity index and enzymatic hydrolysis have negative correlations with cellulase accessibility [132]. Michaelis–Menten based models and enzyme adsorption models are frequently utilized for modeling kinetics [132, 133].

1.6 OBJECTIVES

The cell wall matrix of plants provides an effective barrier to the chemical and/or biological deconstruction to its monosaccharide components for use in biofuels applications. The properties of the cell wall matrix impacting its recalcitrance are set by many features of cell wall macromolecules across a wide range of length scales. Changes in the physical and chemical properties of cell wall polymers and the overall cell wall environment are important outcomes of pretreatments. The overall goal of this research is to fundamentally understand the recalcitrance of plant cell walls through investigating the impact of pretreatment on plant cell walls with diverse properties. To be specific, alkaline and alkaline oxidative pretreatments are applied to alter the plant cell walls in diverse plants. The chemical and structural features will be investigated before and after the pretreatments for the diverse feedstocks. Correlations and empirical models will be developed to in order to understand how the cell wall recalcitrance is impacted by alkaline and alkaline-oxidative pretreatments.

This series of the study will focus on the following four studies:

1. Understand the roles that the abundance and accessibility of non-cellulosic cell wall glycans play in the plant cell wall recalcitrance, through quantitatively identifying how those properties in structurally and taxonomically diverse cell walls are impacted by alkaline oxidative pretreatments and how this correlates to reduced recalcitrance.

2. Investigate the impacts of alkaline oxidative pretreatment on diverse grass cell walls and the relationship between the changes of grass cell wall properties and enzymatic hydrolysis by cellulolytic enzymes, through characterizing the specific grass cell wall components including hydroxycinnamates, xylans, β-glucans, and cellulose accessibility associated with the alkaline oxidative pretreatments with varied severity.

3. Identify correlations between maize cell wall properties and their relationship to the enzymatic hydrolysis yields associated with alkaline pretreatments, in order to identify the features contributing to maize cell wall recalcitrance.

4. Develop chemometric models and multivariate models to predict the cell wall composition and the hydrolysis yields, in order to examine the distribution of the cell wall recalcitrance as well as to provide fast characterization tools on the diverse plant cell walls.

2. PLANT CELL WALL RECALCITRANCE IN DIVERSE BIOENERGY FEEDSTOCKS

2.1 INTRODUCTION

Immunological methods using glycan directed monoclonal antibodies (mAbs) are widely used tools to investigate plant cell wall structure [134, 135]. Besides the cell wall composition and structure, mAbs can be used for qualitative and quantitative detection of carbohydrate epitopes in plant sequential extracts. This has been performed in order to characterize pretreatments using one approach that plotted polysaccharides from three increasingly severe cell wall extracts (CDTA, NaOH, cadoxen) onto a microarray, which was then probed with mABs and CBMs [119] in order to identify changes in content and extractability of xylans, XyGs, and MLGs epitopes in hydrothermal pretreated wheat straw [120]. Recently, Pattathil et al. assembled a collection of glycan-specific antibodies [136] and an ELISA-based screen was used to categorize these structurally related glycans from diverse cell walls [121, 137]. This technique called “Glycome profiling” has been applied to evaluate the structural, accessibility, and extractability changes of cell wall glycans in hybrid poplar during dilute acid pretreatments [122] and to compare differences in the structural features underpinning cellulose digestibility in switchgrass and hybrid poplar [138]. In this chapter, glycome profiling was applied to understand how a mild alkaline oxidative pretreatment impacts the composition and structural organization of the cell wall. Two distinct mechanisms by which this pretreatment overcomes cell wall recalcitrance in either herbaceous dicots or graminaceous monocots were identified. These results highlight both the taxonomic differences in cell wall organization and the differences in their response to pretreatment.

Specifically, in this study the response of cell wall glycans of diverse plants including hybrid poplar (woody dicot), goldenrod (herbaceous dicot), and corn stover (monocot grass) to AHP pretreatment with increasing H_2O_2 loadings has been investigated. The cell wall response to pretreatment was characterized by compositional changes and overall mass loss of the cell wall, glucan and xylan enzymatic conversions using a commercial enzyme cocktail, and glycome profiling of the sequential glycan extracts of the untreated and AHP-pretreated cell walls at 12.5% (w/w) H_2O_2 loading on biomass. Using this information, we draw conclusions about the structural changes associated with AHP pretreatment and additionally are able to gain insights into the role that differences in plant cell wall architecture have on cell wall recalcitrance.

2.2 MATERIALS AND METHODS

2.2.1 Pretreatment

Biomass consisted of a commercial hybrid corn stover (Pioneer Hi-Bred 36H56) provided through the Great Lakes Bioenergy Research Center (GLBRC), debarked hybrid poplar (*Populus nigra var. charkoviensis* x caudina cv. NE-19) grown at the University of Wisconsin Arlington Agricultural Research Station and provided through the GLBRC, and goldenrod (Solidago canadensis) collected locally in East Lansing, MI and obtained from Dr. Jonathan Walton (MSU, Plant Biology). Biomass was initially milled with a Wiley MiniMill (Thomas Scientific) to pass a 2 mm screen and air-dried to ~5% moisture. The milled biomass was subjected to alkaline hydrogen peroxide (AHP) pretreatment using H_2O_2 loadings of either 12.5%, 25%, 50% (g H_2O_2 per g biomass). These were performed in shake flasks with a 100 g total mass and 2% (w/v) solids concentration for 24 h at 30 ºC with orbital shaking at 170 rpm and periodic pH adjustment to 11.5. After pretreatments, the liquid in the samples was removed by vacuum filtration (#113 Whatman filter paper) and the slurry was

washed several times with deionized water to remove solubles and air-dried at room temperature. The mass yield during pretreatment was quantified as the ratio between the mass (dry basis) after pretreatment and the initial mass before pretreatment.

2.2.2 Composition analysis, enzymatic hydrolysis and digestibility determination

The pretreated biomass was subjected to a two-stage acid hydrolysis according to NREL composition analysis [139] to determine neutral polysaccharide content, Klason lignin, and ash with the difference that Aminex HPX-87H (Bio-Rad, Hercules, CA) column was used to quantify the glucan, xylan+galactan+mannan, arabinan, as well as acetate content. The total uronic acids were assayed enzymatically (K-Uronic, Megazyme, Wicklow, Ireland). The extractives content was determined by a sequential 3-step extraction including 70% ethanol, 1:1 (v/v) methanol and chloroform mixture, and acetone in 1 mL total volume at 5% solid loading. Three extraction cycles for each solvent were performed and followed by centrifugation at 10000 x g for 10 minutes for each cycle. Before the enzymatic hydrolysis, the pretreated biomass was ball-milled for 3 cycles using a QIAGEN TissueLyser II equipped with 25 mL Teflon jars and 20 mm diameter Teflon balls at 30 Hz for 2 minutes with liquid nitrogen cooling. The ball-milled samples were incubated with Cellic C-Tec2 (Novozymes, Bagsværd, Denmark) at a loading of 30 mg protein/g glucan at 50 ºC, 10% solid loading and 5 mL total volume in 0.05 M Na-citrate buffer pH 4.8, for 24 hours or 72 hours. The glucan and xylan yields (based on only glucan and xylan remaining after pretreatment) were determined by the HPLC analyzable glucose and xylose concentrations after hydrolysis divided by the original glucan and xylan contents in the pretreated samples.

2.2.3 Sequential extraction, glycome profiling

(Sequential extraction and glycome profiling were done by Sivakumar Pattathil, University of Georgia.)

Sequential cell wall extractions and glycome profiling were carried out as described previously [121, 122, 140]. The six extractions included (in order of extraction) oxalate to remove "loose" pectins, carbonate to remove "more tightly" bound pectins, 1M KOH to remove "loose" hemicelluloses along with tightly bound pectins, 4M KOH to remove "tightly" bound hemicelluloses along with tightly bound pectins, acid chlorite to oxidize and solubilize lignin and release lignin-embedded hemicelluloses, and a 4M KOH post-chlorite treatment to remove additional lignin-bound polysaccharides. 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 Appendix, Table A1, which includes links to a web database, WallMAbDB (http://www.wallmabdb.net) that provides detailed information about each antibody.

2.3 RESULTS AND DISCUSSIONS

2.3.1 Changes in composition and mass loss

Three types of biomass representing three classes of plants that may offer promise as feedstocks for cellulosic biofuels were tested in this study. Corn stover represents the agricultural residue with the highest production and availability for bioenergy applications in the U.S. [141], while short-rotation hybrid poplar has agronomic, logistical, and environmental advantages as a feedstock [142]. "Low-input high-diversity" bioenergy landscapes have many attractive sustainability attributes [143] and comprise mixed communities of plants on marginal or degraded lands, and in this study we use goldenrod (*Solidago canadensis*) as a representative herbaceous dicot that may be present in these landscapes[144]. The composition of the untreated biomass is presented in Table 2.1.

Notable differences include the low content of pectic polysaccharides (as uronic acids) in the corn stover, which is 5-fold lower than the goldenrod and 2-fold lower than the hybrid poplar. The goldenrod has a substantially higher extractives content (23.5%) relative to the other two biomass types. Additionally, lignin content of the corn stover is nearly half that of the goldenrod and poplar.

	Hybrid Poplar (wt/wt %)	**Goldenrod (wt/wt %)**	**Corn Stover (wt/wt %)**
Glucan	48.3±2.0	27.1±0.8	38.4±0.3
Xylan+Mannan+Galactan	16.9±0.6	13.6±0.8	25.2±0.2
Arabinan	1.33±0.07	2.96±0.03	3.92±0.02
Acetate	4.14±0.09	2.59±0.06	3.20±0.10
Total Uronic Acids	1.88±0.10	4.75±0.03	0.88±0.02
Lignin (Klason)	20.85±1.1	19.92±0.7	12.57±1.2
Extractives	5.79±0.22	23.5±0.23	12.0±0.74
Ash	1.95±0.48	6.08±1.02	3.03±0.29

Table 2.1 Composition of untreated biomass on a whole sample basis rather than an extractives-free basis. Errors represent data range of duplicate measurements.

AHP pretreatment was performed at increasing H_2O_2 loadings (12.5%, 25%, and 50% w/w on biomass), of which the two highest loadings would be significantly higher than would be economically practicable industrially. The reason for these high loadings was to compare and analyze how the diverse cell walls differ in their susceptibility to low temperature mild oxidative delignification and hemicellulose extraction and as a screen for overall digestibility differences. The total cell wall mass loss (excluding extractives), xylan loss, and lignin loss for the three biomass types following pretreatment with increasing H_2O_2 loadings show

distinct responses between the biomass types (Figure 2.1 A-C). For poplar, minimal material was solubilized with pretreatment (<1% by mass), while up to 20% and 25% of the mass of the cell walls of the goldenrod and corn stover, respectively, was solubilized by pretreatment at the higher H_2O_2 loadings. The mass of corn stover decreased continuously with increasing H_2O_2 loading, while the sample mass of poplar and goldenrod decreased abruptly with the mildest treatment (12.5% H_2O_2). For individual cell wall components, AHP pretreatments resulted in minimal changes in glucan for all biomass types representing preservation of cellulose (data not shown) which is consistent with our previous findings [145, 146], while the xylan content decreased only for the corn stover (Figure 2.1B). For goldenrod, the Klason lignin content was only slightly reduced by AHP pretreatment and did not change significantly by increasing H_2O_2 loading. The simultaneous removal of xylan and Klason lignin in corn stover was increased by increasing H_2O_2 loading. This is a well-known property of grass cell walls and is due to the higher solubility of grass lignins [25] and alkali-only extraction of lignin and xylans in grasses is known to be significantly higher in grasses than in woody dicots [138, 147]. Besides alkali solubility, cleavage of ester and ether cross-links between xylan and lignin or lignin and lignin mediated by ferulate [31, 148] in grasses are thought to be an important target of AHP pretreatment [146] and likely contribute to these outcomes.

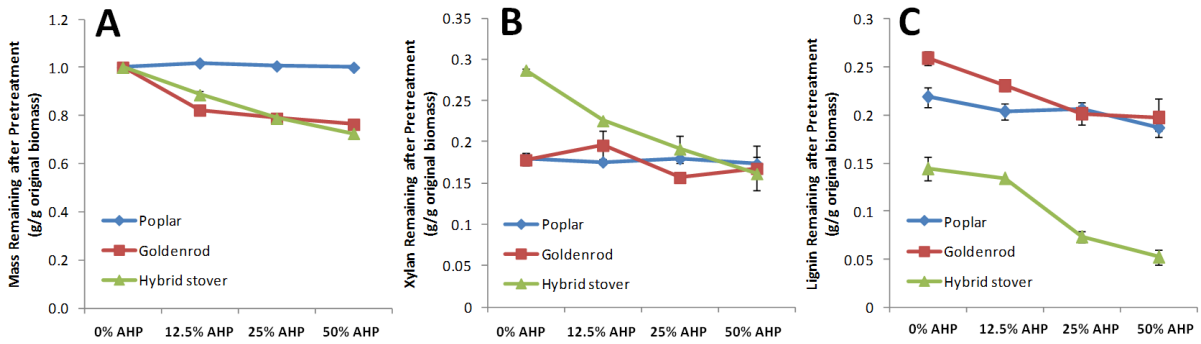

Figure 2.1 Compositional changes (extractives-free basis) associated with AHP pretreatment with increasing H_2O_2 loading showing solubilization of total cell wall mass (A), xylan (B), and lignin (C).

2.3.2 Digestibility of poplar, goldenrod, and corn stover

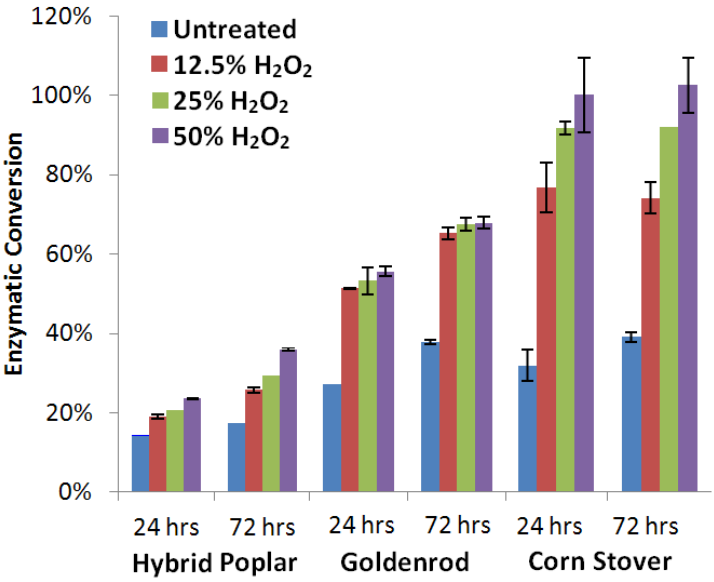

Figure 2.2 Enzymatic conversion of cell wall glucan to glucose in untreated and AHP pretreated biomass with increasing H2O2 loading for poplar, goldenrod, and corn stover with cellulase (Cellic CTec2) for 24 h and 72 h.

Figure 2.2 shows the enzymatic digestibility of the poplar, goldenrod, and corn stover subjected to increasing H_2O_2 loadings for 24 and 72 hr hydrolysis times using a commercial cellulase (Cellic CTec2) with no xylanase supplementation. These results represent screening

only and were not focused on optimizing enzyme cocktail or loading. As such, improved glucan (and xylan) conversions at lower enzyme loadings would be observed if xylanase and pectinase were added. These results show that the glucan enzymatic digestibilities were increased with increasing H_2O_2 loading for the three types of biomass. Similar to the results for compositional changes (Figure 2.1), the digestibility changes with pretreatment are significantly different between the three classes of plants tested. For poplar, the digestibilities were significantly lower than goldenrod and corn stover with the highest glucan conversions approaching 40%. For goldenrod, the glucan digestibilities "saturate" at approximately 70% at the mildest pretreatment condition. For corn stover, the glucan digestibility approaches 100% with increased H_2O_2 loading. It is known that corn stover as well as many other cereal stovers are often considerably more digestible than many undomesticated grasses [146, 148]. Gould [60] determined that diverse graminaceous monocots responded considerably better to AHP pretreatment at 100% (w/w) H_2O_2 loadings than herbaceous dicots/forbs (including goldenrod) and that the seven grasses tested had on average more than double the improvement in digestibility of the 11 forbs tested following AHP pretreatment. He determined that the goldenrod showed the largest improvement in digestibility of the dicots tested and was among the highest in terms of absolute glucose release per gram of biomass.

2.3.3 Glycome profiling of poplar, goldenrod, and corn stover

Glycome profiling (GP) provides quantitative information on both how pretreatment impacts the strength of association between cell wall glycans and other cell wall matrix polymers and how pretreatment impacts cell wall glycan composition. For GP, the samples were subjected to increasingly severe extractions to sequentially remove the cell wall polymers, followed by quantification of recovered materials in each extraction step, and probing of the binding strength for the diverse array of antibodies covering a range of non-cellulose cell wall

polysaccharide epitopes [121, 122, 149]. For the sequential extractions, the strength of association between individual glycans and other cell wall matrix polymers may be hypothesized to be mechanistically due differences in: (1) the strength of non-covalent association or physical entanglement between polymers or "encrustation" within lignin, (2) location within the cell wall (surface vs. interior), and (3) tissue type (e.g. lignified parenchyma and sclerenchyma versus low lignin pith tissues). The six extractions included (in order of extraction) oxalate to remove "loose" pectins, carbonate to remove "tightly" bound pectins, 1M KOH to remove "loose" hemicelluloses, 4M KOH to remove "tightly" bound hemicelluloses, acid chlorite to oxidize and solubilize lignin and release lignin-embedded hemicelluloses, and a 4M KOH post-chlorite treatment to remove additional lignin-bound polysaccharides. The role of lignin in setting the alkali-extractability of cell wall glycans was recently demonstrated by Pattathil et al. [150], whereby it was identified that alfalfa lines with disrupted monolignol synthesis resulting in a low-lignin phenotype contained considerably more alkali-extractable glycans than the control line where much more of the cell wall glycans were extractable only after chlorite delignification.

The GP results from this work are presented in Figures 2.3 for the three biomass categories for either no pretreatment or AHP pretreatment at 12.5% (w/w) H_2O_2 loading on the biomass. Substantial differences can be observed between biomass types and for biomass subjected to pretreatment as quantified for both mass partitioning of extracted glycans (top panel of Figure 2.3) and differences in the abundance of glycan epitopes in these extracts (heat map in the lower part of Figure 2.3). For the extract mass partitioning of glycans, it is clear that the two dicots have similar profiles for the four most severe extracts, i.e. the 1 M KOH, 4 M KOH, chlorite, and 4 M KOH PC. The goldenrod shows a very high content of the oxalate- and carbonate-extractable polysaccharides relative to the other two types of biomass. This may be a consequence of the goldenrod having a higher fraction of pectin-rich leaves compared to the

poplar which consists of only stem heartwood, and the corn stover, which as a graminaceous monocot is known to have low pectic polysaccharide content [151]

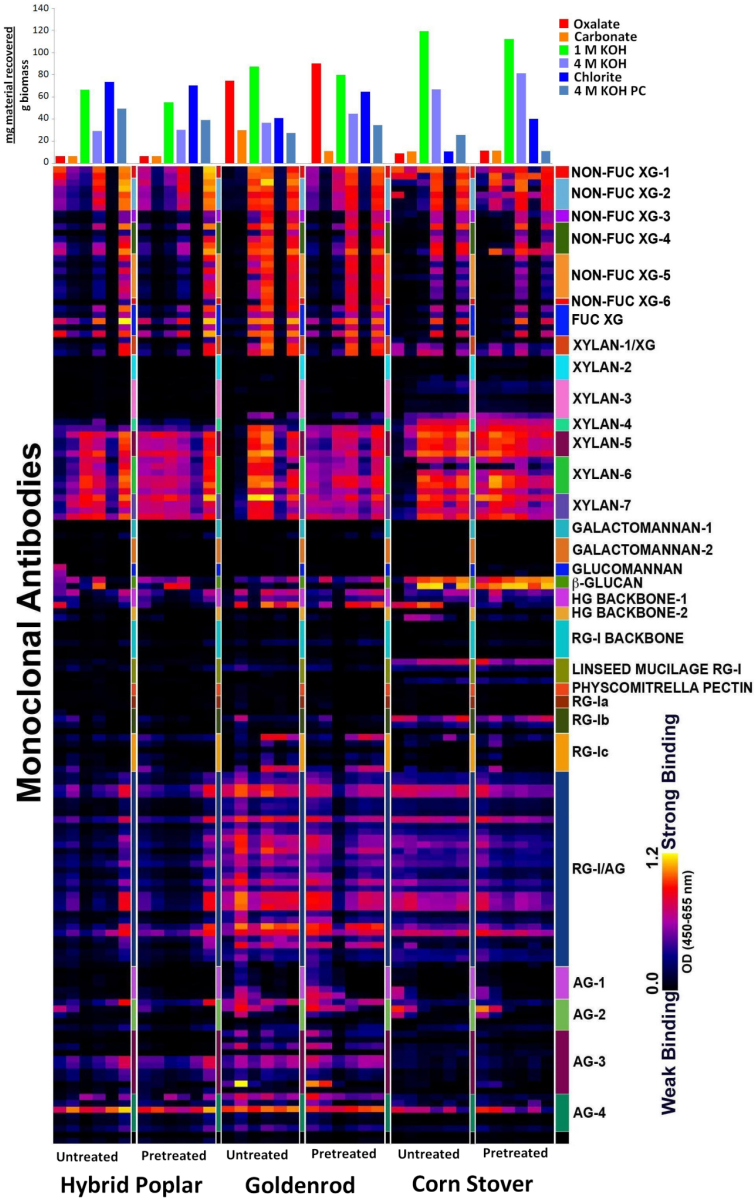

Figure 2.3 Glycome profiling of Poplar, Goldenrod and Corn stover biomass samples before and after AHP pretreatment (12.5% H_2O_2 loading). (Sequential extraction and glycome profiling were done by Sivakumar Pattathil, University of Georgia.)

