INTERACTIONS BETWEEN BIOMASS FEEDSTOCK CHARACTERISTICS AND BIOENERGY PRODUCTION: FROM THE LANDSCAPE TO THE MOLECULAR SCALE

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ABSTRACT

INTERACTIONS BETWEEN BIOMASS FEEDSTOCK CHARACTERISTICS AND BIOENERGY PRODUCTION: FROM THE LANDSCAPE TO THE MOLECULAR SCALE

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The choices that are made with respect to the efficient development and operation of any bioenergy conversion process are inherently linked to the physical and chemical properties of the feedstock. These interactions can be examined at a variety of scales ranging from the landscape scale where the availability of feedstock can affect the appropriate energy generation method for a given region, to the molecular scale where small variations in cell wall components can have a large impact on process yields. Because biomass is an inherently heterogeneous material, it is necessary to develop a broad understanding of how different characteristics impact the conversion process: how differences in biomass classification can help us make generalizations about new feedstocks, whether different varieties of the same species have inherent differences that alter their relative efficiencies of conversion, and what variations are possible depending on which portion of the plant is used for a feedstock.

At the landscape scale, the distribution of usable crop residues would be one factor influencing the decision of where to locate a lignocellulosic biorefinery. In Mainland China, 594 million metric tons of crop residues are produced each year, however only 125 million tons are available for energy generation, either in rural homes or in a larger facility. Based on residue availability, Henan in particular would be the most likely site for a biorefinery, with potential for other locations in central, eastern, and northeastern China.

Plant materials can interact with pretreatment and enzymatic hydrolysis at a variety of scales. Plant classification largely determines cell wall chemistry, and there are distinct differences between the way that dicots and grasses interact with pretreatment and enzymatic hydrolysis. Mixed-species feedstocks that have a higher mass contribution by grasses are more digestible and also generate higher sugar yields compared to those dominated by dicots. In contrast, the differences between different varieties of the same species, in this case switchgrass (when grown under the same environmental conditions), showed much smaller differences in digestibility, optimal pretreatment conditions, and enzyme combinations. At the next scale down, the different portions of the plant also have distinctly different structures and compositions that affect their amenability toward pretreatment. This could be one consideration toward determining how to best harvest lignocellulosics on the field. For corn stover, the best scenario involved harvesting the fractions in order of decreasing lignin content: husk > leaves > stem > cob, an order that is feasible using currently available harvesting equipment and methods.

Klason lignin content was related to decreased glucan digestibility in both the mixed-species materials and in corn stover fractions. However, no effect due to lignin content or lignin monomer composition was observed for genetically modified poplar. Of the samples tested, the C4H::F5H poplar that was modified to have highly linear and extractable lignin with mostly syringyl residues showed the greatest improvement following AFEX^{TM} pretreatment. However the increase was not substantial and for these types of materials it may be more effective to employ an liquid ammonia pretreatment that is able to extract the lignin and simultaneously modify the cellulose crystal structure.

DEDICATION

I dedicate this dissertation to the people I love most and who have generously helped to keep me sane during my doctoral work: my husband **Benjamin Ong**, who loved, supported, encouraged, and perhaps most importantly, made sure I ate while writing this dissertation; to my parents **Dayle and Nancy Garlock**, sister **Robin**, and brother **Kevin**, who have given me unconditional support and love my entire life and expressed their pride in me for accomplishing this grand and sometimes painful task; to my grad school roommate of four years, **Sarah Coefield**, who took me out to pet owls, helped me laugh when I was discouraged, and is one of my best friends; and most of all to **Jesus Christ, my savior**, who taught me during my time in graduate school more about humility, patience, and what to do when everything goes wrong (i.e. pray like crazy) than I ever realized that I needed to learn.

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I would also like to acknowledge Dow Chemical and the Hong Kong University of Science and Technology for funding my project on crop residues in China and giving me the once-in-a-lifetime opportunity to live overseas in a cross-cultural environment. I am especially grateful to Christopher Chao and Moses Ng for hosting me during my stay at HKUST, as well as Chris Chan from Dow, David Zweig and Meggy Wan from HKUST, and Glenn Shive from the Hong Kong-America Center, for their assistance and involvement during my time in Hong Kong.

