UNDERSTANDING PLANT CELL-WALL RECALCITRANCE TO IMPROVE LIGNOCELLULOSIC BIOREFINERY BY CHARACTERIZING NON-CELLULOSIC CARBOHYDRATES AND LIGNIN-DERIVED METABOLITES STRUCTURES

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ABSTRACT

The success of an economically feasible and environmentally sustainable lignocellulosic biorefinery has been largely impeded because of the native recalcitrance of lignocellulosic plant cell walls to thermochemical treatment (e.g. chemical pretreatment) and biochemical processing (e.g. enzymatic hydrolysis). Several leading pretreatment technologies, including Ammonia Fiber Expansion, Extractive Ammonia, Ionic Liquid, Steam Explosion and Dilute Acid, significantly help enhance the enzyme digestibility of pretreated biomass. Comprehensive enzyme cocktails have also been developed to maximize the biomass hydrolysis to fermentable sugars. However, due the complex nature of plant cell wall, inefficient sugar conversion remains to be a universal problem for various pretreatment technologies. The mechanism of recalcitrant oligosaccharides accumulation is poorly understood. The goal of this dissertation is to understand the limiting factors that contribute to unconverted carbohydrates in bio-based chemical industry, and provide information for the rational design of enzyme cocktail, fermentation and pretreatment process adjustments.

We first proposed the methodology for large-scale purification of oligosaccharides using charcoal fractionation and size exclusion chromatography. The carbohydrate composition of recalcitrant oligosaccharides, their mass and molecular weight distribution profiles were determined. Enzyme activity assay revealed that sugar inhibition and the lack of enzyme activity in commercial enzyme mixtures are major reasons for inefficient monomeric sugar conversion.

A novel glycome profiling method (Elisa screening), combined with biotinylation derivatization, was used as a rapid, high-throughput tool to identify recalcitrant non-cellulosic glycan structures for small-molecule oligosaccharides and insoluble polysaccharides. 4-*O*-methyl-D-glucuronic acid substituted xylan and pectic-arabinogalactan were found to be the most abundant epitopes recognized by monoclonal antibodies in liquid hydrolysate and unhydrolysed solids. These structures were further validated by MALDI-TOF and TMS composition analysis. Based on the epitope information, novel accessory enzymes were supplemented into existing enzyme cocktails with the required activities for achieving complete sugar deconstruction.

An artificial, chemically-defined Synthetic Hydrolysate was used to study the inhibitory effect

of water-soluble components of crude lignin stream produced extractive ammonia pretreatment (EA) on yeast fermentation. Key inhibitory compounds that were identified using LC-MS and GC-MS included p-coumaroylamide, feruloylamide and p-coumaroylglycerol. Chemical genomics was employed to show the fingerprints of genes deletion response to inhibitors. The different sensitive/resistant genes clusters confirmed the variability and similarity of inhibitors in water-soluble components with real hydrolysate.

I dedicate this thesis to my father, who not only gave me life but also instilled in me the courage to be true to myself, has always been a beacon of light in my life, and will forever hold a special place in my heart.

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LIST OF ABBREVIATIONS

α-ArbF α-Arabinofuranosidase

α-Glu α-Glucuronidase

ACS AFEX Pretreated Corn Stover

ACSH AFEX pretreated Corn Stover Hydrolysate

AFEX Ammonia Fibre Expansion

AG Arabino-Galactan

βG β-Glucosidase

βX β-Xylosidase

CS Corn Stover

DA Dilute Acid

DP Degree of Polymerization

EA Extractive Ammonia

EG Endoglucanase

Elisa Enzyme-linked immuno sorbent assay

EX Endoxylanase

FPLC Fast Protein Liquid Chromatography

GalA Galacturonic Acid

GC Gas Chromatography

GluA Glucuronic Acid

HG Homo-Galactoronan

HPAEC High Performance Anion Exchange Chromatography

HPLC High Performance Liquid Chromatography

IL Ionic Liquid

LC Liquid Chromatography

mAbs Monoclonal Antibodies

MALDI Matrix-Assisted Laser Desorption Ionization

MP Multifect Pectinase

MS-MS Tandem Mass Spectrum

MS Mass Spectrum

NMR Nuclear Magnetic Resonance

OD Optimal Density

RG Rhamno-Galactan

SE Steam Explosion

SEC Size Exclusion Chromatography

SynH Synthetic Hydrolysates

TMS Trimethylsilyl

UA Uronic Acid

UV Ultraviolet

WSC Water-Soluble Components

XG Xylo-Glucan

CHAPTER 1

INTRODUCTION

Declining crude oil reserves and environmental concerns associated with greenhouse gas emissions due to petroleum products has provided an impetus to transition from the current fossil fuel scenario to a more sustainable renewable energy system [36]. Inedible plant biomass, also known as lignocellulosic biomass, includes agricultural residues, forestry residues, herbaceous and woody crops. These are the most abundant sources of potential feedstocks for producing renewable liquid transportation fuels [89]. Structural carbohydrates from the plant cell walls represent a vast untapped energy source. Attempting to economically convert these carbohydrates to biofuels, particularly via the biochemical route, will be a step forward in creating a more sustainable liquid transportation fuel. In the past few decades, significant research efforts have been undertaken in the field.

Nevertheless, several major issues impede the successful commercialization of cellulosic biomass conversion to liquid fuels [6]. These barriers include cost of biomass degrading enzymes, incomplete conversion of biomass to fermentable sugars and low ethanol productivity [19, 90, 163]. Cellulose and hemicelluloses in the plant cell wall are embedded in a complex matrix with lignin that make biomass highly recalcitrant [28]. Thus, numerous pretreatment technologies have been developed to overcome biomass recalcitrance, such as: hot water, steam explosion, lime, phosphoric acid, dilute acid (DA), ammonia [such as, ammonia fibre expansion (AFEX), soaking in aqueous ammonia (SAA) and ammonia recycled percolation (ARP)] and ionic liquid (IL)-based pretreatments [34, 51]. DA, IL and AFEX pretreatments are of the most leading technologies studied at the three US Department of Energy (DOE) funded bioenergy research centers, namely Great Lakes Bioenergy Research Center (GLBRC), Joint Bioenergy Institute (JBEI) and the BioEnergy Science Center (BESC). Considerable research has been done to streamline these processes to make them cost effective. In previous work, comparative studies of commercial enzyme mixture optimization and ethanol production of DA, IL and AFEX- pretreated corn stover have been performed to improve downstream processing conditions and to understand factors contributing to

the glucose and xylose yields during enzymatic hydrolysis [49, 142]. Unlike acidic pretreatments which require a wash stream, AFEX pretreatment is a dry-to-dry process that keeps the carbohydrate composition unaltered and preserves most of the sugars intact in a single solid biomass stream [7, 27, 29, 34, 139]. The presence of hemicellulose and pectin, however, requires more complex enzyme cocktails relative to acidic pretreatments, where hemicellulases, pectinases and other accessory enzymes must be added to cellulases to maximize overall sugar yields. Non-cellulosic polysaccharides, which account for 25-35% of plant cell walls, have branched cross-linkages with varying levels of substitution [46, 48, 146, 160]. Thus, a higher degree of synergy between a diverse set of enzyme activities is required to completely depolymerize such complex and highly branched carbohydrate structures into monomeric sugars [80, 96]. Though enzyme cocktail complexity is increased for deconstructing ammonia pretreated biomass, the overall enzyme loading required does not change significantly in relation to dilute acid pretreatment [142]. One approach for studying this problem is to rationally design the enzyme cocktail by understanding the limiting factors that contribute to oligosaccharide and polysaccharide accumulation during hydrolysis. For example, if some of the required biomass-degrading enzymes are not present or present at low levels in the commercial enzyme cocktail, some glycosidic linkages will tend to accumulate during the hydrolysis process. Thus, by carrying out detailed composition analysis and identifying structural features of enriched recalcitrant cell wall components, one can rationally determine the enzymes that are limiting the hydrolysis process. In addition to incomplete sugar conversion due to the native recalcitrant of plant cell wall, the degradation products generated during pretreatment processes strongly inhibit microorganisms and impede ethanol yield [40]. Under acidic conditions, carbohydrates present in the biomass degrade into furfural or hydroxymethylfurfural and the lignin degrades into a variety of phenolic compounds [75]. Instead, AFEX process produces many ammoniated compounds, which are significantly less inhibitory than their acid counterparts [29, 140]. However, the efficiency of sugar utilization in ethanol production of AFEX pretreated biomass hydrolysates (enriched in both pentoses and hexoses) still requires improvement. One of the major issues faced during mixed hexose/pentose fermentation is the low xylose consumption rate, which

is related to the presence of pretreatment-derived biomass decomposition products, ethanol, and other fermentation metabolites [37, 122, 128, 140]

1.1 Objectives

The goal of this dissertation is to understand the limiting factors that contribute to unconverted carbohydrates in bio-based chemical industry, and provide information for the rational design of enzyme cocktail, fermentation and pretreatment process adjustments.

Objective 1: Understanding the mechanism for recalcitrant carbohydrates in UHS and hydrolysate, by analyzing their sugar compositions, mass distribution and recalcitrance, studying their effect on enzyme inhibition, to help provide information for novel enzyme discovery and development.

Objective 2: Focusing on the structure determination and method development small-molecule water-soluble oligosaccharides, which have been overlooked in the past studies, but have great potential to be easily converted to fermentable sugar.

Objective 3: Using Synthetic Hydrolysate (SynH) to identify key inhibitors produced in optimized extractive ammonia process, to help optimize fermentation and pretreatment processes.

CHAPTER 2

LITERATURE REVIEW

Cell walls provide essential functions that are important for structure, intercellular communication, defense responses, growth, and development in plants [73]. Cell walls are also dynamic in that they change in their structure and composition depending on the tissue and cell type being examined, its developmental stage, and other biotic and abiotic factors. Plant cell walls are the major component of plant biomass and its natural resistance to biodegradation, otherwise termed "cell wall recalcitrance ". This is a major hurdle that must be overcome if lignocellulosic biomass is to be used effectively for biofuel production [89, 156]. Thus, understanding the features of plant cell walls that underlie recalcitrance is of prime interest to cell wall researchers. Such an understanding will facilitate fine-tuning the properties of plant biomass and optimizing biomass processing in order to more efficiently and cost-effectively break down the biomass to its component sugars for subsequent conversion to biofuel and other valuable products.

2.1 Compositional and structural complexity of plant cell wall

Plants are composed of at least 35 different cell types that are distinct in composition, structure, and ultrastructure [32]. However, all cells have a 0.1 to 10 um cell wall that provides rigidity to the cell and prevents attack by pathogens. Cell walls typically are composed of three layers, the middle lamella, primary cell wall, and secondary cell wall. Secondary cell walls, which have further sublayers (S1, outer; S2, middle; and S3, inner), are present only in certain tissues (e.g., thickened cells that constitute the vascular bundles) and mature generally after cessation of growth, unlike the primary walls that are ubiquitous to all cells. Composition of cell wall changes from species to species, and different stages of their growth. Cellulose (20-50% on a dry weight basis), hemicellulose (15-35%), and lignin (10-30%) are the primary constituents of cell walls, whereas proteins (3-10%), lipids (1-5%), soluble sugars (1-10%), and minerals (5-10%) are minor components [108]. This chemical composition of cell walls differs significantly between monocots.

Cellulose is a complex macromolecule composed of linear β-1,4-glucan chains that tightly aggregate into microfibrils (3 to 5 nm in diameter) held together via strong intra- and intermolecular

hydrogen bonds and van der Waals forces resulting from pyranose ring stacking. The degree of polymerization of cellulose varies, depending on its source, between 100 and 10,000 [101, 138]. Native cellulose is degraded to a length of approximately 150 nm fairly rapidly, beyond which severe chemical or enzymatic treatment is necessary to hydrolyze it completely [97]. The steric hindrance of glucan chains packed tightly in this solid, crystalline morphology is responsible for the low saccharification rate of cellulose [165]. The most abundant crystalline polymorph found in higher plants is cellulose Iβ, which has a two-chain monoclinic unit cell [98]. Thermochemical treatments can transform cellulose Iβ into other polymorphs [101], namely, cellulose II by NaOH [148], cellulose III by amines or ammonia [63, 147], and cellulose IV by glycerol [50]. Differences in glucan chain packing in these polymorphs have been shown to influence their hydrolysis rates [26, 63, 148]; however, a molecular-level explanation of the observed differences in digestion rates between cellulose polymorphs remains an open question.

Hemicelluloses are polysaccharides that are extractable by alkaline solutions. In contrast, pectins are a major component of the compound middle lamella that can be extracted with hot water and chelating agents. We now know that these complex heteropolysaccharides can be classified into four structurally distinct classes: (a) xylans(β-1,4-xylosylbackbone with arabinose, uronic acid, and acetyl side chains), (b) mannans (β-1,4-mannosyl or glucosyl-mannosyl backbones with galactose side chains), (c) β-glucans with mixed linkages (β-1,3-1,4-glucosyl backbone), and (d) xyloglucans (β-1,4-glucosyl backbone with xylose side chains) [8]. Unlike cellulose, hemicellulose composition varies depending on cell tissue and plant species and differs in type of glycosidic linkages, side chain composition, and degree of polymerization [42, 66].The most abundant hemicelluloses found in monocots (e.g., corn stover, switchgrass) and dicots are glucuronoarabinoxylans and galactoglucomannans, respectively.

Lignins are complex, phenyl-propanoid polymers derived from three basic monomeric units (monolignols): p-hydroxyphenyls (H), guaicyls (G), and syringyls (S), which vary between species and cell tissue type [13, 53]. Lignin structures are hypothesized to arise from free-radical polymerization of phenoxy radicals (β-O-4-linked aryl ether linkages are most common) formed by oxida-

tive enzymes in the cell wall [13]. Hardwood (e.g., poplar, aspen, oak) lignins are predominantly G and S monolignols with trace amounts of H units. Softwood (e.g., cedar, pine, spruce) lignins are composed of mostly G units, whereas monocots (e.g., corn, swichgrass, wheat) incorporate equivalent amounts of G and S units along with significantly higher amounts of H monolignols.

The composition and structural features of plant cell walls are so complex that the structural make up of cell walls varies in different plants, tissue and cell types, and even at subcellular levels [73, 109]. Many of the polysaccharides found in plant cell walls are themselves highly heterogeneous in their composition. Multiple structural forms have been demonstrated for most of the major cell wall hemicellulosic polysaccharides [126, 161], pectic polysaccharides [95], and arabinogalactan glycoproteins [76].

2.2 Novel immunological method for cell wall characterization

Cell walls play important roles in the structure, physiology, growth, and development of plants [24]. Plant cell wall materials are also important sources of human and animal nutrition, natural textile fibers, paper and wood products, and raw materials for biofuel production [137]. Many genes are thought to be responsible for plant wall biosynthesis and modification have been identified [20, 85, 95], and 15% of the *Arabidopsis (Arabidopsis thaliana)* genome is likely devoted to these functions [23]. However, phenotypic analysis in plants carrying cell wall-related mutations has proven particularly difficult. First, cell wall-related genes are often expressed differentially and at low levels between cells of different tissues [124]. Also, plants have compensatory mechanisms to maintain cell wall function in the absence of a particular gene [138]. Thus, novel tools and approaches are needed to characterize plant cell wall structures and the genes responsible for their synthesis and modification.

2.2.1 Monoclonal antibodies and glycome profiling

Monoclonal antibodies (mAbs) developed against cell wall polymers have emerged as an important tool for the study of plant cell wall structure and function [77]. Previous studies have utilized mAbs that bind epitopes present on rhamnogalacturonan I (RG-I) [2, 30, 44, 68, 92, 152], homogalacturonan [31, 153], xylogalacturonan [155], xylans and arabinoxylans [91], xyloglucan

[43, 44], arabinogalactan(protein) [39, 110, 115, 136], and extensins [135] to localize these epitopes in plant cells and tissues. In addition, mAbs have been used to characterize plants carrying mutations in genes thought to be associated with cell wall biosynthesis and metabolism [25, 99, 111, 129, 162]. Despite their utility, the available set of mAbs against carbohydrate structures is relatively small given the structural complexity of wall polymers [119], and knolwillats1998generationwledge of their epitope specificity is limited. Thus, additional mAbs specific to diverse epitope structures and methods for rapid epitope characterization are needed [138].

2.2.2 Biotinylation: Derivatization for studying carbohydrate-protein interactions

One way of optimizing mAbs-based Elisa assay is the derivatization of substrates to improve or enable effective binding with the plate. Biotin has been widely used for investigating receptor-ligand interactions [58, 151]. The use of biotin for labeling biological compounds was first described for localizing membrane proteins by electron microscopy, making use of the very high affinity of avidin for biotin [54]. It has since been used to study the expression and cellular distribution of membrane proteins and glycoproteins, thereby largely replacing the hazardous radioactive iodine labeling. A number of biotinylation procedures in which the label is attached to carbohydrates using oxidative methods have been developed [10, 100]. These methods, however, affect the structural integrity of the glycan and therefore may influence the biological function of carbohydrates and their binding properties to lectins. During recent years, a number of non-destructive biotinylation procedures involving the coupling of a biotin group via an amine- or hydrazide-containing linker to the reducing end of purified or synthesized carbohydrates have been described [64, 86, 120, 133, 141, 157]. In the first article describing such a method, the label BAP, containing both a biotin and a fluorescent group, was coupled via reductive amination to the reducing ends of carbohydrates and permitted detection of carbohydrates during HPLC analysis [120, 141]. A drawback of this method, however, is the elaborate preparation of BAP, which is synthesized by coupling biotin-N-hydroxysuccinimide ester (NHS) to diaminopyridine (DAP), requiring a preparative chromatography step to separate the formed label from the reactants. Also, the coupling efficiency of BAP to carbohydrates was rather low. Recently, an alternative to this method in which

DAP is first coupled to the reducing ends of carbohydrates was presented. Sample purification is obtained by paper chromatography. In a second reaction step, DAP-labeled carbohydrates were coupled to biotin-NHS and purified by HPLC [157]. A much-simplified procedure was developed using the label BNAH, which was coupled to carbohydrates in a one-step reaction, eliminating the elaborate label synthesis. Sample purification again was performed by HPLC [86]. This label showed excellent properties in studying carbohydrate-protein interactions.

2.3 Incomplete sugar conversion in lignocellulosic biorefinery

Incomplete sugar conversion is one major bottleneck in ligonocellulosic biorefinery due to the recalcitrance of cell wall polysaccharides to enzymes during saccharification. A recent study by Uppugundla et al. showed that inefficient sugar conversion is a problem for various thermochemical pretreatment technologies, including AFEX, dilute acid, and ionic liquid pretreatments [142]. Using the advanced commercial cocktails containing Cellic Ctec2, Cellic Htec2, and Multifect Pectinase with optimized ratio, nearly 22% of total carbohydrates from AFEX-pretreated biomass were left behind as polymeric and oligomeric sugars after seven days of hydrolysis, at high enzyme loading (20 mg protein/g glucan) and solids loading (6% glucan loading). Since the sugar polymers are not completely converted to monomeric sugars, the biofuel production potential is significantly reduced, which negatively impacts the overall economics of the biorefinery [81]. This is a universal problem faced by researchers when using hardwood and agricultural residues/grasses [142]. Recalcitrant cell wall polysaccharides not only resistant to commercial enzymes, they also block the accessibility of cellulases to cellulose.

2.3.1 Oligosaccharides accumulation cause sugar loss in biorefinery

Oligosaccharides are generated by incomplete digestion of glucan or xylan sugar polymers by water-soluble endo- and exo-enzymes. Most oligosaccharides are ultimately converted to glucose or xylose by β-glucosidase and β-xylosidase [46, 48] . However, some oligomeric sugars are not converted and thus accumulate in the hydrolysate. Oligosaccharides are produced by AFEX pretreatment and during high solid loading enzymatic hydrolysis [29, 51]. Gluco-oligosaccharides (DP 2-24) were found in AFEX corn stover (ACS) water extracts [146]. When pre-soaked wheat

straw was treated with pressurized water at 195 °C for 12 min at a water-straw ratio of 5:1, most hemicelluloses were solubilized into water-soluble oligosaccharides as a mixture of xylo-oligomers and gluco-oligomers with DP ranging from 7-16 [79]. The soluble oligosaccharides present in switchgrass hydrolysates produced using commercial enzyme cocktails were a combination of both linear (gluco-) and branched (xylo-) oligomers, with DP ranging from 2-6 [12]. These unconverted oligosaccharides in hydrolysate reduce biofuel yields because most industrial ethanol-producing strains, including yeast and bacteria, consume only monomeric sugars (glucose and xylose) and do not have machinery to use oligomeric sugars [3, 45, 52, 140].

2.3.2 Mechanistic understanding of oligosaccharide accumulation

The exact reasons for oligosaccharide build-up in high-solids loading (HSL) hydrolysis remain open, but there are several possible mechanisms to explain this phenomenon. Monomeric sugars (glucose, xylose, mannose, galactose, etc.) and cellobiose have been determined to exert strong end-product inhibition on cellulase and β-glucosidase [74, 81], and oligomeric sugars (xylo-oligomers and gluco-oligomers) can inhibit cellulases during hydrolysis even more strongly [79, 82, 117]. Accumulation of xylo-oligomers reduces ethanol yields by inhibiting cellulase enzymes, especially CBH I and CBH II [9, 164], thereby reducing cellulose hydrolysis to glucose. Competitive inhibition of cellulases by high DP xylo-oligomers is greater than other end products (glucose, xylose) [30]. Oligosaccharides with a DP ranging from 7 to 16 resulting from wheat straw hydrothermal pretreatment are approximately 100-fold stronger inhibitors of *Trichoderma reesei* CBHs than cellobiose [79]. A possible explanation was proposed by the author that the xylo-oligomers and gluco-oligomers may mimic the structure of the cellulose chain and bind to more glucose unit binding sites in the active site tunnel than cellobiose. Alternatively, the presence of side-chain substituents on arabinoxylan, including acetyl, arabinofuranosyl and glucopyranosyl uronic acid, may hinder the formation of enzyme-substrate complexes, and thus impede enzymatic hydrolysis [158]. Current commercial enzyme cocktails require accessory enzymes that can cleave these linkages [96]. Such accessory enzymes including glucuronidases, β-xylosidases, α-l-arabinofuranosidases and acetyl esterases are essential in achieving complete degradation of

heteroxylans [14, 16, 48, 83, 144, 158].

2.3.3 Oligosaccharide purification and characterization

Other studies have explored oligosaccharide recalcitrance, including the purification and/or characterization of oligosaccharides [14–17, 33, 150]. Neutral gluco-oligosaccharides in ACS water extracts were enriched by solid-phase extraction (SPE), followed by high-performance liquid chromatography (HPLC) separation and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) [146]. High-purity xylo-oligosaccharide fractions with DP ranging from 2 to 14 were isolated from hydrothermal pretreatment hydrolysate of birchwood xylan by gel permeation chromatography [88]. For structural elucidation on the chromatographic time scale, nonselective multiplexed collision-induced dissociation was performed for quasi- simultaneous acquisition of oligosaccharide molecular and fragment masses in a single analysis [146]. Hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS) was successfully used to characterize reducing end-labelled xylo-oligosaccharides [15]. Feruloylated xylo-oligomers from thermochemically treated corn fibre were pooled and fractionated by a solid-phase C-18 column and a Bio-Rad P2 gel column and further purified with reverse-phase high-performance liquid chromatography (RP-HPLC). Electrospray ionisation mass spectrometry (ESI-MSn) and nuclear magnetic resonance (NMR) were then used for structure elucidation. Interestingly, several oligosaccharide analogues contained an α-L-galactopyranosyl-(1-2)-β-D-xylopyranosyl-(1-2)-5-O-trans-feruloyl-l-arabinofuranose side chain attached to the O-3 position of a xylose comprising the β-1-4 linked backbone [45]. Arabino-xylooligomers derived from switchgrass xylan were characterised by RP-HPLC-MSn [14]. Side chain substitutions of (1-2)-β-xylose-(1-3)-α- arabinose were identified from the products of swichgrass xylan hydrolysis using commercial enzymes with supplementation of α-arabinofuranosidase, indicating that required activity for this linkage is lacking from commercial enzyme preparations [17]. With all these research efforts, however, only a few studies have focused on the nature of oligosaccharide buildup during HSL hydrolysis in terms of release of oligomeric sugars, changes in the oligomer chain length during the course of hydrolysis, ratios of different DP oligomers and their distribution profiles [12, 116, 146].

CHAPTER 3

SEPARATING OLIGOSACCHARIDES AND EVALUATING THE SUGAR LOSS AND ENZYME INHIBITION

3.1 Large scale oligosaccharides separation and understanding the accumulation mechanism

Accumulation of recalcitrant oligosaccharides during high-solids loading enzymatic hydrolysis of cellulosic biomass reduces biofuel yields and increases processing costs for a cellulosic biorefinery. Recalcitrant oligosaccharides in AFEX-pretreated corn stover hydrolysate accumulate to the extent of about 18-25% of the total soluble sugars in the hydrolysate and 12-18% of the total polysaccharides in the inlet biomass (untreated), equivalent to a yield loss of about 7-9 kg of monomeric sugars per 100 kg of inlet dry biomass (untreated). These oligosaccharides represent a yield loss and also inhibit commercial hydrolytic enzymes, with both being serious bottlenecks for economical biofuel production from cellulosic biomass. Very little is understood about the nature of these oligomers and why they are recalcitrant to commercial enzymes. This chapter presents a robust method for separating recalcitrant oligosaccharides from high solid loading hydrolysate in large quantities. Composition analysis, recalcitrance study and enzyme inhibition study were performed to understand their chemical nature.

3.1.1 Abstract

Oligosaccharide accumulation occurs during high solid loading enzymatic hydrolysis of corn stover (CS) irrespective of using different pretreated corn stover (dilute acid: DA, ionic liquids: IL, and ammonia fibre expansion: AFEX). The methodology for large-scale separation of recalcitrant oligosaccharides from 25% solids-loading AFEX-CS hydrolysate using charcoal fractionation and size exclusion chromatography is reported for the first time. Oligosaccharides with higher degree of polymerization (DP) were recalcitrant towards commercial enzyme mixtures [Ctec2, Htec2 and Multifect pectinase (MP)] compared to lower DP oligosaccharides. Enzyme inhibition studies using processed substrates (Avicel and xylan) showed that low DP oligosaccharides also inhibit commercial enzymes. Addition of monomeric sugars to oligosaccharides increases the inhibitory

effects of oligosaccharides on commercial enzymes.

The carbohydrate composition of the recalcitrant oligosaccharides, ratios of different DP oligomers and their distribution profiles were determined. Recalcitrance and enzyme inhibition studies help us to determine whether the commercial enzyme mixtures lack the enzyme activities required to completely de-polymerize the plant cell wall. Such studies help us to understand the reasons for oligosaccharide accumulation and contribute to strategies by which oligosaccharides can be converted into fermentable sugars and provide higher sugar conversion and biofuel yields with less enzyme.

3.1.2 Background

Concerns about energy security and environmental problems due to petroleum consumption provide an impetus to transition from the current fossil fuel system to a more sustainable energy system [36]. Nonfood plant biomass or cellulosic biomass, including agricultural residues (e.g., corn stover (CS), wheat straw), perennial grasses (e.g., switchgrass, miscanthus), forestry residues, herbaceous and woody crops, is the most abundant source that could be used as potential feedstock for producing renewable liquid fuels [89]. Since cellulosic biomass is likely to play an important role in future energy portfolios, numerous research efforts are underway globally to economically produce biofuels.

Nevertheless, several major issues impede the successful commercialization of cellulosic biomass conversion to liquid fuels [6]. These barriers include cost of biomass degrading enzymes, incomplete conversion of biomass to fermentable sugars and low ethanol productivity [19, 90, 163]. Cellulose and hemicelluloses in the plant cell wall are embedded in a complex matrix with lignin which make biomass highly recalcitrant [28]. Thus, numerous pretreatment technologies have been developed to overcome biomass recalcitrance, such as, hot water, steam explosion, lime, phosphoric acid, dilute acid (DA), ammonia [such as, AFEX, soaking in aqueous ammonia (SAA) and ammonia recycled percolation (ARP)] and ionic liquid (IL)-based pretreatments [34, 51]. DA, IL and AFEX pretreatments are of the most leading technologies and core programms studied at the three US Department of Energy (DOE) funded bioenergy research centers, namely Great Lakes

Bioenergy Research Center (GLBRC), Joint Bioenergy Institute (JBEI) and the BioEnergy Science Center (BESC). Considerable research has been done to streamline these processes to make them cost effective. In the previous work, comparative studies of commercial enzyme mixture optimization and ethanol production of DA, IL and AFEX-pretreated corn stover have been performed to improve downstream processing conditions and to understand factors contributing to the glucose and xylose yields during enzymatic hydrolysis [49, 142]. To reduce the cost in cellulosic ethanol production, high sugar concentrations are required to achieve high ethanol titers and to reduce the energy required to distill the ethanol from fermentation broths [69, 70]. However, as the solids loading increases, the viscosity of the hydrolysate also increases, contributing to mixing and mass transfer problems that reduce sugar conversion [4]. High solids loadings also leads to unproductive binding of enzyme to substrate [47] and product inhibition [56, 81], which are stumbling blocks for converting biomass to high concentrations of fermentable sugars.