A number of noteworthy differences are apparent in comparing the glycan epitope abundances within the six extracts for the three types of untreated biomass. One difference is that the XyG epitopes show significantly different partitioning between the three biomass types and the goldenrod is the only biomass exhibiting abundant XyG epitopes in the 1 M KOH extract. The xylan epitopes are more abundant in the corn stover extracts and more abundantly distributed into the two most severe extracts that might correspond to "lignin-bound" xylan. Pectic polysaccharides and AG domains show different partitioning behavior between the biomass types as well, with the cell wall extracts from goldenrod exhibiting the most abundant content of these classes of epitopes. Two intense MLG epitopes are present in all the corn stover cell wall extracts corresponding to antibodies LAMP2H12H7 and BG1 and the abundance of these epitopes increase in extracts (particularly in the two mildest and the chlorite delignification) following AHP pretreatment. Weak epitope binding for both of these antibodies is present in some of the poplar extracts and goldenrod extracts. These observations are consistent with the primary cell wall models proposed by Carpita [31], where for grasses (Type II cell wall), the MLGs and GXs have a more important structural role and may act in the capacity that XyGs and pectins in dicots (Type I cell wall).

Pretreatment can conceivably alter the binding of mABs to their glycan epitopes from the same extraction condition in three ways by: (1) altering the cell wall structural integrity to shift the glycan epitope into a more easily (or more difficult) extractable category, (2) solubilizing the glycan epitope during pretreatment, and (3) structurally altering the glycan epitope, for example, through alkali-induced deacetylation or demethylesterification. These three modes of action are used to interpret the changes associated with pretreatment. To better visualize the effects of pretreatment on glycan extractability, the GP data are replotted in Figures 2.4 and 2.5 after normalizing to epitope abundance per mass of original cell wall. In this representation, glycan epitopes that are increased in their abundance in individual

extracts after pretreatment will appear to the left of the x-y line, while epitopes that are decreased will appear to the right. It can be observed that for the poplar, pretreatment has very little effect on the total extractable glycans in most of the six fractions. The apparent increase in the xylan epitopes in oxalate and carbonate extracts suggest that the extractability of xylan by mild solvents may be enhanced by pretreatment (Figure 2.4, subplots A-C). However, considering that the total content of carbohydrates in these extracts are unchanged and that the xylan-specific antibodies were developed for deacetylated, alkali-extracted xylans, this result likely indicates that easily extractable xylans were deacetylated by pretreatment and that the abundance of deacetylated xylan epitopes increase as a consequence. The slight differences in other epitopes in the 4 harshest extracts (Figure 2.5, subplots A-C) suggests that AHP pretreatment results in only minor alterations in the extractability of other major cell wall glycans indicating minimal impact on the structural and compositional organization of the cell wall in agreement with the results in Figures 2.1 and 2.2. An exception is the xyloglucan epitopes in the 1M KOH extract (Figure 2.5A) which are slightly improved in their extractability by pretreatment.

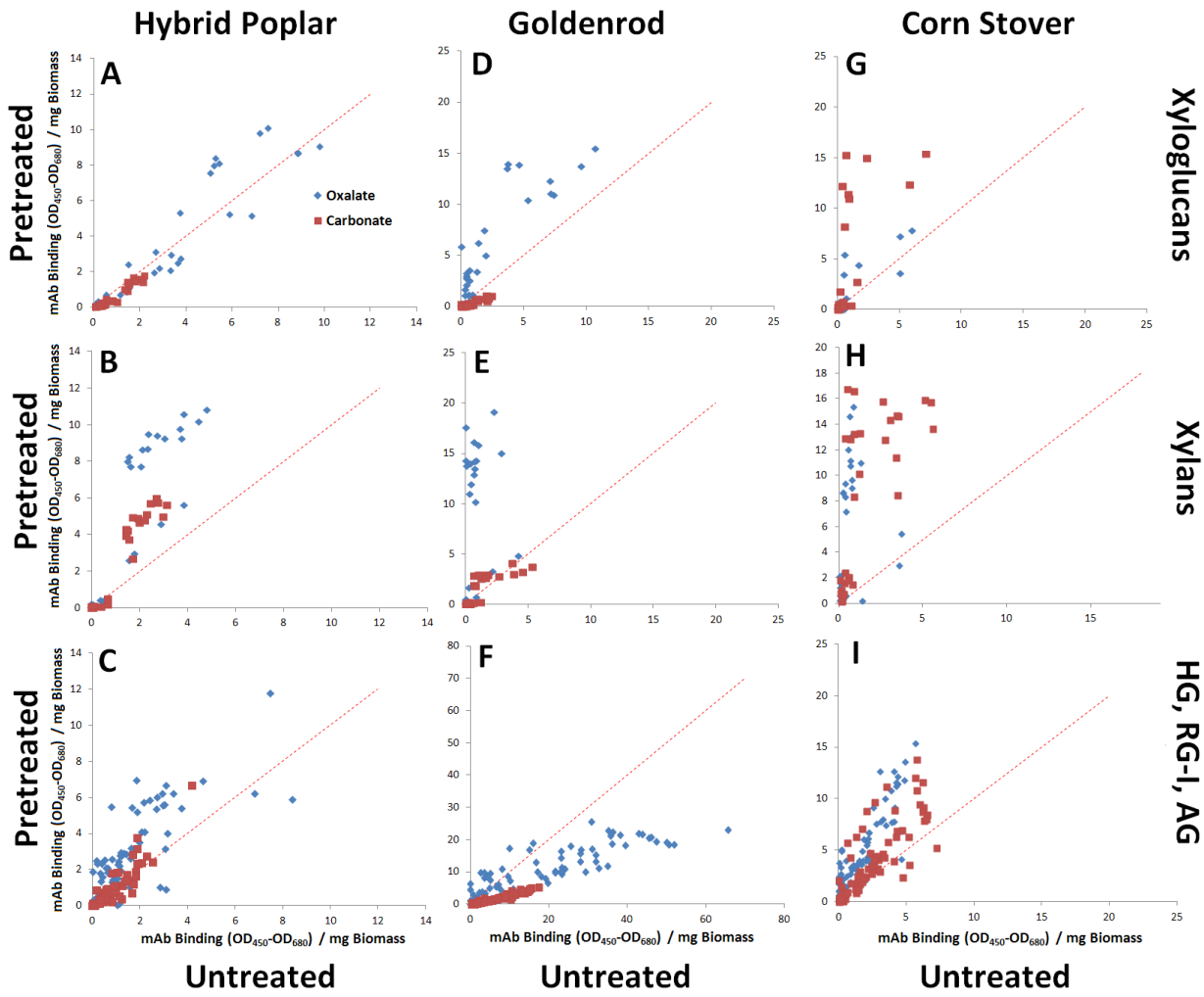

Figure 2.4 Comparison of the changes in the relative abundance of three glycan epitope categories due to AHP pretreatment from the oxalate (blue dots) and carbonate (red dots) extracts in glycome profiling, for poplar (A, B and C, for xyloglucans, xylans and pectic polysaccharides), goldenrod (D–F for xyloglucans, xylans and pectic polysaccharides) and corn stover (G–I for xyloglucans, xylans and pectic polysaccharides).

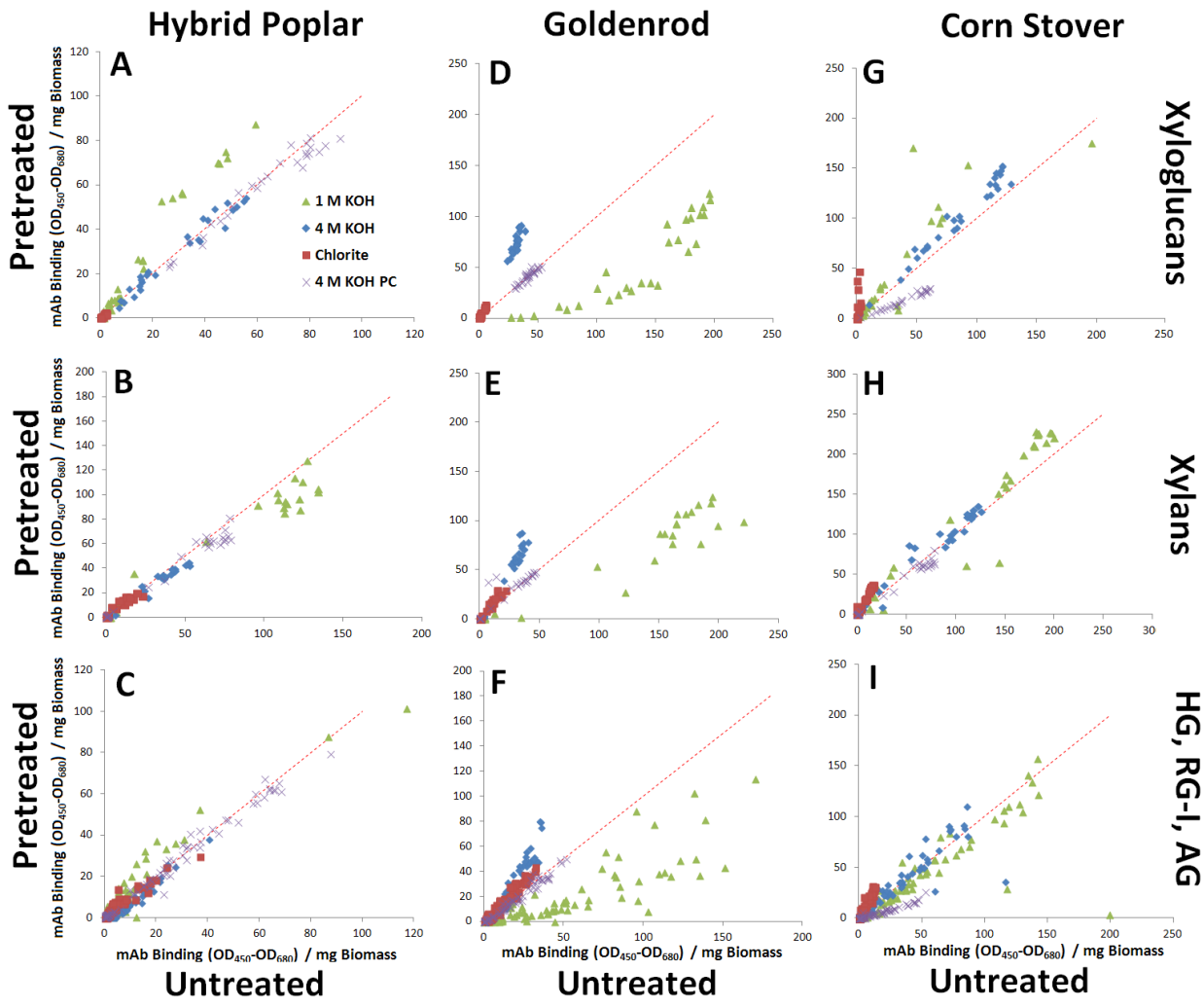

Figure 2.5 Comparison of the changes in the relative abundance of three glycan epitope categories due to AHP pretreatment from each of the four harshest extracts including 1 M KOH (green triangles), 4 M KOH (blue diamonds), chlorite (red squares), and 4 M KOH PC (purple crosses) in glycome profiling.

For goldenrod, the GP results show that xylan epitopes and XyG epitopes in the oxalate extract increased considerably (Figure 2.4, subplots D-F); potentially as a consequence of pretreatment-induced deacetylation or by pretreatment increasing the extractability in agreement with the increase in total glycan mass in the oxalate extract with pretreatment (Figure 2.3). Unlike the poplar, the epitopes for HG backbones, RG-I/AG, and AG are decreased in both the oxalate and carbonate extracts, indicating that these pectic polysaccharides in goldenrod are likely solubilized during AHP pretreatment. In the four

most severe extracts, a number of important trends are apparent (Figure 2.5, subplots D-F). The first is that virtually all epitopes are decreased as a consequence of pretreatment in the 1M KOH extract (corresponding to alkali-soluble glycans not closely associated with lignin), while slight increases in the abundance of epitopes are observed after pretreatment in the 4M KOH, chlorite and 4M KOH post-chlorite extracts (corresponding to lignin-embedded glycans). These results indicate that the likely target of AHP pretreatment for improving digestibility in goldenrod is glycan (XyG and xylan) solubilization to improve cell wall accessibility to glycolytic enzymes and minor delignification which slightly improves the extractability of lignin-embedded polysaccharides.

The corn stover results show considerable differences in both their glycan extraction plots and antibody binding profiles relative to the hybrid poplar and goldenrod (Figure 2.3). From the glycan mass extraction profile at the top of the panel, it can be observed that, unlike the goldenrod, the amounts of extractable glycans in the three most severe extracts were significantly altered by pretreatment. One noticeable alteration is that the glycan epitopes in the 4M KOH post-chlorite extract were shifted into the chlorite extracts after AHP pretreatment. This phenomenon of the pretreatment changing the glycan extractability profile was not shown for dilute acid pretreated hardwood [122]. This result supports the identified changes in composition shown in Figure 2.1, and together with Figure 2.2, make clear that alteration of the non-cellulosic glycan extractability directly impacts the glucan digestibility for the subsequent enzymatic hydrolysis. In the two mildest extracts from corn stover, the XyG and xylan epitopes were increased, which may be a consequence of improving extractability or likely due to deacetylation of these glycans by pretreatment (Figure 2.5, subplots H and I). Unlike goldenrod and poplar, the epitopes for pectic polysaccharides were increased in the two mildest extracts, possibly indicating differences in their structural role between monocots and dicots in pectic polysaccharides [31], Additionally, the results for

changes in epitope abundance as a result of pretreatment for the four harshest extracts were considerably different for the corn stover than for the goldenrod. Compared to goldenrod, where all glycan epitopes were decreased by pretreatment in the 1M KOH extract but slightly increased in the lignin-associated extracts, the corn stover glycan epitopes showed increases across all four extracts except in the 4M KOH post-chlorite extract, in which the lignin-embedded glycan epitodes are decreased by pretreatment (Figure 2.5, subplots H-J). These reductions of corn stover glycans in the harshest extracts are likely a consequence of these epitopes being already removed by less harsh extractions. Additionally, increases in the 2 MLG epitopes were observed with pretreatment for corn stover (Figure 2.3) for all extracts except the 4M KOH post chlorite treatment.

These differing responses to AHP pretreatment between monocots and dicots have important implications for structural features of the cell wall contributing to recalcitrance as well as the mechanism or target of pretreatment. The composition and structure of the cell wall are obviously important and many properties of the cell wall impacting recalcitrance have been described in the literature including cell wall hydrophobicity [152], porosity [153, 154], xylan content [155], lignin content, cross-linking, and higher order structure [130]. Jung et al. [156] noted that lignified secondary cell walls were the primary obstacle hindering ruminant digestibility in dicots with stem secondary xylem (i.e. woody biomass) the most recalcitrant, while increasing lignification in grasses hinders, but does not completely inhibit digestion. The current work identified that AHP pretreatment has a relatively minor impact on the hybrid poplar composition, digestibility, and glycan extractability profiles. Substantial work has been devoted to understanding the cell wall properties contributing to ruminant digestibility of grasses and properties including ferulate content, total lignin content, syringyl:guaicyl ratio of lignin monomers, and degree of arabinosylation of xylans have all been linked to different digestibility [130, 156-158]. Pretreatments may impact any of these

cell wall properties to improve cell wall digestibility. DeMartini et al. [138] found that treatment of switchgrass with alkali alone to solubilize xylan (and lignin) from the cell wall was sufficient to result in glucan digestibilities approaching the theoretical maximum, while for hybrid poplar, chlorite delignification was necessary to improve digestibility significantly past alkali-only treatment. This is consistent with models for grass cell walls that include alkali-labile ferulate ester cross-links between cell wall polymers as an important structural feature controlling lignin integration into cell walls [148]. Our previous work identified that lignin and ferulate removal by AHP pretreatment are important predictors of digestibility in diverse grasses [146]. We have previously shown that AHP pretreatment results in the destruction of β-O-4 bonds in grasses [146] and, for example, the content of free phenolics in grass lignins may enable improved alkali solubilization or potentially participate in the initiation β-O-4 scission reactions.

2.4 CONCLUSION

For a woody dicot (hybrid poplar), an herbaceous dicot (goldenrod), and a graminaceous monocot (corn stover), we found that enzymatic hydrolysis yields, cell wall biopolymer and total mass solubilization, cell wall glycan extractabilities, and glycan epitope abundances differed significantly in their response to AHP pretreatment. Using glycome profiling, we identified different mechanisms for how AHP pretreatment overcomes cell wall recalcitrance in goldenrod versus corn stover, while it was relatively ineffective on poplar. For corn stover, mild alkaline oxidative pretreatment resulted in slight delignification and presumably disruption of cell wall polymer cross-linking. This had the consequence of disrupting the structural integrity of the cell wall, which was manifested through improved extractability of important structural glycans including xylans, MLGs, and XyGs and presumably allowed for improved accessibility for glycolytic enzymes into the cell wall during hydrolysis. Goldenrod

was found to respond differently in the extractability profiles where all classes of glycan epitopes exhibited considerable decreases in the 1M KOH extracts following pretreatment rather than an increase as was observed for corn stover. Besides these differences, it was revealed that the pectic polysaccharides (HG, RG-I, and AG) were not only significantly more abundant in goldenrod than in corn stover, but were solubilized by pretreatment as indicated by their decrease following pretreatment in the three mildest extracts for goldenrod. For corn stover, the pectic polysaccharides as well as the significantly more abundant MLGs showed mild increases in extractability following pretreatment indicating "loosening" from the cell wall rather than solubilization.

3. PLANT CELL WALL RECALCITRANCE IN DIVERSE GRASSES

3.1 INTRODUCTION

Alkaline oxidative pretreatments applied in recent years to enhance enzymatic digestibility [57, 145, 159-161] were derived from the non-delignifying bleaching in pulp and paper processes. Alkaline pre-extraction coupled to alkaline oxidative post-treatment has also been applied to compare the effects of alkaline hydrogen peroxide as a pretreatment and post-treatment [98]. The impacts of alkaline oxidative pretreatment on diverse plant cell walls have been investigated in previous studies [56, 146, 162]. We proposed that the alkaline oxidative pretreatment was effective by removing lignin primarily through breaking β-O-4 bonds [146], introducing carboxylic group on cellulose microfibirils [56] and increasing glycan extractability and enzyme accessibility, and that this pretreatment is especially suitable for grasses [162]. In addition, the metal complexes may be utilized as catalyst for increasing the rate and extent of hydrogen peroxide oxidation by participation in Fenton's chemistry [55, 56]. Alkaline pretreatments usually yield higher digestibility for grasses than the other feedstocks [163] due to the relatively abundant presence of the alkali-labile ferulate or diferulate esters bridges in the grass lignins or glucuronoarabinoxylans [164] and the high phenolic hydroxyl content [97]. Studies have shown that the hydrolysis yield is correlated with lignin content of the cell walls, which is independent on the type of biomass [146], and the glucan hydrolysis yield is not necessary correlated to the xylan content after alkaline pretreatments [165] since the xylan remaining in the cell wall is usually "loosened" by alkaline pretreatments, which is shown the previous chapter. In addition, we proposed that the

metal content in the grasses may be another contributor for improving the efficiency of alkaline oxidative pretreatment.

The purpose of this study was to investigate the relationship between grass cell wall properties and enzymatic hydrolysis through characterization of the specific grass cell wall components including lignin, hydroxycinnamates, xylans and β-glucans. In addition, metal content and cellulose accessibility were analyzed and correlated with the pretreatment severity and hydrolysis yields. In this study, switchgrass (*Panicum virgatum* cv. Cave-In-Rock), two corn stovers (a commercial hybrid and inbred brown midrib lines bm1 and bm3), and *Miscanthus* spp. are examples of cereal stovers and energy perennials were subjected to alkaline oxidative pretreatments at varied hydrogen peroxide loading. In addition, hybrid corn stover was subjected to an alkaline pre-extraction coupled with alkaline oxidative post-treatment. The differences in hydrolysis yields were compared between the grasses and correlated with their composition properties based on sugar analysis, ferulate analysis and metal analysis. "Glycome profiling" [140] was applied to identify changes in the extractability and abundance of different glycan epitopes in the individual species cell walls as functions of pretreatment severity. Glycans released during pretreatment were determined using ELISA screening with the same glycan-directed mAbs library as previously described methods [121]. Cellulose accessibility was examined through assessing the binding characteristics for pretreated and untreated corn stover using *ct*CBM family 3 which binds specifically to crystalline cellulose.

3.2 MATERIALS AND METHODS

3.2.1 Biomass pretreatment, composition analysis and enzymatic hydrolysis

In this study, corn stovers (*Zea mays*) including Pioneer hybrid 36H56, inbred brown midrib bm1 and bm3, switchgrass (*Panicum virgatum* cv. Cave-in-Rock) and miscanthus

(*miscanthus x giganteus*) were subjected to 2.5, 5, 12.5, 25, 50% H_2O_2 loading (w/w biomass) Alkaline hydrogen peroxide (AHP) pretreatments with pH adjusted to 11.5. The pretreatments were carried out in 250 mL Erlenmeyer flasks covered by aluminum foil, with a total reaction volume of approximately 100 mL and 2% (w/v) solid loading, 30 ºC and 180rpm for 24 hours. The pretreated samples were washed with DI water until pH reached neutral, and then air-dried overnight. The mass loss during pretreatment was measured gravimetrically. Two-stage sulfuric acid hydrolysis was carried out according to NREL composition analysis protocol to determine the structural carbohydrates by HPLC installed with an Aminex HPX-87 H (Bio-Rad, Hercules, CA) column. Pretreated samples were ball-milled with a QIAGEN Tissuelyzer II for 3x2 minutes, which was followed by enzymatic hydrolysis performed at pH 4.8, 50 ºC and 180 rpm, with 30 mg/g protein loading of C-Tec 2 (Novozymes, Denmark) for 72 hours. The glucan hydrolysis yield (digestibility) was determined by the glucose released after enzymatic hydrolysis divided by the original glucan content in the pretreated samples.