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KEY TO SYMBOLS AND ABBREVIATIONS

ABSL = acetyl bromide soluble lignin

4CL = 4-coumarate:CoA ligase

AFEX^{TM} = ammonia fiber expansion pretreatment

ANOVA = analysis of variance

CAD = cinnamyl alcohol dehydrogenase

COMT = caffeic acid o-methyltransferase

CCR = cinnamoyl-CoA reductase

CS = corn stover

DM = dry matter

dwb = dry weight basis

F5H = ferulate-5-hydroxylase

GLBRC = Great Lakes Bioenergy Research Center

HPLC = high-performance liquid chromatography

KBS = W.K. Kellogg Biological Station

LSF = late successional old field combined sample

MANOVA = multivariate analysis of variance

MESP = minimum ethanol selling price

MTSY = maximum theoretical sugar yield

NDF = neutral detergent fiber

LTER = Long-Term Ecological Research

R1-R5 = GLBRC intensive site old field replicates

SF1-SF3= LTER late-successional old field treatments

S:G = syringyl:guaicyl lignin ratio

SHCS = selectively harvested corn stover

SOC = soil organic carbon

CHAPTER 1 :
INTRODUCTION

The issues related to the development, acquisition, and use of fossil fuels are becoming increasingly global as third world countries understandably attempt to become more developed and worldwide primary energy consumption continues to climb (**Figure 1.1**).

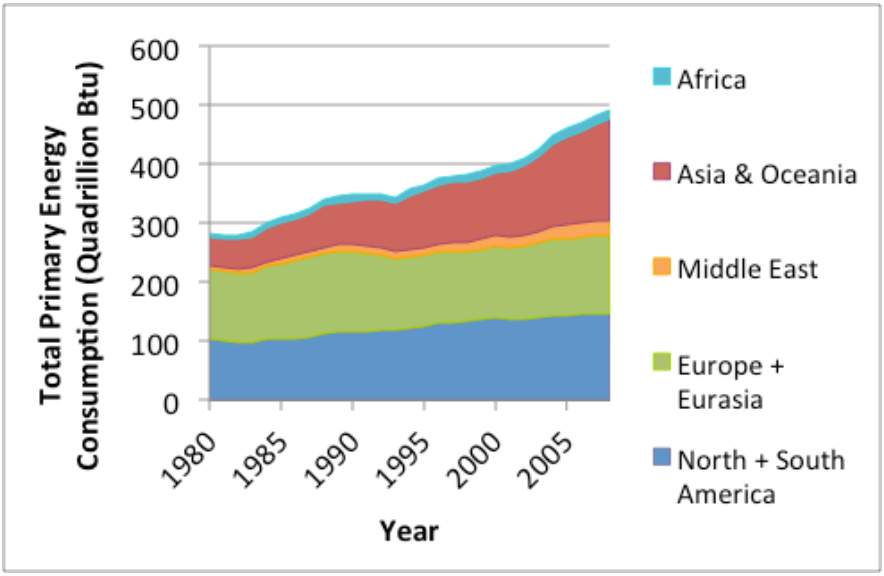

Figure 1.1: Worldwide primary energy consumption: 1980 – 2008. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

These issues, including climate change, energy security, and pollution, are compounded by the increasing global population. Bioenergy, or the production of energy from plant and algal feedstocks, is one form of alternative energy and perhaps the one most surrounded by confusion and controversy. Barriers to large-scale bioenergy production include the supposed competition between food and fuel production [1-3] and uncertainty surrounding the long-

term effects of growing bioenergy crops on global emissions [4-6]. But there are a number of benefits surrounding bioenergy production that should not be overlooked. First, bioenergy produced from lignocellulosic plant materials, such as waste paper; agricultural and forestry residues; and dedicated herbaceous and woody energy crops; does not necessarily have to compete with or displace food production. Second, the generation of usable energy from lignocellulosic materials is a near-term solution. If progress on current projects continues, there could be significant quantities produced within the next five years [7]. Finally, certain methods, infrastructure, and supply chains for biofuel production from lignocellulosics, could readily transition into markets such as animal feed, bio-based chemicals, polymers, and pharmaceuticals, for which renewable sources other than biomass do not exist [8-11].