For the first time, we present a methodology for large-scale purification of recalcitrant oligosaccharides from AFEX treated corn stover hydrolysate (ACSH) prepared at high solids loading. Composition analysis, recalcitrance studies, and investigating enzyme inhibition by recalcitrant oligosaccharide on commercial enzyme cocktail have helped us define the characteristics of the mechanism of oligosaccharide accumulation.

3.1.3 Materials and Methods

See Appendix A.1 - A.13.

3.1.4 Results and Discussions

3.1.4.1 Oligosaccharides accumulation

Glucan and xylan conversion of CS using three different leading pretreatment technologies (dilute acid: DA, ionic liquids: IL, ammonia fiber expansion: AFEX) under commercial enzymes treatment were tested to see if oligosaccharides accumulation is a universal phenomenon or pertains only to particular pretreatments (Figure 3.1). The optimal commercial enzyme combinations (Ctec2: Htec2: Multifect Pectinase) for different pretreated biomass was shown to be different in

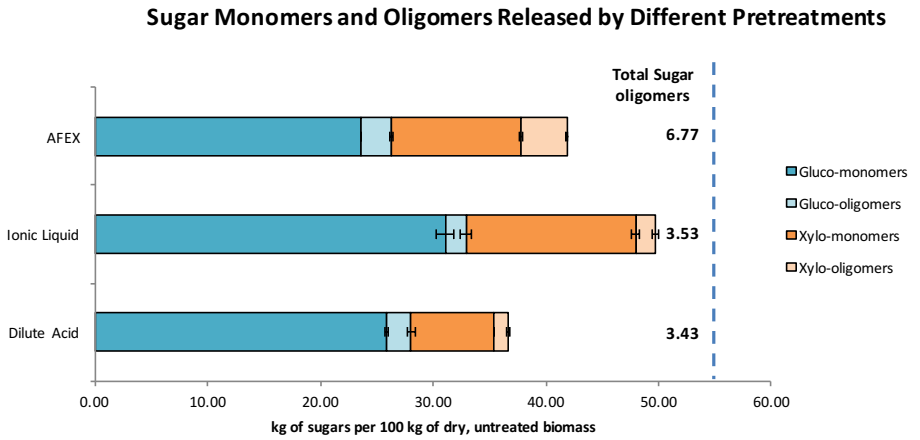


Figure 3.1 Sugar monomers and oligomers released by different pretreatments (dilute acid: DA, ionic liquid: IL, ammonia fiber expansion: AFEX). Enzymatic hydrolysis conditions: 6% glucan loading, 72 hrs hydrolysis, 15 mg/g enzyme loading (Ctec2: Htec2: MP - 1:1:1).

our previous work [142]. Nevertheless, to reduce factors that might affect the sugar conversion and have a comparative results between three different pretreatment methods, we chose 1:1:1 (percentage total protein loading basis) for our experiments. Regardless of the pretreatment method used, 3.4 - 6.1% of sugars (glucose and xylose) in dry untreated corn stover accumulated as oligomers. These recalcitrant oligosaccharides are not converted to fermentable sugars, and thereby reduce biofuel yields. The oligosaccharides also inhibit both fermentation rate and biomass hydrolysis as stated above. AFEX is a thermochemical biomass pretreatment that cleaves the lignin-carbohydrate complex (LCC) linkages, and improves the digestibility of biomass by relocating lignin and creating pores [7, 27]. It is a dry-to-dry process without any washing step, and no exogenous nutrition supplementation is needed for downstream fermentation. Nevertheless, highest content of oligosaccharides (gluco-oligomers and xylo-oligomers) were produced during AFEX pretreatment (Figure 3.1). Therefore, rather that handling different pretreated biomass, we decided to choose AFEX pretreated corn stover to broadly represent alkaline pretreatment process and try to have more in-depth understanding of recalcitrant oligosaccharides produced during enzyme hydrolysis. We chose to use ACSH produced under high solids-loading (25%) enzymatic hydrolysis to study the accumulation profile of recalcitrant oligosaccharides and their structure characterization.

As more than 25% of the initial xylan content remains as xylo-oligomers after enzymatic

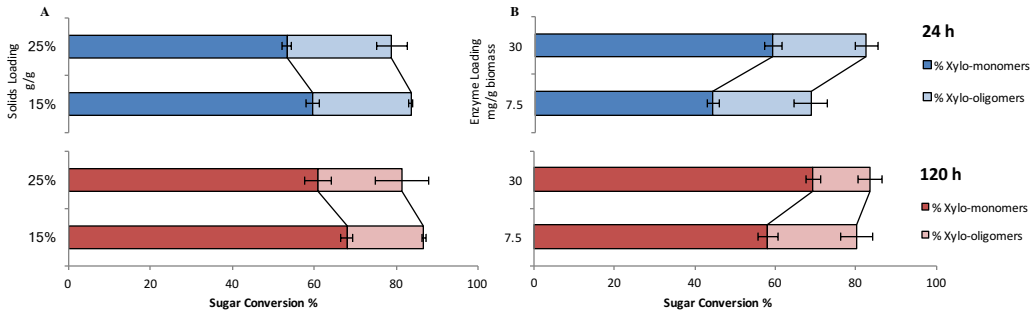


Figure 3.2 Effect of solids loading and enzyme loading on xylo-oligomers accumulation in ACS enzymatic hydrolysis. Here, A, Enzyme loading was fixed at 18.75 mg/g glucan (optimized mixture, Ctec2: Htec2: MP - 1:1:1). Two solids loadings (15% and 25%) were compared at 24 h and 120 h; and B, Solids loading was fixed at 20%. The effects of two enzyme loadings (7.5 mg/g glucan and 30 mg/g glucan, Ctec2: Htec2: MP - 1:1:1) were compared at 24 h and 120 h.

hydrolysis of ACS while gluco-oligomers accumulate (<10%) to a lesser extent, different solids loadings (Figure 3.2-A) and enzyme loadings (Figure 3.2-B) were tested for their effects on xylo-oligomer accumulation. Here solids loading (g/g) is defined by the amount of dry material that enters the process divided by the total mass of material and water added to the material [94], and enzyme loading (mg/g) is defined as the amount of protein added in the process divided by the amount of glucan in pretreated biomass. Sugar conversion is defined as the amount of xylose or xylo-oligomers produced after enzymatic hydrolysis divided by the amount of xylan in ACS (AFEX-pretreated corn stover). In Figure 3.2-A, approximately 80% of total xylan conversion was achieved in the first 24 h at both 15 and 25% solids loadings with 18.75 mg/g enzyme loading. This value does not change significantly after 120 h of enzymatic hydrolysis. The monomeric xylose content increased by 7-10% for both solids loadings when the hydrolysis time was extended from 24 h to 120 h. For 15% solids loading, the xylo-oligomers content decreased from 24% to 18% after 120 h hydrolysis. At 25% solid loading, xylo-oligomers content decreased from 28% to 24% after 120 h hydrolysis, indicating that more xylo-oligomers accumulated in high solids loading enzymatic hydrolysis. The effect of enzyme loadings at a fixed solid loading (20%) was also tested (Figure 3.2-B). At 20% solids loading, enzymatic hydrolysis with 7.5 mg/g enzyme loading achieved 70% xylan conversion, while 30 mg/g enzyme loading achieved over 80% in the first 24 h. At 120 h, 7.5 mg/g of enzyme achieved nearly the same overall conversion (80%) as did under

Table 3.1 Sugar monomers and oligomers concentration in 25% solids loading ACS hydrolysate and inlet untreated biomass. Oligomeric sugar percentage in soluble sugar is based on individual sugar (glucose, xylose and arabinose), while the oligomeric sugar percentage in total sugar is based on the sum of all three sugars.

	Glucose	Xylose	Arabinose	**Total Sugar**
Monomers (g/L)	77	43	6	**126**
Oligomers (g/L)	8	14	3	**25**
Oligo percentage in soluble sugar	9%	25%	33%	**17%**
Oligo percentage in soluble sugar	2%	4%	1%	**7%**

the higher enzyme loading (83%). However, higher level of xylo-oligomers (22%) is still present at 7.5 mg/g enzyme loading than at 30 mg/g enzyme loading (13%). As more xylo-oligomers accumulate at the high solids loading, we chose 25% solids loading (equivalent to 7.9% glucan loading) ACSH to produce oligosaccharides in a larger scale.

3.1.4.2 Methodology and mass balance for oligosaccharides purification

To better understand these oligosaccharides, the first step is to separate them from the hydrolysate by removing monomeric sugars, proteins, salts and other lignin degradation products present (Figure 3.3). High solids loading (25%, i.e., 7.9% glucan loading) ACS hydrolysate was prepared for large-scale separation of oligosaccharides. Enzymatic hydrolysis of ACS was performed using a commercial enzymes mixture including Ctec2, Htec2 and Multifect Pectinase (20 μg protein/mg glucan, 2:1:1 ratio). After 96 h hydrolysis, the hydrolysate was centrifuged, filtered and stored at 4 °C prior to charcoal fractionation. Oligosaccharides analysis were performed to determine the composition of glucose, xylose and arabinose. The composition of sugar monomers and oligomers in 25% solids loading ACSH is shown in Table 3.1. The soluble sugar percentage is the weight percentage of oligomers in each individual sugars (monomers plus oligomers), and the biomass percentage is the weight percentage of oligosaccharides based on starting material (ACS).

An activated charcoal and celite column was used to adsorb oligosaccharides from 25% solids loading ACS hydrolysate [1, 93]. After oligosaccharides being fully adsorpted, most neutral oligosaccharides was eluted from the charcoal/celite column by using 50% acetonitrile (v/w), followed by desorption of more acidic oligosaccharides using 50% acetonitrile containing 1%

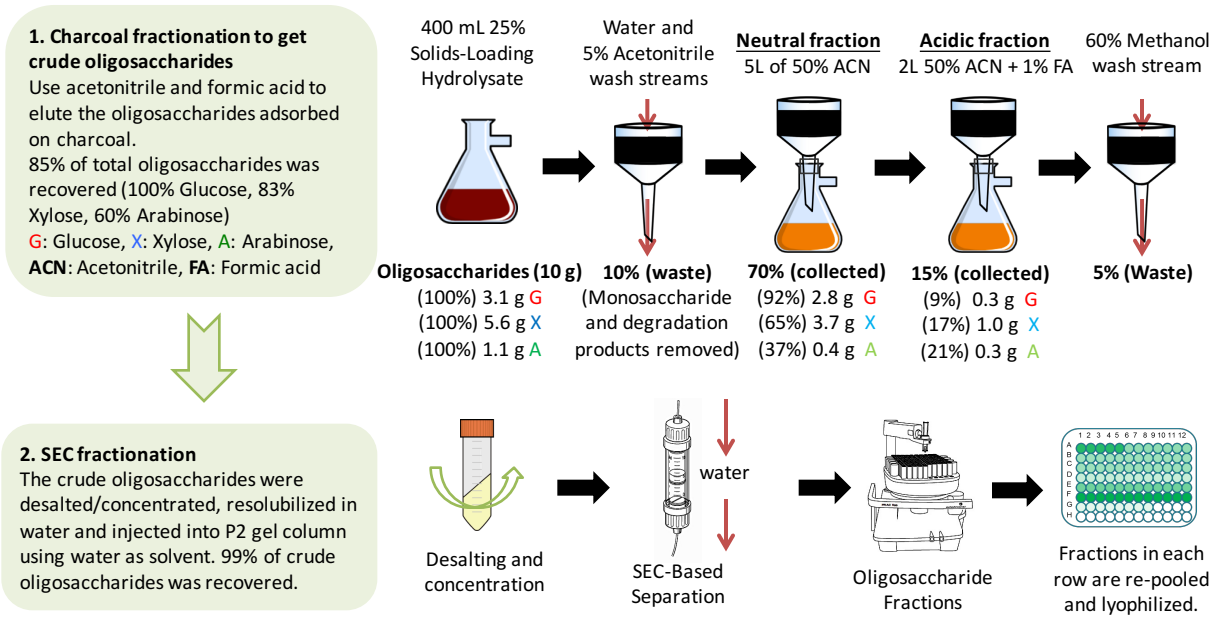


Figure 3.3 Mass balance and methodology of oligosaccharides separation. Oligosaccharides were separated from ACSH by activated charcoal bed and further fractionated by size exclusion chromatography (SEC). Dilute sulfuric acid hydrolysis was performed for sugar composition and mass balance. G: Glucose; X: Xylose; A: Arabinose, ACN: acetonitrile, FA: Formic acid.

formic acid (v/w). The neutral and acidic oligosaccharide elution streams were collected as crude oligosaccharide fractions, and were processed for further fractionation by size exclusion chromatography (SEC). Crude oligosaccharides were concentrated to remove the organic solvent and additives prior to SEC fractionation. The concentrated fractions were re-suspended in water (50 mL of neutral fractions/55 mL of formic acid fractions) and used for sugar compositional analysis by acid hydrolysis, recalcitrance studies, and SEC fractionation. A mass balance for oligosaccharides separated in the process and sugar recovery were calculated based on these acid hydrolysis results.

The concentrated neutral and acidic fractions were further fractionated based on their molecular weight using SEC. Five mL of concentrated neutral fractions (containing 670 mg oligosaccharides) and acidic fractions (containing 140 mg oligosaccharides) were injected into the SEC column separately. About 90 fractions (A1-12, B1-12, C1-12, D1-12, E1-12, F1-12, G1-12, H1-6, 10 mL in each tube) were collected from each run and characterized using acid hydrolysis and enzyme digestion assays. Fractions in the same row were re-pooled and lyophilized to produce dry samples

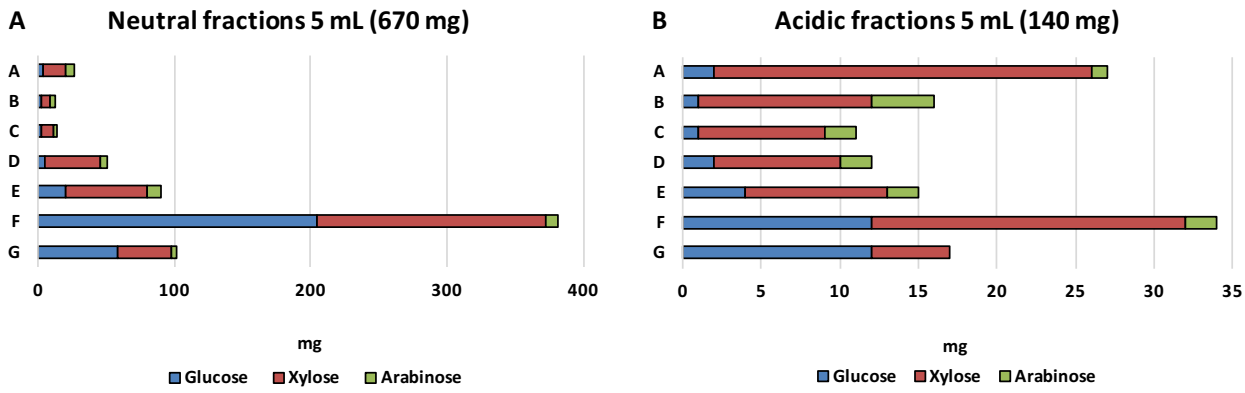


Figure 3.4 Mass distribution profile and monomeric compositions of SEC oligosaccharides fractions. Here, 5 mL of concentrated (A) neutral fractions containing 670 mg oligosaccharides and (B) acidic fractions containing 140 mg oligosaccharides were injected into the SEC column. Fractions were re-pooled from row A to G and lyophilized. G: Glucose; X: Xylose; A: Arabinose.

A to G (H fractions are predominantly monomers according to composition analysis). Mass distribution of a total of 14 pooled samples was generated (Figure 3.4, row A-G for both neutral and acidic oligosaccharides). The composition of oligosaccharides separated using SEC fractionations were also analyzed in the same way as crude oligosaccharides.

A complete mass balance was carried out following the charcoal fractionation process (Figure 3.3 and Figure 3.4). Using this oligosaccharide separation process we were able to separate 400 mL of 25% solids loading hydrolysate containing about 10 g of mixed oligosaccharides (in accordance with the sugar concentration in Table 3.1) in one activated charcoal bed. About 15% (1.5 g) of 10 g oligosaccharides in the hydrolysate were lost due to co-elution with monomeric sugars and irreversible binding to the charcoal matrix. About 85% (8.5 g) of oligosaccharides were recovered containing 6.9 g neutral oligosaccharides and 1.7 g acidic oligosaccharides. Acid hydrolysis showed that acidic oligosaccharides had higher xylose content (63% vs. 54%) and arabinose content (19% vs. 6%) than neutral oligosaccharides. After SEC fractionation, more than 99% of these crude oligosaccharides were recovered The mass distribution profile in Figure 3.4 shows that most neutral fractions were enriched in low DP oligosaccharides (row F and G) while the acidic fractions were distributed more evenly in high and low DP (row A and F) oligosaccharides. Acid hydrolysis revealed that high DP oligosaccharides (A, B, C rows) contain more xylose than low

DP oligosaccharides (F, G rows) for both neutral and acidic fractions.

3.1.4.3 Enzyme recalcitrance of oligosaccharides

The oligosaccharides extracted from the initial hydrolysate (8.5 g) by charcoal fractionation were further fractionated by SEC chromatography. A series of hydrolysis using commercial enzyme mixtures (Ctec2 + Htec2 + MP) were performed on both charcoal fractionated oligosaccharides and SEC-fractionated oligosaccharides to determine the level of recalcitrance in each fraction (0.5 mL reaction volumes with Ctec2: Htec2: MP in 1:1:1 ratio for 24 h hydrolysis at 50 °C and pH 4.8 and 20 mg/g oligosaccharides enzyme loading). Here, we define recalcitrance as the percentage of oligosaccharides not hydrolysed into monomeric sugars. Figure 3.5-A shows that among the three monomeric sugars (glucose, xylose and arabinose), the gluco-oligomers were most digestible (20%-40% recalcitrance), followed by xylo-oligomers (50%-80% recalcitrance), and arabino-oligomers were found to be highly recalcitrant (85%-95%). Additionally, acidic oligosaccharides (70% recalcitrant) were more recalcitrant than neutral oligosaccharides (49% recalcitrance). The higher recalcitrance of acidic fractions when compared to neutral ones is consistent with a previous study that enrichment of certain substituents (arabinose, acetyl group, glucuronic acid and uronic acid) correlated with increasing recalcitrance characteristic in corn residues [80]. It is reasonable to believe that multiple substitutions on the arbinoxylan backbone, especially in acidic fraction, can hinder the accessibility of oligosaccharides to commercial enzyme mixtures, leading to accumulation of unhydrolysed oligosaccharides in hydrolysate.

Based on the sugar conversion of diluted SEC-fractionated oligosaccharides incubated with commercial enzyme mixtures, we were able to separate the crude oligosaccharides into 3 different groups (Figure 3.5-B). They are: 1) highly recalcitrant (100% recalcitrant, row A, B and C); 2) moderately recalcitrant (30-60% recalcitrant, row D and E); and 3) less recalcitrant (10-20% recalcitrant, row F and G). The highly recalcitrant oligosaccharides accounted for 15% (1.3 g) of the crude oligosaccharides separated from the hydrolysate. The remaining oligosaccharide fractions (7.2 g) were found to be partially digestible when subjected to commercial enzymes (Ctec2, Htec2 and MP). The recalcitrance results from Figure 3.5-B show that more than 80% (w/w) of crude

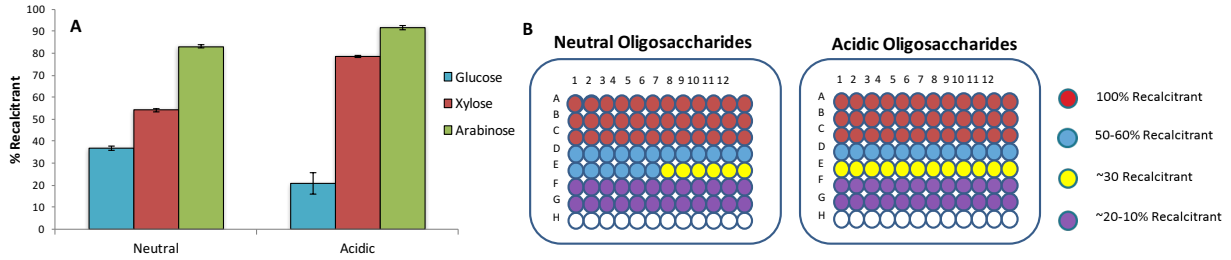


Figure 3.5 Recalcitrance study of neutral and acidic oligosaccharides. Here, (A) recalcitrance of crude oligosaccharides after charcoal fractionation based on monomeric sugar composition, and (B) recalcitrance of oligosaccharides fractions separated by SEC was determined. Enzymatic hydrolysis was performed in 0.5 mL reaction volumes with commercial enzymes (Ctec2: Htec2: MP) in 1:1:1 ratio for 24 h hydrolysis at 50 C. Maximum sugar concentration per well was 3 g/L while the minimum was 0.2 g/L (determined by acid hydrolysis). Enzyme loading is 60 μg/well.

oligosaccharide mixtures can be partially digested after separation from monomeric sugars and being diluted to concentrations lower than 10 g/L. Oligosaccharide accumulation in the hydrolysate may result either from inhibition of glycosyl hydrolases by degradation products produced during pretreatment or oligosaccharides, or because of the high concentrations of monomeric sugars that are produced during enzyme hydrolysis.

3.1.4.4 Enzyme inhibition effect from oligosaccharides

It has been reported that oligosaccharides inhibit commercial enzyme mixtures [74, 79, 82, 117, 164]. To study the inhibition on commercial enzymes by fractionated oligosaccharides with different degree of DPs and degree of recalcitrance, the activities of commercial enzymes on Avicel and beechwood xylan, with and without SEC fractionated acidic or neutral oligosaccharides, were measured with micro-plate assay (enzyme loading of 10 mg/g substrate, i.e., 62.5 μg enzyme per well, for 24 h hydrolysis at 50°C, 10 rpm and pH 4.8, types and ratio of enzymes are stated below). Inhibition tests were performed with varying concentrations of crude oligosaccharides (0.5 g/L to 10 g/L). The enzyme activities in the commercial enzyme preparations are different: Ctec2 is cellulases mixture blended with high level of β-glucosidases and some hemicellulases; Htec2 is a endoxylanase with cellulase background (Novozyme: http://bioenergy.novozymes.com/en/); Multifect Pectinase contains high levels of pectinase, mannanase, and some b-glucosidase [52].

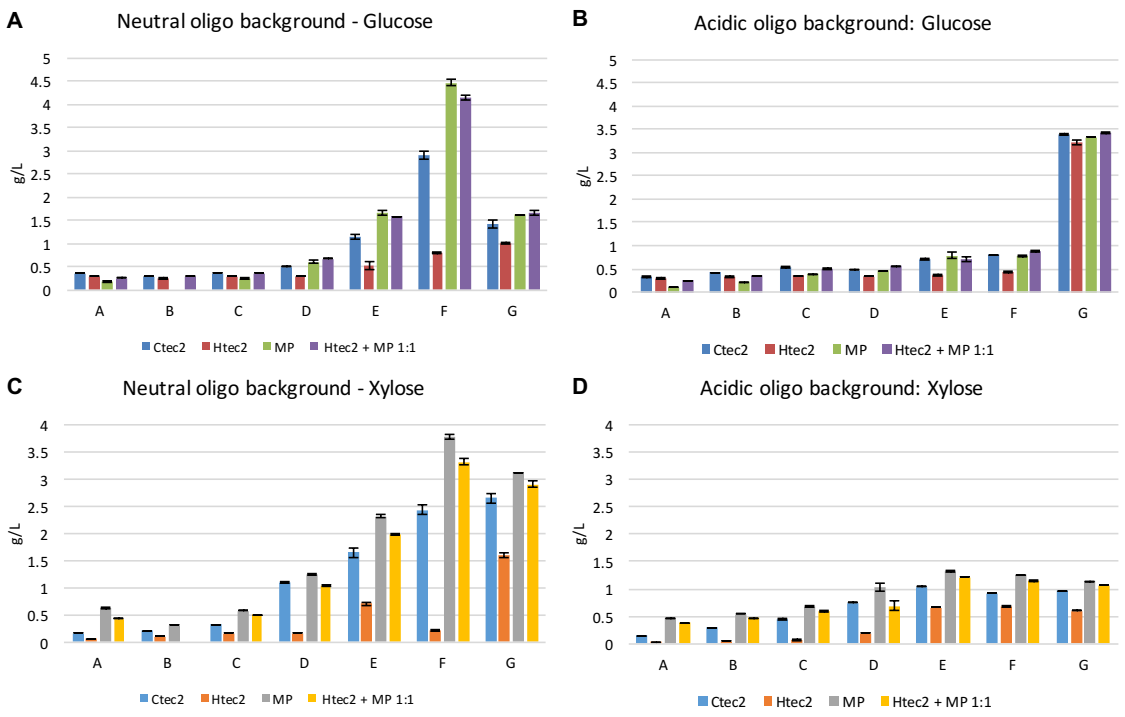


Figure 3.6 Glucose and xylose background for oligosaccharides under commercial enzyme mixtures. Here, clusters labelled with A, B - G represented different fractions after SEC separation. Each fraction was treated with 4 enzyme preparations (Ctec2, Htec2, MP and Htec2+MP 1:1). Microplate enzymatic hydrolysis condition: Pure substrates (Avicel and beechwood xylan) were added in 1.25% solids loading 500 μL reaction volume. Enzyme loading was 10 mg/g substrate with 24 h hydrolysis at 50 degrees celsius, 10 rpm and pH 4.8.

Four groups of inhibition experiments were performed, namely: 1). Enzyme + Substrate (blank control); 2). Enzyme + Substrate + Oligosaccharides (to test the inhibition of oligosaccharides on enzymes); 3). Enzyme + Substrate + Oligosaccharides + Monomeric sugars (to test the inhibition of both oligosaccharides and monomeric sugars on enzymes); 4). Enzyme + Oligosaccharides (for background subtraction). The sugar levels from Group 2 experiments were subtracted by the background oligosaccharides (Group 4) sugar level, and the levels from experiment Group 3 were subtracted by both the oligosaccharides background (Group 4) and the supplemented monomeric sugar levels. The monomeric sugars produced (glucose, xylose and arabinose) in experimental Groups 2 and 3, after subtraction, were compared with control Group 1 (Figure 3.7 and 3.8). If the sugar conversion levels are lower than the control group, the oligosaccharides fraction is considered inhibitory to commercial enzymes.