3.2.2 *p*-hydroxycinnamic acids determination and metal analysis

The hydroxycinnamic acid (HCA) content of the untreated maize and mild alkaline treated maize was determined by a severe alkaline extraction method which represents the content of both the esterified and etherified HCAs [130, 166, 167]. In this, 0.1g biomass was treated with 5 mL of 4M NaOH in sealed Teflon tubes and held at 170 ºC for 2 hour in an oven. After cooling down in room temperature, 50 μL of 10 mg/mL methanol solution of o-coumaric acid was added as an internal standard for each sample. The mixture was transferred to Eppendorf tubes and centrifuged at 13,000 rpm for 10 minutes. The pH of the supernatant was adjusted to 2 with concentrated HCl, and the samples were then placed into a 4 ºC fridge overnight. After the overnight storage, the supernatant of the samples were analyzed in a HPLC stalled with a C18 column (Discovery HS C18 HPLC Column, 5 cm ×

2.1 mm, 5 μm). Standards containing ferulic acid, *p*-coumaric acid and o-coumaric acid were run in parallel to determine the concentration of the acids in the samples. The metal analysis results of the samples was provided by A & L Great Lakes Laboratories, Inc. (Fort Wayne, IN).

3.2.3 Glycome profiling and data processing

(Sequential extraction and glycome profiling were done by Sivakumar Pattathil, University of Georgia.)

The extractives-free cell wall samples were fractionated by successive extraction with six increasingly harsh solvents including oxalate, carbonate,1 M KOH, 4 M KOH, Chlorite and post-Chlorite 4 M KOH. These six extracts were collected, weighed and screened with the 156 glycan-directed mAbs [162]. The relative mAbs binding strength was determined from UV absorbance at OD 450-OD 680 on the basis of 20 μg glucan equivalent. The extractives content was determined gravimetrically by three cycles of organic solvent extraction performed sequentially with 70% ethanol, methanol and chloroform 1:1 (v/v) solution and pure acetone, which was followed by a 10-minute centrifuge at 10,000 rpm. The specific cell wall epitopes abundance in each extract was estimated by the following equation:

Epitope abundance = [(OD 450-OD 680) / (g original biomass)] = $N_1*N_2*N_3*N_4/20$

N_1 = [(OD 450-OD 680)/(20 μg glucan equivalent)]

N_2 = [(μg glucan equivalent)/(mg extracted material)]

N_3 = [(mg extracted material/(g extractive-free biomass)

N_4 = [(g extractive-free biomass)/(g original biomass)]

3.2.4 ELISA screening of pretreatment liquors

(ELISA screening was done by Sivakumar Pattathil, University of Georgia.)

AHP pretreatment liquor for each grass was collected and dried at 50 ºC in a convection oven. The ELISA screening was performed as described in previous work [98]. Plant glycan-directed monoclonal antibodies were obtained 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). Further description of the mAbs used in this study can be found in the Supporting Information Table S1, which includes links to a web database, WallMAbDB (http://www.wallmabdb.net) that provides detailed information about each antibody.

3.2.5 Enzyme binding experiments

Enzyme binding experiments were conducted at 1 mL total liquid volume at 4 ºC, pH 7.0 for 4 hours with end-to-end rotation using bacterial CBM family 3 from *Clostridium thermocellum* (Prozomix, UK), which bind specific to crystalline cellulose was used to test the accessible crystalline cellulose in the pretreated and untreated corn stover. The binding isotherms were developed by a Langmuir isotherm model:

$$E_{ads} = \frac{E_{max} K_{ads} E_f S}{\left(1 + K_{ads} E_f\right)}$$

The parameters of the model were estimated based on the binding data using Excel Solver.

3.3 RESULTS AND DISCUSSIONS

3.3.1 Correlations between grass cell wall properties and digestibility

The digestibility profiles associated with H_2O_2 loading were investigated and results are shown in Figure 3.1. Results showed that the corn stovers had higher hydrolysis yields than the two perennial grasses at comparable pretreatment conditions. This is further supported by the composition data shown in Figure 3.2, which shows that glucan content is almost constant

for all samples, the corn stovers have higher xylan and lignin removal than the perennial grasses, and the three corn stovers generally have similar removal of xylan and lignin. Other researchers have found that corn stover generally have higher glucan hydrolysis yields than switchgrass following alkaline or neutral pretreatments such as lime and liquid hot water pretreatment [163]. The results in this study suggested that the lignin and xylan removal both contribute to the increased glucan hydrolysis for the diverse grasses.

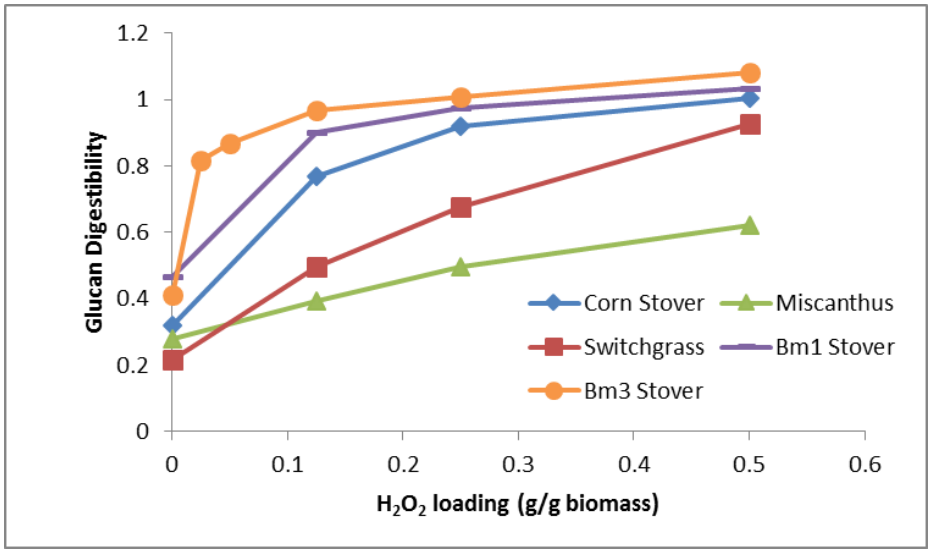

Figure 3.1 Glucan digestibility (hydrolysis yield) profile associated with H_2O_2 loading after AHP pretreatment for the diverse grasses (0% AHP are untreated samples).

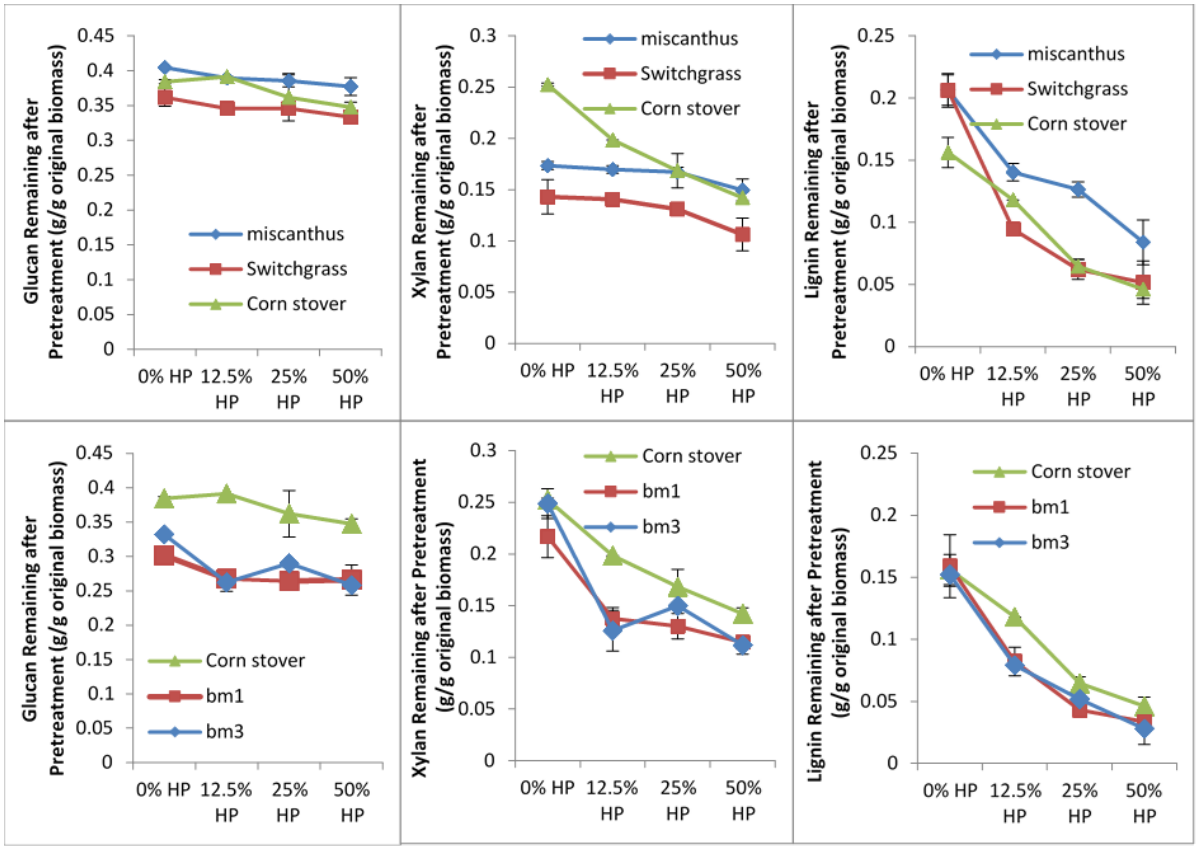

Figure 3.2 Composition and weight loss of switchgrass, miscanthus, bm1, bm3 and hybrid corn stover.

Transition metals, including iron (Fe), copper (Cu) and manganese (Mn), play important roles in Fenton's reactions. They can catalyze the hydrogen peroxide delignification [168] and are also essential micronutrients for growth and development of plants. Transition metal complexes have been utilized as catalysts for improving the rate and extent of alkaline hydrogen peroxide pretreatment for poplar [55, 56]. However, the metals contained in the plant cell walls may also contribute to Fenton's reactions during the hydrogen peroxide pretreatment. Table 3.1 shows the concentrations of metals in the five grasses. It is worth noting that the iron content is higher than the other metals and the amount is diverse between different types of feedstocks. Figure 3.3A plots the relationship between the initial iron content and glucose yield of the diversely AHP pretreated grasses, and shows a clear positive trend, which implies that the contribution of the iron in the plants is considerable for

improving the peroxide oxidation of lignin. The synergistic effects of the iron and hydrogen peroxide on improving the hydrolysis yields of diverse grasses indicate that the *in vivo* iron participates as a Fenton reagent in alkaline oxidation pretreatment.

Sample Name	K (%)	Mg (%)	Ca (%)	B (ppm)	Zn (ppm)	Mn (ppm)	Fe (ppm)	Cu (ppm)	Al (ppm)
Corn stover	1.06	0.13	0.24	3	31	44	255	5	138
Switchgrass	0.4	0.15	0.4	3	10	49	64	3	21
Miscanthus	1.06	0.11	0.18	3	28	35	43	2	27
bm1 stover	1.34	0.33	0.42	8	33	58	425	5	118
bm3 stover	1.53	0.25	0.3	7	21	27	171	5	54

Table 3.1 Metal analysis of grasses

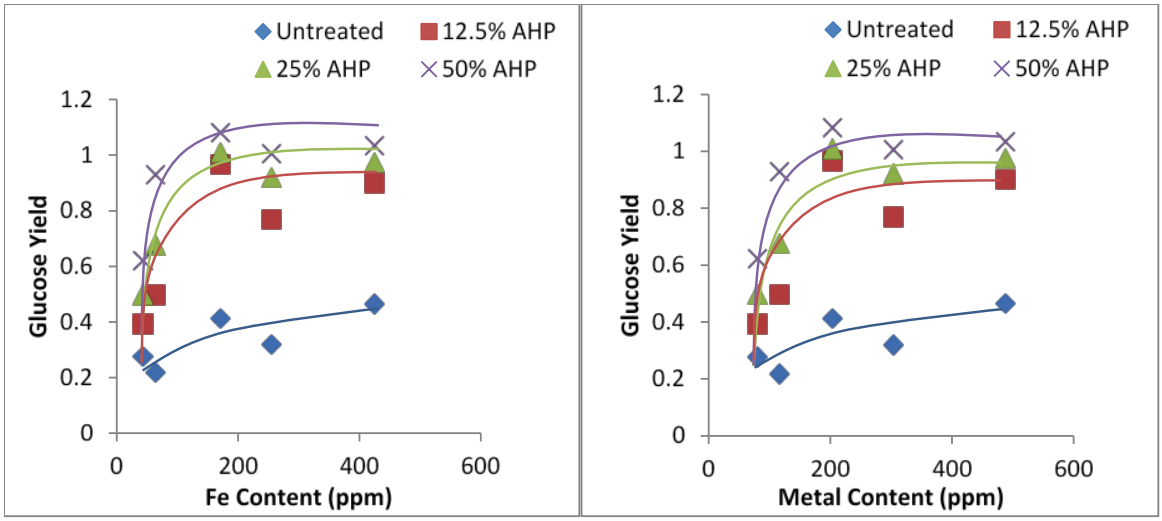

Figure 3.3 The correlation between the metal content (A. Fe content, B. Total metal content) and glucose yield for the diversely AHP pretreated grasses including corn stover, bm1 stover, bm3 stover, switchgrass and miscanthus.

Inbred brown midrib maize lines are the result of natural mutations in corn, and are noted for their visible brown color in leaves, have been studied for over 50 years [169]. Brown midrib lines bm1 and bm3 are well-known for their characteristics in lignin composition [170-173]. The lower amounts of cinnamyl alcohol dehydrogenase (CAD) in bm1 results in reduced lignin, ferulic acid and *p*-coumaric acid esters as well as enriched carbon-carbon monolignol linkages, and bm3 is deficient in caffeic acid O-methyl transferase (COMT), which results in

decreased total lignin and syringyl monolignol incorporation [173, 174]. These characteristics are thought to result in higher digestibilities relative to other corn stovers [146, 169]. In this study, bm3 stover shows the highest hydrolysis yields following pretreatment but does not have a significant compositional difference compared to the three corn stovers, indicating structural differences other than lignin content and composition, for instance, the secondary cell wall thickness and deposition [175].

Monocot lignins are rich in hydroxycinnamates (HCAs), including ferulate (FA) and *p*-coumarate (*p*CA), and it was found that the ferulic acid crosslinks grass lignins primarily with ether links, while *p*CA bonds with grass lignins with both esters and ethers[176, 177]. Many analytical methods for quantify HCAs in grasses apply different severities of alkaline extraction to break either ester bonds or ether bonds along with ester bonds based on the alkali-labile properties of these bonds [22, 130, 178, 179]. Previous research has found that the pyrolysis products derived from ferulate and p-coumarate both show strong inverse correlation with digestibility [146]. The HCAs concentrations in the pretreatment liquors have also been found to increase with increased H_2O_2 loading during pretreatment (data not published). In this study, for the 12.5% AHP pretreated and untreated corn stover, switchgrass and miscanthus, the correlation between digestibility and major cell wall properties including lignin, xylan and ferulate content have been analyzed and the heap map of correlation coefficients generated by MATLAB is shown in Figure 3.4. The hierarchical clustering on the top of Figure 3.4 developed by MATLAB based on Euclidean geometry shows the degree of distance between values, visualizing the relationships between these major grass properties. For the untreated and 12.5% AHP pretreated diverse grasses, results showed that both ferulate and lignin content are negatively correlated with glucan digestibility with correlation coefficients of -0.96 and -0.81, while xylan content and *p*CA content are not correlated well with glucan digestibility with correlation coefficients of 0.21

and -0.50. The strong correlation between ferulate and lignin revealed in the results indicate that both lignin and FA are solubilized in pretreatment, thus FA content would be an indicator of pretreatment effectiveness in terms of cell wall digestibility [146].

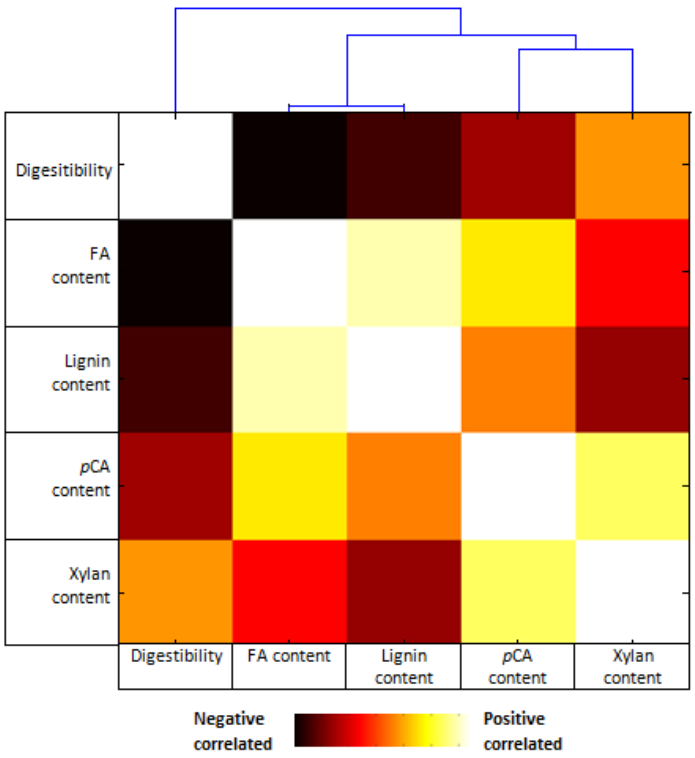

Figure 3.4 Heat map of correlation coefficients between cell wall properties and digestibility. The color blocks in the map reveal the relationship between the specific two individual cell wall properties as horizontally and vertically labeled, where the darker colors indicate positive correlations while the lighter colors indicate negative correlations. Note: this only includes data for 12.5% AHP pretreated and untreated corn stover, switchgrass and miscanthus.

However, metal content was not selected to be included in the correlation coefficient heat map. Since metals participate in the Fenton's reaction and synergistically affect the hydrogen peroxide delignification, the hydrogen peroxide loading is critical for the correlation between the metal content and the digestibility. In addition, metal content of woody biomass has been correlated with the alkaline oxidative pretreatment (data not published yet), and it was shown that the poplar with the lowest metal content responses the worst with the alkaline oxidative

pretreatment but responses the best with Cu(bpy) catalyzed alkaline oxidative pretreatment [55, 56]. Thus, we can conclude that lignin removal is the major contributor of the increased hydrolysis yields following alkaline oxidative pretreatment, and the lignin removal is correlated with the cleavage of HCAs in the cell walls and the improved hydrogen peroxide oxidation catalyzed by metals.

3.3.2 Glycome profiling derived xylan/β-glucan extractability of cell walls

Glycome profiling is based on the analysis of the binding abundance of glycan-directed monoclonal antibodies in sequential cell wall extracts. The glycome profiling results here demonstrated the alteration of extractability for non-cellulosic glycans for 12.5% AHP pretreatment (Original data shown in Appendix Figure A1-A2). The non-cellulosic glycans were grouped into four major types of glycans including xyloglucan, xylan, β-glucan and pectins based on a previous study [162]. Among them, glucuronoarabinoxylans (GAX) are the major grass hemicellulose in the primary and secondary cell wall with ferulate substitutions [180, 181]. β-glucans are abundant in grasses where they play a cross-linking role comparable to that of pectins in dicots [181, 182]. Thus, this part of the study is focused on comparing the changes of these two cell wall glycans between types of pretreatment and feedstock.

In order to visualize the effects of pretreatment on cell wall glycans, the epitope abundance (calculated as described in the material and methods section) before and after pretreatment have been respectively plotted on the X and Y axes of the same diagram [162]. Accordingly, Figure 3.5 illustrates the pretreatment effects on xylan epitope abundance of diverse grasses including switchgrass (A), miscanthus (B), corn stover (C), bm1 stover (D), bm3 stover (E) and alkali-extracted corn stover (F). Among the six sequential extracts, since the first two milder extractions using oxalate and carbonate only remove extractives and loosely bound

pectins, and these two extracts only contain a small fraction of the recovered materials, we only focused on the epitopes in four of the other harsher extracts including 1 M KOH, 4 M KOH, chlorite, post-chlorite 4 M KOH in these plots. In these plots, most of the trend lines are linear and relatively close to the X=Y line. It is shown in Figure 4 that the epitope abundance in the 1 M KOH extract and the 4 M KOH extract have been unchanged or slightly increased by pretreatment for switchgrass, miscanthus and corn stover. However, the brown midribs seem to have different xylan epitopes since the mAbs in 4 M KOH for bm1 and 1 M KOH for bm3 do not follow the linear trend, which indicates the xylan extactability is different from the other grasses. This means that the xylan is more extractable in bm1 and bm3 following pretreatment relative to other grasses, which can be attributed to improved lignin removal (Figure 3.2) relative to other grasses. Vermerris et al. also showed that the reduced lignin and ferulate content in brown midribs has been compensated by increased hemicelluloses, especially GAX, which results in a thickened secondary cell wall [175]. For the corn stover, epitope abundance in PC 4 M KOH has been shifted to chlorite extracts, indicating that the xylan epitopes possibly have been "loosened" by pretreatment [162]. Alkali pre-extracted corn stover is the only sample that has reduced epitope abundance after pretreatment, indicating xylan epitopes are removed from the cell wall instead of "loosening" for the other untreated grasses, possibly due to the already "loosened" xylan structure by alkali pre-extraction. Interestingly, as early as 1956 it was recognized that xylans were solubilized from hardwood cell walls according to two rate regimes during dilute acid hydrolysis [183]. These two pools of "fast-reacting" and "slow-reacting" xylan have subsequently been identified in kinetic studies of dilute acid hydrolysis of corn stover and switchgrass as well [184-186], although with significantly different kinetic parameters. It is likely that these pools of xylan identified from hydrolysis kinetic studies are related to the same properties that control xylan extractability from the cell wall. In this study, that a pool

of xylan is easier to be extracted by alkali-pre-extraction and that another pool of xylan is only solubilized in the post-pretreatment, might be an analogous phenomenon during alkaline pretreatments to these two pools of "different-reacting" xylan solubilized in acid pretreatments.