The term lignocellulose refers to the three main classes of compounds found within plant cell walls: cellulose, hemicellulose, and lignin. Ethanol produced from cellulosics is derived from the long-chain carbohydrates, cellulose and hemicellulose. Except for the source material and greater complexity of the process, the ethanol that is produced from a cellulosic material is identical to ethanol produced from starchy materials, such as corn or wheat grain, or from sucrose-rich materials, such as sugar cane or sugar beets. The biochemical process for conversion of cellulosics to ethanol requires three key steps: pretreatment (a chemical and/or mechanical process necessary to disrupt the cell wall structure), followed by enzymatic saccharification to release cell wall sugars and subsequent or simultaneous fermentation to convert these sugars into ethanol (**Figure 1.2**).

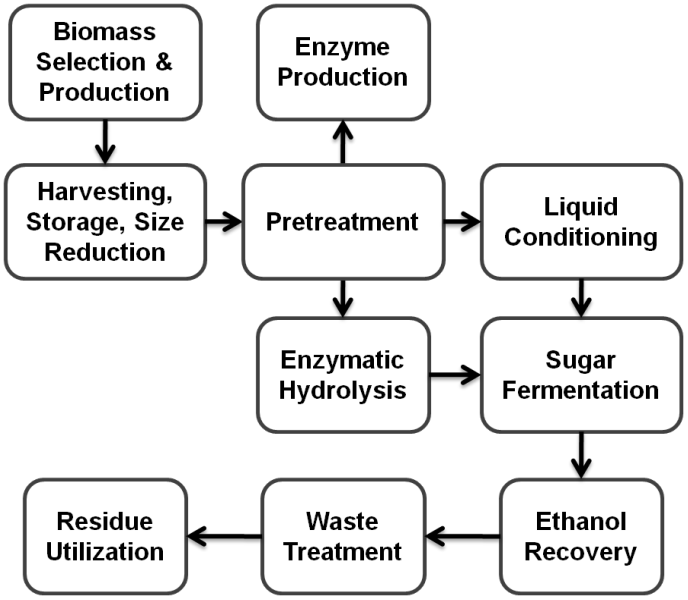

Figure 1.2: Generic process flow diagram of the cellulosic ethanol conversion process.

Ammonia fiber expansion (AFEX™)[1] is one pretreatment that is used to disrupt the plant cell wall prior to enzymatic conversion. AFEX™ is an alkali pretreatment that uses anhydrous or conc-entrated ammonia as a reactant. Ammonia and water react with the biomass, cleaving internal bonds via ammonolysis and hydrolysis reac-tions and solubilizing components within the cell wall that are later deposited on the biomass surface. This effectively opens the cell wall structure, allowing greater enzyme access. AFEX™ reactions are typically operated at temperatures from 60 – 180°C, for less than an hour, and at pressures ranging from 100 – 600 psi. At the end of the residence time, the reactor is vented,

[1] AFEX™ is a registered trademark of trademark of MBI International, Lansing, Michigan

releasing excess ammonia gas and water vapor to be recycled, and the system is cooled. AFEX^{TM} is a unique pretreatment process in that it uses a much smaller amount of water compared to many of the other forms of pretreatment. Because of this, there are no separate liquid streams produced and unless the biomass is washed following pretreatment, all of the biomass components are present in the subsequent enzymatic hydrolysis step.