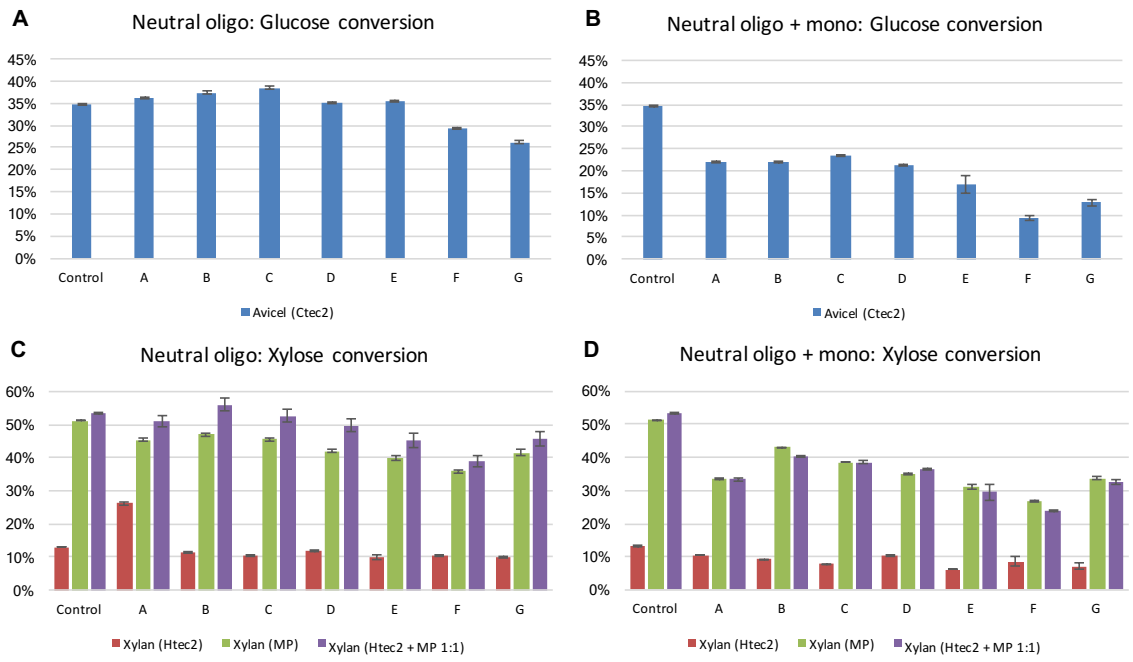


Figure 3.7 Neutral oligosaccharides inhibition on pure substrate enzymatic hydrolysis with or without monomeric sugars inhibition. Here, clusters labelled with A-G represented different pooled fractions after SEC separation. Avicel was treated with Ctec2 for glucose conversion; beechwood xylan was treated with Htec2, MP and Htec2+MP 1:1 for xylose conversion. Microplate enzymatic hydrolysis condition: Pure substrates (Avicel and beechwood xylan) were added in 1.25% solids loading 500 uL reaction volume. Enzyme loading was 10 mg/g substrate with 24 h hydrolysis at 50 degree celsius, 10 rpm and pH 4.8. Oligosaccharides concentration was at 10 g/L; monomeric sugars were at 20 g/L glucose, 10 g/L xylose.

We started with oligosaccharides concentration ranging from 0.1-2.5 g/L and no inhibition was observed on the enzymes; this is not surprising since the concentration of oligosaccharides in high-solids loading ACSH is approximately 20 g/L. Therefore, we tested the inhibitory effect of these oligosaccharides at higher concentrations (10 g/L). The enzyme preparations used for different substrates were also different. Avicel was tested using Ctec2 to test inhibition effect of oligosaccharides on glucose conversion with cellulases; beechwood xylan contains mixture of xylose and glucuronic acid and was treated with Htec2 and MP individually, and then using enzyme mixture of Htec2 and MP in 1:1 mass ratio at 10 mg/g to test the inhibition of oligosaccharides xylose conversion with xylanase, pectinase, and other hemicellulases. Monomeric sugars (20 g/L glucose, 10 g/L xylose) based on 50-60% conversion of xylan and glucan from CS were also added to the system to evaluate the role of monomeric sugars on enzyme inhibition.

The concentration of sugars released at the same oligosaccharide concentration (10 g/L) and the same enzyme dosage (10 mg/g) is a measure of the recalcitrance of the given oligosaccharide mixture (Figure 3.6). The background concentrations of glucose and xylose shown in Figure 3.6 were consistent with the recalcitrance study results showed in Figure 3.5-B. For both neutral and acidic fractions, high DP fractions (A, B and C) are more recalcitrant and low DP oligosaccharides (row E, F and G) could be partially digested to monosaccharides after separation from crude oligosaccharides. We believe that neutral fraction G consists of mostly monomers and degradation products impurities, resulting in a much lower glucose release than fraction F. Arabinose conversion was not shown here because of its low abundance in these oligosaccharides and no conclusions can be drawn due to large standard deviation. For both glucose and xylose conversion, it was clearly shown that acidic fractions are more recalcitrant than the neutral fractions. The resulting glucose conversions for most high to medium DP fractions (row A-F) for acid fractions were less than 1 g/L, Only the lowest DP fraction G is partially convertible (3.5 g/L) (Figure 3.6-B). Interestingly, Multifect Pectinase exhibited higher activity on glucose and xylose conversion than Ctec2 and Htec2. For example, when neutral fraction F was treated with MP, 4.5 g/L glucose was released (4.1 g/L for Htec2 + MP 1:1 treatment), while, only 2.9 g/L glucose was released when treated with Ctec2, and treatment with Htec2 gave the lowest sugar concentration of 0.8 g/L glucose (Figure 3.6-A). Same phenomena can be observed on the xylose release on both neutral and acidic fractions (Figure 3.6-C/D), indicating that Htec2 (mainly endoxylanases) lacks the enzyme activity to digest the unhydrolysed oligosaccharides.

Inhibitory effects from neutral oligosaccharides fractions (Figure 3.7-A/C), and from a combination of oligosaccharides and monomeric sugars (Figure 3.7-B/D) were further studied. The micro-plate activity assays were performed under similar condition as shown in Figure 3.6, with the only difference that both oligosaccharides and monomeric sugars were added to the reaction mixture containing pure substrates and enzymes to test their inhibitory effects. At least four important trends can be deduced from the sugar inhibition profiles given in Figure 3.7. They are: 1). Low DP oligosaccharides were more inhibitory (especially fraction F) than high DP oligosaccharides

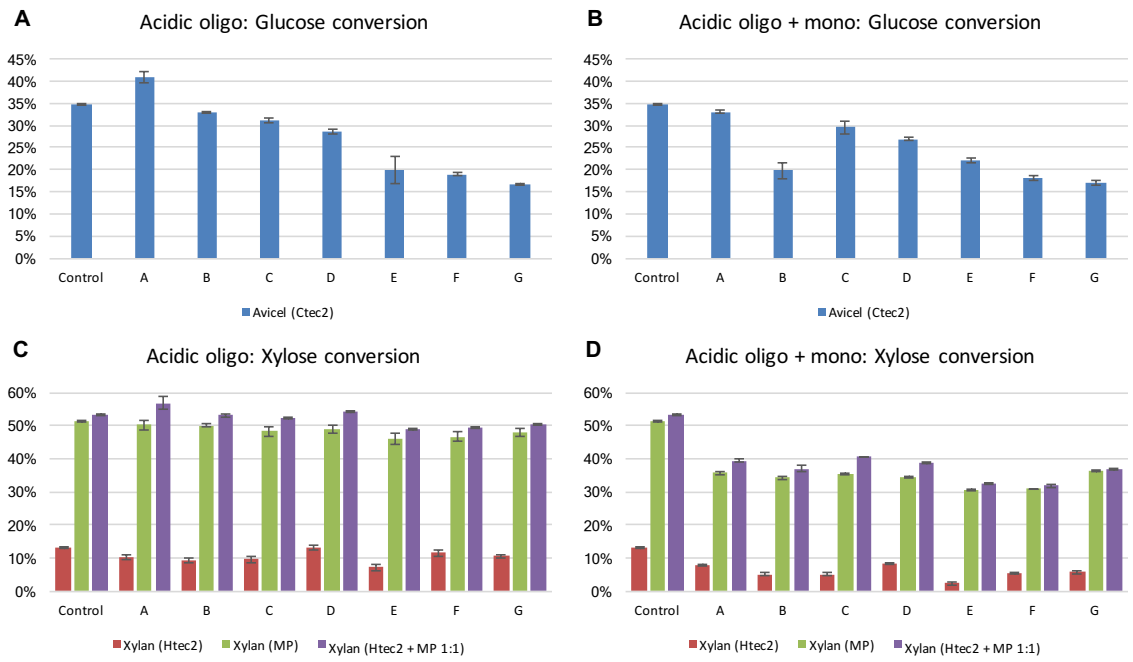


Figure 3.8 Acidic oligosaccharides inhibition on pure substrate enzymatic hydrolysis with or without monomeric sugars inhibition. Here, clusters labelled with A, B - G represented different fractions after SEC separation. Avicel was treated with Ctec2 for glucose conversion; beechwood xylan was treated with Htec2, MP and Htec2+MP 1:1 for xylose conversion. Microplate enzymatic hydrolysis condition: Pure substrates (Avicel and beechwood xylan) were added at 1.25% solids loading 500 uL reaction volume. Enzyme loading was 10 mg/g substrate with 24 h hydrolysis at 50 degree celsius, 10 rpm and pH 4.8. Oligosaccharides concentration was at 10 g/L; monomeric sugars were at 20 g/L glucose, 10 g/L xylose.

on glucose and xylose conversion; 2) Neutral oligosaccharides were more inhibitory toward xylose conversion (Figure 3.7-C) while their inhibitory effect on glucose conversion is not as significant. With neutral fraction F, the xylose conversion of xylan treated with MP is 50% (13% reduction from control) while the glucose conversion of Avicel treated with Ctec2 is 37% (only 6% reduction from control); 3) The activity of Htec2 on beechwood xylan is much lower compared with MP and their 1:1 mixtures (Figure 3.7-C/D), indicating that xylanases are made more effective in sugar conversion because of the action of accessory enzymes; 4). Supplementing monomeric sugar into the hydrolysis solution led to stronger inhibition of both glucose conversion (15-20% reduced) and xylose conversion (10-15% reduced, comparing Figure 3.7-A/C with B/D).

The inhibitory effect of acidic oligosaccharides were different from those of neutral oligosaccharides (Figure 8) in the following ways. Low DP oligosaccharides were more inhibitory to

glucose conversion than high DP oligomers. With acidic fraction G, the glucose conversion of Avicel treated with Ctec2 was 20% (23% reduction from control, Figure 3.8-A), while xylose conversion of xylan under all three enzyme preparations with different DPs of oligosaccharides were similar with control group (60% with MP and 15% with Ctec2, Figure 3.8-C). The inhibitory effect of acidic oligosaccharides on xylose conversion increased with the addition of monomeric sugars (20% reduction for MP and 5% reduction for Ctec2, Figure 3.8 C/D), but no significant conversion decrease was observed for glucose conversion (Figure 3.8 A/B). Overall, the inhibitory effect of both neutral oligosaccharides and acidic oligosaccharides were observed on commercial enzyme cocktails (Ctec2, Htec2 and MP). Both cellulases and hemicellulases in the commercial enzymes were inhibited by oligosaccharides. Neither the oligosaccharides nor pure substrates could be degraded completely, augmenting the missing enzyme activities lacking in commercial enzyme cocktails would help break down the recalcitrant linkages in the oligosaccharides. Integrated biological processes like simultaneous saccharification and co-fermentation (SSCF), and using high cell density fermentations with cell recycle, enhance the process productivity by removing sugar inhibition [67, 123].

3.1.5 Conclusions

In this chapter, a method of separating recalcitrant oligosaccharides from high solids-loading ACSH at a large scale was first developed. A series of acid hydrolysis and enzymatic hydrolysis with commercial cocktails were performed to understand the composition, recalcitrance and inhibitory effect of these oligosaccharides. As oligosaccharides accumulation in high solids-loading enzymatic hydrolysis was shown to be a universal problem for different pretreatment technologies (IL, DA and EA). This chapter also provides a method to isolate and analyze oligosaccharides from different pretreated biomass, and permitting studies to understand the mechanism behind their accumulation to develop strategies to improve sugar yield during enzyme hydrolysis.

Using charcoal fractionation we were able to separate and recover approximately 85% of unhydrolysed oligosaccharides. The crude oligosaccharides were further fractionated based on their structural and molecular weight properties (neutral/acidic, different DP and molecular weights)

using size exclusion chromatography. Acid and enzymatic hydrolysis showed that the low DP oligosaccharides became more digestible after being separated from monomeric sugars and degradation products in hydrolysate. High DP oligosaccharides; however, remained recalcitrant. Xylo-oligomers and arabino-oligomers were shown to have much higher recalcitrance compared to gluco-oligomers, giving clues about finding the potential unhydrolysed cross-linkages and targeting enzymes. Inhibition studies on commercial enzymes (Ctec2, Htec2 and MP) revealed that oligosaccharides have inhibitory effect on commercial enzymes. Low DP oligosaccharides were more inhibitory than high DP ones and the addition of monomeric sugars would further contribute to the inhibitory effect. Multifect pectinase possessed the highest digestibility over both neutral and acidic oligosaccharides, indicating the significance of adding appropriate accessory enzymes to work in synergy with cellulases and hemicellulases to fully convert the recalcitrant oligosaccharides into monomeric sugars. Overall, the mass distribution profile, recalcitrant study and enzyme inhibition study help us explore the complexity of recalcitrant oligosaccharides, and to search for additional enzyme activities that are currently missing in commercial enzyme cocktails thereby generating higher sugar conversion.

CHAPTER 4

COMPREHENSIVE CHARACTERIZATION OF THE RECALCITRANT NON-CELLULOSIC CELL WALL STRUCTURES IN AFEX-PRETREATED CORN STOVER

4.1 Characterizing non-cellulosic recalcitrant cell wall carbohydrates in AFEX corn stover

AFEX pretreatment has improved enzyme digestibility of plant cell walls. Although complex sets of enzymes have been identified to effectively break down and generate simple sugars from pretreated lignocellulosic biomass, there are recalcitrant enzyme-resistant linkages remain in the cell walls that cause the accumulation of unhydrolyzed polysaccharides in residual solids (UHS) and dissolved un-deconstructed oligosaccharides in the hydrolysate. Together, for the case of AFEX-treated corn stover, these effects cause a 7-10% yield loss of inlet untreated dry biomass. A better fundamental understanding of the nature of these recalcitrant cell wall structures after enzyme hydrolysis can help identify the missing enzyme activities, thereby enabling more effective enzyme cocktails for better sugar release.

Use of glycan-directed monoclonal antibodies (glycome profiling or ELISA based mAb screening) is a powerful tool for in-depth characterization of most major cell wall glycans constituting plant biomass. Glycome profiling, however, only facilitates comprehensive characterization of larger cell wall-glycans (>20 kDa) as majority of oligosaccharides immobilization to ELISA plate is inefficient. In this study, AFEX pretreated corn stover was subjected to enzymatic hydrolysis at high solid-loading and resulting unhydrolyzed solids (UHS) were collected. Glycome profiling was employed to determine the composition and extractability of large cell wall glycans present in UHS and hydrolysate. Analysis of smaller oligosaccharides (<15 kDa) in the hydrolysate using mAbs, however, necessitating additional tools for facilitating efficient oligosaccharides immobilization to ELISA plates. Herein, we report a novel and efficient method to immobilize oligosaccharides for mAb screening by combining oligosaccharide biotinylation and subsequent ELISA screening on NeutrAvidin^{TM} coated plates. The immobilized biotinylated oligosaccharides showed great affinity to the antibodies enabling rapid and efficient structure elucidation of recalcitrant oligosac-

charides. MALDI and glycosyl-residue composition analysis of these recalcitrant oligosaccharides substantiated the results obtained using this novel immuno-screening method. These studies thus demonstrate that combining oligosaccharide biotinylation and subsequent ELISA screening on NeutrAvidin coated plates with glycan-directed mAbs is useful in identifying the cross-linkages in oligosaccharides and can be widely applied to other biochemical studies of oligosaccharides structure characterization.

4.1.1 Abstract

Inefficient carbohydrate conversion has been an unsolved problem for various lignocellulosic bio-mass pretreatment technologies, including AFEX, dilute acid and ionic liquid pretreatments. Nearly 22% of total carbohydrates are typically unconverted, remaining as soluble oligomers or insoluble after prolonged hydrolysis with excess commercial enzyme loadings (20 mg enzyme/g biomass). Nearly half (10 out of the 22%) of these total unconverted carbohydrates are present in unhydrolyzed solid (UHS) residues. The presence of these unconverted carbohydrates lead to a considerable sugar yield loss, which negatively impacts the overall economics of the biorefinery. Current commercial enzyme cocktails are not effective to digest specific cross-linkages in plant cell wall glycans, especially some of those present in hemicelluloses and pectins. Thus, obtaining information about the most recalcitrant non-cellulosic glycan cross-linkages becomes a key study to rationally improve commercial enzyme cocktails, by supplementing the required enzyme activities for hydrolyzing those unconverted glycans.

In this chapter, cell wall glycans that could not be enzymatically converted to monomeric sugars from AFEX-pretreated corn stover were characterized using compositional analysis and glycome profiling techniques. The pretreated corn stover was hydrolyzed using commercial enzyme mixtures comprised of cellulase and hemicellulase at 7% glucan loading (20% solid loading). The carbohydrates present in unhydrolyzed solids (UHS) and liquid hydrolysate were evaluated over a time period of 168 h enzymatic hydrolysis. Cell wall glycan-specific monoclonal antibodies (mAbs) were used to characterize the type and abundance of non-cellulosic polysaccharides present in UHS over the course of enzymatic hydrolysis. substituted xylan (4-O-methyl-D-glucuronic acid-)

and pectic-arabinogalactan were found to be the most abundant epitopes recognized by monoclonal antibodies (mAbs) in UHS and liquid hydrolysate, suggesting that the commercial enzyme cocktails used in this chapter are unable to effectively target those substituted polysaccharide residues. To our knowledge, this is the first report using glycome profiling as a tool to dynamically monitor recalcitrant cell wall carbohydrates during the course of enzymatic hydrolysis. Glycome profiling of UHS and liquid hydrolysates unveiled some of the glycans that are not cleaved and enriched after enzyme hydrolysis. The major polysaccharides include 4-O-methyl-D-glucuronic acid substituted xylan and pectic-arabinogalactan, suggesting that enzymes with glucuronidase and arabinofuranosidase activities are required to maximize monomeric sugar yields. This methodology provides a rapid tool to assist in developing new enzyme cocktails, by supplementing the existing cocktails with the required enzyme activities for achieving complete deconstruction of pretreated biomass in the future.

4.1.2 Background

Cellulose, hemicelluloses and pectins in the plant cell wall are embedded in a complex matrix with lignin. Plant cell walls are highly recalcitrant to biomass degrading enzymes, which are responsible to cleave glycosidic bonds and produce monomeric sugars for fermentation [28, 29]. Obtaining high yield of monomeric carbohydrates at minimal enzyme loading is quite challenging and it is one of the key bottlenecks for obtaining cost-effective biofuels [19, 163]. Due to the recalcitrant nature of the cell wall, pretreatment is required to improve the accessibility of enzymes to their substrates and thereby improve the efficiency of biomass deconstruction [34, 51, 142]. AFEX is a pretreatment process in which ammonia reacts with biomass at elevated temperatures and pressures. Ammonia can be used in liquid or gaseous forms and about 97% of ammonia can be recovered and reused in the process [21, 22, 145]. The AFEX process loosens the plant cell wall ultrastructure, cleaving lignin-carbohydrate complexes (LCCs), partly relocating lignin to the surface of the cell wall, leaving behind porous structures that help to improve enzyme accessibility to carbohydrates [7, 28]. Due to their physiochemical nature, AFEX is most effective on grasses, including corn stover, switchgrass, sugarcane bagasse and miscanthus. As corn stover is the most

abundantly available feedstock in the United States, AFEX could be a promising option for biofuel production in the US [61, 71, 84].

Unlike acidic pretreatments which require a wash stream, AFEX pretreatment is a dry-to-dry process that keeps the carbohydrate composition unaltered and preserves most of the sugars intact in a single solid biomass stream [7, 27, 29, 34, 139]. The presence of hemicellulose and pectin, however, requires more complex enzyme cocktails relative to acidic pretreatments, where hemicellulases, pectinases and other accessory enzymes must be added to cellulases to maximize overall sugar yields. Non-cellulosic polysaccharides, which account for 25-35% of plant cell walls, have branched cross-linkages with varying levels of substitution [46, 48, 146, 160]. Thus, a higher degree of synergy between a diverse set of enzyme activities is required to completely depolymerize such complex and highly branched carbohydrate structures into monomeric sugars [80, 96]. Though enzyme cocktail complexity is increased for deconstructing ammonia-pretreated biomass, the overall enzyme loading required does not change significantly in relation to dilute acid pretreatment [142].

One approach for studying this problem is to rationally design the enzyme cocktail by understanding the limiting factors that contribute to oligosaccharide and polysaccharide accumulation. For example, if some of the required biomass-degrading enzymes are not present or present at low levels in the commercial enzyme cocktail, some glycosidic linkages will tend to accumulate during the hydrolysis process. Thus, by carrying out detailed composition analysis and identifying structural features of enriched recalcitrant cell wall components, one can rationally determine the enzymes that are limiting the hydrolysis process. To facilitate such fundamental understanding of recalcitrant cell wall components, we require rapid analytical tools that provide in-depth structural information about non-cellulosic glycans at the molecular level. One of the methods currently available is glycome profiling. Glycan profiling takes advantage of a worldwide collection of more than 200 plant cell wall glycan-directed mAbs to evaluate the glycan composition of plant cell walls. These mABs enable monitoring of carbohydrate epitopes found in most major non-cellulosic cell wall glycans [105]. Recent studies have employed glycome profiling to

better understand cell wall modifications in plant biomass during genetic modifications, biomass pretreatments and microbial fermentations [5, 38, 65, 87, 105–107, 118, 132]. This information is essential to better understand the glycan linkages contributing to biomass recalcitrance and develop strategies for overcoming this problem.

In this chapter, we used glycome profiling to identify the cell wall components that remain intact after prolonged enzymatic hydrolysis by commercial enzymes, including specific glycan residues from AFEX-pretreated corn stover (ACS) (Figure 4.1). The glycan epitopes in both UHS and hydrolysates after high solids-loading enzymatic hydrolysis were analyzed in order to determine which groups of polysaccharides are most abundant and resistant to commercial enzyme cocktails. To our knowledge, this is the first study using glycome profiling to understand unhydrolyzed cell wall constituents present in UHS and liquid hydrolysate after intensive enzymatic hydrolysis. The structural information obtained from this study provides insights about important enzyme activities that are needed to make better commercial enzyme cocktails, compared to the cocktail used in this study. Improved cocktails will help increase sugar conversion during enzyme hydrolysis, increase biofuel yield and reduce biofuel cost in a biorefinery.

4.1.3 Materials and Methods

See Appendix A.14 - A.22, Figure B.1

4.1.4 Results and Discussions

4.1.4.1 Time profile of unhydrolyzed solids (UHS) composition

To understand how cellulose and hemicellulose-derived sugars are released from AFEX-CS during the course of enzymatic hydrolysis, the composition of UHS was periodically analyzed during 168 h hydrolysis. The details about mass balance for AFEX pretreatment and enzyme hydrolysis can be found in previously published work [9]. The amount of insoluble solids continuously decreased throughout the course of hydrolysis as shown in Figure 4.2. The hemicellulose, which includes xylan, arabinan, and galactan, was rapidly hydrolyzed, decreasing from 0.22 g/g corn stover (CS) at the start of hydrolysis to 0.051 g/g CS within the first 3 h. This dramatic

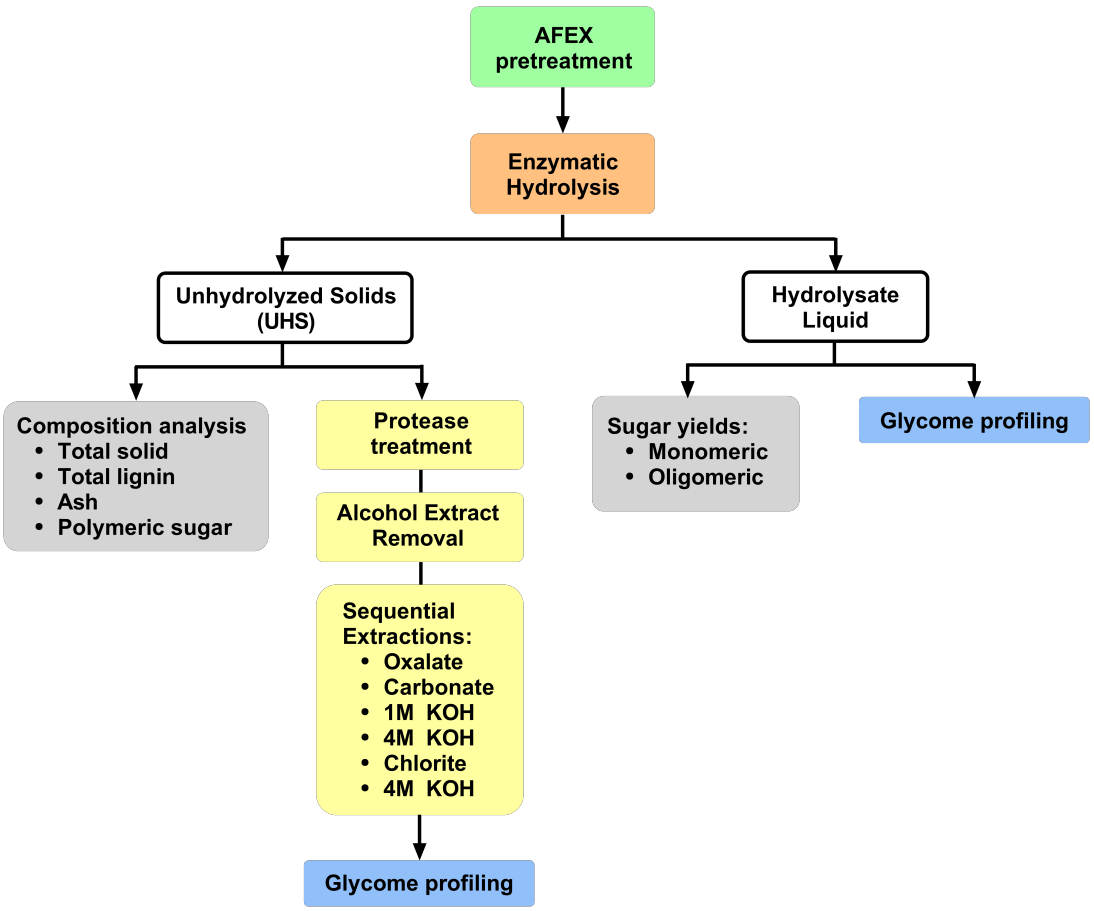


Figure 4.1 Process of characterizing the recalcitrant plant cell wall components in AFEX treated corn stover (AFEX-CS).

reduction shows that the majority of the digested hemicellulose was converted into soluble sugar (i.e., oligomers and monomers). The hemicellulose polysaccharides further decreased to 0.03 g/g CS after 24 h. During the remaining 6 days period of hydrolysis, little amounts of hemicellulose polysaccharides were further solubilized. In contrast, the reduction in cellulose content was more gradual throughout the entire course of hydrolysis, whereas the amount of insoluble lignin and ash (which includes soil that is brought in with the biomass) remained practically constant. These results were confirmed by the mass balance result summarized in Figure 4.3, where monomeric and oligomeric sugars present in the liquid phase (the hydrolysate) and the insoluble polysaccharides present in the solid phase (UHS) throughout the course of hydrolysis are shown. All results are normalized as a percentage of the amount present in the UHS and a mass balance closure over 95%

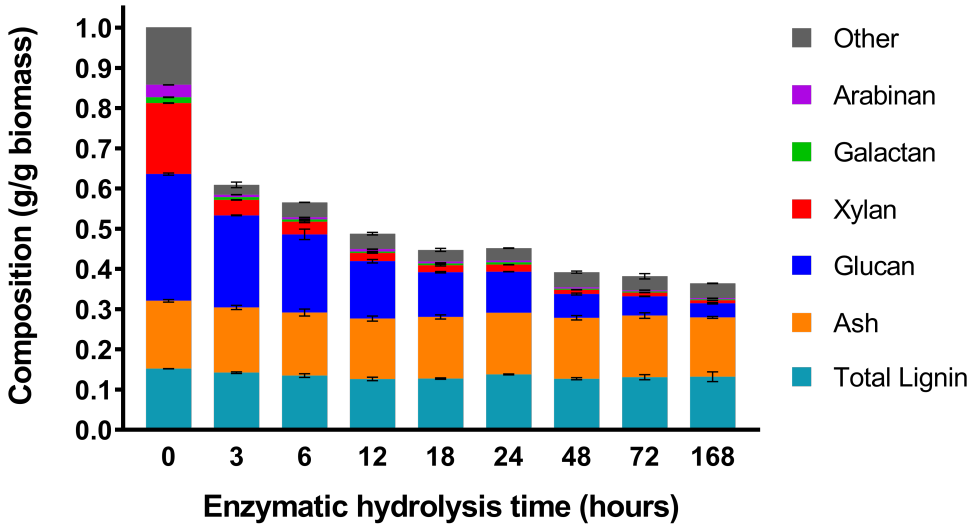


Figure 4.2 Composition of insoluble solids throughout enzymatic hydrolysis. Total height is normalized to the original amount of corn stover prior to hydrolysis. Composition includes all the insoluble structural carbohydrate, combined acid soluble and insoluble lignin, and ash.

was obtained for all sugars analyzed in this study. The combined amount of soluble (oligomeric and monomeric) hemicellulose, including xylose, arabinose, and galactose, reached 70-80% of the total sugars within the first 3 h. The amount of monomeric hemicellulose-derived sugars continued to increase slightly throughout the rest of the hydrolysis, although the total soluble sugars remained constant between 24 and 168 h. In contrast, mostly glucose monomers were produced within the first 24 h, which continued to increase slowly for the rest of the six-day period. Glucose oligomers also increased slowly over the course of hydrolysis while hemicellulose-derived oligomers decline slowly. These results are consistent with other studies showing that most biomass is solubilized within the first 24 h of enzyme hydrolysis under high enzyme loading hydrolysis. Also, it confirms the differences between cellulose and hemicellulose degradation patterns by enzymes [49, 142].