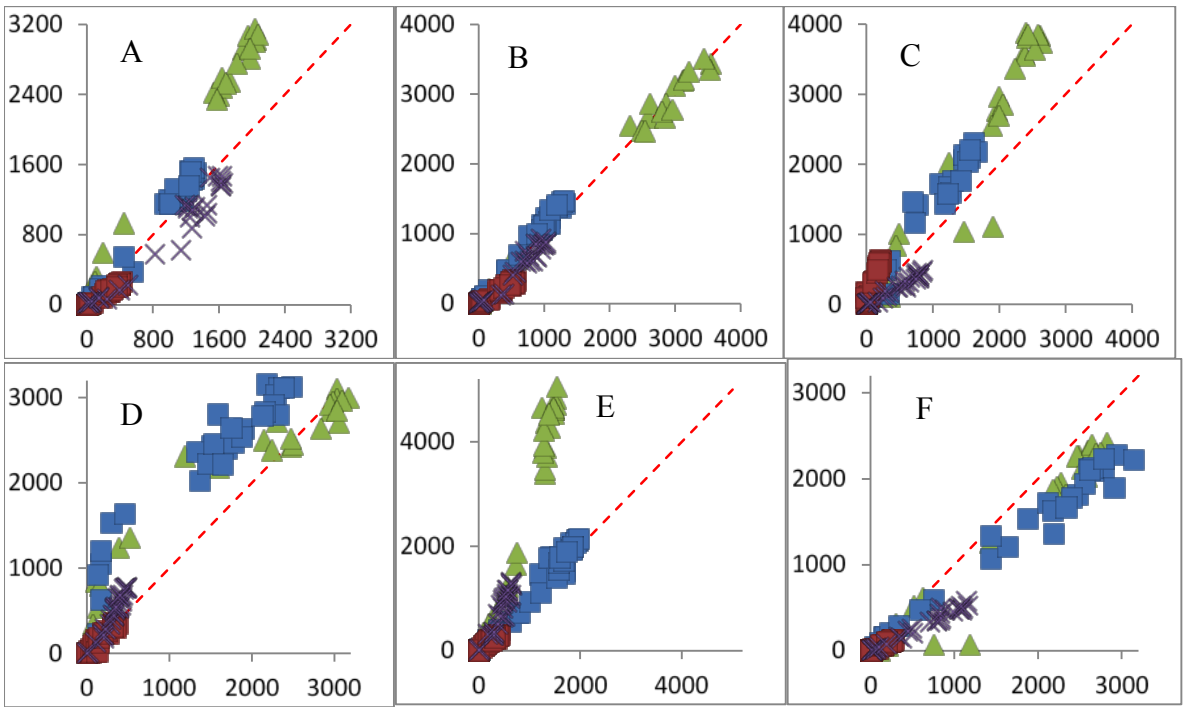

Figure 3.5 Plots of binding quantity of individual mAbs for xylan epitopes in extracts of 1 M KOH, 4 M KOH, chlorite, and post chlorite 4 M KOH. Values on X or Y axis are respectively the binding strength of xylan specific mAbs on untreated or pretreated switchgrass (A), miscanthus (B), corn stover (C), bm1 stover (D), bm3 stover (E) and alkali-extracted corn stover (F)

In addition to the xylan epitopes, another epitope in grasses that of interest are the β-glucans, since these mixed-linkage glucans are abundant and play a role in cross-linking in the primary cell wall of cereal grasses [181]. Two mAbs, including LAMP and CCRC-M154 are known to bind to the β-glucan and the xylan epitope, were chosen to compare the extractability of β-glucan and xylan [121]. Figure 3.6 shows the binding strengths of these individual mAbs (LAMP and CCRC-M154) on untreated and AHP pretreated switchgrass, miscanthus, corn stover, bm1 and bm3 stovers, and also includes alkaline pre-extracted corn

stover and alkaline pre-extracted coupled with AHP post-treated corn stover. These result show that xylan and β-glucan have similar binding alterations induced by AHP pretreatment. Interestingly, the AHP pretreatment increases the epitope abundance for switchgrass, miscanthus, corn stover, bm1 and bm3, but decreases the epitope abundance for alkaline pre-extracted corn stover. This phenomenon suggests the xylan and β-glucan in the primary cell wall have been altered to different extents by 5% alkaline extraction and 12.5% AHP. AHP pretreatment can increase the extractability of xylan and β-glucan in grasses, while the role of AHP after alkaline pre-extraction is to oxidize or remove the xylan and β-glucan on the already alkali-"loosened" cell wall. Another notable observation is the extractaibility alteration in bm3. In bm3, the increases in epitope abundance for xylan and β-glucan in the sequential extracts are the most significant among five types of grasses, indicating bm3 possibly has a "looser" or more extractable structure of cell walls than the other grasses, which should be the reason why bm3 always has the highest hydrolysis yields compared to the others shown in Figure 3.1.

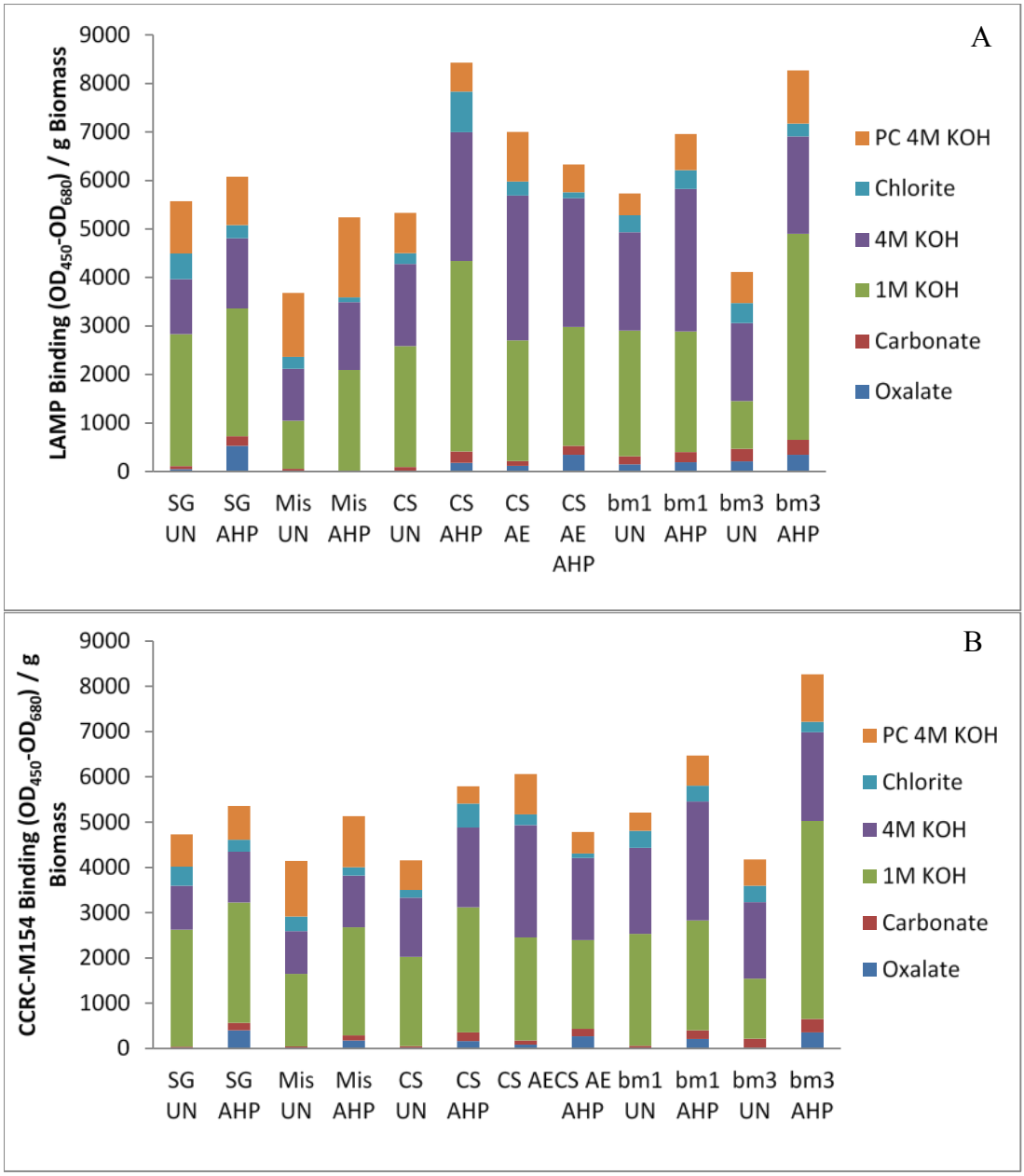

Figure 3.6 Plots of individual mAbs (LAMP and CCRC-M154) for β-glucan epitodes binding strength on untreated and pretreated switchgrass (SG), miscanthus (Mis), corn stover (CS), bm1 and bm3 stovers.

Figure 3.7 shows a summary of all of the glycome profiling results, including epitopes other than those for xylan and B glucan, (Appendix Figures A1-A2) in terms of the epitope abundance alteration by pretreatments of diverse grasses for three major cell wall epitopes (xyloglucan, xylan and pectin). In order to quantify the epitope abundance alteration, each series in Figure 4 has been fitted to a linear line, and the slope for each extract for each

biomass has been recorded as an absolute value. The color in the heat map was determined as the logarithm of the values of these slopes with respect to base 10 for all of the epitopes in every extract, for all biomass. The warmer the color is, the larger the fold difference of mAbs for the specific epitope has been increased. This result illustrates three responses of AHP on glycan alterations quantified by glycome profiling. Firstly, binding quantity shifting from milder extract to harsher extract for corn stover shows that glycans have possibly been "loosened" by pretreatment and made more extractable. Secondly, the largest range of warmer colors for bm3 stover suggests bm3 has the highest increased glycan extractability after AHP pretreatment, which contributes to higher digesitibility than the other grasses under varied H_2O_2 loading (Figure 3.1). Thirdly, the largest range of cooler color for alkaline pre-extracted corn stover shows a reduction of glycan epitopes by AHP post-treatment, possibly due to the direct removal of glycans on the alkali-"loosened" materials.

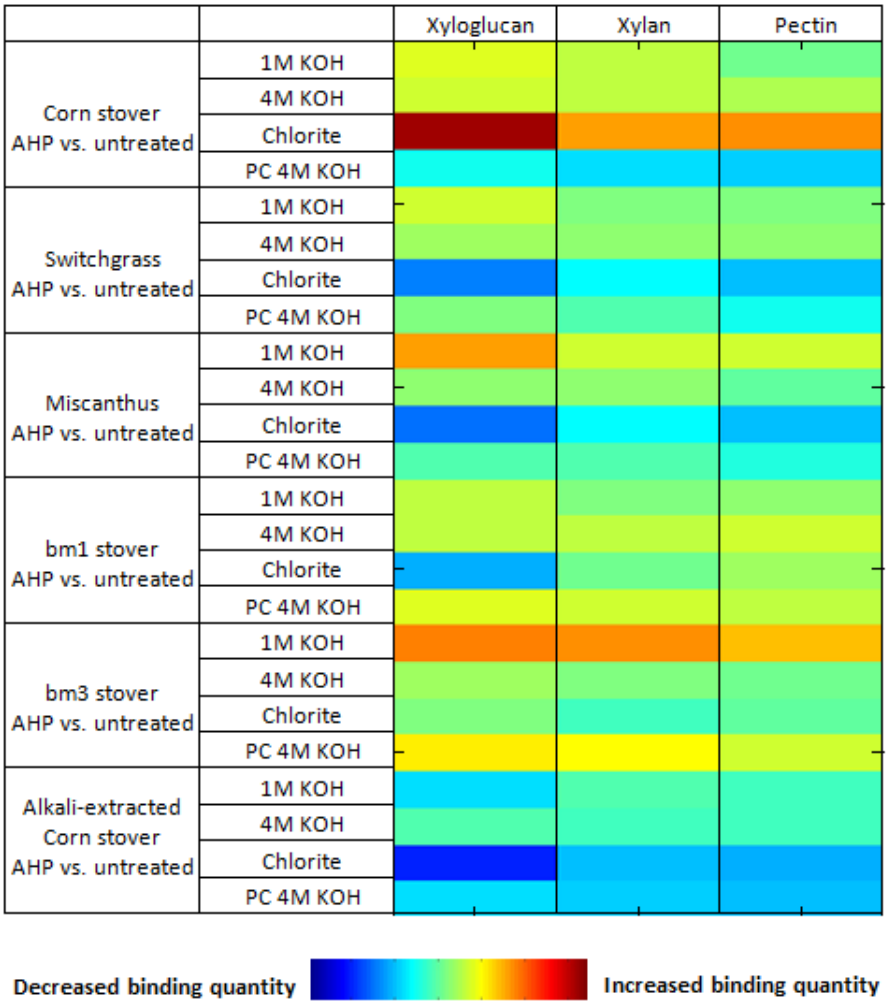

Figure 3.7 Heat map of major cell wall non-cellulosic glycans extractability alteration by AHP pretreatment.

3.3.3 ELISA screening of the pretreatment liquor

ELISA screening using cell wall glycan-directed mAbs could also be applied to characterize the glycans solubilized in pretreatment liquors generated from diverse grasses according to previous research [98]. Figure 3.8 shows the screening results of 12.5% AHP pretreatment liquors for five types of grasses including corn stover (A), bm1 stover (B), bm3 stover (C), miscanthus (D) and switchgrass (E). The brightness showing the high abundance of xylan epitopes indicates that AHP pretreatment removes xylans from grass cell walls into the liquid phase. In contrast with the significant amount of the xyloglucan epitopes in the glycome profiling results for all types of grasses, the mostly dark region in the top portion of Figure 8

shows there are much less xyloglucan epitopes in these pretreatment liquors, which indicates that AHP pretreatment either removes minimal xyloglucans to the liquid phase, or solubilizes xyloglucans to compounds that do not bind to the mAbs. Additionally, the very similar pectin epitopes abundances in the pretreatment liquors to the glycome profiling extracts indicates the AHP pretreatment also removes pectins from the grass cell walls with the similar quantity as it does for the sequential extractions.

According to Figure 3.2, xylan removal of the three stovers is much more significant than the two perennials. However, the ELISA screening results did not show large differences between the diverse species in terms of xylan epitope abundance, and only showed slightly higher xylan epitope abundance in the liquors from the bm1 and bm3 stovers. This is because of lower concentrations of the binding sites for the mAbs in the liquid phases than in the extracts [121]. The lower resolution of the quantification for the liquid phase makes the results indistinguishable.

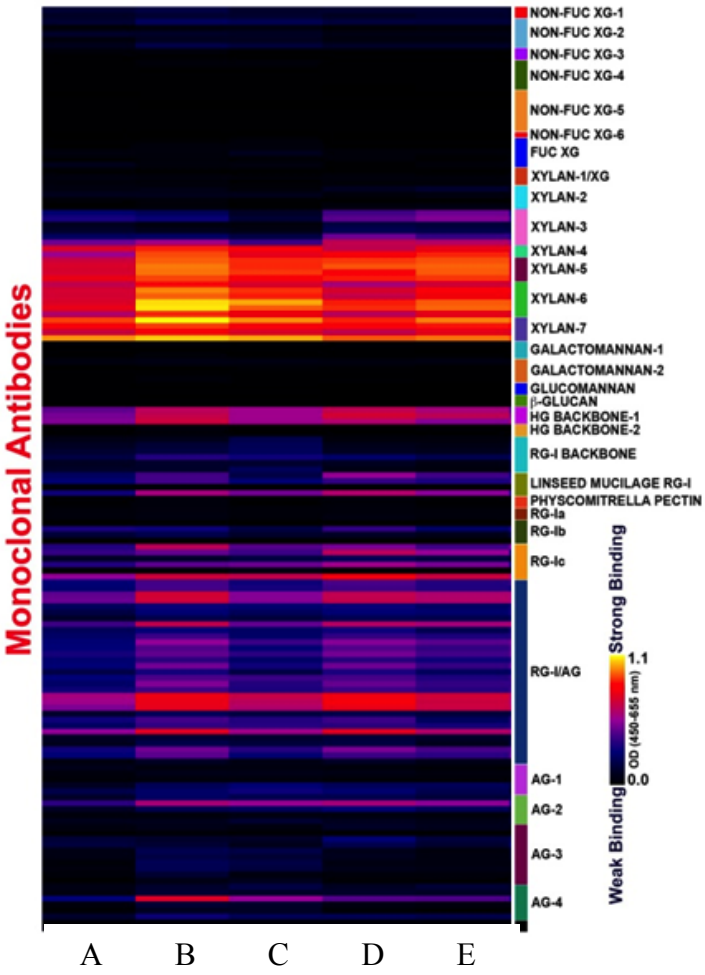

Figure 3.8 ELISA screening of glycans solubilized in 12.5% AHP pretreatment liquors of (A) corn stover, (B) bm1 stover, (C) bm3 stover, (D) miscanthus and (E) switchgrass using 155 cell wall glycan-directed mAbs. (ELISA screening was done by Sivakumar Pattathil, University of Georgia.)

3.3.4 Enzyme accessibility of grass cell walls

Carbohydrate binding modules (CBMs) from microbial cell wall polymer hydrolases [187] are another important category of molecular tools to measure the accessibility of cellulose and non-cellulosic glycans [119, 120]. In this study, enzyme binding assay was developed based on previous work [188, 189]. In order to quantify the accessibility of cellulose microfibrils, a family 3 CBM [190] from *Clostridium thermocellum,* which is specific to crystalline cellulose, was used to determine the celluloase accessibility of corn stover under

different severities of AHP pretreatment. The binding curve generated using CBM3 (Figure 3.9) on untreated and pretreated corn stovers could then be fit with a Langmuir model [132]:

$$E_{ads} = \frac{E_{max} K_{ads} E_f S}{(1 + K_{ads} E_f)}$$

The calculated model parameters are shown in Table 3.2. The binding capacity and affinity of CBM3 increased with increased pretreatment severity in term of the hydrogen peroxide loading.

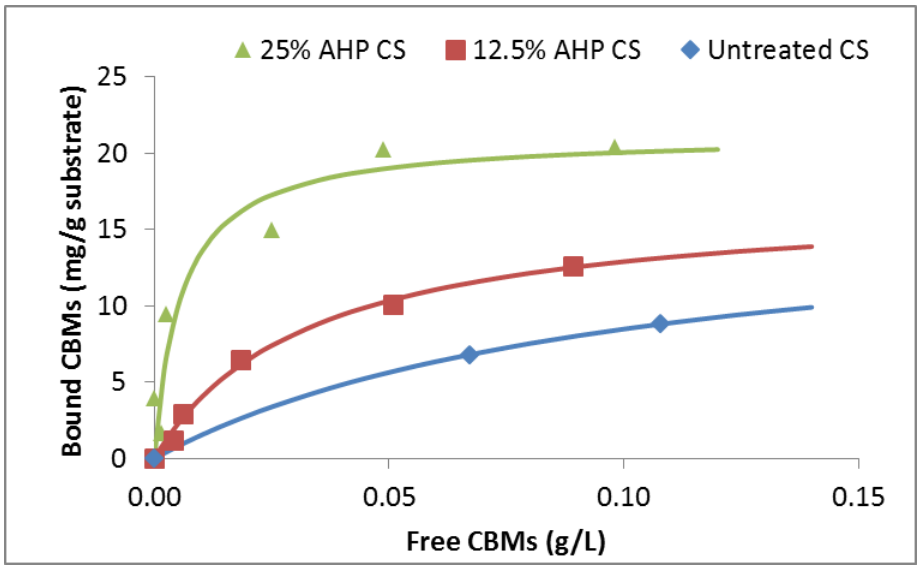

Figure 3.9 Binding isotherms of *Ct*CBM3 on untreated, 12.5% and 25% H2O2 (w/w) AHP pretreated corn stover

Biomass	Binding capacity Emax (mg/g substrate)	Binding affinity Kads^{-1} (M^{-1})
Untreated corn stover	8.57	0.66×10^6
12.5% AHP corn stover	10.86	1.14×10^6
25% AHP corn stover	21.22	3.94×10^6

Table 3.2 Parameters fitting based on Langmuir adsorption model showing binding capacity and affinity of *Ct*CBM3 on corn stovers under different treatments.

Binding characteristics of single celluases [191], cellulase mixtures [188], and CBMs [190, 192, 193] have been investigated on model cellulose such as Avicel, bacterial cellulose and

other regenerated cellulose [191], as well as on untreated and pretreated biomass [188, 194-196], and on isolated lignin[197, 198], to investigate the interaction between cellulolytic enzymes and substrates. Previous study showed that the hydrogen peroxide loading increases the bound fraction of an enzyme cocktail on AHP pretreated corn stover, and the cell wall hydrophilicity quantified by WRV and enzymatic hydrolysis yield are correlated with the bound fractions [195]. In this study, a commercial CBM3 from *Clostridium thermocellum* specific to crystalline cellulose was shown to bind with the untreated and pretreated corn stover under different pretreatment severities, indicating the cellulose accessibility of corn stover was increased by the pretreatment due to the removal of lignin and hemicelluloses with increased loading of hydrogen peroxide.