As mentioned earlier, potential cellulosic feedstocks for bioethanol production include waste paper, agricultural and forestry residues, and dedicated woody and herbaceous energy crops. It is important for the biorefinery choose the best feedstocks for their process and to fully make use of any feedstock that is selected. This is because the feedstock and associated handling costs are predicted to contribute the greatest amount to the cost of producing cellulosic ethanol, more than the amount contributed by pretreatment, enzymatic hydrolysis, and fermentation combined [12], and the relative importance of the feedstock will only increase as the liquid biofuel industry matures. So research on improving plant materials and their interactions with processing can decrease the overall costs. Because different feedstocks do not perform equally well with a given pretreatment or energy conversion method [13, 14], it is necessary to know which feedstocks are suitable for a conversion process before a biorefinery location is decided upon. Otherwise a company may make the decision based solely on other important factors such as transportation, available labor force, governmental incentives, and available feedstock supply. Later they may discover that the feedstock that is available does not generate profitable yields.

Unfortunately, it is currently too time-consuming and labor-intensive to test and optimize each potential feedstock that could be used by the biorefinery. It is more realistic to

research the detailed interactions between the conversion process and a handful of feedstocks and then make generalizations for similar feedstocks. The most common way to classify bioenergy feedstocks is based on the relative compositions of lignin, hemicellulose, cellulose, and various minor components. However, while the relative amounts of these components impact the overall yields and feedstock digestibility, the variability between feedstocks cannot be entirely explained by the relative amounts of each component. The exact interactions that are possible between processing conditions and cell wall characteristics are not completely understood and are difficult to predict because of the complexity and variability of the plant cell wall.