Among the soluble sugars, hemicelluloses are generally more difficult to convert to monomeric sugars and therefore, we observed considerable levels of xylan, galactan and arabinan containing oligomers during enzymatic hydrolysis. Nearly 25% of the total xylan was present as oligomers after 72 h, while only 9% of the glucan is present as oligomers at this time point. Likewise, a large proportion of arabinan and galactan sugars remained as oligomers, as shown in Figure 4.3 C

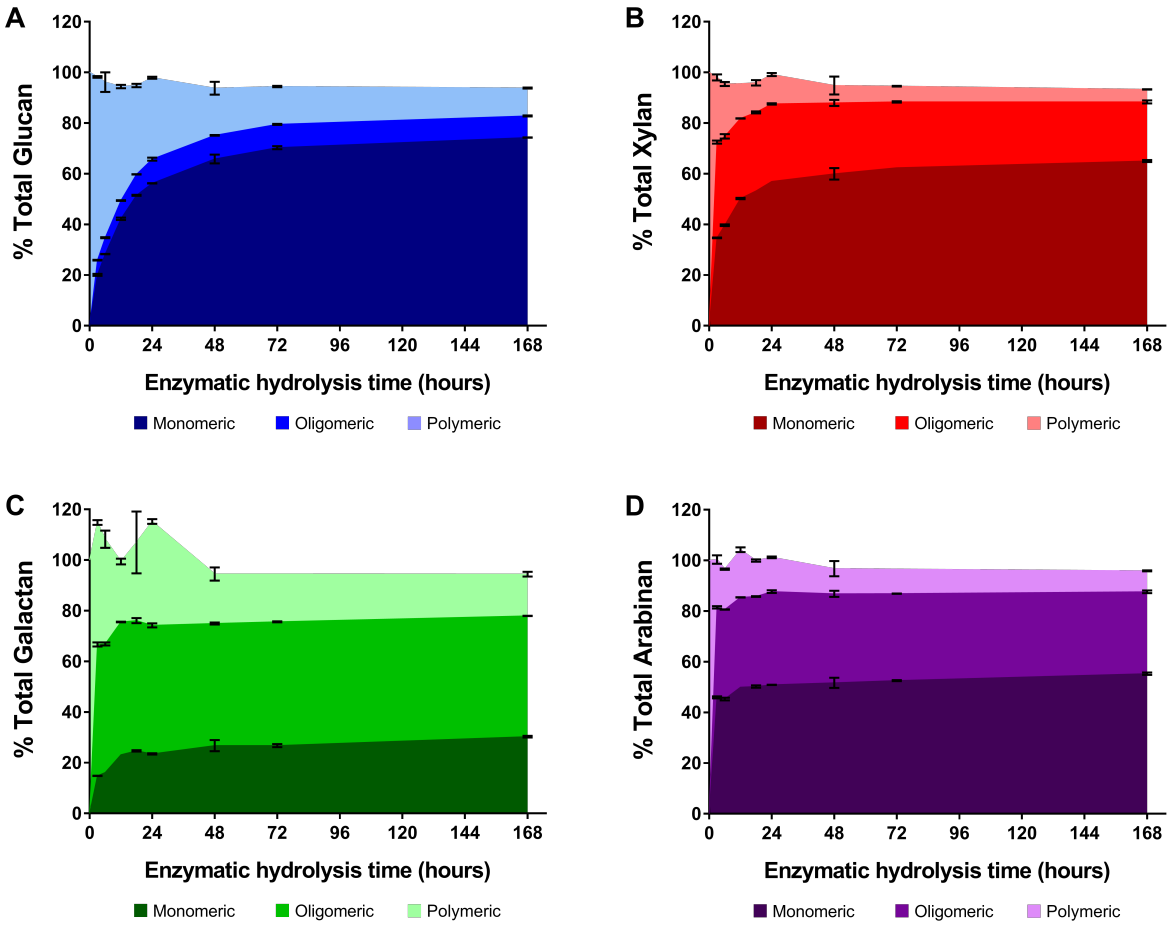


Figure 4.3 Mass balance for different monomeric and oligomeric sugar conversion throughout the course of hydrolysis. Here (A) glucan, (B) xylan, (C) galactan, (D) arabinan. Monomeric and oligomeric sugars are measured in the liquid portion, while polymeric sugar is measured in the unhydrolyzed insoluble material. Total shaded area represents mass balance closure. The Y-axis has been scaled to 100% as the maximum at the beginning of hydrolysis.

and 4.3 D. Most of the recalcitrant carbohydrates in hemicellulose (xylan, arabinan and galactan) were solubilized as oligomers. It appears that the commercial enzymes used here can efficiently solubilize some of the hemicelluloses, but not able to cleave all the hemicellulose linkages to generate monomeric sugars. Cellulose is a relatively simpler structure consisting of glucan chains connected with inter- and intra- molecular hydrogen bonding. However, hemicellulose is highly branched with multiple sugars and cross-linked with other organic moieties (e.g., acetyl, feruloyl, galactronyl, glucoronyl), some of which forming complexes with lignin [41]. Multiple accessory enzymes are required to fully break down these complex hemicellulose linkages [131]. Based on

our results, it is unlikely that all the enzymes are present at sufficient quantities and/or activities in the commercial enzyme cocktails used in this study. To determine the most abundant linkages present in UHS after enzymatic hydrolysis, we have further performed glycome profiling on UHS produced during enzymatic hydrolysis of ACS. As control experiments, we have also performed glycome profiling of untreated and AFEX pretreated corn stover.

4.1.4.2 Glycome profiling of untreated and AFEX pretreated biomass

Prior to glycome profiling, cell wall materials were prepared from biomass residues and were subjected to six sequential extractions with reagents of increasing severity, notably ammonium oxalate (50 mM), sodium carbonate (50 mM), KOH (1M and 4M) and acidic chlorite. These reagents selectively solubilize cell wall matrix polysaccharides on the basis of the relative tightness with which they are integrated into the plant cell walls. The extracts were then subjected to ELISAs against a comprehensive suite of 155 cell wall glycan-directed mAbs, providing responses that were further represented as heat maps. Hierarchical clustering of binding data for these mAbs against 54 structurally known plant polysaccharides allowed classification of these mAbs into the categories used in this work [38]. In previous glycome profiling studies [107], it has been demonstrated that AFEX pretreatment significantly reduces cell wall recalcitrance by inducing structural modifications to the polysaccharide network. In Figure 4.4, AFEX pretreatment induced enhancement in the extractability of non-cellulosic cell wall glycans including xylans and pectins, as indicated by the increased binding of specific groups of mAbs, notably xylan-3 through xylan-7 and pectic backbone (HG backbone-I and RG-I backbone) groups of mAbs, to oxalate and carbonate extracts from ACS (oxalate and carbonate extractions are performed in milder conditions and therefore, extracted glycans are more loosely bound to lignin compared to KOH and chlorite extracts). Normalized data of the sugar intensity (gram per gram of biomass in thousands) (Figure 4.5A) allows a closer examination of the different epitopes extractability from ACS relative to untreated corn stover. From Figure 4.5A, it is clear that the overall intensity of extracted carbohydrates in the untreated samples is much lower than those from ACS for most epitopes, especially for the xylans. From previous reports we know that AFEX pretreatment partially solubilizes hemicellulose,

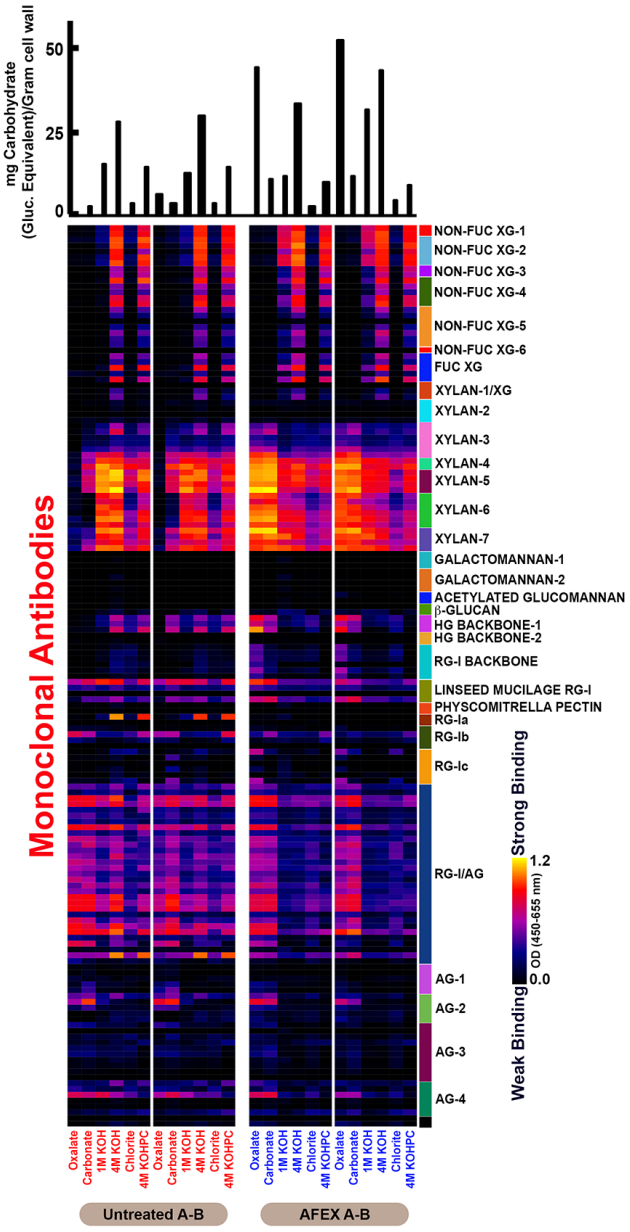


Figure 4.4 Glycome profiling of the cell wall extracts of untreated corn stover versus ACS. Here, A-B, represents replicates of untreated or pretreated biomass prior to hydrolysis. Labels at the bottom of each panel indicate the reagents used for the sequential extractions of the cell wall. The amounts of sugars extracted are shown in the bar graphs above the panel. All the antibody groups used for the ELISA screening are shown on the right side of the heat map.

loosens the cell wall and cleaves lignin-carbohydrate complexes [7, 28]. The cell wall modifications that take place during pretreatment increases enzyme access to cellulose and hemicelluloses chains for subsequent depolymerization. Thus, the increased extractability of major non-cellulosic glycans is thought to be associated with loosening of the cell wall structure, resulting in better enzyme access and digestibility after AFEX pretreatment (Figure 4.5B). This observation may also help explain the rapid hemicellulose solubilization within the first 3 h of enzymatic hydrolysis, as observed in Figure 4.3 B-D. The greatly improved digestibility of ACS during animal feed trials also supports the hypothesis that hemicellulose is quickly digested by ruminant microorganisms, allowing cellulose to be increasingly exposed for subsequent degradation and bioconversion. As a result, ACS releases more energy and nutrients to ruminant animals when compared to untreated corn stover [35, 121].

4.1.4.3 Glycome profiling and structural insights of UHS

In order to elucidate the overall composition and extractability of non-cellulosic cell wall glycans that remained insoluble in UHS after enzymatic hydrolysis of ACS, glycome profiling was applied to UHS as a function of hydrolysis time (Figure 4.6). Overall, fewer carbohydrates were recovered in extracts from UHS subjected to prolonged enzymatic hydrolysis (see bar graphs on the top of Figure 4.6 for sugar extracted per gram of cell wall in each step). Compared with ACS, UHS produced in the first 3 h of enzymatic hydrolysis showed significantly lower carbohydrate recovery among various cell wall extracts, especially hemicellulose and pectins (xylans and pectic arbinogalactans, respectively). This observation is consistent with the results shown in Figure 4.3, where most of the hemicelluloses in the plant cell wall were solubilized within the first 3 h of enzymatic hydrolysis. After 3 h enzyme hydrolysis, a significant amount of xyloglucan and xylan epitopes were converted (including the epitopes non-fucosylated xyloglucan-3 through non-fucosylated xyloglucans-6, fucosylated xyloglucans and xylan-1 though xylan-3). These epitopes completely disappeared after 12 h hydrolysis. Following the same pattern, epitopes recognized by mAbs against RG-I backbone were also converted gradually with time, completely disappearing from the ELISA heat map after 24 h of hydrolysis. In all the UHS analyzed in this study,

most of the xylan epitopes were not detectable in oxalate and carbonate extracts, revealing that the easily-extractable xylans from ACS, which are not strongly associated with lignin and/or other insoluble cell wall polymers, were mostly digested within the first 3 h of hydrolysis. This observation supports the hypothesis that the more loosened cell wall components can be extracted under milder conditions are more accessible to enzymes and therefore, they can be more easily digested. However, xylan epitopes recognized by xylan-4 through xylan-7 groups of mAbs were still present in the oxalate and carbonate extracts after 3 h of hydrolysis, and were further enriched for the harsher extraction conditions (1 M KOH, 4 M KOH and 4 M KOHPC) and after 168 h of hydrolysis. We would like to emphasize that this is a key observation, as it indicates that some highly substituted xylan components in ACS cannot be completely deconstructed with current state-of-the-art commercial enzyme cocktails. When associated with lignin and/or other insoluble cell wall components, these substituted xylans tend to be even more resistant to hemicellulase enzymes. Lignin, which is enriched during enzymatic hydrolysis, acts as a barrier for enzymes to access these carbohydrate linkages, which only become accessible to the mAbs after a harsh base treatment. Apart from xylan epitopes, those comprised of pectic-arabinogalactan, arabinogalactans and non-fucosylated xyloglucans, also remained present in oxalate and alkaline extracts after 168 h enzymatic hydrolysis (Figure 4.6).

The results from Figure 4.6 show that major part of the undigested epitopes present in UHS are only revealed after alkaline or chlorite treatment. Those carbohydrates are typically secondary cell wall components, which are still coupled with lignin. Some of those specific carbohydrates may be totally blocked by the presence of lignin, which does not allow enzymes to access their substrates [11], resulting in a significant epitope accumulation for various groups of polysaccharides (Figure 4.6). Some of the epitope linkages have been identified in previous work. For example, xylan-5 mAb recognizes one of the most recalcitrant non-cellulosic glycans present in UHS and hydrolysate [127]. The epitope for this mAb contains a 4-O-methyl glucuronic acid side residue on an linear xylan backbone (Table 4.2) [143]. It appears that this particular side chain is poorly cleaved during the hydrolysis process, indicating low a-glucuronidase activity. Likewise, RG-

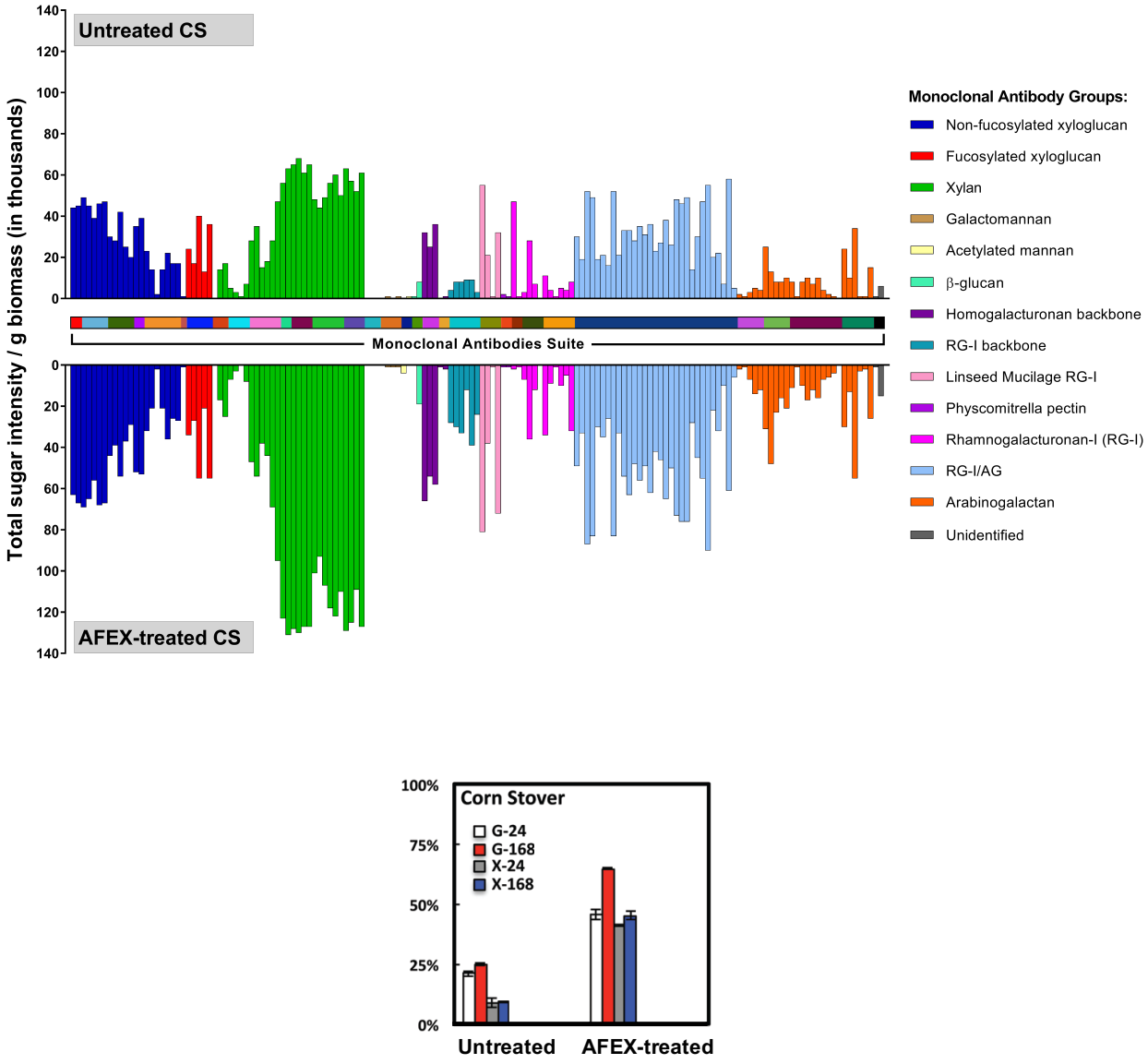


Figure 4.5 AFEX increases the extractability and digestibility of corn stover. Here, **A**. Extractability is measured by total sugar intensity from all extracts from sequential extractions. On the top is the untreated corn stover and in the bottom is the AFEX pretreated corn stover. The Y-axis shows the ratio of total sugar intensity versus g of biomass in thousands (*1000). All antibody groups used for ELISA screening are shown on the right side of the heat map, and, **B**. Digestibility of untreated and AFEX-CS as total glucan-to-glucose yields after 24 h and 168 h are shown in white and red bars, respectively. Total xylan-to-xylose yields after 24 h and 168 h are shown in grey and blue bars, respectively. Error bars depict standard deviations of data from the mean values reported for assays conducted in triplicate. Adapted from Figure 7 in *S. Pattathil et al (2015)* [107].

1/AG epitopes correspond to pectic-arabinogalactan linkages. This is consistent with Figure 4.3, in which 50% of the galactan remains in the oligomeric form through enzymatic hydrolysis. Linseed mucilage RG-1 mAb, which is associated with rhamnogalacturonan-I, had the strongest binding in UHS from AFEX-CS. Rhamnogalacturonan-I often has arabinan and galactan side chains, requiring multiple enzyme activities to be fully decomposed to monomers [31, 78, 154]. Identifying appropriate accessory enzymes that can cleave these side chain residues is required for achieving complete deconstruction of these complex carbohydrates. We hypothesize that the commercial enzyme cocktail used in this chapter requires those supplemental enzyme activities, which will synergize and significantly increase hemicellulose conversion during enzymatic hydrolysis of AFEX-CS. Increasing the levels of these missing enzymes may also increase the rate and extent of cellulose hydrolysis by unmasking cellulose chains more readily and making them more accessible to cellulase enzymes.

Although the heat map from Figure 4.6 indicates the glycans that remain present in UHS during enzymatic hydrolysis, the intensity shown is for a constant amount of extracted sugars rather than representing the relative amount of sugar present as a function of time. When normalized to the initial amount of epitope present in AFEX-CS (Table 4.1), the total amount of each epitope present in UHS as a function of time correlates to the trends of carbohydrate solubilization seen in Figure 4.2. Almost all the non-cellulosic polysaccharide components rapidly decreased within the first 3 h, further decreasing to levels below 4% of their initial value after 24 h enzymatic hydrolysis. The fact that some non-cellulosic glycan linkages remain insoluble throughout hydrolysis could suggest that they may be completely resistant to enzyme digestion by the commercial enzymes used in this study, completely surrounded by lignin (blocking enzyme access) or enzymes could be inhibited. Looking closer to the hydrolysate we could find some evidence that lignin blockage may not explain everything about the recalcitrance of the UHS carbohydrates. In Table 4.1 and Figure A1 (see ESI), it is clear that the liquid hydrolysate contains xylan-5, Linseed Mucilate/RG-I and RG-I/AG epitopes, suggesting that the commercial enzymes were not able to hydrolyze those linkages, even when they are accessible as soluble oligosaccharides, without the presence of

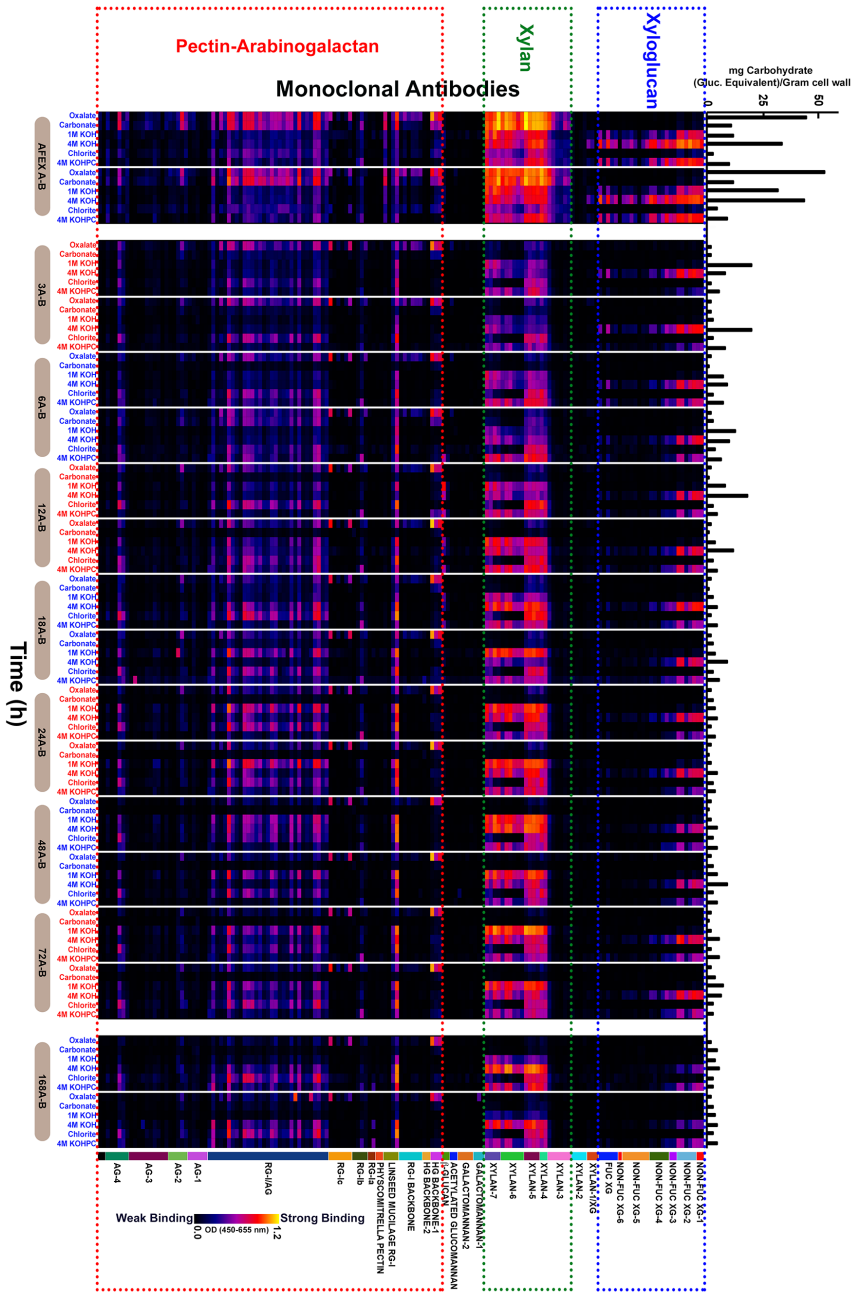
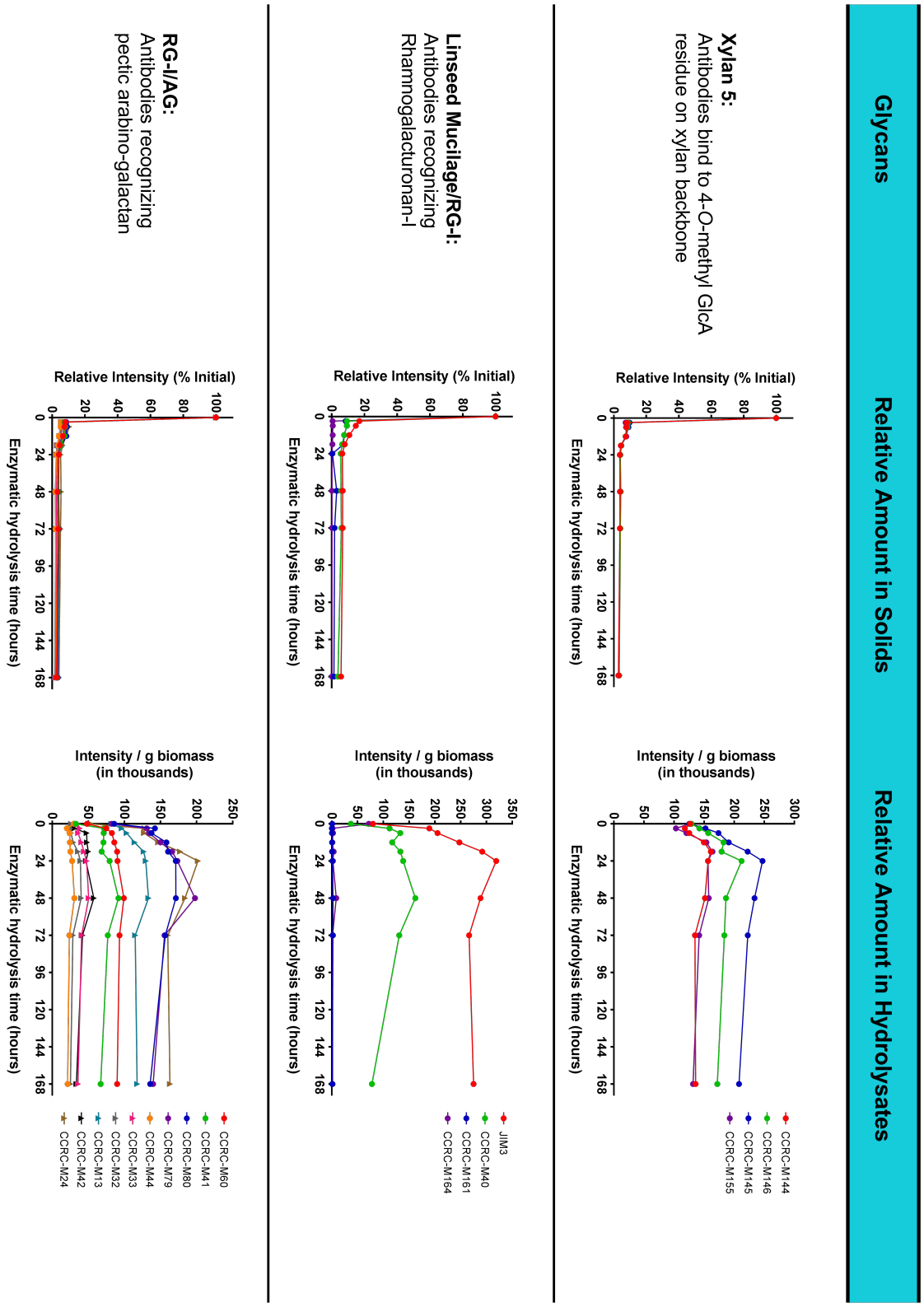


Figure 4.6 Glycome profiling of the cell wall extracts of AFEX-treated corn stover over the course of hydrolysis. Here, A-B represents replicates of extracts. AFEX A-B showed the composition of biomass at the beginning of hydrolysis. The other panels show the time points at which UHS were sampled (3A-B indicates 3 h time point). Labels at the bottom of each panel indicate the reagents used for the sequential extractions of the cell wall. The amounts of sugars extracted are shown in the bar graphs above the panel. All the antibody groups used for the ELISA screening are shown on the right side of the heat map.

enriched insoluble lignin. The reduction of xylan-5, Linseed Mucilate/RG-I and RG-I/AG epitopes in the UHS over the course of enzymatic hydrolysis could potentially be attributed to solubilization of carbohydrate fragments containing those epitopes. The presence of these soluble epitopes in the liquid hydrolysate are likely due to lack of enzyme activity, either by the absence or presence of low levels of specific enzymes, or enzyme inhibition. In all these cases, it is important to increase the ratio of enzymes that could break those epitopes, so that complete conversion of those soluble oligosaccharides to fermentable sugars can be achieved. It is also important to mention that the current glycome profiling method is only effective for detecting oligosaccharides of DP larger than 20 and our previous work has shown that most oligosaccharides have DP lower than 20 [146]. Therefore, it is likely that there are other undigested epitopes that could not be detected by this method. For overcoming this limitation and to have a better representation of the undigested epitopes in solution, the current glycome profiling technique must be modified.