3.4 CONCLUSION

In this study, diverse grasses including three lines of corn stovers and two perennials commonly used as bioenergy feedstocks including switchgrass and miscanthus, were compared based on their responses towards alkaline oxidative pretreatments at increasing hydrogen peroxide loadings. Lignin content, metal content and ferulate content were three main important factors affecting the biomass digestibility profiles associated with hydrogen peroxide loading. The glycome profiling results indicated that alkaline oxidative pretreatments impact grass cell wall glycan by “loosening” the glycans to improve the cell wall digestibility, while impacting the alkali pre-extracted cell wall by directly removing the glycans. The glycans released into the pretreatment liquor mainly consist of xylans and pectins, indicating the removal of these glycans but not the xyloglucans. Based on the binding isotherms generated using cellulase cocktails and cellulose specific CBMs, increased binding capacity and affinity were observed for corn stover under increased loading of hydrogen peroxide, which indicates the AHP pretreatment increased cellulose accessibility. Higher

binding for corn stover following hydrogen peroxide oxidation than switchgrass indicates differences in higher order structure between the two species.

4. CORRELATING MAIZE CELL WALL PROPERTIES TO THE RECALCITRANCE

4.1 INTRODUCTION

It was shown that corn stover compromised 85% of 320 million dry tons of total primary residue by 2030 according to "The Billion Ton Study" , and is considered to be one of the most promising lignocelluosic bioenergy feedstocks in the US [90]. Strategies for breeding maize for its cellulosic biofuel (as well as feed or silage value) have been focused on increasing the non-starch biomass yield and the total carbohydrate content[199, 200] as well as reducing the overall recalcitrance [201, 202] for example through decreasing lignin content [203], altering the lignin monomer content [204], and redirection of carbon to non-cellulosic sugars (e.g. β-glucan, starch) [205, 206]. The cell wall composition and ruminant digestibility changes during plant development stage, and vary by parts of the plant [207], so the cell wall recalcitrance is influenced by environmental or agronomic factors such as maturity [199, 208], harvest method [209] and genetic variation [210]. The ratio of cell types such as the epidermis, sclerenchyma, vascular bundle zone cells and pith parenchyma [93] also results in significant differences in cell wall thickness, porosity and accessibility.

Many studies have been carried out previously to correlate the variability of maize cell walls with *in vitro* digestibility [130, 210-217], *in vivo* ruminant digestibility [214], and cellulolytic enzymatic digestibility with [170] or without pretreatments [218]. It was found that the untreated maize cell wall digestibility is impacted by neutral detergent fiber content, lignin content, S/G ratio, etherified FA and esterified *p*CA [211, 212], and the correlation between S/G ratio and esterified *p*CA suggests the association between *p*CA esters and S lignin [211]. Lignin content is generally regarded as the major factor impacting cell wall degradability

among grass cell walls [214]. However, regression models correlating lignin condensation and cell wall *p*-coumaroylation with ruminant digestibility suggest that uncondensed lignin represented by the β-O-4 yield and S lignin linked *p*CA would be one of the most crucial factor impacting digestibility for untreated maize [130]. Alkaline oxidative pretreatment cleaves β-O-4 bond to remove lignin so as to enhance the hydrolysis yields [146], and studies showed that β-O-4 content is an important impediment to cell wall degradability since it has higher interactions with cellulose [130]. However, the mechanism that S/G ratio impacts hydrolysis yields is not that clear compared with β-O-4 and the results were varied by species [22, 146]. On the other hand, the delignified grass cell wall has been regarded as a hydrogel [100] with lignin prevent the swelling of the lignified cell walls. Grass cell walls swell after alkaline treatment [100, 160] and are more easily accessible to both enzymes and water [195], which is another reason that alkaline or alkaline oxidative treated monocots yields much higher digestibilities compared to dicots [219]. The water retention value (WRV) can be used as an indirect measurement of a number of cell wall properties including overall hydrophilicity, porosity, and polysaccharides accessibility, and has been used in our recent work as a predictor of enzymatic hydrolysis yields for corn stover and switchgrass subjected to alkali, alkaline-oxidative, and liquid hot water pretreatments [195].

Recently, approaches for high-throughput screening of pretreatment and enzymatic hydrolysis have been developed for the purpose of screening large sets of cell wall material for the purpose of identifying potential reduced recalcitrance phenotypes both with and without a pretreatment [220, 221]. Understanding the cell wall traits associated with reduced recalcitrance phenotypes or phenotypes that exhibit specific responses to pretreatment are an important component of screening. However, typically only composition analysis is performed across the sample sets, limiting the understanding of the cell wall properties, traits, or phenotypes that are also associated with the cell wall's response to enzymatic hydrolysis.

Notably, recent work employing high-throughput screening of panels of promising bioenergy grasses including diverse cultivars of wheat (*Triticum aestivum* L.) [222] and *Miscanthus* spp. [223] subjected to hydrothermal pretreatment and a sorghum (*Sorghum bicolor* (L.) Moench) diversity panel subjected aqueous ammonia pretreatment [224] were able to identify substantial differences in cell wall responses to pretreatment.

The goal of this work is to better understand how maize cell wall properties both impact initial recalcitrance as well as NaOH pretreatment. Specifically, 12 cell wall properties were selected that may influence overall cell wall recalcitrance including the WRV, the xylan and lignin contents before and after pretreatment, initial cell wall acetate content, the *p*CA and FA content before pretreatment, the solubilized *p*CA and FA during pretreatment, as well as the S/G ratio of the lignin in the untreated cell wall. These properties were quantified for a diversity panel of 26 maize lines that was previously identified as exhibiting substantial phenotypic diversity in glucose release following mild NaOH pretreatment (Muttoni *et al.*, 2012). These were subjected to mild NaOH pretreatment, the glucose yields determined for all the different lines before and after the pretreatment. Subsequently, the correlations between the cell wall properties and hydrolysis yields were investigated.

4.2 MATERIALS AND METHODS

4.2.1 Maize diversity panel

(The maize diversity panel was grown and harvested by Marlies Heckwolf, University of Wisconsin.)

The maize diversity set was grown in Arlington, WI in 2012 and harvested at grain physiological maturity with the Case IH® 2144 axial-flow combine, which allows harvesting grain and biomass in a single pass as well as measuring whole-stover and grain weight and

lastly chopping the whole stover. A sub-sample of approximately 1 kg per plot was obtained and dried at 50 ºC. The dried material was ground to a particle size of 1 mm. A complete list of maize lines with accession number or resources is presented in Appendix Table A2.

4.2.2 Water Retention Value (WRV) measurement

The WRV of samples pretreated with alkali were determined as follows: 2 g of biomass were added to 150 mL of 0.08% (w/w) NaOH solution. Biomass samples were soaked for three hours at room temperature, with occasional mixing. Samples were then neutralized and filtered using a fabricated 200-mesh Buchner funnel with 500mL of deionized water. Pellet samples were created by a 200-mesh stainless steel membrane in a ten mL plastic vial, with wet biomass placed on top of membrane. Vials were centrifuged at 900 x G for 15min. Centrifuged biomass was placed in tared aluminum trays, weighed, and dried in a convection oven at 105 ºC for 24 hours. Dried samples were then reweighed and the WRV was determined in triplicate using the following equation [195]:

$$\mathrm{WRV} = \frac{\text{mass of cetrifuged sample}}{\text{mass of dried sample}} - 1$$

4.2.3 *p*-hydroxycinnamic acids determination

The hydroxycinnamic acid content of the untreated maize and mild alkaline treated maize was determined by a modified high-throughput alkaline extraction method. In this, 0.5g biomass was treated with 25 mL of 3M NaOH in sealed pressure tubes and held at 120 ºC for 1 hour in an autoclave. After cooling down in room temperature, 250 μL of 10 mg/mL methanol solution of o-coumaric acid was added as an internal standard for each sample. The mixture was transferred to Eppendorf tubes and centrifuged at 13,000 rpm for 10 minutes. The pH of the supernatant was adjusted to 2 with concentrated HCl, and the samples were then placed into a 4 ºC fridge overnight. After the overnight storage, the supernatant of the samples were analyzed in a HPLC stalled with a C18 column (Discovery HS C18 HPLC

Column, 5 cm × 2.1 mm, 5 μm). Standards containing ferulic acid, *p*-coumaric acid and o-coumaric acid were run in parallel to determine the concentration of the acids in the samples.

4.2.4 Alkaline extraction, composition analysis and enzymatic hydrolysis

Milled samples of 27 maize lines were subjected to a mild alkaline pretreatment. During the alkaline extraction, 2 g biomass was added to a 20 mL 0.8% NaOH aqueous solution in 50 mL centrifuge tube and the tubes were placed in a static water bath at 80 ºC for 1 hour. After pretreatment, the liquid was removed via filtration and the residual biomass was washed by deionized water until neutral. The yield of pretreatment was determined by measuring the difference between the original and air-dried pretreated materials. Two-stage sulfuric acid hydrolysis was carried out according to the NREL composition analysis protocol to determine the structural carbohydrates of the untreated and pretreated maize lines. A HPLC installed with an Aminex HPX-87 H (Bio-Rad, Hercules, CA) column, and the acid insoluble residue was regarded as the lignin content. The enzymatic hydrolysis was performed at pH 5.0, 50 ºC and 180 rpm shaking, with a 30mg/g protein loading of C-Tec 2 (Novozymes, Denmark) on glucan for 6 hours or 72 hours. The glucan yield was determined as the amount of glucan released after enzymatic hydrolysis divided by the original glucan content in the pretreated samples after washing.

4.2.5 S/G ratio prediction

The S/G ratio was predicted by the combination of principal component analysis and partial least square (PLS) regression based on their py-MBMS spectra provided by Robert Sykes (National Renewable Energy Laboratory). The parameters of the prediction model were generated previously by correlating thioacidolysis S/G and py-MBMS spectra of a set of samples including untreated and alkaline oxidative pretreated hybrid corn stover, brown midrib stover bm1 and bm3, switchgrass and *Miscanthus*. This model will be further discussed in next chapter.

4.3 RESULTS AND DISCUSSION

4.3.1 Cell wall properties and hydrolysis yields

12 types of cell wall properties, including initial and final WRV (X1 and X2), initial and final xylan content (X3 and X4), initial and final lignin content (X5 and X6), initial acetate (X7), *p*CA (X8) and FA (X9) contents, solubilized *p*CA (X10) and FA (X11), and S/G ratio (X12), were used in this study as a set of maize cell wall properties. These twelve cell wall properties or traits that may be indicators of cell wall recalcitrance were quantified across the maize diversity set in addition to 6 and 72-hr hydrolysis yields prior to and following mild NaOH pretreatment. The features describing the property data set presented in Table 4.1 and the yields presented in Figure 4.1. As mentioned in the Introduction, the WRV are proposed to act as a proxy variable that may be able to explain a number of phenomenon related to cell wall polysaccharide accessibility to cellulolytic enzymes. As observed, untreated maize has WRVs ranging from approximately 1.9 to 2.4 with the WRV following alkaline treatment ranging from 2.3 to 3.3 (Table 4.1). This phenomenon suggests that the variety of levels of cell wall swelling in alkali for different phenotypes increases compared with cell wall swelling in water, indicating that the alkali removes lignin and other functional groups such as hydroxyl and carboxyl groups at different levels, which results in the variability of hydrophilicity of different maize cell walls after alkaline pretreatment [195]. Xylan concentration also exhibited substantial variability within the data set and increased in relative abundance from an average of 0.200 g/g to 0.266 g/g as a consequence of the removal of lignin and extractives. Wide ranges were observed for initial cell wall acetyl content, *p*CA content, and FA content as well as corresponding *p*CA and FA release. The S/G ratio as determined by py-MBMS coupled to PLS models also shows a diverse range, although within the range reported for maize [225].

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}
Min	1.68	2.22	0.16	0.20	0.13	0.07	26.70	8.66	9.02	4.10	4.36	0.68
Avg	2.02	2.68	0.20	0.27	0.17	0.11	33.10	11.90	11.30	5.87	5.74	0.97
Max	2.39	3.32	0.26	0.31	0.21	0.14	39.20	15.90	13.90	8.49	7.43	1.52
Std	0.17	0.30	0.02	0.02	0.02	0.02	3.25	1.66	1.26	1.27	0.79	0.20

Table 4.1 Variability within the data set for the 12 properties across the 27 maize lines.

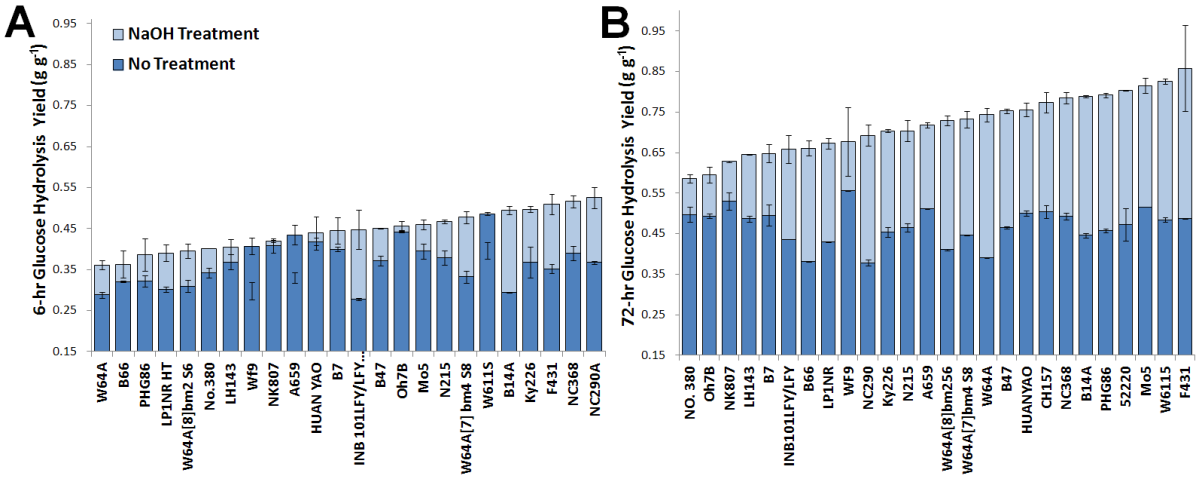

Figure 4.1 Range of hydrolysis yields obtained for untreated and NaOH-pretreated maize for **(**A**)** 6-hr hydrolysis yields and (B) 72-hr hydrolysis yields. Error bars represent data range for duplicate samples. Due to missing data, some samples do not appear.

Glucose hydrolysis yields ranged between 37.8% to 55.5% for untreated biomass and 58.6% and 82.5% for NaOH-pretreated biomass following 72 hours of hydrolysis (Figure 4.1), clearly exhibiting a diverse range of digestibility phenotypes as well as diverse responses to mild NaOH pretreatment. The 6-hr hydrolysis yields are intended to represent the initial hydrolysis rates, while the 72-hr hydrolysis yields represent the extent of hydrolysis as only minimal additional sugar release is observed beyond 72 hours (data not shown). It should be noted that these data are plotted for glucose yield rather than glucose release, such that results are not biased towards cell walls with higher glucan content.

4.3.2 Correlation between cell wall properties

In order to better visualize the relationships between the variables, a correlation map was developed and organized using hierarchical clustering according to the Euclidian distance between sets of the Pearson correlation coefficients (R) (Figure 4.2). The magnitude, scale, and significance of the correlations are presented in Table 4.2. Within the correlation map (Figure 4.2), several multi-property clusters of positive correlations are observed along the top left to bottom right diagonal, while one multi-property cluster of negative correlations stands out in both the bottom left and top right corners. This indicates that there a number of properties that are correlated across diverse maize lines and may be responsible for both differences in the cell wall's response to enzymatic hydrolysis as well as the response to pretreatment. A number of strong positive correlations between related properties or yields are observable along the top left to bottom right diagonal, namely the 6 and 72-hr hydrolysis yields, initial *p*CA content and *p*CA solubilization, initial FA content and FA solubilization, and initial and final WRV. These specific results are not surprising as they may be expected to be related. A number of property correlations that highlight either causal or merely correlative relationships between cell wall properties (but not hydrolysis yields) from within the highlighted clusters were selected and replotted in Figure 4.3. These correlations are plotted using either initial acetate content or initial WRV as the abscissa as these properties appear within two of the important clusters. It should be stressed that acetate content and WRV are not necessarily the properties responsible for the variations in the other properties, but are merely correlated to them.

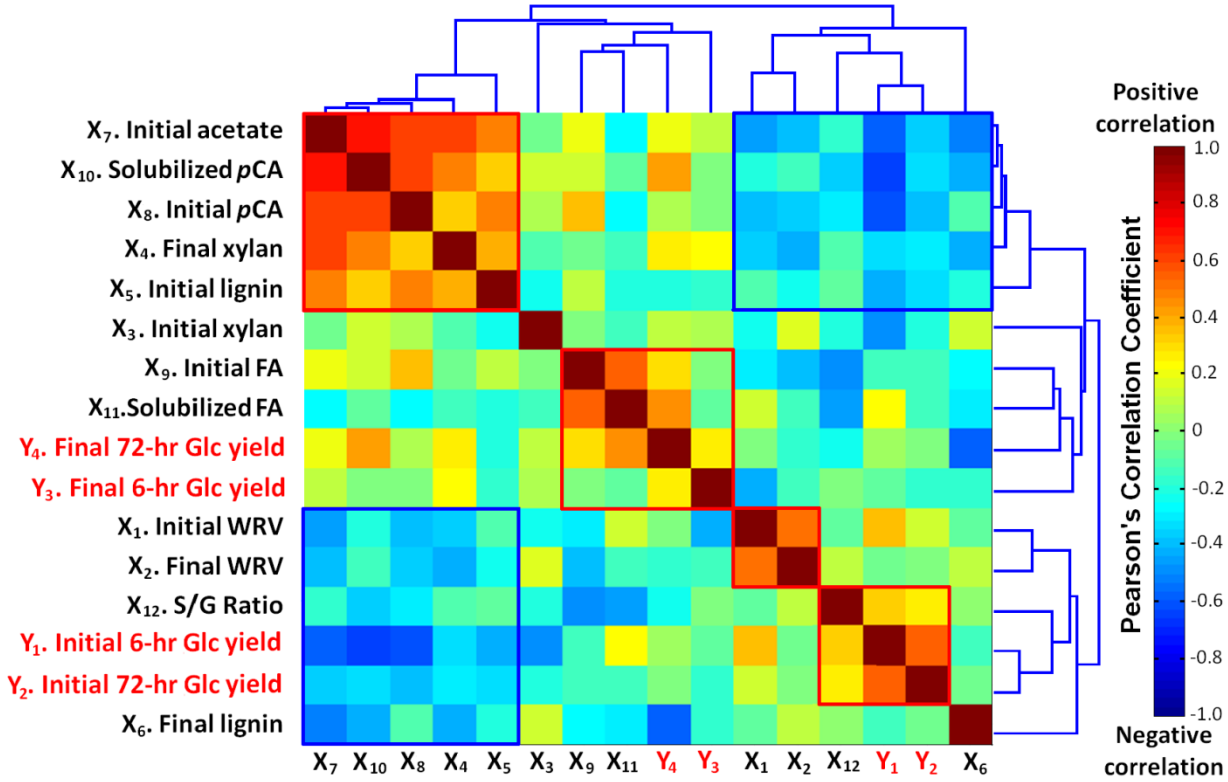

Figure 4.2 Correlation map of the Pearson's correlation coefficients for the 12 cell wall properties and 4 hydrolysis yields (red text) across the 27 maize lines as organized by hierarchical cluster analysis. Clusters of properties and yields exhibiting are highlighted. "Initial" indicates the property in the original untreated biomass sample while "final" indicates the property following pretreatment.

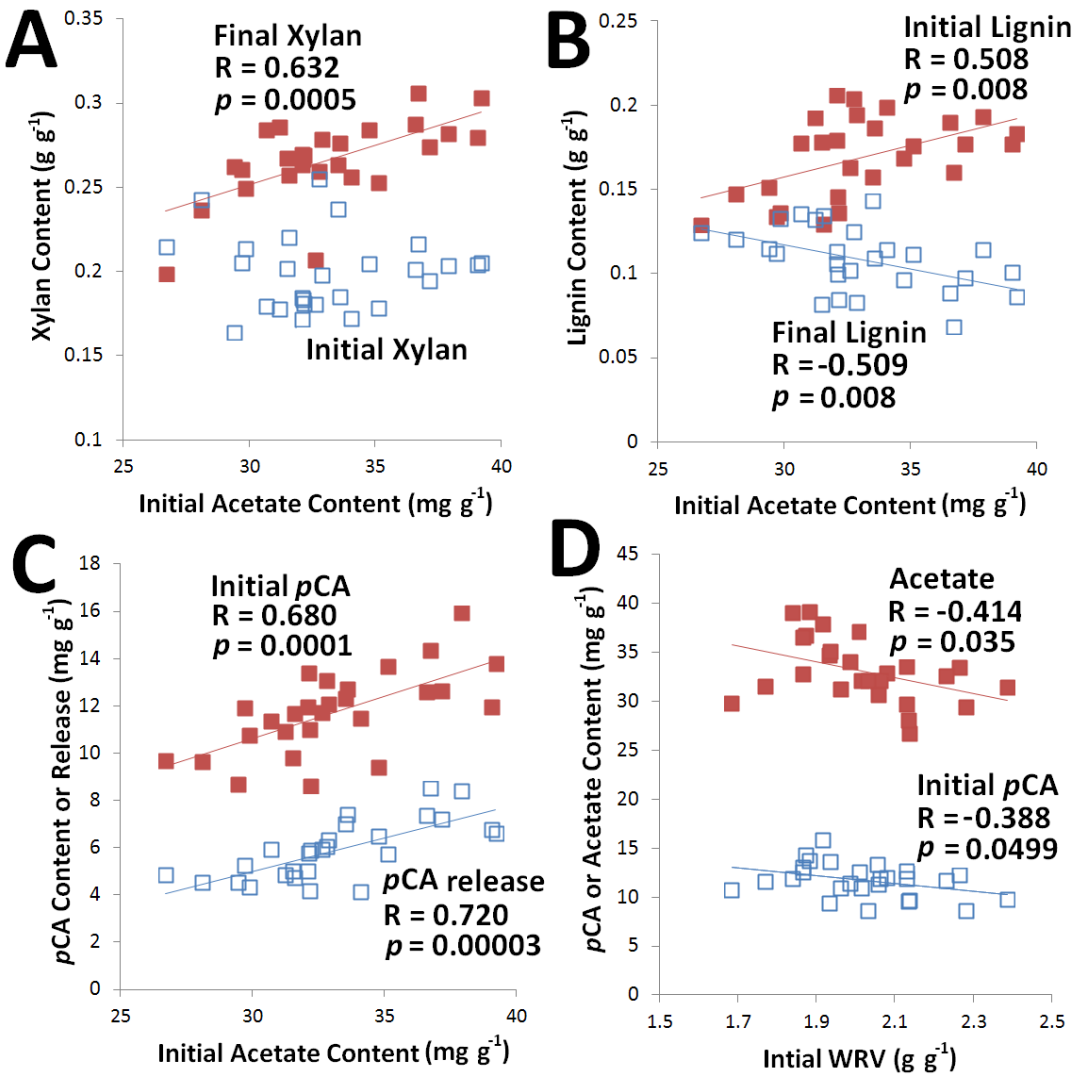

Figure 4.3 Several between-property correlations highlighted to demonstrate relationships within the data set. Each data point represents the property for one of the 27 maize lines. Pearson's correlation coefficients and *p*-values are presented for all property correlations with $p \leq 0.05$.Error bars on individual samples are not shown to improve clarity.