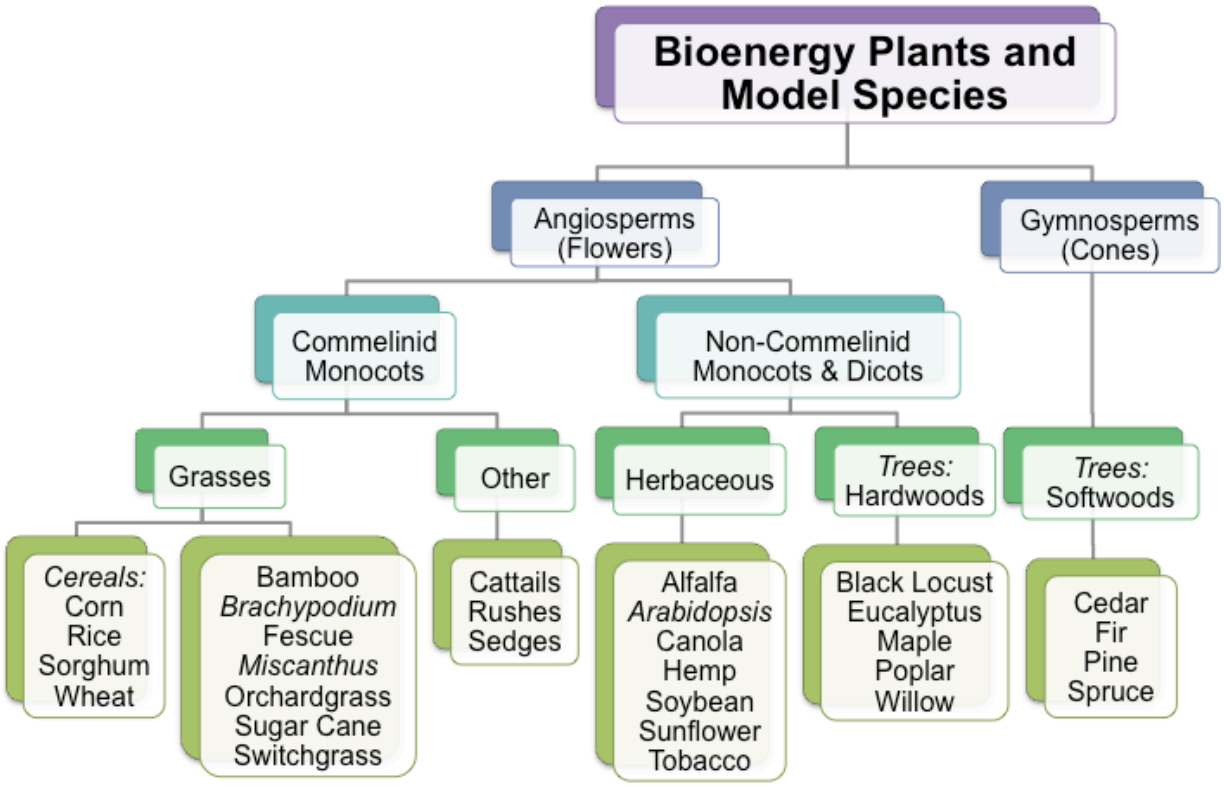

Figure 1.3: Common bioenergy plants and model laboratory species arranged in groups according to botanical classification.

Table 1.1: Key differences in the cell wall chemistry for different plant classifications. Information on hemicelluloses is from [15]. For the lignin subunits, (+) represents relative abundance within the lignin polymer and (-) represents absence.

Class	Major Hemicellulose	Proportion of Hemicellulose in 2° Cell Wall	Are Xylans Esterified with Ferulic Acid?	Lignin Subunits		
				S	G	H
Commelinids (Grasses and Relatives)	Glucurono-arabinoxylan	40-50%	Yes (mostly)	+++	++	+
Non-Commelinid Monocots and Dicots (Forbs and Hardwoods)	Glucuronoxylan	20-30%	No	+++	++	+/-
Gymnosperms (Softwoods)	Galacto-glucomannan	10-30%	No	-	+++	++

Another way to group bioenergy feedstocks is in terms of their botanical classification that automatically incorporates certain distinct, and more detailed, differences in the cell wall structure and chemistry (**Figure 1.3, Table 1.1**). Typical bioenergy feedstocks will fall within one of four major groups: commelinid monocots; non-commelinid monocots and herbaceous dicots; hardwoods; softwoods; and a potential fifth group, "woody" commelinids such as bamboo and palms. Due to these distinct differences in cell wall chemistry that result in differences in the organization and ultrastructure of the cell wall between each of the classes, one would expect species within the same group to behave more alike in terms of interactions with pretreatment chemistry and enzymatic conversion compared to species from a different class.

The most well known differences in cell wall chemistry between the classes are hemicellulose and lignin composition. Hemicelluloses are a diverse class of amorphous carbohydrates that cross-link cellulose microfibrils and lignin chains within the cell wall.

Different classes of plants have distinctly different types of hemicelluloses within their cell walls. In commelinids (grasses and related species), the primary hemicelluloses are heteroxylans such as glucurono-arabinoxylan [16-18], which is cross-linked to lignin via ferulate and diferulate bridges [19, 20]. The ester linkages between the arabinose and ferulate molecules are known to be readily cleaved under alkaline conditions [19] and this is one proposed mode of action for the improved digestibility of alkaline pretreated grasses [21]. The hemicelluloses in non-commelinid monocot and dicot cell walls, including all hardwood tree species, are primarily xylans (4-O-methyl-glucuronoxylans), xyloglucans, and some glucomannans [16, 18]. In softwoods the hemicelluloses are mainly galactomannans or galactoglucomannans [18].

A second difference between the different plant classes, are the ratios of specific lignin monomers that are present in the lignin polymer chains. The three lignin subunits: *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), are synthesized via a complex enzymatic pathway [22] (**Figure 1.4**) and then transferred into the cell wall where they are oxidatively coupled to form the complex lignin polymer [23, 24]. Commelinids have similar levels of G- and S-units with significant amounts of H-units. Non-commelinid monocots and dicots have principally G- and S-units with trace amounts of H-units [23] . Softwoods have primarily G-units and low levels of H-units [25]. While differences in the lignin monomer composition within the plant cell wall have been shown to have little effect on enzymatic digestibility [26], there can be an effect on pretreatment or other chemical processes due to changes in cleavable linkages. The increase in resistance to Kraft pulping by COMT down-regulated poplar was attributed to an increase in proportion of G units that resulted in a decrease in