Overall, the information provided by this chapter shows that specific glycans (e.g., xylans decorated with 4-O-methyl glucuronic acid residues, pectic arabinolactan and rhamnogalacturonan-I) are not completely digested by the commercial enzyme cocktail used in this work, even when those epitopes are completely soluble in the liquid hydrolysate and potentially free from lignin blockage. In contrast, a larger range of glycan epitopes can be detected in the UHS when they are associated with lignin, suggesting that those glycans may not be accessed by enzymes due to lignin blockage. Though these insoluble non-cellulosic carbohydrates represent less than 7% of the total carbohydrates after excessive enzyme treatment with optimized cocktails, the soluble counterpart in hydrolysate represent as much as 15% of the total carbohydrates in pretreated biomass, which is a much more significant fraction of substrate that is not converted to monomeric sugars and biofuel. Some future work includes better understanding the factors that contribute to epitope accumulation in the UHS and liquid hydrolysate. Imaging techniques, such as TEM and florescent microscopy, can be applied to depict the spatial orientation of cell wall components in the UHS and understand phenomena such as lignin blockage. NMR and mass spectrometry can also be used to determine the composition, structure and linkage patterns of purified recalcitrant

Table 4.1 Relative amount of sugar normalized to the amount of AFEX-treated corn stover present in each stage of hydrolysis present at each time point.



carbohydrates (mostly oligosaccharides). All these studies will complement this work and help to comprehensively understand cell wall recalcitrance. The information provided by this work will help us to rationally redesign the enzyme cocktail, by adding a selection of key enzymes for improving monomeric sugar yields, with minimal enzyme usage.

4.1.4.4 Novel immunological method for oligosaccharides

Glycome profiling has been proved to be a useful tool for comprehensive glycan structure analysis on extracts isolated from solid biomass residues. However, Figure B.1 (supplementary material) showed that the water-soluble sugars were underrepresented using this routine method, as the small molecule oligosaccharides were not able to bind to the Elisa plates, and were washed away before antibodies were added. Thus, a novel Immunological method was developed to enable the coating of soluble recalcitrant oligosaccharides on the Avidin-coated Elisa Plates. Biotinylation was used to enhance the binding affinity of oligosaccharides (Figure 4.7A)[55], by adding biotin-LC-hydrazide to the reducing ends of carbohydrates. In solution, semiacetal group was formed at the reducing end of sugar, reacted with the hydrazide group of biotin-LC-hydrazide, and formed a hydrazone linkage. In the presence of the reductant $NaCNBH_3$, the hydrazone linkage was reduced to become the stable biotinylated end product. With sugar reducing end modified, the binding of low-DP oligosaccharides to Elisa plates was made available. Elisa analsis could be performed on Avidin coated plates as shown in Figure 4.7B.

4.1.4.5 Glycome profiling analysis and structural insights of oligosaccharides

We proved the value of this newly-developed technique in a specific application: Characterization of soluble oligosaccharides present in ACSH. Crude and purified oligosaccharides fractions separated from lignocellulosic hydrolysates (Chapter 3) was used. From Figure 4.8, the most abundant epitopes identified in ACSH using the bionylated glycome profiling method are substituted xylan (usually by uronic acid or methyl uronic acid) and pectic arabinogalactan. These non-celllosic structures can not be digested by major cellulases and hemicellulases in commercial enzyme cocktails, therefore required novel accessory enzymes. Comparing the distribution of signals, we can find that the binding epitopes are more prominent in high DP sugar fractions (A, B, C) than low

Table 4.2 Antibodies-binding epitopes of the most recalcitrant glycans and their cross-linkage patterns. Cross-linkages were depicted with GlycoWorkBench developed in CCRC-UGA.

Glycans	Identified Structures	Linkage Patterns	Binding Antibodies
Xylan 5	Antibodies bind to 4-*O*-methyl GlcA side residue on xylan backbone		CCRC-M144 CCRC-M146 CCRC-M145 CCRC-M155 ...
Linseed Mucilage/RG-I	Antibodies recognizing Rhamnogalacturonan-I	Unpublished data	JIM3 CCRC-M40 CCRC-M161 CCRC-M164 ...
RG-I/AG	Antibodies recognizing pectic arabino-galactan		CCRC-M60 CCRC-M41 CCRC-M80 CCRC-M7 ...

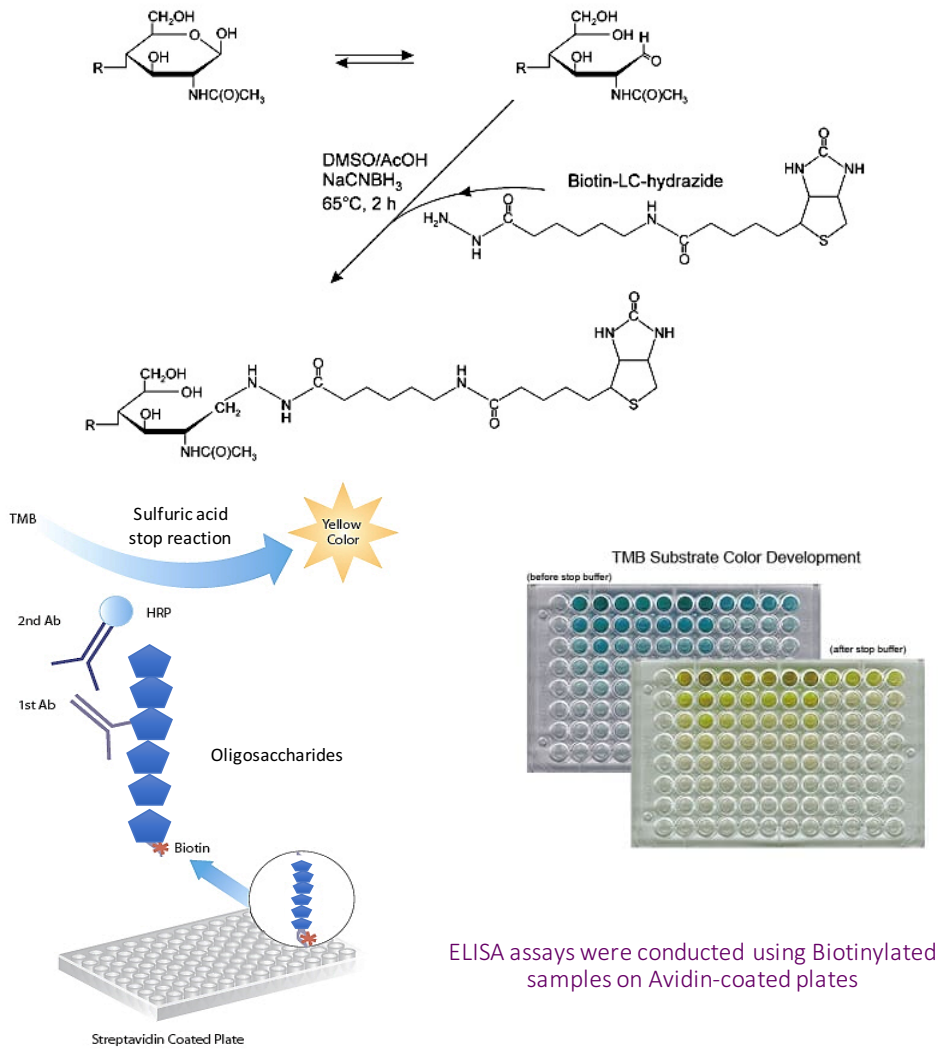


Figure 4.7 One-step biotinylation procedure for oligosaccharides evaluation. Step-by-step procedures for conducting Elisa assay on Avidin-coated plates are shown on the bottom right. Protocol adapted from *Grun, 2006* [55].

DP fractions (D, E, F). The acidic fractions were also shown to be more abundant in non-cellulosic epitopes than neutral fractions. These phenomenon were consistent with the oligosaccharides enzyme recalcitrant study in Chapter 3, where high DP and acidic fractions were more resistant to enzyme hydrolysis. The connection indicated that the existence of non-cellulosic glycan epitopes and uronic acid substitutions might be important reasons of oligosaccharides recalcitrance.

4.1.4.6 Complementary methods: MALDI and TMS sugar analysis

To evaluate how immunological methods can complement other alternative characterization methods for low molecular weight oligosaccharides present in lignocellulosic hydrolysates, we

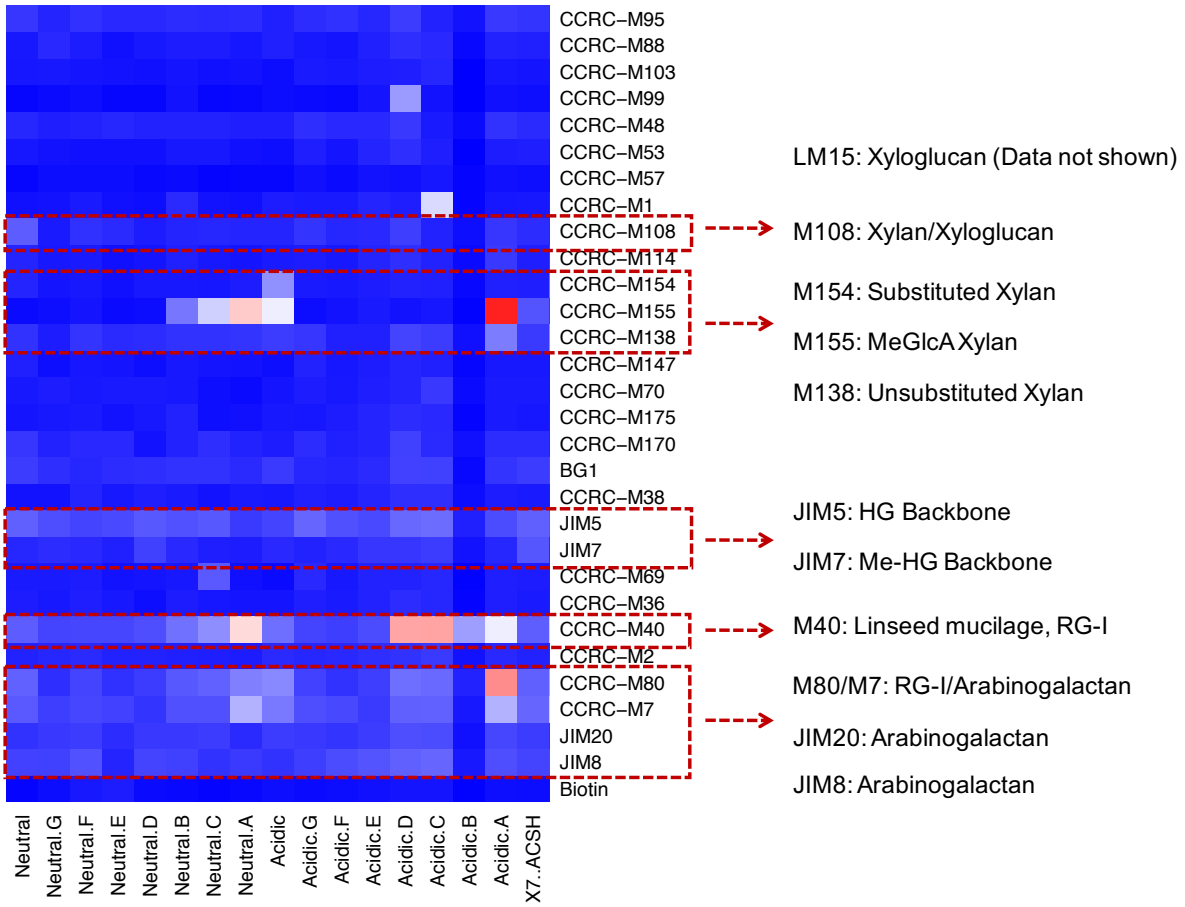


Figure 4.8 Recalcitrant oligosaccharides epitopes being detected using cell wall glycan-directed mAbs. Here, antibody names and binding epitope are given on the right side. The oligosaccharides fractions are on the bottom. Biotin was used in the bottom line as a blank control. The color of the grid is blue with no signal, and turns to white then red with increasing intensity of binding signal. Neutral and acidic fractions are crude oligosaccharides separated from ACSH. Fractions A - G were further purified using SEC. Fraction MW is from high to low for fraction A to G.

performed further compositional analysis, including MALDI and TMS sugar analysis on same sets of oligosaccharides fractions. MALDI was used to compare whether the mass distribution of oligosaccharides molecules fit the hypothesized structure. Figure 4.8 showed the mass spectrum of acidic fraction ACN C. Its mass distribution revealed a (Me)U substituted pentose series with DP 8-15, which was consistent the the substituted xylan epitope detected in Elisa.

Both neural and acidic sugars (GluA and GalA) were analyzed in TMS analysis. Gluronic acid were only found in acidic fractions C and D. And galacturonic acid was only found found in acid fraction A and B. They were all high-DP acidic sugar fractions, which was not only com-

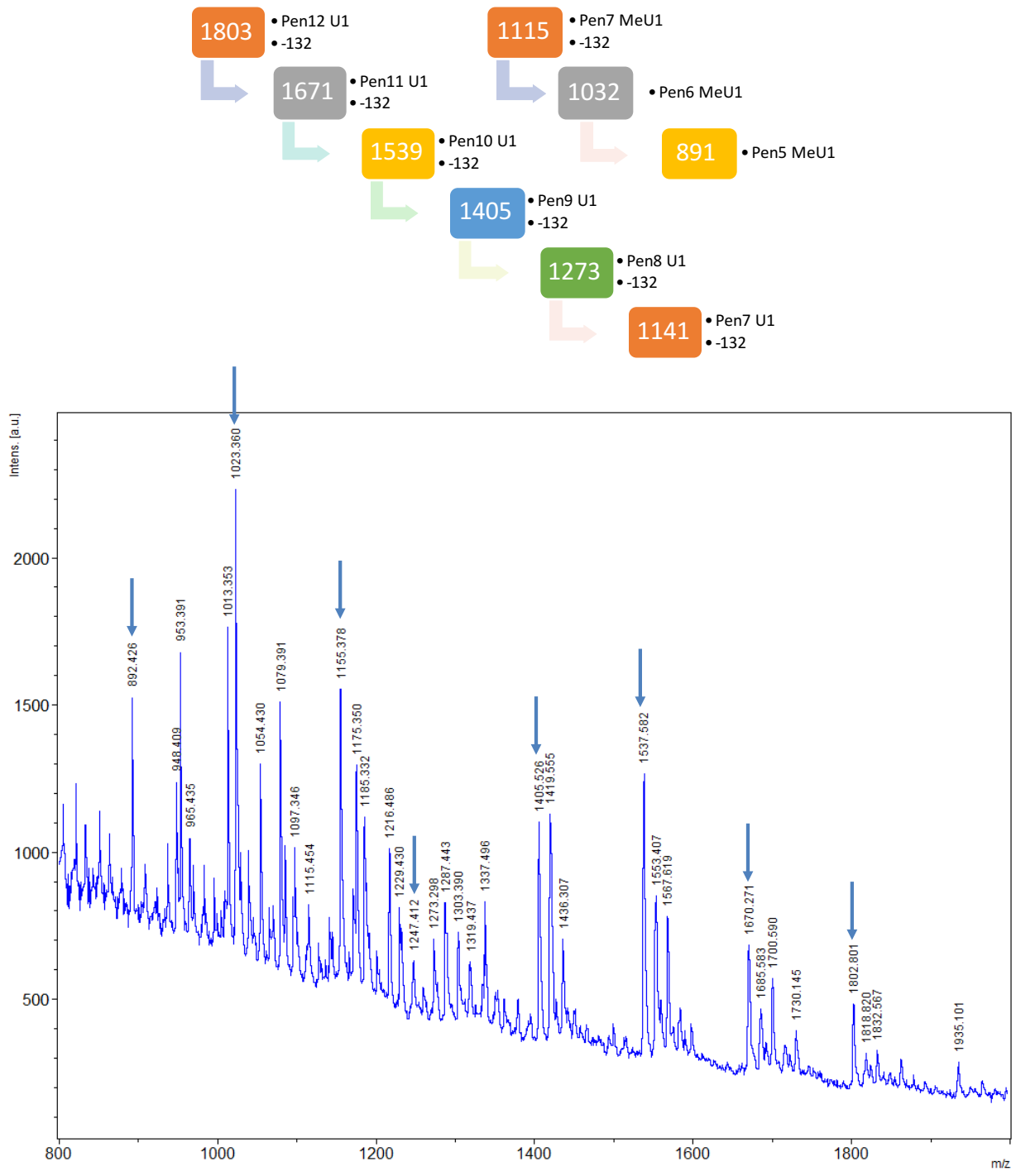


Figure 4.9 MALDI spectrum and identified mass list from acidic oligosaccharides fraction ACN A.

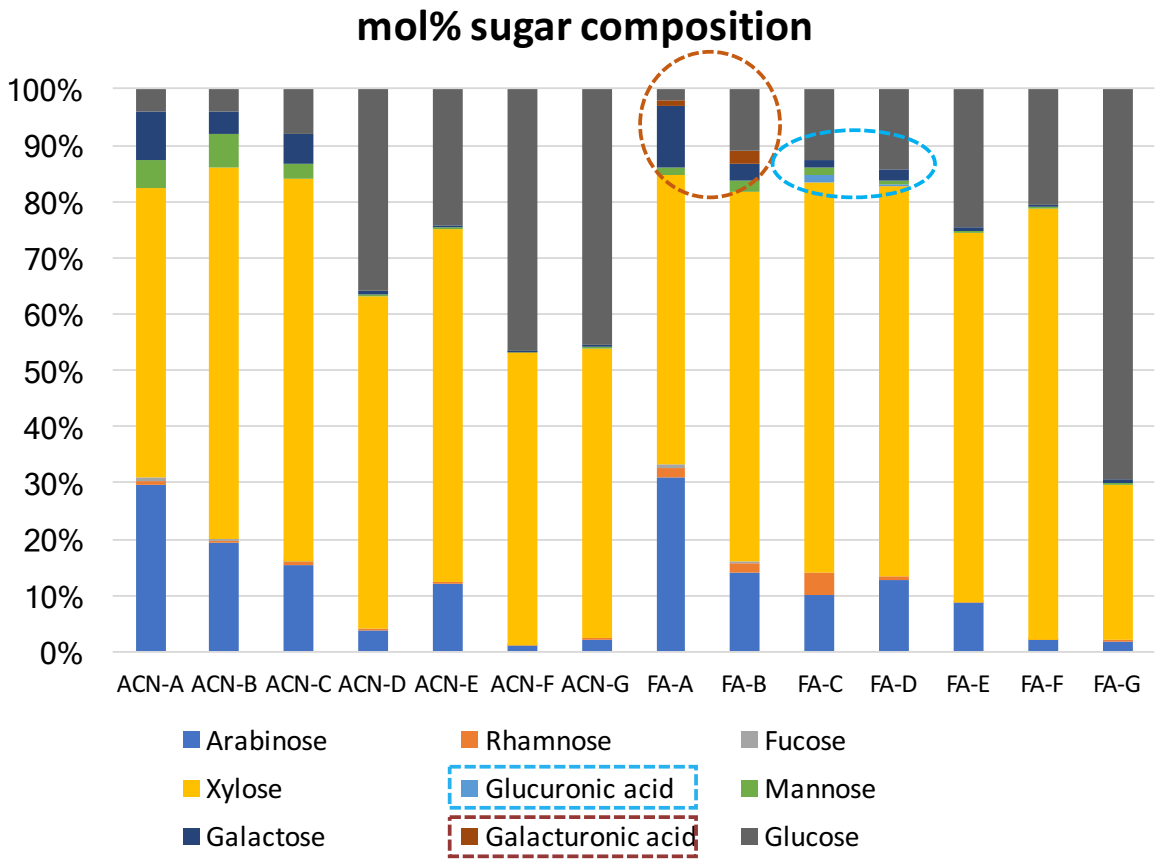


Figure 4.10 TMS sugar composition analysis. Mol % of each sugar composition based on 100% stack bar graph from Arabinose at the bottom to Glucose at the top.

plementary to Elisa and MALDI, but also consistent with the fractionation method and enzyme recalcitrant study in Chapter 3. Through these experimental results, we are confident that an advanced immunological method using biotinylation and subsequent Elisa screening will be a useful method to evaluate soluble recalcitrant oligosaccharides [159]. The method can be applied to broader biochemical oligosaccharides study after being further characterized.

4.1.5 Conclusions

The chemical nature of some of the recalcitrant carbohydrate linkages present in corn stover was studied by analysing UHS and hydrolysates resulting from enzymatic hydrolysis of AFEX-CS. Samples taken at multiple time points over a period of 168 h were analyzed to understand changes in the different cell wall components as a function of time. The polysaccharides that were easier to extract after AFEX treatment were rapidly deconstructed by the enzymes, while

some of the carbohydrates that required harsher alkaline extractions could not be hydrolyzed by the enzymes and accumulated in the UHS. While a wide range of polysaccharides remained in the UHS, the amount remaining in the insoluble form was relatively small (< 5%) after 24 h. However, soluble polysaccharides, particularly those recognized by xylan-5 and RG-I/AG groups of mAbs, remained abundant in hydrolysate and UHS throughout the course of hydrolysis, indicating a lack of appropriate enzyme activities or severe enzyme inhibition. These results show that complete sugar conversion is not possible when using commercial enzyme cocktails (Cellic Ctec2, Cellic Htec2 and Multifect Pectinase) as used in this work, at high solid loading enzymatic hydrolysis conditions.

Future work is needed to find enzymes that hydrolyse these recalcitrant non-cellulosic polysaccharide linkages. For example, accessory enzymes such as pectinase, α-glucuronidase and enzyme activities targeting arabinan and galactan should be identified and added to the enzyme cocktail, so that branched linkages that block enzyme accessibility to backbone polysaccharide chains can be hydrolyzed and potentially be de-coupled from the complex structure of hemicellulose. If successful, this approach could not only increase monomeric xylose yields, but may also synergistically improve cellulose hydrolysis, thus increasing glucose yields and a possible reduction in enzyme loading to lower biofuel production cost. Likewise, this approach could be adapted with other pretreatment technologies and biomass to optimize hydrolysis conditions for maximum sugar output. Moreover, fluorescent labelled antibodies studies using fluorescent microscopy and flow cytometry could be performed for more comprehensive understanding of small molecule oligosaccharides structures. MS-MS could also be applied to supplement with the novel glycome profiling method for getting detailed structures in molecule level. Such studies are underway at the Great Lakes Bioenergy Center (GLBRC).

CHAPTER 5

CHARACTERIZATION OF WATER-SOLUBLE LIGNO-DERIVED COMPOUNDS USING SYNTHETIC HYDROLYSATE

5.1 Water soluble phenolic compounds produced from Extractive Ammonia pretreatment mimic real inhibitors for yeast fermentation using synthetic hydrolysate

5.1.1 Abstract

Biochemical conversion of lignocellulosic biomass to liquid fuels requires pretreatment and enzymatic hydrolysis of the biomass to produce fermentable sugars. Degradation products produced during thermochemical pretreatment, however, are inhibitory to the microbes on both ethanol yield and cell growth. In this chapter, we use synthetic hydrolysates to study the inhibition of yeast fermentation by water-soluble components isolated from lignin stream obtained after extractive ammonia pretreatment (EA). We found that synthetic hydrolysate with 20g/L water soluble compounds mimics real hydrolysate in cell growth, sugar consumption and ethanol production. Nevertheless, a long lag phase was observed in the first 48-h of fermentation of SynH, showing that these compounds had excessive inhibitory effects to microbes. Ethyl acetate extraction was conducted to separate phenolic compounds from other water soluble components which acted as key inhibitors during ethanol fermentation. The most abundant compounds were identified by LC-MS and GC-MS, including coumaroyl amide, feruloyl amide and coumaroyl glycerol. Chemical genomics was employed for the fingerprints of genes deletion response of yeast to different groups of inhibitors in WSC and ACSH, and the sensitive/resistant genes clusters patterns confirmed their similarity.

5.1.2 Background

In the fossil fuel based economy, crude oil is the primary feedstock source for producing transportation fuels and industrial chemicals. Volatility of crude oil prices, which raises energy security concerns and greenhouse gas emissions driving climate change, have triggered worldwide research towards the development of alternative, sustainable sources of energy [60]. Lignocellulosic biofuels are playing a substantial role in the replacement of current-generation fossil-derived liquid fuels such as gasoline and diesel [27, 36]. Unlike corn grain-based ethanol, where the starch can

be readily hydrolyzed to fermentable sugars using enzymes, the lignocellulosic biomass used in second-generation biofuel has naturally evolved to be highly recalcitrant to enzymatic deconstruction by fungi and bacteria [149]. Pretreatment of lignocellulosic biomass is necessary for reducing its native recalcitrance and obtaining efficient conversion to monomeric sugars [6]. Pretreatment processes are commonly performed under high temperature, high pressure, caustic, or acidic conditions, which generate degradation compounds that inhibit microorganisms [40]. Under acidic conditions, carbohydrates present in the biomass degrade into furfural or hydroxymethylfurfural and the lignin degrades into a variety of phenolic compounds [75]. Instead, Ammonia Fiber Expansion (AFEX^{TM}) process produces many ammoniated compounds, which are significantly less inhibitory than their acid counterparts [29, 140]. A previous comparison of AFEX and dilute acid treated corn stover showed that dilute acid pretreatment produces 316% more acidic compounds, 142% more aromatics, 3555% more furans, but no nitrogenous compounds [29].

With less toxic degradation products presented, the sugar utilization efficiency of ethanol production using ammonia pretreated biomass still requires improvement. One major issue is the low xylose consumption rate during hexose/pentose co-fermentation, which resulted from the presence of pretreatment-derived biomass decomposition products, ethanol, and other fermentation metabolites [37, 122, 128, 140]. Thus, novel pretreatment technologies that further reduce toxic degradation products content in biomass are essential for economically producing biofuels. Extractive-Ammonia (EA) is a newly developed pretreatment technology that selectively extracts lignin present in biomass. Compared to AFEX, EA uses higher ammonia-to-biomass loading and lower water loading, generates a separate lignin stream to extract 30-45% of the lignin from lignocellulosic, and removes most of the degradation products [139]. Thus, EA-pretreated corn stover was found to have reduced lignin content, enhanced digestibility and improved hydrolysate fermentability[139].