Xylans (as well as other non-cellulosic cell wall polysaccharides) are known to be substantially O-acetylated and the role of these substitutions are hypothesized to be to control the strength of glycan-glycan cross-linking by preventing H-bonding between cell wall polymers[20, 226, 227]. Of note is that while the acetate content showed a diverse range (26.7-39.2 mg/g), there was no correlation with the cell wall xylan content (Figure 3.3A) indicating either that the xylans exhibited either a wide range of O-acetylation - calculated to be 0.25-0.60 mol Ac per mol Xyl, which is within the reported range for maize [228] - or that

the acetyl groups are substituted on moieties other than xylan (*e.g.* glucomannans, pectins, or lignins). While uncorrelated to the initial xylan content (Figure 3.3A), this diverse range of acetate contents can be observed to be exhibit correlations to a number of other cell wall properties, including a strong, statistically-significant positive correlations to the final xylan content (Figure 3.3A), the initial lignin content (Figure 3.3B), the initial content and release of *p*CA (Figure 3.3C), while showing negative correlations to the final lignin content (Figure 3.3B), a strong positive correlation to both the final lignin content (Figure 3.3B) and the initial WRV (Figure 3.3D).

The acetate results can be interpreted as potentially relating to the lignin release. While it may be expected that high acetate contents would consume more alkali and presumably hinder lignin removal the opposite is the case. As observed in Figure 3B, the initial cell wall acetate content is positively correlated to initial lignin contents and negatively correlated to final lignin content, indicating that high-acetate cell walls are likely to be more recalcitrant yet respond better to alkaline pretreatment with respect to lignin removal due to the cleavage of the ester bond between acetate and hemicelluloses is especially susceptible to cleavage by alkaline pretreatments. Additionally it can be observed that initial *p*CA content and *p*CA released are positively correlated to initial acetate content (Figure 3.3C). This may be a consequence of the correlation of between acetate and lignin since *p*CA is known to be acylated to syringyl lignins and may only represent the correlation to lignin content[79]. The correlation to final xylan content may be a consequence of high acetate corresponding to higher lignin removal, which would enrich the xylan content of the pretreated biomass. Overall, it is not clear whether this cluster of co-varying properties is an indication of differences in the "average" cell wall property or whether these may indicate differences in the abundance of, for example, high-lignin, high-*p*CA, and high-acetate tissues. Recently, work has been targeted at altering of the expression of genes associated with O-acetylation as

a strategy for altering cell wall recalcitrance [229], although it's currently not clear how only alteration in xylan O-acetylation will impact cell wall recalcitrance.

Interestingly, the WRVs did not show a significant correlation to any of the cell wall biopolymer content (*i.e.* lignin and xylan) while it did exhibit significant correlations (albeit weak) to substitutions on these biopolymers (*i.e. p*CA and acetate, Figure 3.3D). Specifically, the initial cell wall *p*CA content is inversely correlated to the initial WRV may provide evidence that highly *p*-coumarylated cell walls may be more hydrophobic (and higher in lignin content), potentially imparting increased recalcitrance to degradation by microbial pathogens or rumen microbiota. The (weak) negative correlation between initial cell wall acetate content and initial WRV (Figure 3.3D) could be potentially due to increasing hydrophobicity of exposed xylans (*i.e.* the glycan will have an exposed ethyl group rather than a hydroxyl group). Also, the hypothesis that highly acetylated cell walls are less porous due to a decreased level of H-bonding between xylans and cellulose does fit this data since increasing acetyl content corresponds to decreasing WRV's (Figure 3.3D). As initial lignin content is positivity correlated to the cell wall acetate content (Figure 3.3B), the decreasing initial WRV with increasing acetate could be a consequence of the increasing lignin content decreasing the cell wall's capacity to sorb water.

4.3.3 Correlations between cell wall properties and hydrolysis yields

Besides between-property correlations, correlations between cell wall properties and hydrolysis yields are important for understanding property contributions to cell wall recalcitrance. All significant ($p \leq 0.05$) correlations between cell wall properties and hydrolysis yields are plotted in Figure 4.4. Notably, this plots only correlations for untreated 6-hr yields and NaOH-pretreated 72-hr yields. The untreated 72-hr yields did not exhibit significant correlations to any properties other than the untreated 6-hr yields (Figure 4.2 and Table 4.2). However, the correlations that were strongest for the untreated 6-hr hydrolysis

yields were not that significant for 72-hour digestibility, exhibiting similar correlations with *p*-values between 0.05-0.15. The differences between the hydrolysis yields obtained in different time points, 6-hr versus 72-hr, may indicate that the initial cell wall composition impacts the hydrolysis rate more strongly than the hydrolysis extent. The 6-hr hydrolysis yields for NaOH-pretreated corn stover were not found to exhibit strong correlations with any other properties and furthermore, were often lower than the untreated 6-hr hydrolysis yields (Figure 4.1B). This contradictory result may be due to the drying of the pretreated material necessitated by the analysis that resulted in its stronger resistance to rehydration which may have introduced more variability in the data for the initial glucose release by hydrolysis, but presumably not the extent of hydrolysis.

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	Y_1	Y_2	Y_3	Y_4
X_1	1	0.54**	-0.21	-0.33	-0.06	-0.04	-0.41*	-0.37*	-0.26	-0.16	0.19	-0.06	0.40*	0.18	-0.40	0.06
X_2		1.00	0.19	-0.40	-0.20	0.15	-0.37	-0.33	-0.36	-0.12	-0.10	0.13	0.00	0.00	-0.11	-0.12
X_3			1.00	-0.09	-0.20	0.19	-0.03	0.12	0.02	0.18	-0.12	-0.18	-0.45*	-0.17	0.10	0.12
X_4				1.00	0.43*	-0.39	0.63**	0.37	-0.02	0.52	-0.11	-0.08	-0.31	-0.26	0.27	0.24
X_5					1.00	-0.16	0.50**	0.52	0.15	0.36	-0.18	-0.06	-0.40*	-0.30	-0.15	-0.18
X_6						1.00	-0.48**	-0.09	-0.25	-0.39	-0.27	0.05	-0.11	-0.03	-0.13	-0.50**
X_7							1.00	0.65**	0.24	0.73**	-0.22	-0.14	-0.53**	-0.34	0.13	0.20
X_8								1.00	0.38	0.65**	-0.25	-0.26	-0.58**	-0.35	0.00	0.03
X_9									1.00	0.16	0.59**	-0.44	-0.11	-0.12	0.02	0.32
X_{10}										1.00	-0.05	-0.32	-0.61**	-0.30	0.02	0.40*
X_{11}											1.00	-0.41*	0.25	-0.11	-0.03	0.50**
X_{12}												1.00	0.36**	0.31	0.03	-0.24
Y_1													1.00	0.59**	-0.05	0.10
Y_2														1.00	-0.15	0.01
Y_3															1.00	0.25
Y_4																1.00

Table 4.2 Calculated proportionality constants (unscaled) between all cell wall properties and hydrolysis yields. * indicates the parameter is significant at $p \leq 0.05$. ** indicates the parameter is significant at $p \leq 0.01$.

A number of important trends can be identified from the data in Figure 4.4. The first is the identification that the WRV is positively correlated to the untreated 6-hr hydrolysis yield (Figure 4.4A). As discussed in the introduction, the WRV was included as a parameter that may offer the potential for consolidating a number of other, unquantified cell wall properties that may be able to be correlated to hydrolysis yields. Our previous work used only two types of biomass (corn stover and switchgrass) subjected to varying levels of delignification by alkaline oxidative pretreatment and found strong correlations between WRV and hydrolysis yields [195]. The current work used a wide range of (similar) biomass that were subjected to a single pretreatment condition which may explain why WRVs following pretreatment were not able to predict different hydrolysis yields following pretreatment.

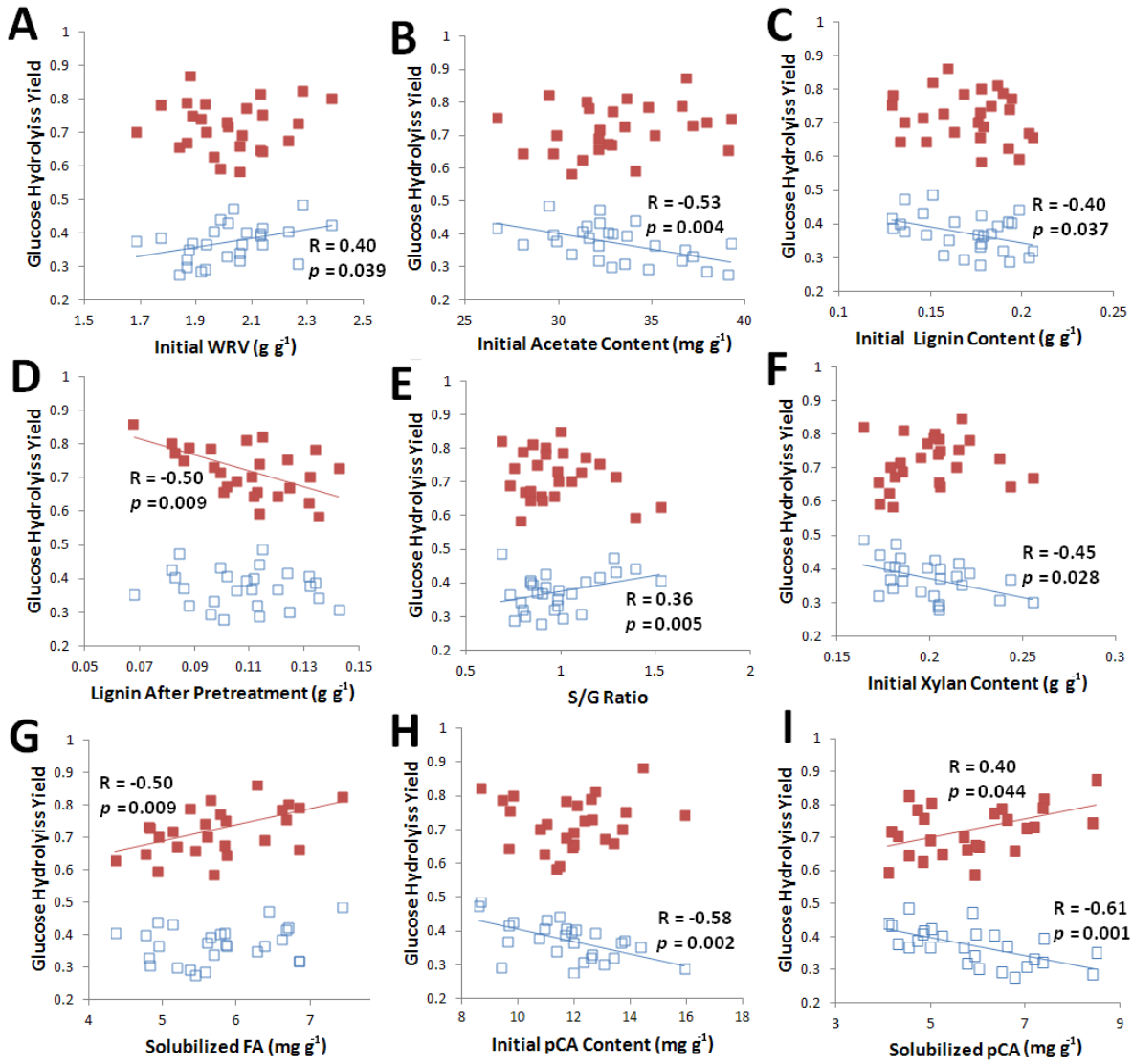

Figure 4.4 Summary of significant property correlations to glucose hydrolysis yields. Open data points represent 6-hr hydrolysis yields for untreated biomass; filled data points represent 72-hr hydrolysis yields for NaOH-pretreated biomass. Each data point represents the value of the property and corresponding yield for one of the 27 maize lines. Pearson's correlation coefficients and *p*-values are presented for all property correlations with $p \leq 0.05$. Error bars on individual samples are not shown to improve clarity.

The initial cell wall acetate content demonstrated a negative correlation to the untreated 6-hr hydrolysis yield although not to the pretreated 72-hr yield (Figure 4.4B). As discussed previously, the acetate content is strongly correlated to many other properties and potentially this cluster of related properties may be able to explain differences in hydrolysis yields rather than a single property. For example, initial acetate content is strongly correlated to initial

lignin content (Figure 4.3B), which may be the property most strongly responsible for the response in the hydrolysis yields. Previously, nonlinear models have been developing relating decreases in acetate and lignin to increases in hydrolysis yields for lime-pretreated corn stover [230] and hybrid poplar [131], although these correlate simultaneous acetate and lignin removal by alkali to improved hydrolysis yields rather than initial variability in these properties as is done in the present work.

Lignin is thought to impact saccharification yields by physical occlusion of polysaccharides [73], providing resistance to swelling [231], and by non-specific binding to cellulases[232] and studies have generally shown strong negative correlation between lignin content and hydrolysis yields for a wide range of untreated grasses [130, 214, 233] with strong correlations following a delignifying pretreatment [146, 230, 234]. As expected, the untreated lignin content is negatively correlated to the untreated 6-hr hydrolysis yield (Figure 4.4C) and the lignin content following pretreatment is negatively correlated to the pretreated 72-hr hydrolysis yield (Figure 4.4D). However, the lignin contents prior to and following pretreatment are not correlated to each other (Figure 4.2 and Table 4.2) indicating that lignin removal for mild NaOH pretreatment is not necessarily dependent on the initial lignin content, but is dependent on many other cell wall properties. This is significant in that many strategies for reduced cell wall recalcitrance have targeted lignin levels [235], although it may be sufficient that the lignin is easily removable by the pretreatment to decrease the final cell wall recalcitrance rather than the initial cell wall recalcitrance.

As we've already identified in this work, the lignin removal is more important than the initial lignin content for mild NaOH pretreatment and consequently, lignin properties that may contribute to improved lignin removal are important. Another interesting lignin-related finding is that the initial S/G ratio is positively correlated to the untreated 6-hr hydrolysis yield (Figure 4.4E), while the trend apparently reverses for the pretreated hydrolysis yield

(although this is not a statistically significant correlation). The correlation between S/G ratio in untreated biomass and *in vitro* ruminant digestibility for grasses has been somewhat contradictory in the literature [22, 130, 211] although a general trend is that increasing S/G ratio may be linked to increasing digestibility.

The initial xylan content is found to be negatively correlated to the untreated 6-hr hydrolysis yield (Figure 4.4F). Considering that both initial lignin and xylan are negatively correlated to the untreated hydrolysis yields, yet, these two properties are uncorrelated to each (Figure 4.2) may indicate that both of these components play an important role in the limited access of cellulolytic enzymes to cellulose. The enzyme cocktail utilized for this study was not supplemented with xylanase, which may be a factor contributing to this result.

Ferulate and diferulate esters are known to be attached to the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues in xylans and be involved in cross-linking between xylans to lignin by incorporation of the aromatic moiety through ether linkages into growing lignin polymers or by cross-coupling [21]. This cross-linking of cell wall polymers by ferulates is generally accepted to play an important role in cell wall recalcitrance [236]. Negative correlations have been found in the literature for total cell wall ferulate content and *in vitro* ruminant digestibility in maize [237] as well as other diverse grasses [96] and decreasing cell wall ferulate content has been investigated as a strategy for improving the forage quality of maize [238]. However, while strong, statistically significant negative correlations were identified between etherified ferulates and *in vitro* digestibility, esterified ferulates content was found to be positively correlated to *in vitro* digestibility in smooth bromegrass (*Bromus inermis* Leyss subsp. inermis), cocksfoot (*Dactylis glomerata* L.), and reed canary grass (*Phalaris arundinacea* L.) [239, 240], and other recent work has validated a strategy for increasing esterified diferulate content in maize to improve its digestibility [241]. The present work did not distinguish between etherified and esterified ferulates although the

method for ferulate content was performed under harsh enough conditions that this term likely comprises both etherified and esterifed as increasing the alkali loading and temperature during saponification did not result in additional ferulate release (unpublished data). The ferulate release during pretreatment may be more representative of esterified ferulate due to mild conditions utilized for pretreatment. Nonetheless, ferulate content and ferulate released were found to be strongly inversely correlated to each other (Figure 4.2 and Table 4.1), and importantly, the ferulate released showed a strong correlation to the 72-hr hydrolysis yields following NaOH pretreatment (Figure 4.4G), although not any of the other hydrolysis conditions. This can be understood as cell walls that are highly cross-linked are more susceptible to alkaline pretreatment. Recently, incorporation of ferulate esters into hybrid poplar has been validated as a strategy to improve the enzymatic hydrolysis following an alkaline pretreatment [242].

Lignin content, *p*CA content, and S/G ratio in maize stem internodes and rinds have been shown to increase with increasing maturity while ferulate content does not change[225]. The simultaneous increase in these properties has been implicated in the decrease in maize digestibility with increasing maturity [18, 91]. Both the initial *p*CA (Figure 4.4H) and solubilized *p*CA (Figure 4.4I) exhibit statistically significant correlations to the hydrolysis yields. These results are notable in that the correlation is of the opposite for untreated biomass compared to NaOH-pretreated biomass, whereby increasing release or content of *p*CA corresponds to decreasing hydrolysis yields for untreated cell walls and increasing hydrolysis yields for pretreated cell walls. High *p*CA content in untreated maize has been correlated to low *in vitro* digestibilities [130, 211], although the finding that high initial *p*-coumarylation of lignin may be related to high hydrolysis yields following pretreatment is novel. As discussed previously, the *p*CA content is positively correlated to the initial lignin content so this could be an indirect measure of the impact of lignin content. As another

alternative, *p*CA-containing lignins may be responsible for sorbing and inactivating cellulolytic enzymes as is known for polyphenolic compounds such as tannins and lignins and phenolic acid monomers [243].

4.4 CONCLUSION

A diversity panel of 26 maize lines were subjected to mild-NaOH pretreatment and a broad set of properties relating to cell wall recalcitrance were characterized. The hydrolysis yields of un-pretreated cell walls were found to be positively correlated to the WRV and the S/G ratio and negatively correlated to the xylan and acetate content. While the initial cell wall xylan and acetate content were uncorrelated to each other, the acetate content was found to exhibit a number of strong correlations to other cell wall properties. The pretreated cell wall hydrolysis yields were positively correlated to the ferulate released by pretreatment, indicating that breaking of ferulate cross-links between cell wall polymers is an important outcome of pretreatment. As expected, statistically significant negative correlations were identified between the cell wall lignin content and the hydrolysis yields for both untreated and NaOH-pretreated maize. It has long been known that cell wall lignin content can be negatively correlated to enzymatic hydrolysis yields, *in vitro* digestibility, as well as *in vivo* digestibility in ruminants. However, the data demonstrated that the initial cell wall lignin content and the pretreated cell wall content were not correlated to each other, and the cell wall lignin content following pretreatment was not correlated to untreated hydrolysis yields. This important finding indicates that while enzymatic hydrolysis yields may be set by the cell wall lignin content, the cell wall's response to a delignifying pretreatment such as mild NaOH pretreatment is not necessarily set by the initial lignin content. The *p*CA that is saponifiable by mild NaOH pretreatment showed a negative correlation to the hydrolysis yields of untreated maize and the inverse response for pretreated maize indicating that cell

walls with a high content of saponifiable *p*CA are more recalcitrant without treatment, yet respond better to pretreatment than cell walls with a low content of saponifiable *p*CA.

5. MODELING OF THE PLANT CELL WALL RECALCITRANCE

5.1 INTRODUCTION

Plant cell wall modification is a consequence of thermochemical or biological pretreatment and is a prerequisite for the enhancement of enzymatic hydrolysis to generate high yields of fermentable sugars. Detailed and accurate quantitative analysis of the quantity or structures of macromolecular cell wall components are essential for selecting the feedstock, monitoring the deconstruction processes, and calculating the details of the final sugar yields. However, compared with other biological macromolecules such as proteins or DNAs, plant cell wall macromolecules are relative difficult to be analyzed in a high-throughput manner due to their complexity. Nondestructive quantitative characterization methods of the cell wall are desired for fundamental research on the cell wall recalcitrance.