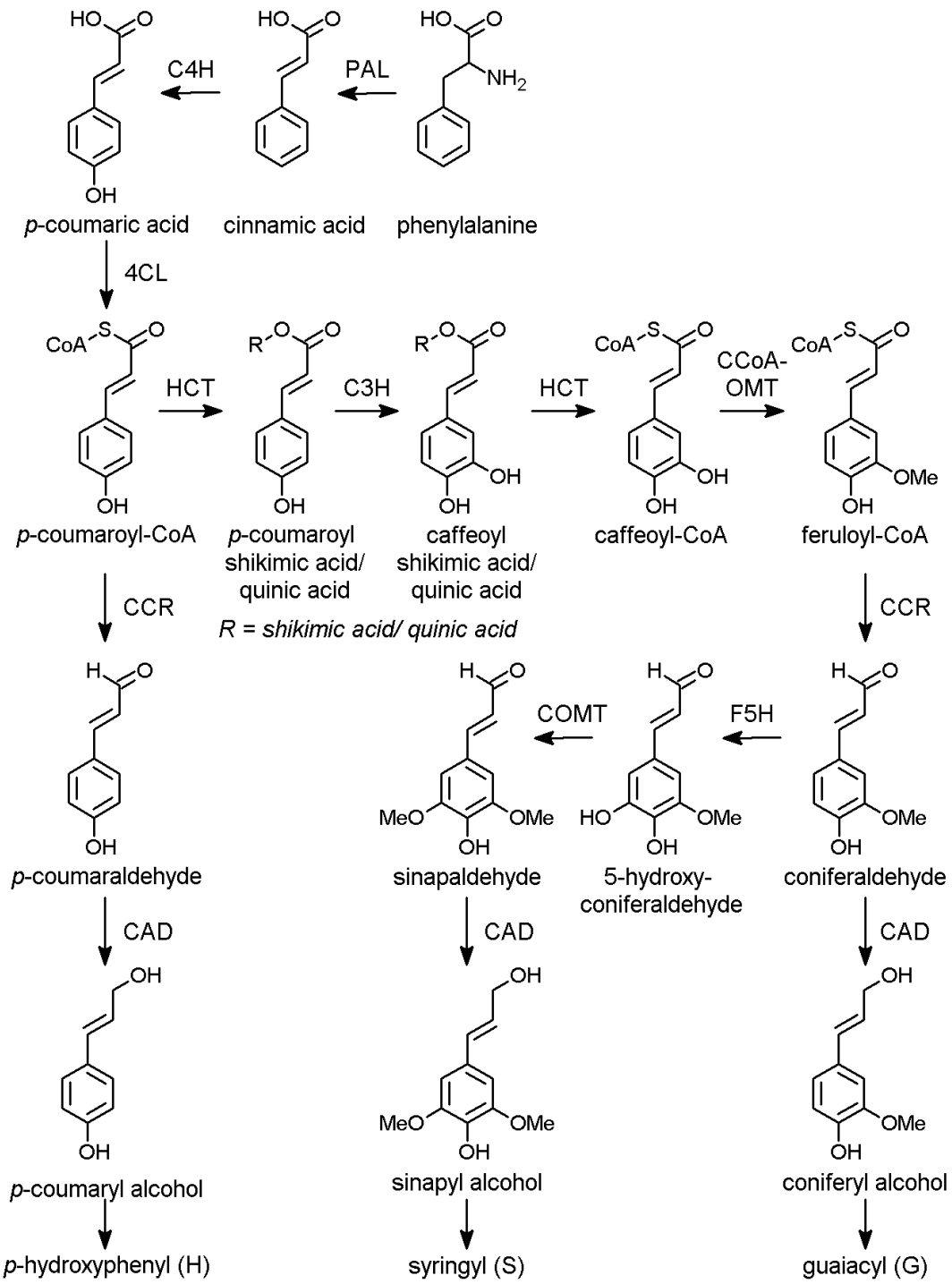

Figure 1.4: Lignin synthesis pathway for the monolignols *p*-coumaryl, sinapyl and coniferyl alcohol. From Vanholme et al. [21]. PAL = phenylalanine ammonia-lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate:CoA ligase; C3H = *p*-coumarate 3-hydroxylase; HCT = *p*-hydroxycinnamoyl-CoA:quinate/ shikimate *p*-hydroxycinnamoyltransferase; CCoAOMT = caffeoyl-CoA *O*-methyltransferase; CCR = cinnamoyl-CoA reductase; F5H = ferulate 5-hydroxylase; COMT = caffeic acid *O*-methyltransferase; CAD = cinnamyl alcohol dehydrogenase.