To study the inhibitory effects of degradation compounds, the DOE Great Lakes Bioenergy Research Center (GLBRC) has formulated a chemically-defined synthetic hydrolysate (SynH) to mimic real AFEX corn stover hydrolysate (ACSH). Synthesized aromatic compounds were added

into the control media based on ACSH composition analysis to clarify the complex inhibitory effect [72, 122, 128, 130, 140]. This artificial media has been proved successful in testing the inhibitory effects of degradation compounds on engineered microbial strains. The synthesized aromatics, however, cannot fully represent the major inhibitory compounds due to the incomplete composition analysis for ACSH, and different conformation between isomers. Therefore, we explored an alternative formulation of SynH using natural compounds alternatives to better represent the inhibitory effects in ACSH. In this study, water soluble components (WSC) were separated from crude lignin stream produced in EA pretreatment. These soluble compounds were supplemented into SynH to mimic real inhibitors in ACSH that affect microbial fermentation. Water-extraction and ethyl acetate extraction were conducted to separate the water-soluble compounds, especially the phenolic nitrogenous compounds in crude lignin stream. We observed dual inhibition effect for WSC on yeast fermentation using synthetic hydrolysate. New inhibitors that were different from ACSH were identified using HPLC and LC/GC-MS. Chemical genomics profiling was performed to further understand the gene response profile and metabolic pathways being inhibited by the existence of WSC.

5.1.3 Materials and Methods

See Appendix A.23 - A.32, Table B.1, Table B.2, Figure B.2, Figure B.3, Figure B.4

5.1.4 Results and Discussions

5.1.4.1 Water extraction and ethyl acetate extraction of lignin streams

As mentioned previously, EA pretreated corn stover yielded a higher fermentable sugar yield compared to the AFEX process while using 60% lower enzyme loading [139]. We believed that removing water-soluble lignin-derived inhibitors greatly relieved the inhibitory effect on enzymatic hydrolysis and microbial fermentation, as these small molecule compounds fully interact with free enzymes and microbes in the hydrolysate [72, 114]. To identify the key inhibitors that affect ethanol production, we performed water extraction to separate the WSC from crude lignin stream, and conducted ethyl acetate extraction on the WSC subsequently (Figure 5.1). Mass balance for

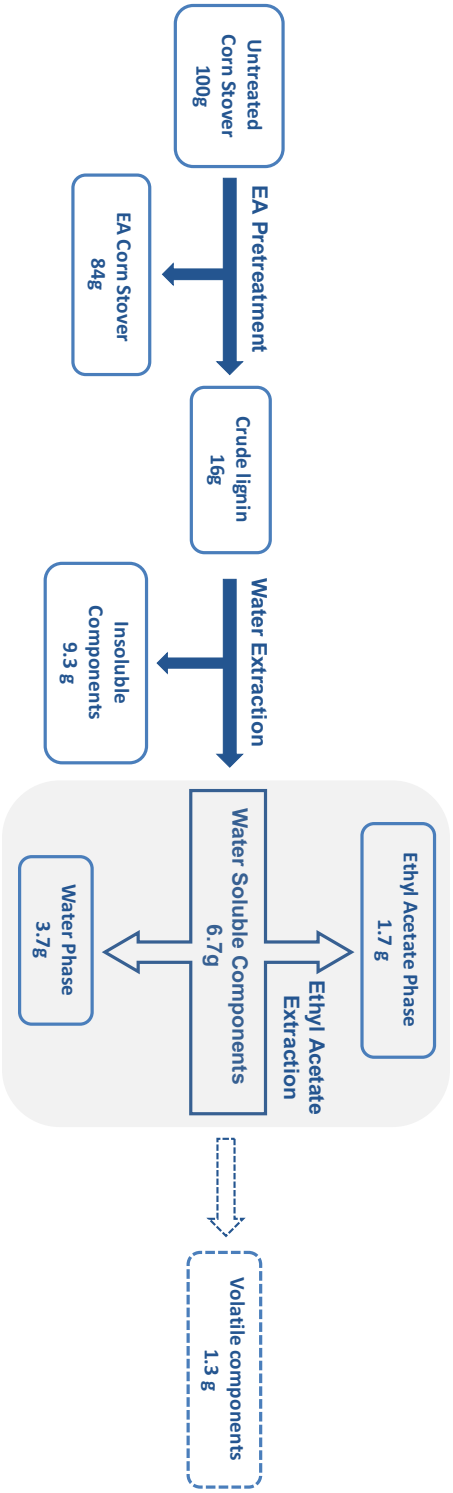


Figure 5.1 Methodology and mass balance of water extraction and ethyl acetate extraction of crude lignin.

water extraction and ethyl acetate extraction were shown in Figure 5.1. 40 wt% of crude lignin stream were water-soluble after extraction. The WSC were subjected to ethyl acetate liquid phase extraction to enrich phenolic compounds, and were used in microbial fermentation and composition analysis. A total recovery of 80 wt% was achieved during ethyl acetate extraction. More than 26% of WSC were extracted as phenolic compounds. The real content of phenolic compounds should be higher as they were volatile and could be lost during vacuum drying. About 55 wt% of WSC remained in water phase after ethyl acetate extractions. To identify major components in WSC and the ethyl acetate fractions, composition analysis, such as SEC, HPLC, LC-MS and GC-MS, were conducted.

To obtain an estimation of the molecule-weight range, we first fractionated the WSC through SEC column, which was packed with P6 gel (separation range 1-6kDA). As shown in Figure 5.2A, five major peaks were eluted. The molecular weight range of peak one to peak five went from high to low. In Figure 5.2B, fractions collected in SEC were subjected to LC-MS screening in the same order as they were collected (from A1 to H12). At least one major compounds were identified in each of the five peaks. Decahexose was detected in peak 1, followed by trihexose being detected in peak 2, showing that oligosaccharides were produced in EA process like AFEX [29, 51, 160]. p-Coumaric acid and p-Coumaroylglycerol were detected in peak 3 and 4. Both of them were major lignin degradation products in corn stover pretreatment. In peak 5, both p-Coumaroylamide and feruloylamide were detected. They are major ammoniated lignin degradation products produced in ammonia pretreatment process. It is worth noting that p-Coumaroylamide itself took more than 80 wt% of total mass being detected, indicating that nitrogenous phenolic compounds were the most abundant and one of the most inhibitory components in WSC. The LC-MS screening also revealed that more than 98% of compounds were below 3kDA, which was consistent with our assumption that most water-soluble inhibitors were small molecules.

As phenolic compounds were enriched in the organic phase after ethyl acetate extraction, UHPLC-MS and GC-MS were employed on the phenolic fractions to identify major components. In Figure 5.3a, phenolic acid, phenolic amides, and lignin derivatives (dimers and trimers) were

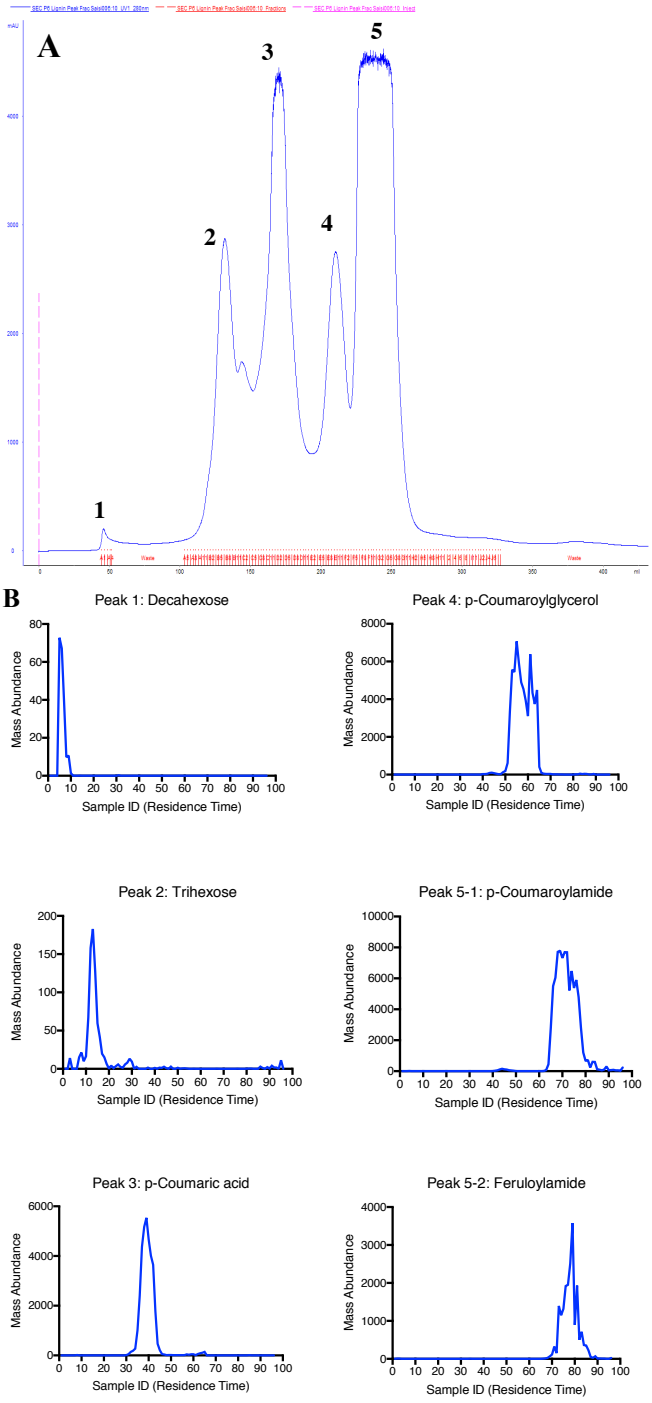


Figure 5.2 Size exclusion chromatography and LC-MS screening of WSC. **A**. SEC of WSC using P6 gel (Polyacrylamide, 1-6kDA, water as mobile phase). 96 samples from A1-A12 were collected in auto-sampler; **B**. LC-MS screening of the fractions collected in SEC in the same order as they were collected (A1-H12). B1-B6. Most abundant components identified from peak 1 to peak 5 in chromatogram.

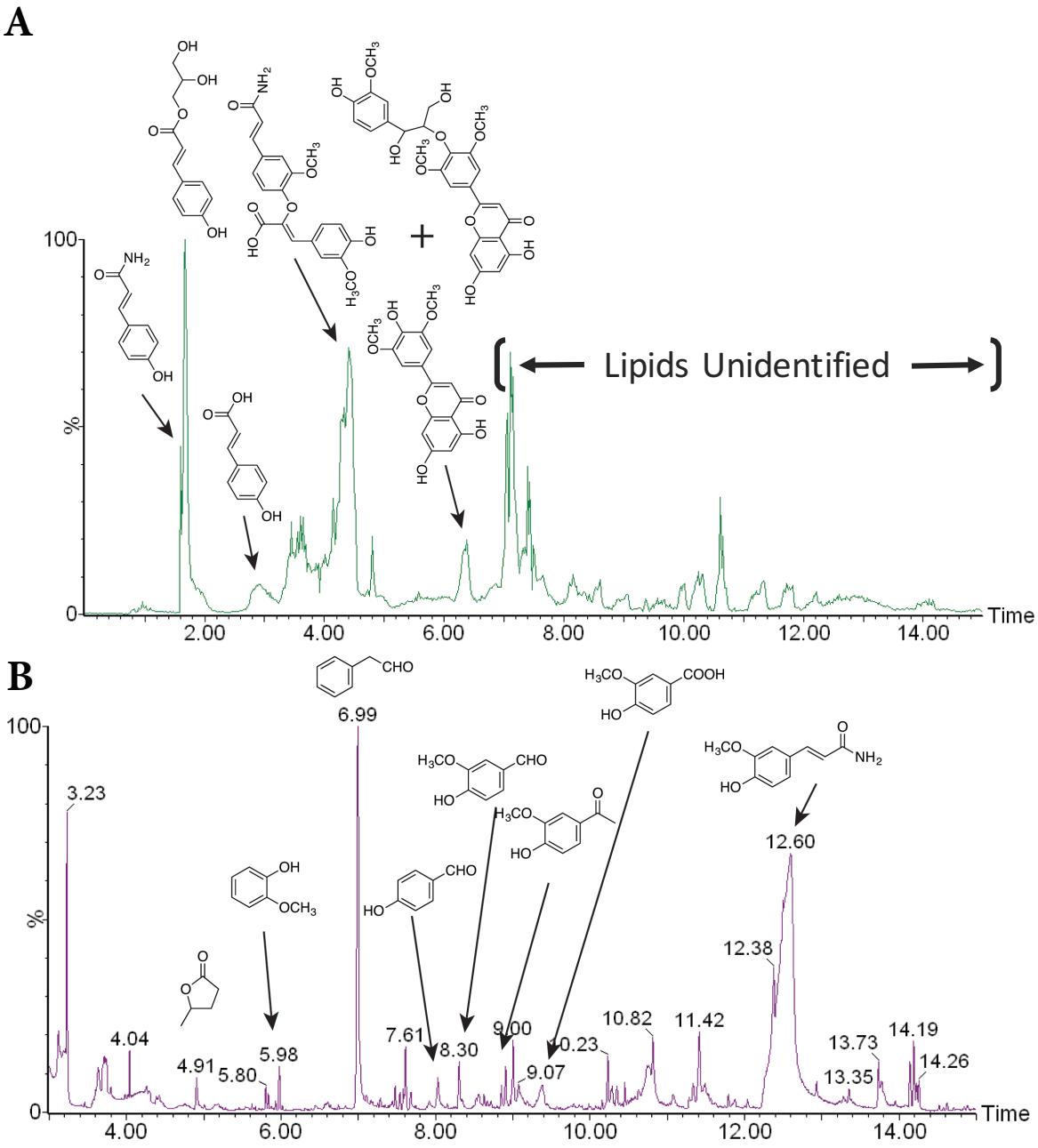


Figure 5.3 UHPLC-MS (**A**) and GC-MS (**B**) chromatograms for phenolic compounds enriched in ethyl acetate fractions of WSC.

identified in UHPLC. Other compounds including aromatic aldehydes/ketones, and furanones were identified in GC-MS (Figure 5.3b), which were produced from Maillard reaction and ammonization of reducing sugars and lignin during ammonia pretreatment [29, 59, 62, 78, 146]. These Maillard reaction products not only accounted for the brown color of ammonia treated biomass and lignin extractives, they also acted as strong inhibitors for microbial fermentation. Identifying phenolic and nitrogenous compounds also explained the reason why EA pretreated biomass had a much better performance in xylose fermentation and ethanol production by removing lignin streams.

The components remained in water phase after ethyl acetate extraction were analyzed. HPLC analysis of sugar and organic acids in both WSC and water phase after ethyl acetate extraction showed small amount of free monomeric sugars (Table B.1) and oligomeric sugars, especially xylo-oligomers (data unpublished). Glycerol and acetate were also detected. LC-MS analysis of water phase fraction showed no signal of phenolic compounds. Instead, esters, fatty acids and oligosaccharides were identified (data not shown), which was consistent with the result of LC-MS screening of WSC. Free amino acids analysis and heavy metal analysis were outsourced and performed by UC-Davis and UW-Soils and Plant Analysis Laboratory (SPAL) respectively (Table B.1). High contents of nitrogen were identified. The distinct composition profiles of components in phenolic phase and water phase after ethyl acetate extraction indicate that they might have variable effects on microbial fermentation performance.

5.1.4.2 Inhibitory effect of WSC

To investigate the inhibitory effect of WSC on microbial fermentation, a chemically-defined hydrolysate media with monomeric sugars and necessary nutrients was needed. GLBRC formulated a synthetic hydrolysate media (SynH) based on the composition analysis of 6% solids loading ACSH [29, 140], which was designed to mimic the composition and behavior of real hydrolysate. This SynH has been successfully employed as a control media to test the inhibitory effects of degradation compounds or engineered microbial strains on ethanol fermentation [6]. Table B.1 and Table B.2 show the recipe of synthetic hydrolysate consisted with six groups of components.

Table 5.1 The major components in SynH recipe.

Carbohydrates	Monomeric sugars (glucose, xylose, arabinose, mannose, galactose, fucose)
Nitrogenous compounds	Ammonium chloride, 20 amino acids, nucleobases
Vitamins	Panthothenic acid, thiamine pyridoxine, etc.
Mineral salts	Zinc chloride, Manganese(II) chloride, etc.
Carbohydrate degradation products/ plant metabolites	Aliphatic acids, Furans, Acetamide, Pyrazines, Imidazoles
Lignin degradation products	Aromatic acids and amides

Carbohydrates, nitrogenous compounds, vitamins and minerals provided carbon source, nitrogen source and necessary nutrients as base media. Degradation products produced from pretreatment process, on the other hand, acted as inhibitory compounds. Two major groups of degradation products were carbohydrates derivatives (aliphatic acids, furans, acetamide, pyrazines, imidazoles, etc.) and lignin derivatives (aromatic acids and amides). The inhibitory degradation components could be replaced with other inhibitory compounds to test their effect on microbes. In this chapter, we used WSC and its ethyl acetate extracts to investigate their inhibition effects on yeast fermentation. Other groups of components were usually retained as control media.

Haploid Y128 yeast strain was developed by GLRBC with the ability to rapidly co-ferment glucose and xylose anaerobically even in the presence of ACSH inhibitors [22]. It was used in this work to investigate the inhibitory effect of WSC. The freeze-dried WSC was re-dissolved into SynH control media with varying concentrations. As shown in Figure 5.4, the concentration of WSC increased from 10-40g/L for SynH 1WSC to SynH 4WSC, respectively. Here 1WSC represents 10 g/L lignin extractions which were re-dissolved in SynH base media. Fermentation was conducted in Erlenmeyer flasks (50 mL) under the conditions of pH 4.8, 30 °C and 150 RPM with inoculum at 2 OD600. The performances of glucose consumption, together with the first 48 hrs of xylose consumption and ethanol production, illustrated that the inhibition effect of WSC was enhanced with the increase in concentration. The addition of WSC resulted in a lag phase in glucose and xylose consumption in first 24 h, especially at higher concentrations. However, once the concentration of WSC reached 40g/L, xylose consumption, ethanol yield and cell growth

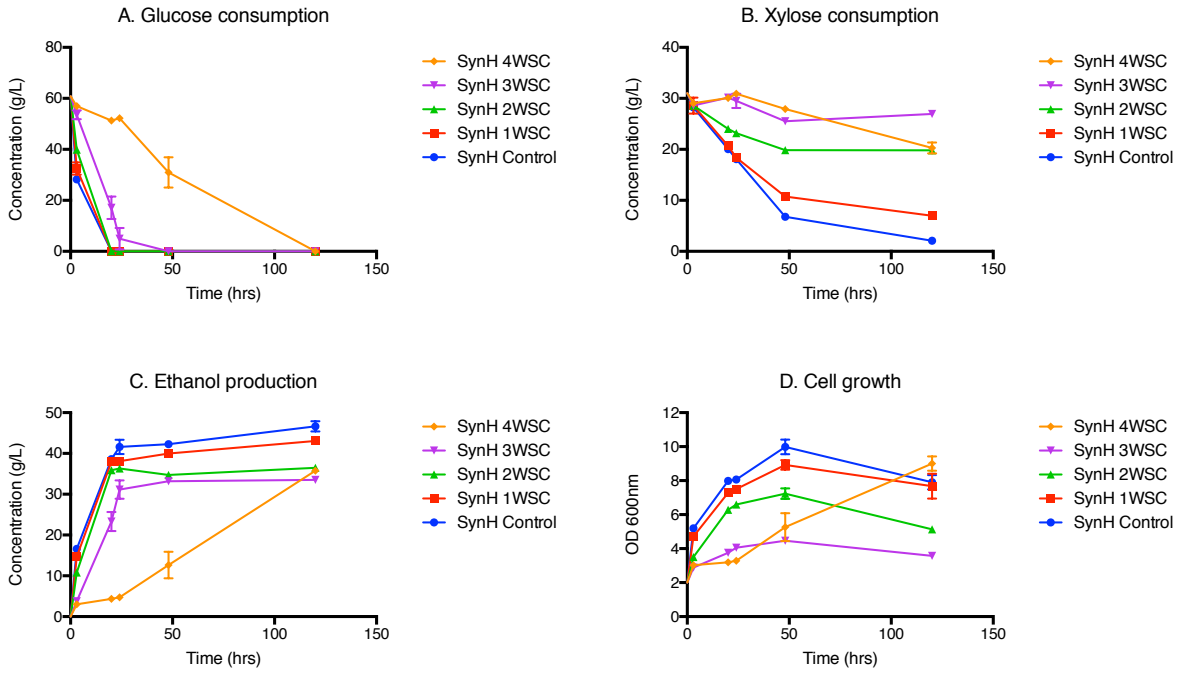


Figure 5.4 Fermentation performance of Y128 under varying concentrations of WSC. **A**. Glucose consumption; **B**. Xylose consumption; **C**. Ethanol production; **D**. Cell growth OD 600. SynH 4WSC: Synthetic hydrolysate with 40 g/L WSC added; SynH 3WSC: Synthetic hydrolysate with 30 g/L WSC added; SynH 2WSC: Synthetic hydrolysate with 20 g/L WSC added; SynH 1WSC: Synthetic hydrolysate with 10 g/L WSC added; SynH Control: Synthetic hydrolysate base media with no inhibitors added. Fermentation was conducted in Erlenmeyer flasks (50 mL) under the conditions of pH 4.8, 30 degree celsius and 150 RPM with inoculum at 2 OD600.

were no longer inhibited strongly after 48 h to 120 h. For example, the xylose concentration at 120h for SynH 4WSC became very close to SynH 2WSC (20.3 g/L versus 19.8 g/L) and much lower than SynH 3WSC (20.3 g/L versus 27.0 g/L), and the ethanol concentration at 120 h for SynH 4WSC was higher than SynH 3WSC (35.8 g/L versus 33.5 g/L). The cell growth of SynH 4WSC in Figure 5.4 even showed a reversed effect. The OD600 reached 9.0 at 120 h, which was the highest among all fermentation media being tested. The components in WSC exerted multiple influences on yeast fermentation. A dual character of early stage lag phase (possibly caused by phenolic compounds) versus late stage cell growth enhancement (possibly caused by free monomeric sugars and cell osmotic protectants) was observed. In addition to fermentation with high initial inoculum at OD 2 (Figure 5.4), we also tested the fermentation with low initial inoculum at OD 0.1. The yeast fermentation at different initial inoculums had similar trends in

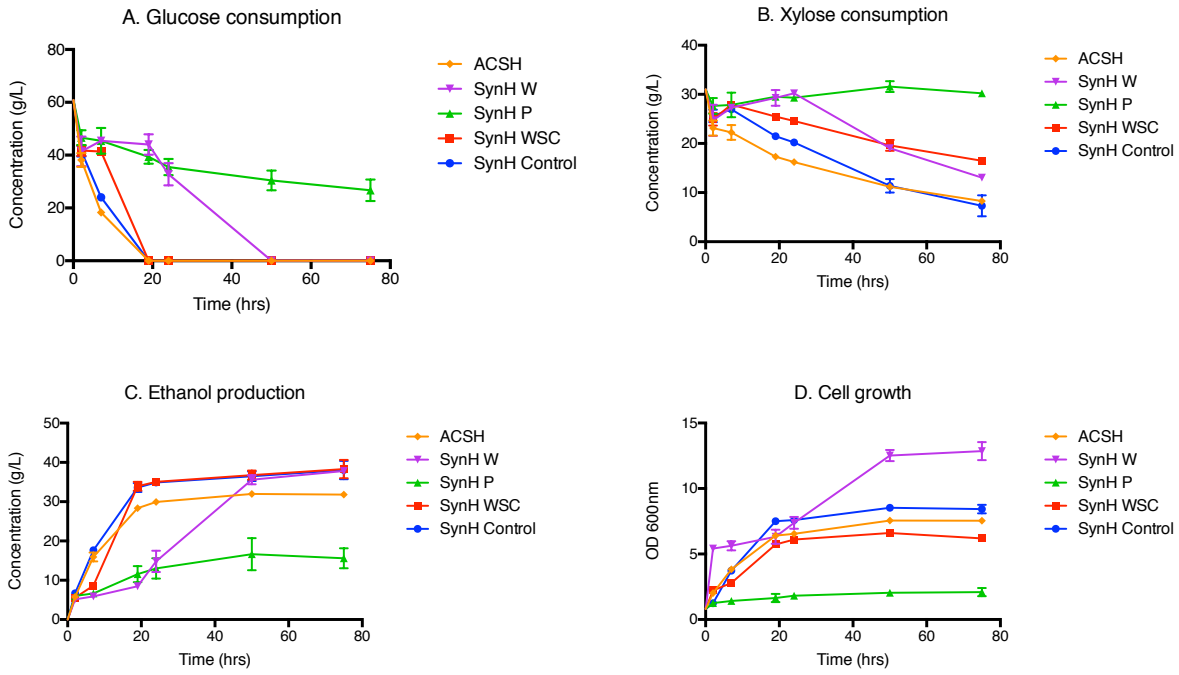


Figure 5.5 Fermentation performance of Y128 under different WSC fractions. **A**. Glucose consumption; **B**. Xylose consumption; **C**. Ethanol production; **D**. ACSH: AFEX corn stover hydrolysate; Syn W: Water phase extract after ethyl acetate extraction where soluble sugars and nutrients exited; Syn P: Ethyl acetate phase extract after ethyl acetate extraction where phenolic compounds were enriched; Syn WSC: Synthetic hydrolysate with 20 g/L WSC; SynH Control: Synthetic hydrolysate base media with no inhibitors added. Both phenolic compounds and nutrient components were re-dissolved in SynH base media at 20 g/L. Fermentation was conducted in Erlenmeyer flasks (50 mL) under the conditions of pH 4.8, 30 degree celsius and 150 RPM with inoculum at 0.8 OD600.

sugar utilization, ethanol production and cell grow. However, it is worth noting that WSC had an amplified effect (both early stage inhibition and late stage improvement) on low inoculum OD 0.1 than high inoculum OD 2, indicating a high inoculum at the beginning of fermentation might help the microbe resist inhibitors in hydrolysate.

5.1.4.3 Inhibitory effect of WSC ethyl acetate fractions

To further investigate the complex dual effect of WSC on the yeast fermentation, we further performed Y128 fermentation on different fractions of WSC after the ethyl acetate extraction. As we found that SynH with 20g/L WSC mimicked real ACSH in cell growth, sugar consumption and ethanol production, both Syn WSC and ACSH were used as positive control in this group. SynH control media was kept as negative control. Syn W represented the water phase extract

after ethyl acetate extraction, in which soluble sugars and nutrients existed. Syn P represented the organic phase extract after the ethyl acetate extraction, in which phenolic compounds were enriched. Both phenolic compounds and nutrient components were re-dissolved in SynH base media at 20 g/L. After separating phenolic compounds from other components in WSC, the binary behaviour observed in previous experiment (Figure 5.4) disappeared. Phenolic compounds exerted the strongest inhibition effect on Y128 fermentation among all media tested. Less than 50% of glucose were consumed and xylose was barely utilized. Ethanol yield after 72 h was 16 g/L compared with 38 g/L for all other media. Cell growth after 72 h was 2.1, which was much lower than all other fermentation media. In contrast, Syn W boosted the cell grow to OD 13 at 72 h which was higher than SynH control media (OD 8,4), even though a short lag phase was observed for the first 24 h. The concentration of monomeric sugars alone in the nutrient fraction was too low to be accounted for increasing cell growth, so we believed that potential cell protectants and oligomeric sugars were major reasons for the cell growth enhancement. Phenolic compounds and water-soluble nutrients together consisted the dual inhibitory effect of WSC on ethanol fermentation.