Data mining and modeling are frequently used in the analytical field of biomass characterization. One high-throughput approach is to apply spectroscopic techniques using multivariate approaches to build up models to convert spectra to compositional properties for biomass. Principal component analysis (PCA) was usually utilized to simplify the spectra, and partial least square regression (PLS) models were applied to correlate the principal components to easily identifiable cell wall features. Prediction of the lignin content in grasses [125, 244] is one application of these models based on pyrolysis-molecular beam mass spectrometry (py-MBMS). Also, PCA/PLS models is capable of predicting other cell wall composition properties including the content of glucan, xylan, mannan, arabinan and galactan, by incorporation of composition analysis with either py-MBMS spectra [245] or NMR spectra [246]. Near-infrared spectroscopy (NIR) has been also frequently used to analysis the composition properties [127, 128] and to predict the digestibility [247] by multivariate

approach for diverse types of biomass including softwoods [127, 128], hardwoods or grasses [247]. Besides those spectroscopic techniques, other types of models have been developed to relate the cell wall properties include cellulose crystallinity[129], lignin content, p-coumaric and ferulic acids content [130], to hydrolysis yields by either neural network[131] or regression models [130]. Li et al. reviewed multivariate data analysis as a high-throughput characterization tool for biomass using py-MBMS and NIR [248].

Pyrolysis is a process rapidly breaks biomass down to small fragments derived from the cell wall components in the condition of high temperature in the absence of oxygen [249-251]. Pyrolysis has been used as an analytical tool since the 1980s for characterizing plant cell wall composition [252, 253] by profiling the major pyrolysis derived cell wall fragments by mass spectrometry in a high-throughput manner. Although lignin macromolecules are relatively difficult to be depolymerized by traditional wet chemistry methods, pyrolysis-GC/MS can provide estimates of lignin properties such as S/G ratio and hydroxycinnamic acid content by quantifying these pyrolyzable lignin-derived fragments [146]. However, since the lignin-derived fragments maybe re-condensed during transferring to GC column due to the low temperature in the transfer line, the mass spectrometer only quantifies a small fraction of the lignin-derived fragments [146]. Pyrolysis molecular beam mass spectrometry (py-MBMS) is another high-throughput method which profiles all of the mass peaks without GC separation, and PCA/PLS models are frequently applied to convert the unseparated peaks to identifiable cell wall properties.

Researchers have done a lot of studies trying to find the relationship with the composition of lignin, such as the S/G ratio and the hydroxycinnamic content, and the digestibility of the lignocellulosic feedstock [254]. A well-developed method to quantify the S/G ratio of lignin is thioacidolysis, an acid –catalyzed reaction to depolymerize lignin [255], which was first

utilized to estimate the S/G ratio by Lapierre et al. at 1980s [256, 257]. However, thioacidolysis yield is based on the content of b-O-4 linkage in lignin [146] because it proceeds mainly by cleavage of the ether linkages in lignin by boron trifluoride etherate in ethanethiol-dioxane [255]. Since b-O-4 is the most preferable linkage in syringyl-rich lignin, thioacidolysis yields lower amount of lignin monomers in grasses for its lower S lignin content than dicots [18, 258]. Besides the roughly estimation of the content of lignin and carbohydrates by the varied types of spectroscopies, py- MBMS and py-GCMS allow to analyze more details of the cell wall lignin properties, such as S/G ratio and hydroxycinnamic acids content. Gerber et al. developed multivariate models to estimate the proportion of lignin as S/G ratio in the wood samples by py-GCMS [259]. Py-MBMS is frequently used to estimate the S/G ratio by summing the syringyl peaks intensity devided by guaiacyl peaks intensity for a large group of samples [260, 261]. However, this method usually overestimate S/G in grasses because the peak derived from ferulates cannot be distinguished from the peaks derived from G lignin, and grasses was shown to have higher ferulates content [21]. Many previous studies have found that p-coumarate (pCA) and ferulates (FA) can exert a strong influence on cell wall recalcitrance in terms of the ruminant digestibility [237] or the recalcitrance associated with enzymatic hydrolysis [236], and recently scientists are trying to increase the content of ferulate esters into model plants to increase the hydrolysis yields after alkaline pretreatments [242]. The content of esterified or etherified of pCA and FA can be quantitatively determined by alkaline extractions in different severity [167], and they were shown to impact differently on the cell wall degradability [130].

Maize (*Zea mays* L.) stover has the largest production area in the United States as a bioenergy feedstock. The environmental and agronomic factors such as maturity, nutrients and genetics may impact the maize cell wall properties. Maize breeders for bioenergy production proposes have focused on increasing the total biomass, increasing carbohydrate

content, or reducing recalcitrance [199]. High-throughput screening has been applied to select maize lines with higher enzymatic hydrolysis yields before or after hydrothermal pretreatments [222]. In this work, a diversity panel of 30 maize lines was utilized to develop chemometric models to predict cell wall properties based on pyrolysis-MBMS spectra through principal component analysis coupled with partial least square regression (PCA/PLS) model. A mild alkaline pretreatment, which removes recalcitrant components in monocot grasses in previous study [254], was utilized to provide the maize diversity panel with different responses under pretreatment. Py-MBMS was applied to develop the chemometric models to predict cell wall hydrolysis yields from the MS spectra through PCA/PLS models for the maize diversity panel before and after alkaline pretreatment. Also, multiple linear regression (MLR) models were developed to predict the hydrolysis yields for maize before and after alkaline pretreatment based on a series of measured cell wall properties including the water retention value (WRV), representing porosity and hydrophilicity, and the other compositional properties including lignin, xylan and *p*-hydroxycinnamic acids content. In addition, the S/G ratio predicted by py-MBMS and quantified by thioacidolysis have been compared and both utilized to incorporate to the prediction model for enzymatic hydrolysis.

5.2 MATERIALS AND METHODS

5.2.1 The determination of the maize cell wall composition properties

Structural carbohydrates in maize cell walls were determined by the NREL two-stage composition analysis, and the acid insoluble solids after the two-stage acid composition analysis were regarded as Klason lignin content [139]. The contents of ferulic acid (FA) and *p*-coumaric acid (*p*CA) were determined by a modified alkaline saponification method introduced in 4.2.3. However, the development and significance of this method will be

discussed in the following section. In general, Chapter 4 and Chapter 5 shared the same data set for quantifiable maize cell wall properties and hydrolysis yields.

5.2.2 Py-MBMS analysis and modeling

The py-MBMS analysis was performed using a pyrolysis furnace coupled to a free-jet molecular beam mass spectrometer without gas chromatographic column separation by Robert Sykes (NREL) according to previous procedure [125]. In py-MBMS spectra, peaks across the span of 51 to 450 m/z at interval of 1.0 m/z were subjected to a principal component analysis [128]. For the prediction model $Y=\beta X$, X was the matrix of the first 10 principle components of the principal component analysis, and Y was one of the cell wall properties. The parameters in the matrix β of the regression model were calculated by MATLAB using partial least square regression method.

5.2.3 Multivariate model development

12 types of cell wall properties, including initial and final WRV (X1 and X2), initial and final xylan content (X3 and X4), initial and final lignin content (X5 and X6), initial acetate (X7), *p*CA (X8) and FA (X9) contents, solubilized *p*CA (X10) and FA (X11), and S/G ratio (X12), were used in this study as a set of maize cell wall properties. The correlation coefficients of those properties were calculated by MATLAB. 4 types of hydrolysis yields, including 6-hr and 72-hr glucose yields for untreated maize (Y1 and Y2) as well as 6-hr and 72-hr glucose yields for pretreated maize (Y3 and Y4), were used as the response variables. To normalize the units and distribution of measurements, the Z-score transformation was applied to manipulate the raw data by the formula:

$$\text{new score} = \frac{\text{raw score} - \text{mean average}}{\text{standard deviation}}$$

Partial least square regressions were done by MATLAB between the property data set and individual hydrolysis yields. Multiple variable selection models were compared and the top 3 models were selected by Cp and BIC criteria by R (free software environment for statistical computing and graphics), and they were compared by the correlation between the predicted values and measured values. Modified models for the hydrolysis yields were developed based on the above gathered information.

5.3 RESULTS AND DISCUSSIONS

5.3.1 *p*CA/FA determination and prediction

Monocot lignins are rich in hydroxycinnamates (HCAs) including ferulate (FA) and p-coumarate (*p*CA), which can be identifiable markers of the grass cell walls in the pyrolysis products [146]. Analytical methods to quantify these compounds in grasses typically involve the application alkaline extraction to break ester and/or ether bonds [22, 130, 178, 179]. The relationship between digestibility, lignin content and ferulate content of the residue cell wall determined semi-quantitatively by py-GC/MS were correlated in previous work, and it was found that the percentage of the pyrolysis products derived from ferulate was correlated with Klason lignin content, which had a strong negative correlation with the cell wall digestibility [146]. The HCA concentrations in the liquid phase after pretreatment also have been found to be increased by the increasing H_2O_2 loading applied in pretreatment (data not published). In order to quantify the HCAs in the residual cell walls, the severe alkaline extraction using 4M NaOH under 170 ºC for 2 hours extract both ester and ether linked FA and *p*CA [22, 130, 178]. Figure 5.3 shows the amount of these acids released by alkaline treatment at different severities for corn stover. It shows that the 3M NaOH condition yields the maximum amount of HCAs among other alkaline conditions, and the HCA yields at 170 ºC is similar as the

HCA yields at 120 ºC with the same NaOH loadings. This result suggests that degradation or oxidation of HCAs possibly happens in high severity conditions.

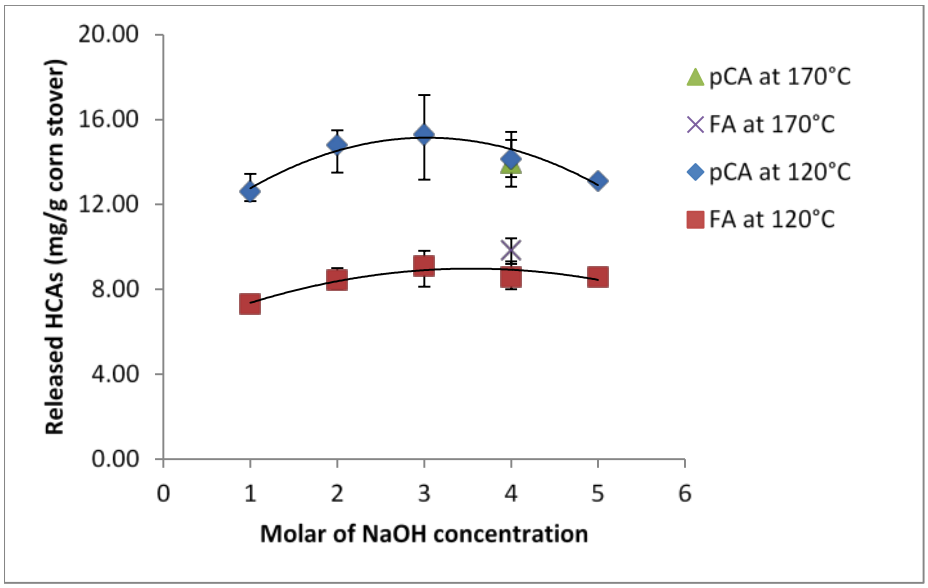

Figure 5.1 *p*CA and FA solubilization in different severity of alkali treatments.

The HCA content determined by the above mentioned method was found to be strongly correlated with the specific peaks 120 m/z and 150 m/z in the py-MBMS spectra with R-squared of 0.47 and 0.33 of the linear correlation (Figure 5.4) [262]. The first ten principal components of the py-MBMS spectra of the maize diversity set were correlated to the quantified HCAs content to obtain a series of parameters β. And the dot product of β and the principal components of the py-MBMS spectra were regarded as the predicted values of HCA content. Figure 5.5 plots the predicted values vesus the measured values for HCAs contents, indicating py-MBMS spectra is capable of making an accurate prediction of HCAs content in the maize cell wall.

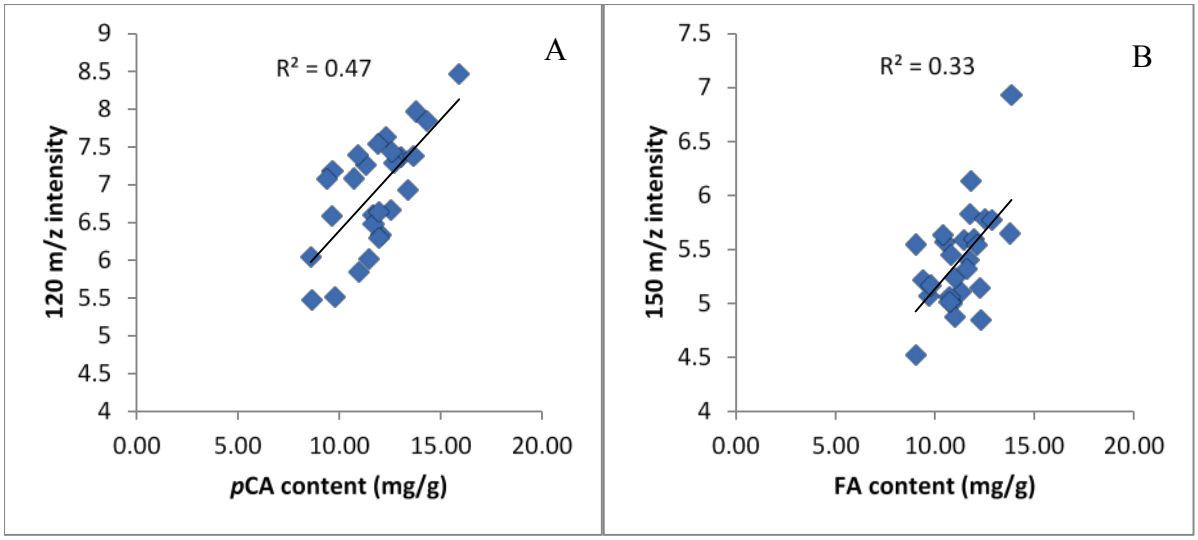

Figure 5.2 Correlation between the mass peak intensity of 120 m/z (A) and 150 m/z (B) with *p*CA (A) and FA (B) content.

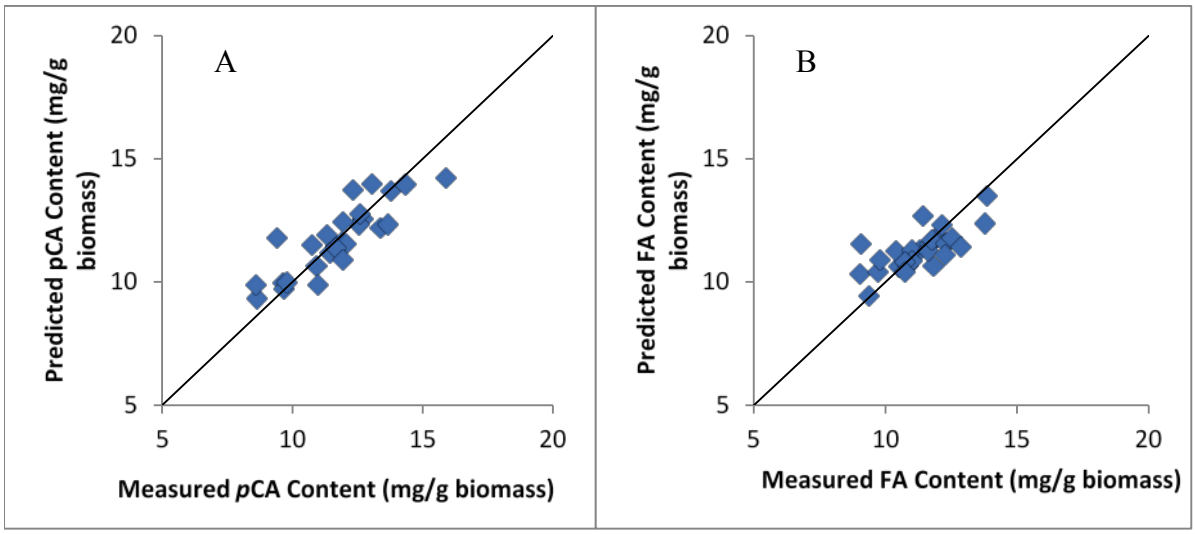

Figure 5.3 Comparison between the measured values vs. predicted values based on PLS regression coupled with PCA analysis of py-MBMS spectra for the cell wall *p*CA content (A) and FA content (B) of 27 maize lines.

5.3.2 PCA/PLS models for predicting hydrolysis yields

PCA/PLS models for predicting hydrolysis yields were also developed based on correlating the first ten principal components to the 6-hr and 72-hr hydrolysis yields of untreated and pretreated maize. Figure 5.6 A-C shows that the PCA/PLS model provides an accurate prediction of the 6-hr hydrolysis yields for untreated biomass (Figure 5.6A), relative weaker prediction for 72-hr hydrolysis yields for untreated biomass (Figure 5.6B), and the poorest prediction for 72-hr hydrolysis yields after alkaline pretreatment (Figure 5.6C). The improved enzymatic hydrolysis yields in plant cell walls after alkaline pretreatments has been explained by the increases in the cell wall porosity and hydrophilicity due to the removal of lignin, hydroxycinnamic acids and hemicelluloses [59, 263]. The previous chapter has shown that initial and final lignin content and HCAs content are negatively correlated with the initial and final hydrolysis yield, respectively, while the removal of these components is related with the increasing hydrolysis yield. Clearly, after alkaline pretreatment, the mass peaks of components including lignin and HCAs as "markers" are eliminated due to removal of these compounds, which resulting in poor prediction for hydrolysis yield of pretreated maize based on the model developed using the py-MBMS spectra of the untreated maize.

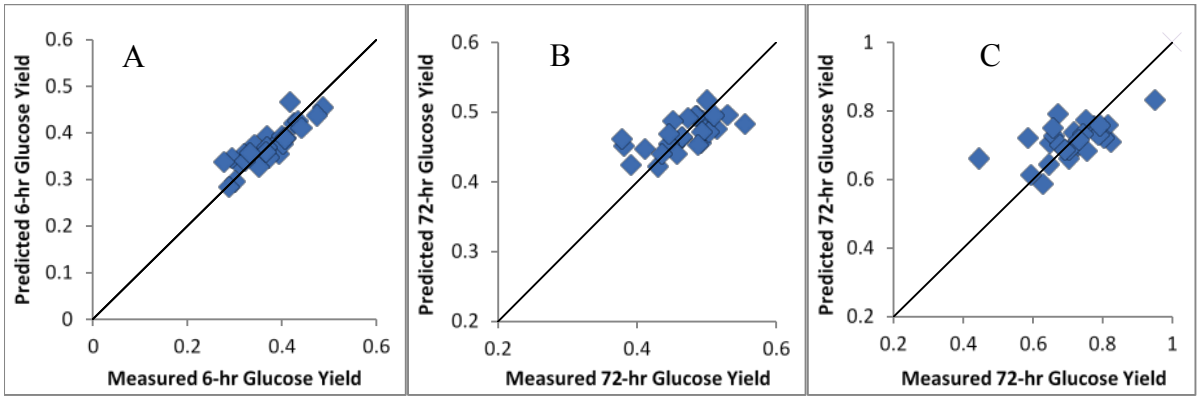

Figure 5.4 Comparison between the measured values vs. predicted values based on PLS regression coupled with PCA analysis of py-MBMS spectra for the 6-hr (A) and 72-hr (B)

hydrolysis yield for 27 maize lines before pretreatment and 72-hr hydrolysis yield after NaOH pretreatment (C).

5.3.3 MLR models for predicting hydrolysis yields using quantified properties

In order to further understand the relationship between the cell wall properties and the response to hydrolysis, multiple linear regression (MLR) models were developed in this study. To select the properties, multiple variable selection criteria have been compared including Akaike information criterion (AIC) [264], R^2, adjusted R^2, Mallow's C*p* [265], and Bayesian information criterion (BIC) [266]. To prevent overfitting, Mallow's C*p* and BIC were chosen for model selection, which are more restrictive and limit additional variables to ones that impact the dependent variable [267]. The top three models based on these criteria were selected using the Leaps function in R, which performs an exhaustive search for the best subsets of the variables in Xs for predicting Ys in linear regression, and the top 3 models with the smallest C*p* and BIC values were selected for each response variable (Table 5.1). The results showed that Y_3 has a limited number of variables in all of the selected models. As stated in previous chapter, hornification [268] of the treated materials may result in difficulties in comparing the 6-hour hydrolysis yield among diverse maize lines, therefore the model was not able to predict 6-hour hydrolysis yield of pretreated maize.

	Y_1	Y_2	Y_3	Y_4
Top 3 models based on C*p* and BIC criteria	$X_3+X_6+X_7+X_9+X_{11}+X_{12}$	X_5+X_{12}	X_1	$X_5+X_6+X_{10}+X_{11}+X_{12}$
	$X_3+X_5+X_6+X_7+X_9+X_{11}+X_{12}$	X_8	X_4	$X_1+X_5+X_6+X_{10}+X_{11}+X_{12}$
	$X_3+X_5+X_{10}$	$X_3+X_5+X_{12}$	X_1+X_5	$X_1+X_5+X_{10}+X_{11}+X_{12}$
Modified model	$X_1+X_3+X_5+X_{10}$	X_5+X_{12}	N/A	$X_5+X_{10}+X_{11}+X_{12}$

Table 5.1 Models based on C*p* and BIC criteria and the modified models.