β- O-4 linkages, which are easily cleavable by chemical means [27], and increase in resistant 5-5 biphenyl structures [24].

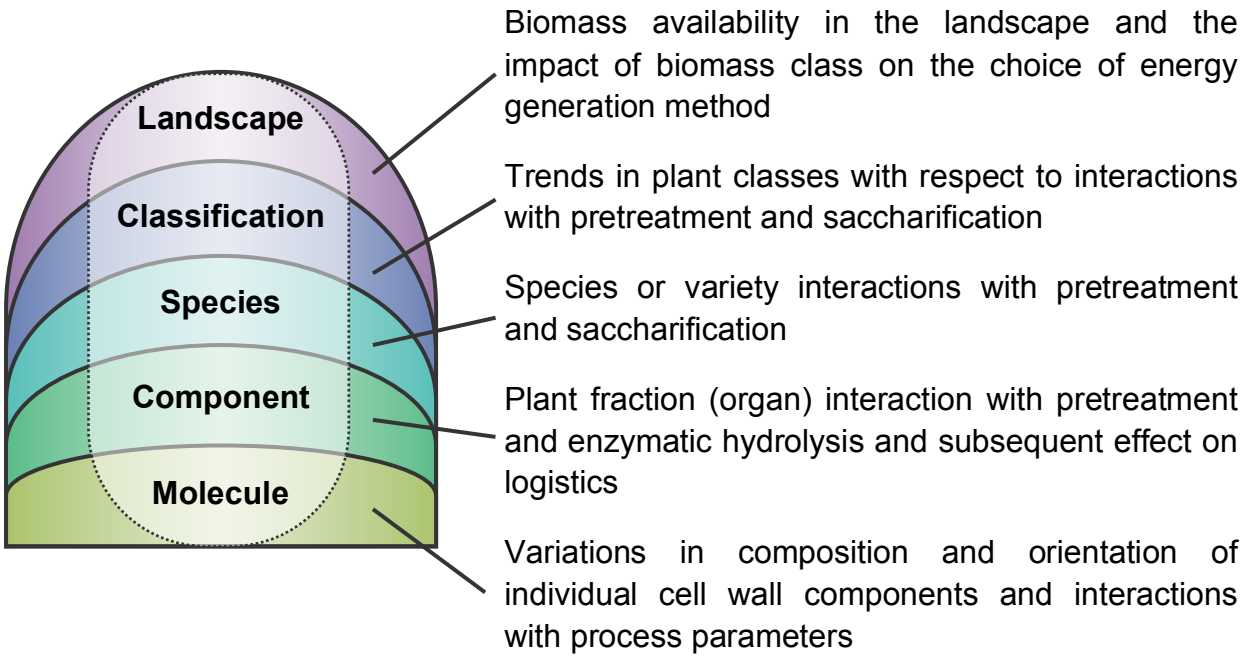

Figure 1.5: Scales of interaction between plant feedstocks and energy production processes.

The interaction of the feedstock with the conversion process can be examined at a number of different scales (**Figure 1.5**). At the largest scale, the landscape scale, the availability of different feedstocks can determine the choice of energy generation method for a region and the best location for a new facility based on the economics, logistics and environmental impacts. At the classification scale, feedstocks from the different botanical classes can be compared and generalizations and trends related to their ease of conversion can be determined. This could allow one to predict which related species would perform well or poorly with a given process, assuming similar environmental factors, location and maturity.