5.1.4.4 Chemical genomics of WSC

Chemical genomics was performed using a genome-wide deletion mutant collection of *S. cerevisiae* to show the fingerprints of genes deletion response to inhibitors. The sensitive and resistant genes clusters could reveal the metabolic pathways that were affected by WSC, provide information for microbe genetic engineering, and confirm the variability between different hydrolysates and fermentation media (e.g. ACSH, SynH control media). Figure 5.6a showed the profile of gene response to WSC. A resistant mutant has a positive interaction score on Y axis, whereas a negative score indicates a sensitive mutant. For example, EEB1(fatty acid biosynthesis) deletion was high interaction score conferred resistance to WSC. Other resistant mutants included gene deletions of ADE 5,7 (the 'de novo' purine nucleotide biosynthetic pathway) and EPS1(ER protein with chaperone and co-chaperone activity). Both the gene deletions, together with EEB1, could be points of engineering to help the yeast strain withhold inhibitory effects. Whereas, ERJ1 (ER Protein folding), LSC1 (Succinyl CoA ligase) and TRP2 (Tryptophan biosynthesis) deletions with

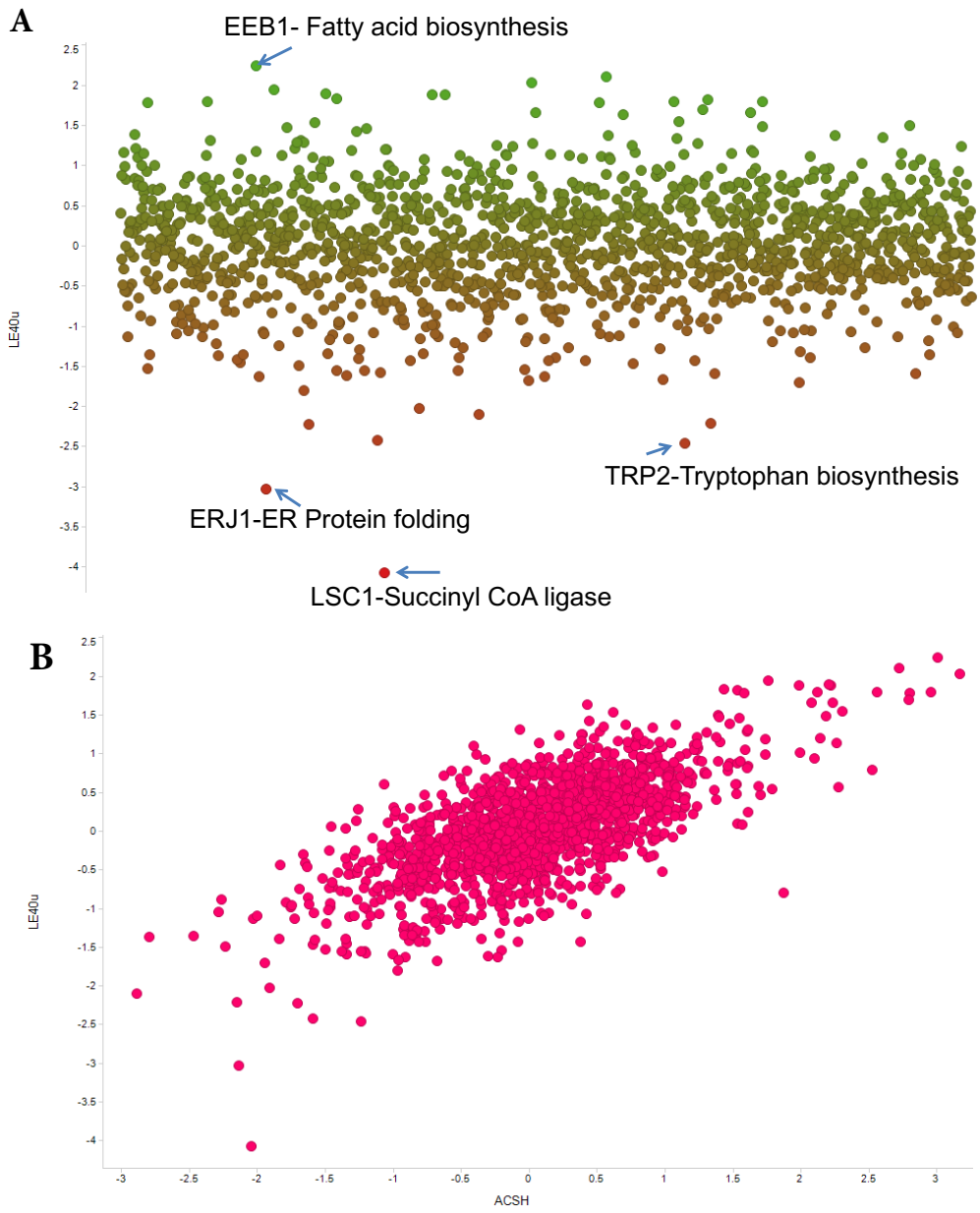


Figure 5.6 Chemical genomic profiling of WSC on using yeast deletion strain library. (**A**). Responsive gene profile; (**B**). Gene clusters correlation between SynWSC and ACSH.

low interaction score showed that these genes were important for resisting the inhibitory effect of WSC and could possibly be upgraded to enhance the resistance. Cell membranes were likely target of WSC toxicity. The fingerprint of WSC also showed similarity to compounds like Latrunculin (actin disruption) and Brefeldin A (Golgi transport disruption), therefore WSC had the potential to be developed into herbicide.

Quantitative analysis of the clustergram data is shown in Figure 5.6B and supplementary Figure

B.3. When comparing all hydrolysate to each other, the R2 correlation value between SynWSC to ACSH is 0.48, which was much higher than 0.02 between SynH control media with ACSH. It appeared that the degradation compounds in water-soluble lignin extractives can represent the real inhibitors in ACSH to a large extent.

5.1.5 Conclusions

Lignocellulose-derived degradation products produced during thermochemical pretreatment could cause strong inhibitory effects in microbial fermentation, and have been a major bottleneck for cost-effective lignocellulosic biorefineries. To optimize the pretreatment process to obtain biomass and hydrolysate with higher digestibility and fermentability, and engineer microbial strains for improved performance, it is critical to identify major inhibitory compounds and understand the mechanisms of inhibition that affect fermentative organisms. In this work, we used SynH to study the inhibition effect of WSC, which was isolated from EA pretreament crude lignin stream, on yeast fermentation. The natural lignin-dervied compounds in WSC could better represent and mimic real inhibitory compounds in ACSH. We found that SynH with 20g/L WSC would mimic real hydrolysate in yeast fermentation. The inhibitory effect of WSC increased with higher dosage. However, when the concentration of WSC reached 40g/L, the inhibitory effect was reversed after 48 hrs, indicating that the additional nutrients that existed in WSC became actively contributing at higher concentrations. To dissociate this dual character, ethyl acetate extraction was conducted to separate phenolic compounds from WSC. Major compounds identified in the organic phase of WSC included coumaroyl amide, feruloyl amide and coumaroyl glycerol. These and other nitrogenous phenolic compounds acted as key inhibitors in microbial fermentation of ACSH. On the other hand, esters and oligosaccharides were identified in the water phase compounds after ethyl acetate extractions. They were shown to improve cell growth in fermentation after 48 hours, which explains the dual character of WSC. Chemical genomic profiling of WSC was conducted on yeast deletion library to identify responsive genes and compounds with similar fingerprints. The gene clusters between WSC and ACSH was much higher than synthesized phenolic compounds, confirming that the natural nitrogenous phenolic compounds better represent the inhibitors in real

hydrolysate. This chapter also proved that EA pretreatment improved hydrolysate fermentability partially by removing highly toxic lignin-derived nitrogenous and phenolic compounds. The fundamental knowledge described in this chapter can be applied to characterize a variety of inhibitory compounds, optimize fermentation process, and be used to design pretreatment process adjustments.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

We first proposed the methodology for large-scale purification of oligosaccharides using charcoal fractionation and size exclusion chromatography. The carbohydrate composition of recalcitrant oligosaccharides, their mass and molecular weight distribution profiles were determined. Enzyme activity assay revealed that sugar inhibition and the lack of enzyme activity in commercial enzyme mixtures are major reasons for inefficient monomeric sugar conversion. A novel glycome profiling method (Elisa screening), combined with biotinylation derivatization, was used as a rapid, high-throughput tool to identify recalcitrant non-cellulosic glycan structures for small-molecule oligosaccharides and insoluble polysaccharides. 4-*O*-methyl-D-glucuronic acid substituted xylan and pectic-arabinogalactan were found to be the most abundant epitopes recognized by mAbs in hydrolysate and UHS. These structures were further validated by MALDI-TOF and TMS composition analysis.

An artificial, chemically-defined SynH was used to study the inhibitory effect of WSC from EA pretreatment on yeast fermentation. Key inhibitory compounds that were identified using LC/GC-MS included p-coumaroylamide, feruloylamide and p-coumaroylglycerol. Chemical genomics was employed to show the fingerprints of genes deletion response to inhibitors. The different sensitive/resistant genes clusters confirmed the variability and similarity of inhibitors in WSC with real ACSH.

Future work is needed to find enzymes that can hydrolyse these recalcitrant non-cellulosic polysaccharide linkages. For example, accessory enzymes such as pectinase, β-glucuronidase and enzyme activities targeting arabinan and galactan should be identified and added to the enzyme cocktail, so that branched linkages that block enzyme accessibility to backbone polysaccharide chains can be hydrolyzed and potentially be decoupled from the complex structure of hemicellulose. If successful, this approach could not only increase monomeric xylose yields, but may also synergistically improve cellulose hydrolysis, thus increasing glucose yields and a possible reduction in enzyme loading to lower biofuel production cost. Likewise, this approach could be

adapted with other pretreatment technologies and biomass to optimize hydrolysis conditions for maximum sugar output. Moreover, since routine glycome profiling only detects large polysaccharides, more advanced techniques such as MS-MS, fluorescent labelled antibodies studies using fluorescent microscopy and flow cytometry which will be able to detect short-chain oligosaccharides that are abundant in the hydrolysate. Quantitative modeling of novel Elisa method is also under development. In terms of better fermentation with existing phenolic and nitrogenous compounds, directional evolution of microbial strains could be performed using WSC to selectively screen resistant strains. Gene responses and metabolic pathway fingerprints could also be used as gene mutation targets. Such studies and collaborations are underway at the Great Lakes Bioenergy Center and Michigan State University.

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APPENDIX A

MATERIALS AND METHODS

A.1 Biomass

Corn stover (CS) of Pioneer hybrid seed variety (33A14) was harvested in 2010 from Kramer farm in Wray (CO). Composition analysis was performed using the NREL protocol [134]. The composition of 2010 CS was 31.4% glucan, 18.7% xylan, 3.3% arabinan, 0.0% mannan, 1.2% galactan, 2.2% acetyl, 14.3% lignin, 1.74% protein, and 13.39% ash. Unless otherwise stated, ACS was used as is for enzymatic hydrolysis experiments without washing, conditioning, nutrient supplementation or detoxification.

A.2 Enzymes

CELLIC CTec2 (138 mg protein/mL, batch number VCNI 0001), a complex blend of cellulase, β-glucosidase and hemicellulase, and CELLIC HTec2 (157 mg protein/mL, batch number VHN00001) were generously provided by Novozymes (Franklinton, NC, USA). Multifect Pectinase® (72 mg protein/mL, batch number 4861295753) was a gift from DuPont Industrial Biosciences (Palo Alto, CA, USA). The protein concentrations of the enzymes were determined by estimating the protein (and subtracting the nonprotein nitrogen contribution) using the Kjeldahl nitrogen analysis method (AOAC Method 2001.11, Dairy One Cooperative Inc., Ithaca, NY, USA).

A.3 Biomass pretreatment

DA pretreatment was performed at BESC (University of California, Riverside, CA, USA) at 160 °C for 20 minutes with 10% w/w solid loading and 0.5% w/w sulfuric acid using a 1 L Parr reactor with two stacked pitched blade impellers (Model 4525, Parr Instruments Company, Moline, IL, USA). It took 2 minutes for the reactor to reach 160 °C and another 2 minutes to bring the biomass temperature down to ambient conditions after pretreatment completion. The heating system was a 4 kW model SBL-2D fluidized sand bath (Techne, Princeton, NJ, USA). After the pretreatment, the residual solids were washed with water to remove acid and other degradation compounds produced during the process.

IL pretreatment was performed at JBEI (Berkeley, CA, USA) using 1-ethyl-3-methylimidazolium acetate, abbreviated as [C2mim][OAc], at 140 °C for 3 hours using 15% (wt/wt) loading of biomass to IL in a controlled tem- perature oil bath using a sealed stirred vessel. It took 30 minutes for the reactor to reach 140 °C and 20 minutes to cool down to 60 °C The residual IL was removed and pretreated biomass material was recovered with a series of water and ethanol washes.

AFEX pretreatment was performed at the GLBRC (Biomass Conversion Research Laboratory, MSU, Lansing, MI, USA). The conditions were 140°C for 15 minutes at 60% (wt/wt) moisture with 1:1 anhydrous ammonia to biomass loading in a bench-top stainless steel batch reactor (Parr Instruments Company) [7]. It took 30 minutes for the reactor to reach 140 °C and the ammonia was rapidly released, which immediately brought the biomass to room temperature. After the treatment, ammonia was removed by evaporation, leaving an essentially dry material. Hence AFEX is a dry to dry process, while IL and DA are dry to wet processes, as noted above.

A.4 Chemicals

Celite 545 was purchased from EMD Millipore (Billerica, MA). Activated charcoal (DARCO, 100 mesh particle), Avicel (PH-101), beechwood xylan and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

A.5 Biomass compositional analysis

Extractive-based compositional analyses of the biomass samples were performed according to the NREL LAPs: Preparation of Samples for Compositional Analysis (NREL/TP-510-42620) [57] and Determination of Structural Carbohydrates and Lignin in Biomass (NREL/TP-510-42618) [134]. The biomass was extracted with water and ethanol prior to the acid hydrolysis step.

A.6 Oligosaccharide analysis

Oligomeric sugar analysis was conducted on the hydrolysate liquid streams using an autoclave-based acid hydrolysis method at a 2 mL scale. Hydrolysate samples were mixed with 69.7 μL of 72% sulfuric acid in 10 mL screw-cap culture tubes and incubated in a 121 °C bench-top hot plate for 1 hour, cooled on ice and filtered into HPLC vials. The concentration of oligomeric sugar was

determined by subtracting the monomeric sugar concentration of the non-hydrolyzed samples from the total sugar concentration of the acid hydrolyzed samples. Sugar degradation was accounted for by running the appropriate sugar recovery standards along with the samples during acid hydrolysis.

A.7 High solids-loading enzymatic hydrolysis

High solids loading 25% (w/w) (approximately to 8% glucan loading) ACSH was prepared as starting material for the large-scale production of oligosaccharides. Enzymatic hydrolysis of ACS was performed using a commercial enzymes mixture including Cellic Ctec2 10 mg protein/g glucan (in pretreated biomass), Htec2 (Novozymes, Franklinton, NC), 5 mg protein/g glucan and Multifect Pectinase (Genencor Inc, USA), 5 mg protein/g glucan. Enzymatic hydrolysis was carried out in a 5L bioreactor with 3L working volume at pH 4.8, 50 °C, and 250 rpm. After 96 h hydrolysis, the hydrolysate was harvested by centrifugation at 6,000 rpm for 30 min and then 14,000 rpm for 30 min to remove unhydrolyzed solids. Hydrolysate was then sterile filtered through a 0.22 μm filter cup. The filtered hydrolysate was stored at 4 °C in a sterile bottle prior to charcoal fractionation (described below). Samples obtained from compositional analysis were subjected to HPLC using Bio-Rad Aminex HPX-87H column to determine sugar concentrations as described below.

A.8 Micro-plate based enzyme recalcitrance study

Micro-plate based enzymatic hydrolysis was performed in 0.5 mL reaction volumes with commercial enzymes (Ctec2: Htec2: MP) in 1:1:1 ratio for 24 h hydrolysis at 50 °C and pH 4.8. Maximum sugar concentration per well was 3 g/L while the minimum sugar concentration per well was 0.2 g/L (determined by acid hydrolysis). Enzyme loading is 60 μg/well i.e., 20 mg/g-300 mg/g glucan.

A.9 Charcoal fractionation of oligosaccharides

An activated charcoal and Celite mixture was used to extract oligosaccharides (affinity and polarity based extraction) from the hydrolysate prepared above [50, 51]. Activated charcoal (250 g) and Celite powder (250 g) were thoroughly mixed and packed in a 1 L sintered glass filter funnel

connected to a 2 L vacuum flask. The charcoal/celite mix was first preconditioned in 100% acetonitrile overnight to clean and activate the charcoal mixture. After the charcoal matrix being soaked in acetonitrile overnight, 6-8 L of water was added with vacuum filtration to prepare the matrix for hydrolysate incubation. Before the last of this water drained out, 500 mL of hydrolysate was added the charcoal matrix and left overnight to allow adsorption of oligosaccharides to the charcoal matrix. After incubation, another 5 L of water followed by 2 L of 5% acetonitrile (v/w) were used to wash the un-adsorbed and loosely-bound materials. Following this wash step, acetonitrile (ACN) and formic acid (FA) were used as eluents to desorb the oligosaccharides from the charcoal matrix. 3 L of 50% acetonitrile (v/w) were used to separate neutral oligosaccharides, followed by 2 L of 50% acetonitrile with 1% formic acid (v/w) to separate the acidic oligosaccharides. After collecting both the neutral and acidic fractions separately, 2 L of 60% methanol (v/w) was used to wash the charcoal matrix. Vacuum can be applied to fasten the fractionation process but longer interaction times between the hydrolysate and elution solvent (acetonitrile and formic acid) with charcoal matrix are beneficial for higher recovery. The charcoal matrix should be submerged in solvent (not allowed to dry out) throughout the process.

A.10 Desalting and concentration of crude oligosaccharides fractions

Crude oligosaccharides from charcoal fractionations were concentrated using a SpeedVac concentrator (SC210P1-115, Thermo Scientific) before SEC fractionation. Acetonitrile was removed first and the oligosaccharides were re-suspended with water (50 mL of neutral fractions/55 mL of formic acid fractions). Ammonium hydroxide was used to neutralize formic acid (pH adjusted to 5.5) in acidic samples and volatile ammonium formate was removed with water addition using same repeated SpeedVac cycles. The desalting procedure was repeated at least five times to remove most of the salts.

A.11 Micro-plate based enzyme inhibition study

Micro-plate enzyme activity assay experiments were performed at enzyme loading of 10 mg/g substrate, i.e., 62.5 μg enzyme per well, for 24 h hydrolysis at 50 °C, 10 rpm and pH 4.8 (adjusted by adding 1M citrate buffer pH 4.3). Reaction volume for each well is 500 uL. Pure substrates (Avi-

cel and beechwood xylan) were added in 1.25% solids loading, i.e., 6.25 mg per well. Oligosaccharides concentration was at 10 g/L; monomeric sugars were at 20 g/L glucose, 10 g/L xylose. Avicel was tested using Ctec2; beechwood xylan was treated with Htec2 and MP individually, and another mixture of Htec2 and MP in 1:1 mass ratio.

A.12 Analytical Method

Glucose, xylose and arabinose concentrations were analyzed using a Shimadzu HPLC system equipped with a Bio-Rad Aminex HPX-87H column equipped with automatic sampler, column heater, isocratic pump, and refractive index detector (RID). The column was maintained at 50 °C and eluted with 5 mM H_2SO_4 in water at 0.6 mL/min flowrate. Monomeric sugars were identified and quantified by comparison to authentic standards using a five-point calibration curve.

A.13 Fractionation based on size exclusion chromatography (SEC) column

The concentrated neutral and acidic samples were fractionated using size exclusion chromatography (FPLC Amersham-Biosciences, Akta system 890) with an XK 260 -1000 mm column (GE Healthcare) packed with P2 gel (200-400 mesh, separation range of 100-1800 MW, Biorad Laboratories, Hercules, CA). The instrument was equipped with both Ultra-Violet and conductivity detectors (GE Healthcare). Water was used as eluent solvent. A flow rate of 1.0 mL/min was used and 5 mL sample was injected for each run. 5 mL of concentrated neutral fractions (containing 670 mg oligosaccharides) and acidic fractions (containing 140 mg oligosaccharides) were injected into the SEC column after a 500 mL void volume. 90 fractions (A1-12, B1-12, C1-12, D1-12, E1-12, F1-12, G1-12, H1-6, 10 mL in each tube) were collected. Fractions in the same row were pooled and lyophilized into dry sample A to H by a freeze-dryer (FreeZone Plus 6 Liter Cascade Console Freeze Dry System, LABCONCO).

A.14 Corn stover and AFEX pretreatment

The corn (Pioneer 36H56) was planted on May 20th, 2010 in field 436 of Arlington Agricultural Research Station, Wisconsin. The field was fertilized with 340 lbs/acre urea three days prior to planting. In October 22nd, 2010, the corn stover was harvested and milled to a particle size of

5 mm. AFEX pretreatment was performed on the corn stover (CS) at 100 °C for 30 minutes with 0.6 g ammonia and 1 g water per g biomass loading in a bench-top stainless steel batch reactor [7, 21, 22] (Parr Instruments Company). It took 30 minutes for the reactor to reach 100 °C and this condition was maintained for 30 minutes. Then the ammonia was rapidly released, which immediately brought the biomass to room temperature. After the treatment, the biomass was transferred to aluminum tray and kept in hood overnight to remove residual ammonia, leaving behind dry material. The AFEX-pretreated corn stover contained 31.4% glucan, 18.7% xylan, 1.4% galactan, 3.3% arabinan, 14.3% lignin (1.23% acid soluble lignin, absorption wavelength 320 nm, absorptivity coefficient 30 L/g cm) and 13.4% ash.

A.15 Enzymatic hydrolysis

Enzymatic hydrolysis was performed in duplicate using baffled Erlenmeyer flasks. The AFEX-pretreated corn stover was loaded at 20% dry solids in a fed-batch manner. Half of the biomass was loaded at t = 0 h, and the remaining biomass was loaded at t = 45 minutes. Commercial enzymes CELLIC CTec2 (Novozymes), CELLIC HTec2 (Novozymes), and Multifect Pectinase (Genencor) were loaded at 10, 5, and 5 mg protein/g glucan at t = 0 h, respectively. Flasks were incubated in a shaking incubator set at 250 rpm and 50 °C. During the sampling process, the flasks were taken out of the incubator and immediately set on ice for approximately 30 minutes to arrest the hydrolysis reaction at each time point (3, 6, 12, 18, 24, 48, 72 and 168 h). Separate pairs of flasks were used for each time point. The pH was adjusted to 5.0 using 12 M hydrochloric acid at the start of the hydrolysis process.

A.16 Post hydrolysis solids recovery

The contents of the flasks were transferred into centrifuge bottles and centrifuged at 10,000 g at 4 °C for 30 minutes. The supernatant was decanted, the volume measured, and filtered through 0.22 μm PES membrane and stored at 4 °C for future sugar analysis. The solid was re-suspended in a known amount of water (approximately 8:1 water to solid ratio) and centrifuged. The supernatant was decanted to a separate tube and a 1 mL sample was taken for sugar analysis. This process was repeated two more times to remove any residual soluble material present in the solids. The

moisture content of the wet solids was measured in triplicate by drying samples at 110 °C overnight in aluminum tray.

A portion of the washed solid was treated with protease from Streptomyces griseus (Sigma Aldrich P5147) at 5% (w/v) solid loading according to the procedure by Berlin et al. (2006)[11]. This helped to remove protein and residual enzymes bound to the cell walls prior to glycome profiling. The remaining solid was freeze-dried and stored in a refrigerator for further analysis.

A.17 Liquid and solid composition analysis

The hydrolysate supernatants were diluted and analyzed for monomeric and oligomeric sugar contents. Monomeric sugars were analyzed using an HPLC equipped with a Bio-Rad (Hercules, CA) Aminex HPX-87P column and de-ashing guard column. Column temperature was held at 80 °C and water was used as the mobile phase flowing at 0.6 mL/min. Oligomeric sugars were determined via dilute acid hydrolysis at 121 °C according to the method of [57]. Hydrolysis samples were neutralized and analyzed using the HPLC method given above for total sugars estimation following acid hydrolysis. The oligomeric sugars were calculated as total sugars after subtracting the monomeric sugars present in hydrolysate.

Freeze dried solids were homogenized using mortar and pestle. Composition analysis was performed on the solids using the standard National Renewable Energy Laboratory (NREL) method for determination of structural carbohydrates and lignin according to [134].

A.18 Mass balance

Mass balances were performed on the major structural components of corn stover cell walls at different time points throughout the course of the enzymatic hydrolysis process. All mass balances were based on one gram of AFEX-treated corn stover (AFEX-CS). Total UHS were collected and measured as described above, while the liquid supernatant was analyzed for monomeric and oligomeric sugars. Because the hydrolysis was performed at a high solid loading, the volume of liquid was not constant throughout hydrolysis and was measured or calculated at the end of hydrolysis. The volume of the hydrolysate was calculated using the following equation:

$$V_H = V_S + \frac{V_w * C_w}{C_H - C_W}$$

Where V_H is the total volume of hydrolysate to be calculated, V_S is the measured volume of the supernatant of the hydrolysate, V_w is the measured amount of water added to the first wash step (as described in the post-hydrolysis recovery section above), C_w is the glucose concentration of the washed supernatant, and C_H is the glucose concentration of the hydrolysate. Here glucose was used to calculate the volume as it was the most abundant sugar.

A.19 Glycome profiling

Glycome profiling of untreated, AFEX^{TM} pretreated and all unhydrolyzed biomass residues (involving preparation of sequential cell wall extracts and their mAb screenings) were carried out using the SOP previously described [38, 106]. To conduct glycome profiling, Alcohol Insoluble Residue (AIR) cell wall materials were prepared from biomass residues and were subjected to sequential extractions with increasingly harsh reagents such as ammonium oxalate (50 mM), sodium carbonate (50 mM), KOH (1 M and 4 M) and acidic chlorite as explained previous [80]. The extracts were then subjected to ELISAs against a comprehensive suite of cell wall glycan-directed mAbs [38] and the mAb binding responses were represented as heat maps. The amounts of different cell wall materials recovered during each extraction are depicted as bar graphs above respective heat map panels. Plant cell wall glycan-directed monoclonal antibodies (mAbs) were received from laboratory stocks (CCRC, JIM and MAC series) maintained by the Complex Carbohydrate Research Center (available through CarboSource Services; http://www.carbosource.net) or were obtained from Bio-Supplies (Australia) (BG1, LAMP). Information on mAbs used in this study can be found in the Table 4.1, including the link to Wall MabDB (http://www.wallmabdb.net) that provides detailed information for each antibody.

A.20 One-step biotinylation of oligosaccharides

A.20.1 Coupling of carbohydrates to biotin-LC-hydrazide

Biotin-LC-hydrazide (4.6 mg/12 μmol) was dissolved in dimethyl sulfoxide (DMSO, 70 μL) by vigorous mixing and heating at 65 °C for 1min. Glacial acetic acid (30 μL) was added, and the mixture was poured onto sodium cyano- borohydride (6.4 mg/100 μmol), which dissolved completely after heating at 65 °C for approximately 1 min. Then 5 to 8 μL of the reaction mixture was added to the dried oligosaccharides (1-100 nmol) to obtain a 10-fold or greater molar excess of label over reducing ends. The reaction was carried out at 65 °C for 2 h, after which the samples were purified immediately. In labeling experiments without reduction, sodium cyanoborohydride was omitted and the samples were allowed to react at 65 °C for 2.5 h.

A.20.2 Sample cleanup

Sample purification cartridges were prepared by cutting two filter discs (12 mm in diameter) from Whatman QM-A Quartz Microfiber Filter using a hole punch and placing them in a holder (e.g., the housing of a 5 mL syringe). Each cartridge was washed successively with water, 30% acetic acid in water, and acetonitrile before applying the sample. The sample was distributed uniformly on the filter surface and allowed to bind for 15min before reactants were removed by rinsing with acetonitrile (1ml) and 4% water in acetonitrile (6×1 ml). Labeled products were eluted with water (4×0.5ml). Filtered through 0.2 μm syringe filters, and dried in a SpeedVac.

A.21 Elisa of biotinylated oligosaccharides samples

A.21.1 Coating and wash

Add 25 μL of each biotinylated samples (100 μL each concentrated samples diluted in 5 ml 0.1M TBS separately) to respective wells on Avidin-coated plates. Coat control wells with 50 μL biotin at 10 μg/ml in 0.1M TBS. Coat DI water for blank readings. Incubate the plates at room temperature for 2 hours in dark (CAUTION: Light sensitive samples). Wash the plates 3 times with 0.1% milk in 0.1M TBS using plate washing program #11 for Grenier flat 3A.