In order to normalize the variables with varied units and ranges, Z-score transformations [269] were applied to transform the raw data to a new data set with similar range for the different

variables. The top three models were regressed in MATLAB and the relative parameter magnitudes of the variables were compared. For the prediction model of Y_1, X_6 and X_7 (definitions of the variables are in 5.2.3) were shown to have a small impact on the hydrolysis yield compared to X_3, X_5 and X_{10}. The variable X_1, which was shown to have a p value less than 0.05 in the individual linear correlations with Y_1, has also been added to the modified model. Thus, the variables selected for prediction model Y_1 are X_1, X_3, X_5 and X_{10}, with parameters for the Z-transformed model as:

$$Y_1 = 0.24X_1 - 0.41X_3 - 0.33X_5 - 0.38X_{10}$$

This model indicates that, besides WRV (X_1) that positively impacts hydrolysis yield, xylan content (X_3), lignin content (X_5) and solubilized *p*CA (X_{10}) have similar magnitude of negative impacts on the initial rate of the hydrolysis of untreated maize. Previous results have shown that WRV, lignin and xylan content are negatively correlated to hydrolysis yields for alkaline hydrogen peroxide (AHP) pretreated grasses [146, 195]. This model also includes solubilized *p*CA content and the solubilized *p*CA was shown to correlate with lignin content in the Table 1. X_5 and X_{12} was shown to be able to fit Y_2 as using the least number of variables, with parameters for the Z-transformed model as:

$$Y_2 = -0.28X_5 + 0.29X_{12}$$

X_1 and X_6 were shown to have minimal impact on Y_4. Thus Y_4 would be fitted using X_5, X_{10}, X_{11}, and X_{12}, would be:

$$Y_4 = -0.38X_5 + 0.74X_{10} + 0.64X_{11} + 0.35X_{12}$$

These two models demonstrate the predictions of 72-hour hydrolysis yields for the pretreated (Y_1) and untreated (Y_4) maize include both initial lignin content (X_5) and S/G ratio (X_{12}), with the only difference that the solubilized *p*CA (X_{10}) and FA (X_{11}) have been also utilized

in the prediction model of the pretreated maize. In the previous research demonstrating that alkaline pretreatments enhance digestibility of maize cell wall, it was shown that the enzymatic degradability is highly correlated with the solubilized amount of *p*CA and FA after alkaline pretreatment [170], which is consistent as the findings in this study. Although the multivariate model depends on the total number of variables, the relative parameter magnitude is not comparable between different treatments, and the model was only able to compare the contribution of different cell wall properties for one of the hydrolysis yields.

Figure 5.5 compares the predictions of the three hydrolysis yields based on PCA/PLS models and MLR models, and shows that the predictions based on MLR models are with comparable accuracy while the predictions based on PCA/PLS models are with incomparable accuracy. Unlike the MLR models, which are developed based on the three sets of maize properties as the variables, the PCA/PLS models are all based on initial composition of the cell walls, which is profiled by py-MBMS spectra. However, the cell wall changes that occur during the pretreatment and enzymatic hydrolysis impact the hydrolysis yields, but are not predictable by the initial composition.

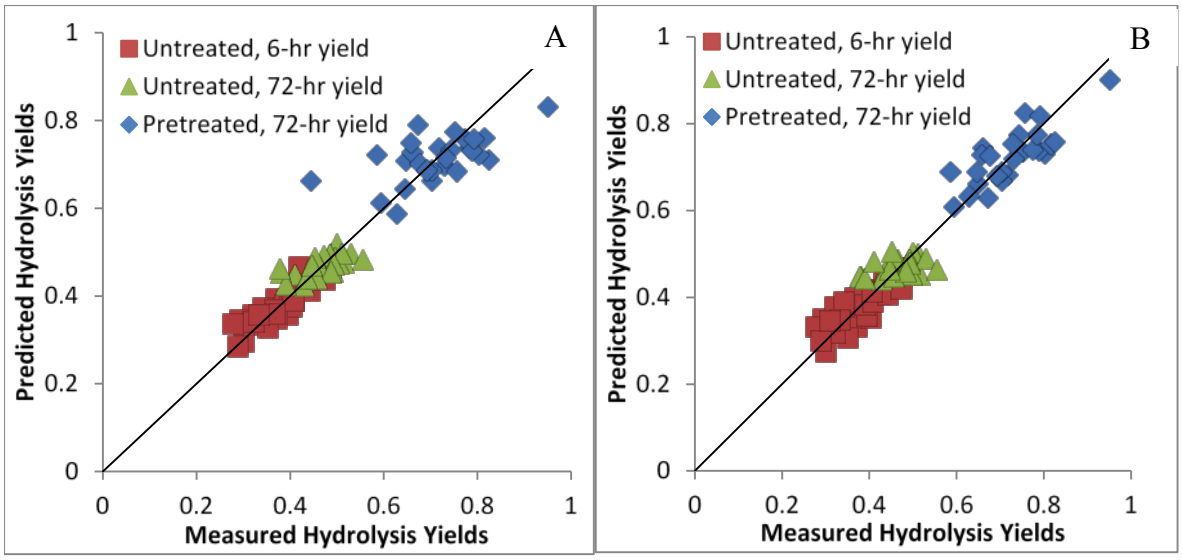

Figure 5.5 The correlation between the predicted yields and the measured yields for 6-hour hydrolysis yield of untreated maize (red square), 72-hour hydrolysis yield of untreated maize (green triangle) and 72-hour hydrolysis yield of NaOH pretreated maize (blue diamond) using PCA/PLS model (A) and MLR model (B).

These models suggest that, the higher cell wall hydrophilicity represented by WRV, initial xylan and lignin content can set the initial cell wall recalcitrance and impact the cell wall degradability. However, only lignin has strong impacts on the hydrolysis extent and pretreatment effectiveness. Initial acetate and *p*CA content have a stronger negative impact on the original cell wall hydrolysis extent (72-hour yield) instead of the initial hydrolysis rate (6-hour yield), which have been eliminated in the model since both of them are strongly correlated with lignin content. Additionally, the solubility of *p*CA and FA quantified as solubilized *p*CA and FA in the liquid phase after pretreatment positively impacts the final hydrolysis yield.

The correlation between S/G ratio and *in vitro* ruminant digestibility for monocot grasses has been shown in many of the previous research with contradictory findings [22, 130, 211]. We have found that S/G ratio did not change during the alkaline oxidative pretreatments since the thioacidolysis method can only quantify the β-O-4 linked monolignols thus the thioacidolysis yields decreased significantly after alkaline oxidative pretreatment without changing the S/G ratio[146]. Characterization of lignin based on pyrolysis techniques have problems with that the ferulates and G lignin derived compounds are not distinguishable, which usually results in underestimation of S/G. In this study, we predicted S/G based on a PCA/PLS model developed previously by correlating pyrolysis-MBMS spectra and thioacidolysis S/G results of another grass set, which avoids the overestimation of G lignin and estimates S/G in a high-throughput manner. Our results showed that S/G ratio is one of the variables in the models to predict the hydrolysis yields for alkaline pretreated maize.

5.4 CONCLUSION

In this study, chemometric models and MLR models were developed and compared for predicting cell wall properties and hydrolysis yields of diverse maize and grasses. A high throughput method for quantifying the cell wall HCAs content is developed in this study. The PCA/PLS model gave a more accurate prediction of hydrolysis rates than hydrolysis yields, and a very poor prediction for the hydrolysis yields after alkaline pretreatment. This indicates that the initial cell wall composition profiled in py-MBMS impacts the hydrolysis rates more than the yields, and the removal of the alkali-labile markers such as HCAs leads to less predictable hydrolysis yields after pretreatment by py-MBMS spectra. The MLR model of the prediction on hydrolysis yields also suggested similar findings. The relative parameter magnitudes of the MLR model showed that the hydrolysis rate was set by the composition properties and WRV, and the hydrolysis yields of the maize cell wall before pretreatment are highly dependent on the lignin content and S/G ratio. For the hydrolysis yields after pretreatment, lignin content, S/G ratio and the releasing amounts of *p*CA and FA in pretreatment liquor are the most influential factors. These findings provide insights for maize breeding for bioenergy production based on stovers-suitable alkaline pretreatments.

6. CONCLUSIONS

Untreated and alkaline oxidative pretreated biomass from phylogenetically diverse plants were compared in this study to understand fundamental features impacting cell wall recalcitrance. The roles that glycans play in the plant cell wall recalcitrance was investigated using taxonomically and structurally diverse biomass feedstocks. Their response to alkaline oxidative pretreatment and how differing features of the cell wall matrix contribute to its recalcitrance was assessed by glycome profiling, and we identified different mechanisms for how AHP pretreatment overcomes cell wall recalcitrance in goldenrod versus corn stover, while it was relatively ineffective on poplar. Furthermore, grass cell wall features were characterized to understand how those features may impact recalcitrance reduced by alkaline oxidative pretreatment. Lignin removal in grasses induced by cleavage of hydroxycinnamic esters and internal metal-expedited peroxide oxidation in the alkaline oxidative pretreatment is the major contributor to improve the following hydrolysis yields. The results call attention to the important role that differences in cell wall structure and organization play in establishing cell wall recalcitrance to deconstruction by pretreatment and enzymatic hydrolysis.

Based on a maize diversity panel, a number of both expected as well as non-intuitive correlations between cell wall properties and recalcitrance were identified for untreated and alkaline pretreated maize. It was found that the properties contributing to a “reduced recalcitrance” phenotype following a specific pretreatment are not necessarily the same properties that contribute to recalcitrance in untreated cell walls. In order to compare and validate the findings, multivariate models including PCA/PLS models and MLR models were applied to predict the hydrolysis yields based on cell wall properties which were predicted using chemometric models or directly quantified. It was found that the initial composition sets the cell wall recalcitrance which impacts the initial hydrolysis rate, but the hydrolysis

extent before and after pretreatment is highly impacted by the content, composition and removal of lignin. Pretreatment conditions that are feedstock-specific are likely to be more effective than general approaches, and future work in breeding and engineering plants with cell walls designed for specific deconstruction approaches should make use of positive synergistic interactions between specific pretreatments and particular cell wall features.

APPENDIX

Glycan Group Recognized	mAb Names
Non-Fucosylated Xyloglucan-1	CCRC-M95
	CCRC-M101
Non-Fucosylated Xyloglucan-2	CCRC-M104
	CCRC-M89
	CCRC-M93
	CCRC-M87
	CCRC-M88
Non-Fucosylated Xyloglucan-3	CCRC-M100
	CCRC-M103
Non-Fucosylated Xyloglucan-4	CCRC-M58
	CCRC-M86
	CCRC-M55
	CCRC-M52
	CCRC-M99
Non-Fucosylated Xyloglucan-5	CCRC-M54
	CCRC-M48
	CCRC-M49
	CCRC-M96
	CCRC-M50
	CCRC-M51
	CCRC-M53
Non-Fucosylated Xyloglucan-6	CCRC-M57
Fucosylated Xyloglucan	CCRC-M102
	CCRC-M39
	CCRC-M106
	CCRC-M84
	CCRC-M1
Xylan-1/XG	CCRC-M111
	CCRC-M108
	CCRC-M109

Table A.1 Listing of plant cell wall glycan-directed monoclonal antibodies (mAbs) used for glycome profiling analyses. The groupings of antibodies are based on a hierarchical clustering of ELISA data generated from a screen of all mAbs against a panel of plant polysaccharide preparations that groups the mAbs according to the predominant polysaccharides that they recognize. The majority of listings link to the Wall*Mab*DB plant cell wall monoclonal antibody database (http://www.wallmabdb.net) that provides detailed descriptions of each mAb, including immunogen, antibody isotype, epitope structure (to the extent known), supplier information, and related literature citations.

Table A.1 (cont'd)

Xylan-2	CCRC-M119
	CCRC-M115
	CCRC-M110
	CCRC-M105
Xylan-3	CCRC-M117
	CCRC-M113
	CCRC-M120
	CCRC-M118
	CCRC-M116
	CCRC-M114
Xylan-4	CCRC-M154
	CCRC-M150
Xylan-5	CCRC-M144
	CCRC-M146
	CCRC-M145
	CCRC-M155
Xylan-6	CCRC-M153
	CCRC-M151
	CCRC-M148
	CCRC-M140
	CCRC-M139
	CCRC-M138
Xylan-7	CCRC-M160
	CCRC-M137
	CCRC-M152
	CCRC-M149
Galactomannan-1	CCRC-M75
	CCRC-M70
	CCRC-M74
Galactomannan-2	CCRC-M166
	CCRC-M168
	CCRC-M174
	CCRC-M175
Acetylated Mannan	CCRC-M169
	CCRC-M170
β-Glucan	LAMP
	BG1
HG Backbone-1	CCRC-M131
	CCRC-M38
	JIM5
HG Backbone-2	JIM136
	JIM7
RG-I Backbone	CCRC-M69
	CCRC-M35
	CCRC-M36

Table A.1 (cont'd)

	CCRC-M14
	CCRC-M129
	CCRC-M72
Linseed Mucilage RG-I	JIM3
	CCRC-M40
	CCRC-M161
	CCRC-M164
Physcomitrella Pectin	CCRC-M98
	CCRC-M94
RG-Ia	CCRC-M5
	CCRC-M2
RG-Ib	JIM137
	JIM101
	CCRC-M61
	CCRC-M30
RG-Ic	CCRC-M23
	CCRC-M17
	CCRC-M19
	CCRC-M18
	CCRC-M56
	CCRC-M16
RG-I/Arabinogalactan	CCRC-M60
	CCRC-M41
	CCRC-M80
	CCRC-M79
	CCRC-M44
	CCRC-M33
	CCRC-M32
	CCRC-M13
	CCRC-M42
	CCRC-M24
	CCRC-M12
	CCRC-M7
	CCRC-M77
	CCRC-M25
	CCRC-M9
	CCRC-M128
	CCRC-M126
	CCRC-M134
	CCRC-M125
	CCRC-M123
	CCRC-M122
	CCRC-M121
	CCRC-M112
	CCRC-M21

Table A.1 (cont'd)

	JIM131
	CCRC-M22
	JIM132
	JIM1
	CCRC-M15
	CCRC-M8
	JIM16
Arabinogalactan-1	JIM93
	JIM94
	JIM11
	MAC204
	JIM20
Arabinogalactan-2	JIM14
	JIM19
	JIM12
	CCRC-M133
	CCRC-M107
Arabinogalactan-3	JIM4
	CCRC-M31
	JIM17
	CCRC-M26
	JIM15
	JIM8
	CCRC-M85
	CCRC-M81
	MAC266
	PN 16.4B4
Arabinogalactan-4	MAC207
	JIM133
	JIM13
	CCRC-M92
	CCRC-M91
	CCRC-M78
Unidentified	MAC265
	CCRC-M97

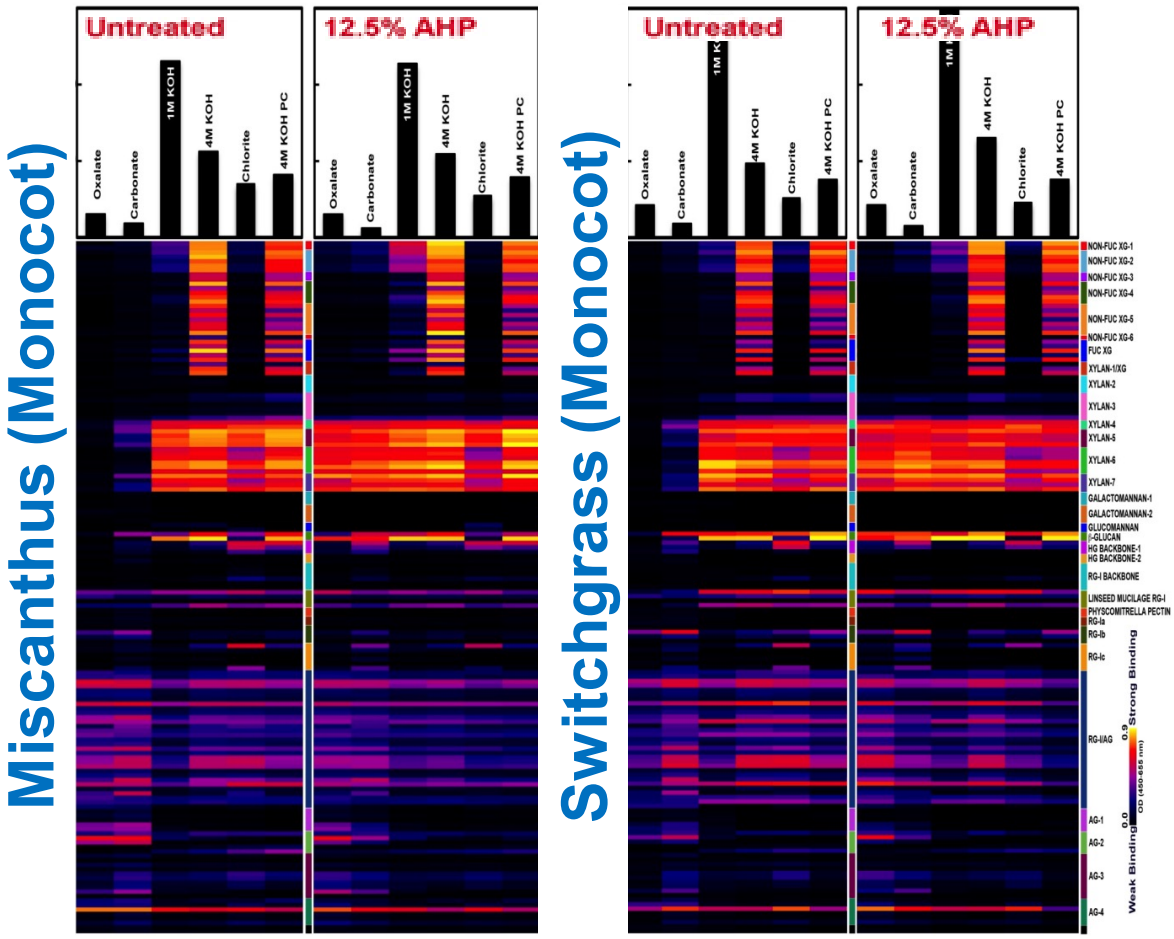

Figure A.1 Glycome profiling results of untreated and 12.5% AHP pretreated miscanthus and switchgrass.

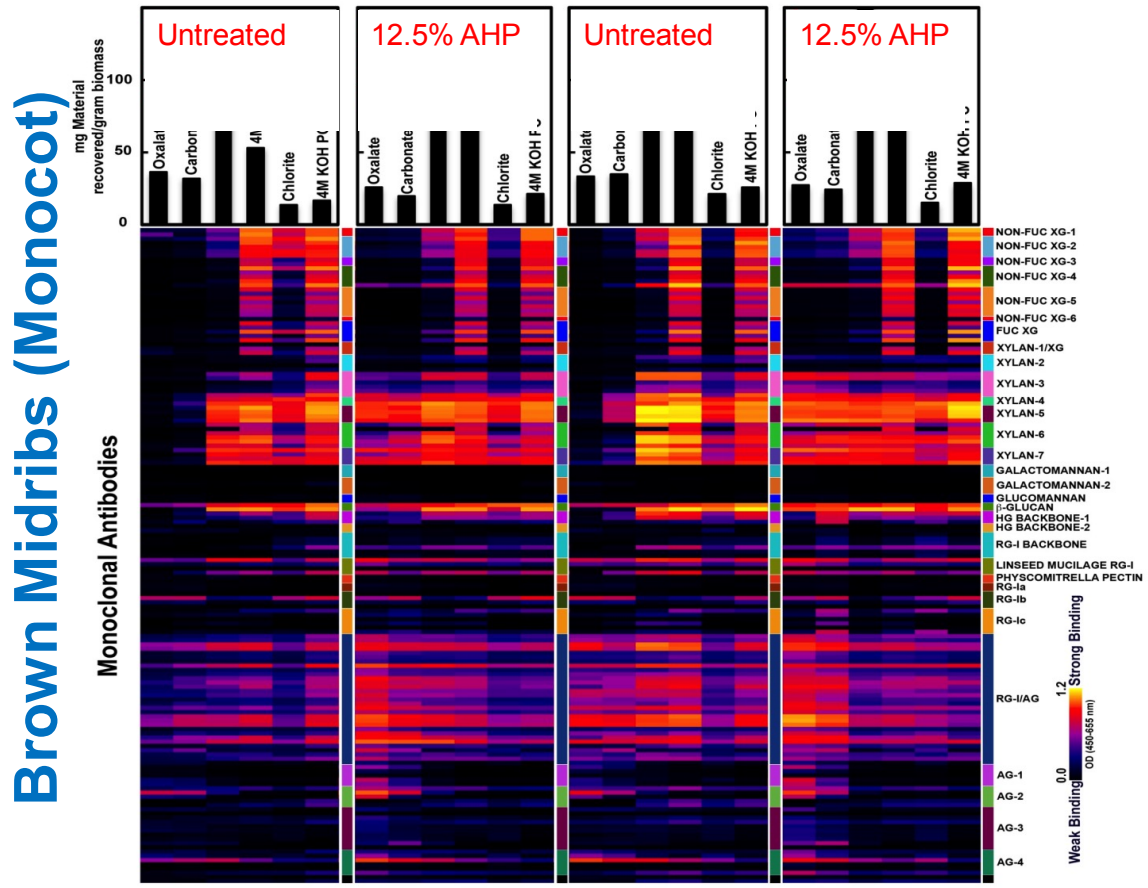

Figure A.2 Glycome profiling results of untreated and 12.5% AHP pretreated bm1 and bm3 stover.

Genotype	Accession Number or Source
52220	Ames 2336
A659	NSL 81599
B14A	PI 550461
B47	PI 601009
B66	PI 550464
B7	NSL 65863
CH157	Ames 22441
F431	PI 257515
HUANYAO	Ames 2337
INB 101LFY/LFY (A632 X M16 S5)	NSL 174429
Ky226	Ames 27131
LH143 (Maintainer)	Ames 27450
LP1NR HAT	PI 600729
Mo5	PI 558523
N215	PI 595367
NC290A	Ames 27141
NC368	Ames 27180
NK807	PI 601430
NO. 380	PI 303924
Oh7B	Ames 19323
PHG86	PI 601442
W611S	[a]
W64A	NSL 30058
W64A[7] bm4 S8	[a]
W64A[8]bm2 S6	[a]
Wf9	Ames 19293
YE 4	Ames 2335

Table A.2 Genotype and reference source for the 27 maize lines used in this work.

(The maize diversity panel was grown and harvested by Marlies Heckwolf, University of Wisconsin.)

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