A.21.2 Primary antibody addition and wash

Add 40 μL primary antibody to respective wells. Incubate the plates at room temperature for 1 hours in dark. (CAUTION: Time sensitive) Wash the plates 3 times with 0.1% milk in 0.1M TBS using plate washing program #11 for Grenier flat 3A.

A.21.3 Secondary antibody addition and wash

Add 50 μL Mouse/Rat secondary antibody to respective wells (dilute secondary antibody in 1:5000 proportion using 0.1% milk in 0.1M TBS). Incubate the plates at room temperature for 1 h in dark. (CAUTION: Time sensitive) Wash the plates 5 times with 0.1% milk in 0.1M TBS using plate washing program #12 for Grenier flat 5A.

A.21.4 Substrate Addition

Add 50 μL of TMB substrate (Prepare TMB substrate by adding 2 drops of buffer, 3 drops of TMB, 2 drops of hydrogen peroxide in 15 mL DI Water and vortex before use). Incubate the plates at room temperature for 30 minutes in dark. (CAUTION: Time sensitive).

A.21.5 Termination step and reading the plate

Add 50 μL of 1N Sulphuric acid to each well. Read the plate using program "ELISA 450 - 655 nm" in ELISA reader.

A.22 TMS composition analysis for neutral and acidic sugars

A.22.1 Standard TMS mix preparation (in duplicates)

Prepare 1 mg/mL solutions of these analytes in deionized water: arabinose, rhamnose, fucose, xylose, galacturonic acid (GalA), glucuronic acid (GlcA), mannose, glucose, galactose, N-acetyl-mannosamine (manNAc), N-acetyl-glucosamine (glcNAc), N-acetyl-galactosamine (galNAc), myo-inositol (internal standard). Prepare 2 standards by addding the 1 mg/mL sugar solutions as **Table C.1** (In duplicates).

Put the caps on the tubes. Freeze at -80 °C until completely frozen. Turn on the lyophilizer and hit "Auto" button to get the lyophilizer ready. When the standards are frozen, loosen the cap, or replace it with aluminum foil with holes or miracloth. Place in lyophilizer flask and attach

Table A.1 Standard TMS mix preparation.

Std TMS 1	Std TMS 2
50 μL arabinose	50 μL rhamnose
50 μL fucose	50 μL xylose
50 μL galA	50 μL glcA
50 μL glucose	50 μL mannose
50 μL glcNAc	50 μL galactose
50 μL manNAc	50 μL galNAc
20 μL inositol	20 μL inositol

the adapter. Open the vacuum knob; lyophilize the samples until all water is removed (usually it takes about 12 - 18 h). When samples are completely dried, close the vacuum knob, detach the lyophilizer flask carefully. Remove all the tubes containing samples from the flask and place the caps back on tightly. Standard is ready to be used for TMS procedure.

A.22.2 Sample preparation (in duplicates)

Add 100-500 μg of sample into a screw cap tube on analytical balance. Record the amount added. It is best to have the samples dissolved in solvent at certain concentration, added into the tube as liquid aliquots. Add 20 μL of 1 mg/mL inositol for internal standard into each sample tube. The amount of internal standard added to sample has to be the same as the one added into the standard tube. Lyophilize the samples as in step 8.1.3 - 8.1.9.

A.22.3 Preparation of 1M methanolic HCl (to perform up to 20 tubes)

Add 8 mL of anhydrous methanol into a screw cap test tube. Add 4 mL of 3N methanolic HCl. Place the cap back on. Vortex. No water allowed in this process!

A.22.4 Hydrolysis

Add 500 μL 1M methanolic HCl into sample and standard TMS tubes. Incubate overnight (16-18 h) at 80 °C heating block. Dry down methanolysis product at room temperature using the drying manifold. Add 200 μL MeOH and dry again. Repeat 2 times more.

Table A.2 Recommended GC temperature program.

No.	Rate	Final temp	Hold time
1.	20 °C/min	140 °C	2 min
2.	2 °C/min	200 °C	0 min
3.	30 °C/min	250 °C	5 min

A.22.5 N-acetylation

Add 200 μL methanol, 100 μL pyridine, and 100 μL acetic anhydride to sample. Mix well. Incubate at room temperature for 30 min. Dry down. Add 200 μL methanol and dry completely.

A.22.6 Silylation

Add 200 μL Tri-Sil and heat for 20 min at 80 °C. Cool down to room temperature to avoid evaporation. Dry down reagents until almost dry (~50 μL left). TMS derivatives are volatile, take pre-caution not to over dry, otherwise you will lose some of the derivatives.

A.22.7 GC-samples preparation and injection (Table C.2)

Add 2 mL hexane and mix well by vortexing. Pack a bit of glass wool into Pasteur pipette tip (5-8 mm). To do this, insert the glass wool from the top of 5-3/4 -inch pipette, push down with 9-inch pipette, it will be packed about 1-inch from the end of the tip. Centrifuge the samples at 3,000 x g for 2 minutes to precipitate any insoluble residue. Pipette out the supernatant and filter through a glass wool packed Pasteur pipette into another screw cap tube. Dry down until 100-150 μL. Inject 1μL samples to GC with initial temp 80 °C and initial time 2.0 min.

A.22.8 Calculation

If running standards for first time, perform TMS derivatization of each sugar separately and run them individually to procure the profile of retention times, spectra and distributions of peaks for each monosaccharide. This will be used to identify the monosacchride in standards mixture and samples. From the GC-MS, obtain the peak area and retention time of each sugar in standards and samples. Each sugar can have as many as 4 peaks, hence total all the area from peaks corresponding to 1 type of sugar. Assign the label of sugar identity to the unknown peaks in samples according to the standards retention time. Because each sugar can have as many as 4 peaks, total all the area

from peaks corresponding to 1 sugar. Calculate the detector response factor (RF) value of each sugar in the standard mixture against internal standard (in this case, inositol). For example:

$$RF\ of\ glucose = \frac{total\ peak\ areas\ of\ glucose\ in\ standard\ /\ inositol\ standard\ peakarea}{glucose\ weight\ in\ standard\ /\ inositol\ weight\ in\ standard}$$

Glucose weight in standard is 50 μg if 50 μL of 1 mg/mL glucose was added into standard tube. Inositol weight in standard is 20 μg if 20 μL of 1 mg/mL inositol was added into standard tube.

Calculate the amount of each sugar in your sample in μg using the RF value for corresponding sugar. For example:

$$Glucose\ amount\ (\mu g) = \frac{sample\ glucose\ peak\ area\ *\ inositol\ weight\ in\ sample}{RF\ of\ glucose\ *\ samples\ inositol\ peak\ area}$$

Inositol weight in sample is 20 μg if 20 μL of 1 mg/mL inositol was added to the sample.

Calculate the mol% of each sugar Calculate the number of moles of each sugar in sample.

$$No.\ of\ umoles = \frac{mass\ of\ sugar\ (in\ \mu g)}{MW\ of\ sugar\ (in\ \frac{\mu g}{\mu mol})}$$

MW = molecular weight. (e.g. MW of glucose = 180.16 g/mol) Calculate the total no. of μmoles of all the sugars present in the samples. Calculate the mol% of each sugar per total number of moles of sugars For example:

$$Glucose\ mol\% = \frac{\mu moles\ of\ Glucose}{\sum \mu moles\ of\ sugars} * 100\%$$

Calculate the sugar composition in sample Obtain the total amount of sample in μg from step 8.2.1. If samples were added in liquid, calculate the amount of sample as follows:

$$sample\ amount = conc\ \frac{\mu g}{\mu L} * volume\ added\ to\ tube\ (\mu L)$$

Calculate the % sugar composition for each sugar. For example:

$$\%\ sugar\ composition = \frac{sugar\ amount\ (\mu g)}{sample\ amount\ (\mu g)}$$

A.23 Obtaining the lignin stream from Extractive Ammonia pretreatment

Lignin stream was separated and collected in the Extractive Ammonia pretreatment (EA) of corn stover. EA pretreatment was applied to corn stover (CS) as previously described in the literature [139] using 6:1 ammonia-to-biomass weight ratio (NH_3 : BM) for 30 min at 120 °C. After the pretreatment, 16 wt% of the biomass was extracted by ammonia, which account for 44 wt% of total lignin in the biomass. This lignin stream was used as starting material for water extraction and ethyl acetate extraction in this work. Composition analysis of lignin stream can be found in our previous work [139].

A.24 Water extraction of EA crude lignin stream

WSC are extracted from crude lignin stream produced during EA pretreatment process. Distilled water was added and vortex-mixed with the lignin stream in a 10:1 (volume to mass) ratio. Water extraction was conducted in shake flask in an incubator under the condition of 250 rpm, 50 °C for 2 h. After the 2-h washing and extraction, the slurry was cooled down in 4 °C, and was then centrifuged and filtered through glass fiber filter (pore size 10 μm). The filtrate was collected and freeze-dried as WSC, and was further used for composition analysis, ethyl acetate extraction, and yeast fermentation.

A.25 Ethyl acetate extraction of WSC

To separate and enrich the phenolic compounds from the WSC, ethyl acetate was conducted on the WSC produced in the water extraction of EA lignin stream. The pH of WSC (supernatant) was adjusted to 2-3 to get ethyl acetate protonated. Ethyl acetate and supernatant (1:1 volume ratio, ethyl Acetate to supernatant) was added into the separation funnel. The liquid was fully mixed in the funnel for 10 min. Let it sit for about a half hour for phase separation. The top ethyl acetate phase where the phenolic compounds were enriched was transferred into another bottle, while the bottom water phase were collected and centrifuged under 4,400 rpm for 5 min. The newly-formed top layer of ethyl acetate fraction were collected and combined with the ethyl acetate phase removed in the previous step. Same volume of ethyl acetate was added into the water fraction again, and the extraction procedure was repeated twice. After three rounds of ethyl

acetate extraction, all the ethyl acetate fractions were combined and dried using the rotor-vap to concentrate the phenolic compounds. The liquid was concentrated until 5-10 mL liquid were left in the round bottom flask. The rest of fractions was air-dried in room temperature to avoid overheating the phenolic compounds. The rest of liquid (water phase) in the separation funnel was dried using the rotor-vap for mass balance and other microbial inhibition study. Water fractions cannot be processed using freeze dryer if it is not equipped with organic trap.

A.26 Fractionation of water soluble extraction using size exclusion chromatography

Size exclusion chromatography were performed to fractionate WSC depending on the molecule weight. An empty GE healthcare XK column (150 mL bed volume, Tube Height: 400 mm, Column i.d.: 16 mm) was packed with Bio-Rad P6 gel (Polyacrylamide, 1-6 kDA). Water was used as mobile phase with 1ml/min flow rate. 0.70 mL of WSC was injected for each run. 96 fractions (A1-H12) were collected in auto-sampler, with the molecule range went from high to low.

A.27 Mass spectrometry of WSC

LC-MS and GC-MS of WSC fractions were conducted in Mass Spectrometry and Metabolomics Core at Michigan State Univerisy. LC-MS was equipped with Supelco Ascentis Express C18 column (2.1 * 50 mm, 2.7 um particles) with Shimadzu LC-20AD ternary pumps and controller with high pressure mixer, Shimadzu column oven and CTC PAL auto-sampler. Column temperature was hold at 55 °C 0.15%, with aqueous formic acid as solvent A and methanol as solvent B.

GC-MS was conducted using a 30 m VF5MS column plus a 10 m EZ-Guard guard column, on a Waters GCT Premier mass spectrometer coupled to Agilent 6890 gas chromatograph with auto-sampler. Scan parameters were set at m/z 40-600, 0.2 seconds/scan (with dynamic range extension). The temperature program was set with an initial of 40 °C (2 min hold), 6 °C/min to 300 °C, and hold for 5 min. Helium was used as carrier gas with a flow rate of 1.3 mL/min.

A.28 Composition analysis of sugars and lignin extractives

Sugars and organic acid in the liquid supernatant ofWSC and hydrolysate were analyzed using an HPLC equipped with a Bio-Rad (Hercules, CA) Aminex HPX-87P column and de-ashing guard

column. Column temperature was held at 80 °C and water was used as the mobile phase flowing at 0.6 mL/min. Mobile phase (5 mM H_2SO_4) flow rate was 0.6 mL/min.

Free amino acids analysis was outsourced and analyzed by Molecular Structure Facility, UC Davis, using Hitachi L-8900 Amino Acid Analyzer.

Heavy metal analysis was outsourced and analysed by the UW Soil and Plant Analysis Lab using standard protocol. The Standard Operation Procedures of ICP-OES/MS are available from the following links: http://uwlab.soils.wisc.edu/files/procedures/ICPMS.pdf

A.29 High solid loading enzymatic hydrolysis

The AFEX-pretreated corn stover were hydrolyzed at 6% glucan loading in a fermenter equipped with a pitched blade impeller. Hydrolysis was performed over a period of 3 days with 20 mg protein/g glucan enzyme loading at 50 °C and 1,000 rpm. Samples were taken every 24 h and the sugar concentrations were measured by HPLC. After 3 days of hydrolysis, the overall mass balances for the pretreated solids were determined as described previously [130].

A.30 Synthetic hydrolysate

The formulation of SynH is based on the composition of ACSH produced in the enzymatic hydrolysis, with glucose concentration at 60 g/L and xylose concentration at 30 g/L. The development of SynH recipe was described elsewhere [72, 114, 140]. Detailed composition information of updated SynH being used in this work was listed in the supplemental material Table 5.2.

A.31 Microorganism and seed culture preparation

Saccharomyces cerevisiae GLBRC-Y128 was used for this study having previously been genetically modified and adapted for xylose utilization [104, 125]. Xylose isomerase and xylulokinase genes were introduced to facilitate xylose utilization. Seed cultures were prepared from glycerol stocks stored at -80 °C. Seed culture media contained 100 g/L dextrose, 25 g/L xylose, 20 g/L tryptone, and 10 g/L yeast extract. Erlenmeyer flasks (250 mL) containing 100 mL of seed culture media were inoculated with 0.1 OD600. The cultures were incubated at 30 °C and 150 RPM in shaker incubators under micro-aerobic conditions for 22 h before inoculation in SynH.

A.32 Chemical genomic profiling hydrolysate and lignin extractives

Chemical genomic analysis of these hydrolysate and lignin extractives was performed as described previously using a collection of 4000 yeast deletion mutants [112, 130]. 200 μL cultures with the pooled collection of S. cerevisiae deletion mutants were grown in the different Synthetic Hydrolysate or yeast extract (10 g/L), peptone (20 g/L), and dextrose (20 g/L) (YPD) medium in triplicate for 48 h at 30 °C under aerobic conditions. Genomic DNA was extracted from the cells and mutant-specific molecular barcodes were amplified using specially designed multiplex primers as described previously [103, 113]. The barcodes were sequenced using an Illumina HiSeq2500 in rapid run mode (Illumina, Inc., San Diego, CA). The average barcode counts for each yeast deletion mutant in the replicate hydrolysates were normalized against the YPD control to define sensitivity or resistance of individual strains (chemical genetic interaction score). Strains with low read counts were omitted from analysis. A resistant mutant has a positive interaction score, whereas a negative score indicates a sensitive mutant. The pattern of genetic interaction scores for all mutant strains represents the chemical genomic profile or biological fingerprint of a sample [103, 112, 113]. Correlations of the chemical genomic profiles across cycles were calculated using Spotfire 5.5.0 (Tibco, Boston, MA, USA). The clustergram of the chemical genomic profiles were created in Cluster 3.0 [26], and visualized in Treeview (v1.1.6r4) [102]. A Bonferroni-corrected hypergeometric distribution test was used to search for significant enrichment of GO terms among the top 10 sensitive deletion mutants [18].

APPENDIX B

SUPPLEMENTARY FIGURES AND TABLES

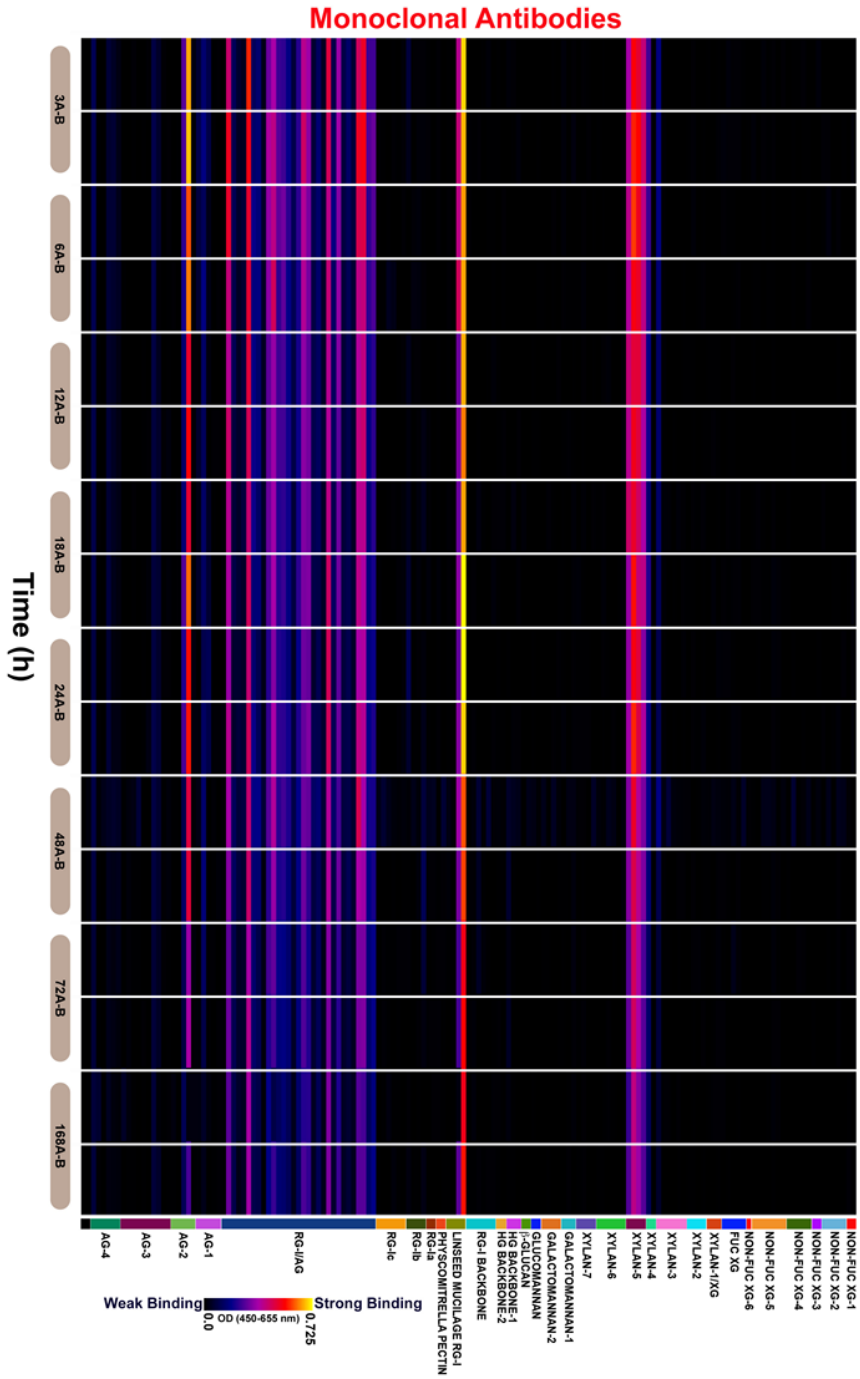


Figure B.1 Glycome profiling of the hydrolysates of AFEX-treated corn stover over the course of hydrolysis. A-B represents replicates of hydrolysate samples at different time points. Antibody groups used for the ELISA screening are shown on the right side of the heat map.

Table B.1 Composition analysis of different fractions of WSC. **A**. HPLC analysis of sugar and salts in WSC; **B**. HPLC analysis of sugar and salts in water phase after ethyl acetate extraction; **C**. Free amino acids analysis of WSC; **D**. Mineral salts (heavy metal) analysis of WSC.

A. Composition analysis of water-soluble components

HPLC (g/L)	Glucose	Xylose	Arabinose	Lactate	Glycerol	Formate	Acetate	Galactose
LE	2.88	1.80	0.55	0.04	1.50	0.41	1.76	0.12

B. Nutrients analysis of water phase in ethyl acetate extract

HPLC (g/L)	Glucose	Arabinose	Lactate	Glycerol	Galactose
Water phase	0.44	1.10	0.12	0.64	0.14

C. Free amino acids (uM)

Asp	**Asn**	**Thr**	**Ser**	**Glu**
1.49E+02	0.00E+00	1.05E+02	1.66E+02	5.80E+01
Ile	**Leu**	**Tyr**	**Phe**	**His**
9.39E+01	9.00E+01	8.12E+01	9.79E+01	0.00E+00
Gln	**Pro**	**Gly**	**Ala**	**Val**
1.90E+02	3.36E+02	1.75E+02	6.05E+02	0.00E+00
Lys	**Arg**	**Cystine**	**Methionine**	**Tryptophan**
4.97E+01	0.00E+00	0.00E+00	0.00E+00	0.00E+00

D. Heavy metals/anions (ppm)

P	**K**	**Ca**	**Mg**	**S**	**Zn**	**B**	**Mn**	**Fe**
23.5	1013.7	20.4	110	105.6	0.73	< 0.04	1.4	0.1
Cu	**Al**	**Na**	**Cd**	**Co**	**Cr**	**Mo**	**Ni**	**Pb**
0.28	0.14	9.4	<0.008	0.01	<0.002	0.01	0.02	<0.04
NH4-N	**Total N**	**Cl**	**F**	**NO2**	**Br**	**NO3**	**PO4**	**SO4**
1624	5509	596.4	1148.5	<0.01	<0.01	<0.01	<0.02	348.7

Table B.2 Recipe of SynH being used for fermentation study.

Component	mM (Final Concn.)
KH_2PO_4	5.84
K_2HPO_4	11.15
$(NH_4)_2SO_4$	30
KCl	36.8
NaCl	1.3
CaCl2·2H2O	5.5
MgCl2. 6H2O	12.5
L-Alanine	1.172
L-Arginine.HCl	0.144
L-Asparagine	0.228
DL-Aspartic acid.K	0.594
L-Cysteine.HCl	0.050
L-glutamine	0.259
L-Glutamic acid.K	0.607
Glycine	0.378
L-Histidine	0.037
L-Isoleucine	0.262
L-Leucine	0.371
L-Lysine.HCl	0.175
L-Methionine	0.100
L-Phenylalanine	0.282
L-Proline	0.656
L-Serine	0.369
L-Threonine	0.310
L-Tryptophan	0.050
L-Valine	0.424
L-Tyrosine	0.202
Adenine	0.05
Cytosine	0.05
Uracil	0.05
Guanine	0.05
Thiamine HCl	0.0004
Calcium Pantothenate	0.003

Component	mM (Final Concn.)
$ZnCl_2$	20
$MnCl_2·4H_2O$	91
$CuCl_2·2H_2O$	1.9
$CoCl_2·6H_2O$	0.03
H_3BO_4	23.1
$(NH_4)_6Mo_7O_{24}•4H_2O$	0.31
FeCl3·6H2O	20
Sodium formate	2.8
Sodium nitrate	1.1
Sodium succinate	0.5
Glycerol	4.1
Betaine.H2O	0.7
Choline Chloride	0.3
DL-Carnitine	0.3
Acetamide	80
Sodium acetate	32
L-lactatic acid (90%)	4
D-Mannose	1.2
L-Arabinose	20
D-Fructose	24
D-Galactose	2.9
D-Glucose	60g/l (333 mM)
D(+)Xylose	30g/l (200 mM)
Pyridoxine.HCl	2.14 µM
Nicotinic Acid	26.78 µM
Biotin	0.1 µM
Inositol	0.056 mM
Polysorbate 80 (Tween 80)	1 ml/l
Ergosterol	10 mg/l

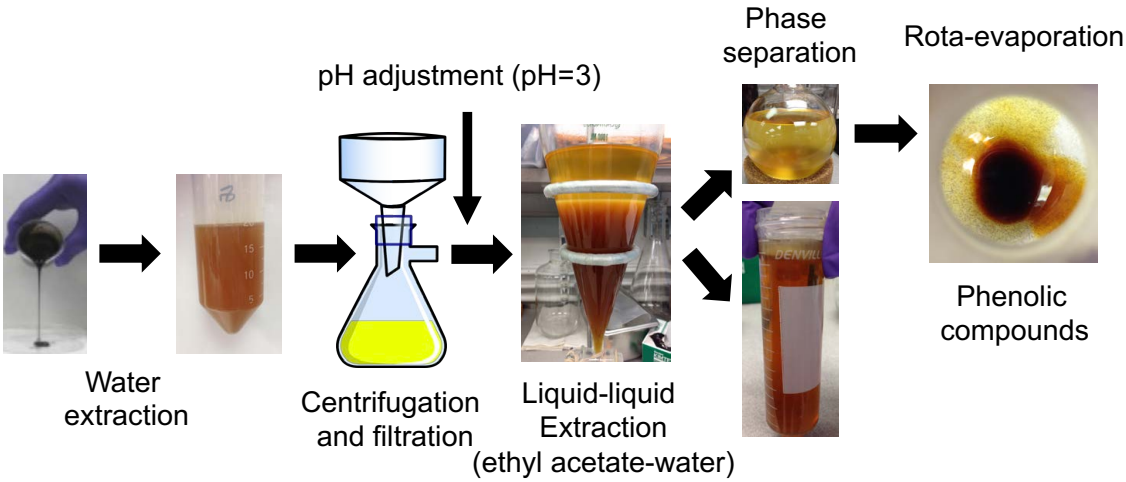


Figure B.2 Methodology and flow chart of water extraction of lignin streams and ethyl acetate extraction of WSC.

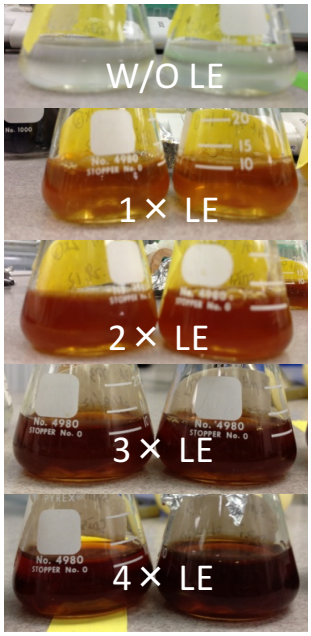
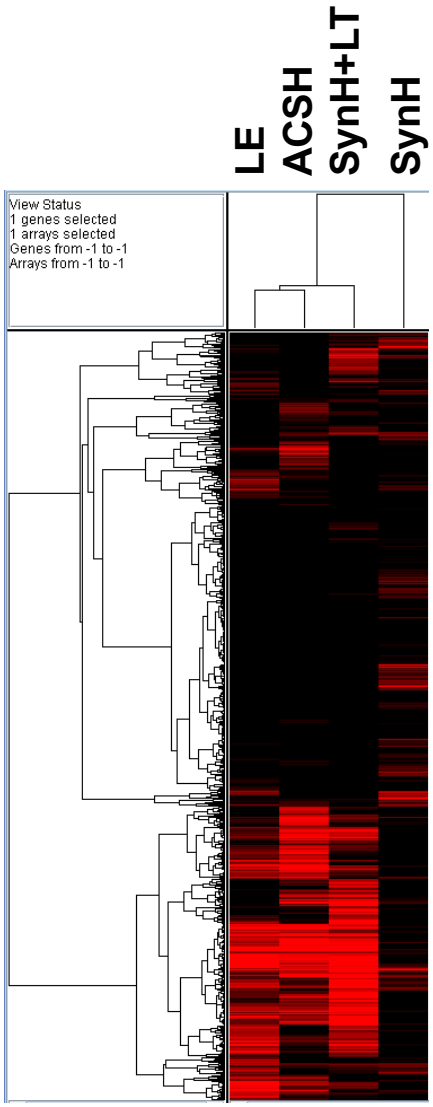


Figure B.3 Fermentation media of SynH with different folds of WSC (unfiltered). 1*LE represents 10 g/L lignin extractions. From top to bottom, the concentrations of WSC were from 0-40 g/L.



Conditions	R^2
SynH+LE vs ACSH	0.48
SynH vs ACSH	0.02
SynH vs SynH+LE	0

* 3 reps, 40g/L unfiltered LE

Figure B.4 Correlation between chemical genomic profiles of WSC, ACSH and SynH control media.