At the smaller scales, feedstock interactions with pretreatment parameters and enzyme mixture and loading can be examined at different levels, comparing different species within the same class or varieties within the same species (species scale), different organs or tissues of the same plant (component scale), and plant materials with specific, divergent ultrastructural or molecular properties (molecular scale).

For this research, one area of potential interest was examined at each scale of interaction:

- **Landscape scale**: For this chapter, the quantity and spatial distribution of key crop residues in Mainland China were determined, as well as the amounts of these residues that are usable for energy generation and their distribution throughout the country. Improved understanding of feedstock distribution and availability allows for a fuller understanding of optimal locations for placement of future biorefineries.
- **Classification scale**: For this chapter, mixed-species feedstocks comprised of different ratios of plants from different species and different plant classifications were compared with respect to their interaction with pretreatment and hydrolysis processing conditions. By understanding how the different classifications of plants interact with processing, one can either focus on species that are amenable to a given method, or seek to alter either the methods or feedstocks in order to better process those materials that are less amenable.
- **Species scale**: For this chapter, optimal pretreatment conditions and enzyme combinations were determined and compared for two varieties of switchgrass grown under the same environmental conditions. Within a given species, there can be a great

deal of variability in composition and processing characteristics. In order to better understand actual differences due to genotype, it is necessary to minimize the environmental differences during plant growth and development.

- **Component scale**: For this chapter, different fractions of corn stover were compared with respect to their response to pretreatment and hydrolysis, and the effect of different selective harvesting scenarios on theoretical ethanol yields was examined. Different parts of the same plant can have very different characteristics. By better understanding how effectively they are processed and the subsequent effect on yields, we can devise better methods for harvesting these materials for biofuel production.
- **Molecular scale**: For this chapter, different poplar samples that had been genetically modified for altered lignin content or composition were tested and compared with respect to their interactions with pretreatment. Certain cell wall components may be more beneficial or more restrictive to biomass processing than others. By using transgenic feedstocks which have been modified it is possible to determine whether 1) using materials that have been modified in this way has value for the biorefinery that could lead to further development and planting of these materials, or 2) whether there are certain traits that should be focused on more carefully for future transgenic work on other potential feedstocks.

The publication status of each chapter and other relevant work by the author are listed in **Table 1.2.**

Table 1.2: Publication status of thesis chapters and other relevant work.

Chapter	Title	Publication Status	Ref.
2	Analysis of key crop residue availability for bioenergy in Mainland China	Not Submitted	-
3	Influence of variable species composition on the saccharification of AFEX™ pretreated biomass from unmanaged fields in comparison to corn stover	Submitted	-
4	Optimization of AFEX™ pretreatment conditions and enzyme mixtures to maximize sugar release from upland and lowland switchgrass	Published	[28]
5	Optimizing harvest of corn stover fractions based on overall sugar yields following AFEX™ pretreatment and enzymatic hydrolysis	Published	[29]
6	AFEX™ pretreatment of poplar modified for lignin content and composition	Not Submitted	-
-	Comparative material balances around pretreatment technologies for the conversion of switchgrass to soluble sugars	Published	[30]
-	AFEX™ pretreatment and enzymatic conversion of black locust (*Robinia pseudoacacia* L.) to soluble sugars	Published	[31]

CHAPTER 2 :
LANDSCAPE SCALE: ANALYSIS OF KEY CROP RESIDUE AVAILABILITY FOR BIOENERGY IN MAINLAND CHINA

2.1. Introduction

Based on international statistics, the top three world energy consumers are the United States, the European Union, and Mainland China, each contributing to roughly one-fifth of the world's primary energy consumption [32]. However, while the demand for petroleum has been steadily decreasing in most developed countries, in the developing world demand has been increasing. Mainland China is currently ranked second behind the United States in world petroleum demand [33] however limited petroleum reserves are forcing increased reliance on imports [34]. Coal is the most abundant source of energy in China, and coal consumption has also increased rapidly over the last decade [32], however acute environmental pollution and human health problems accompany its use. In response to these concerns, the Chinese government has implemented a number of policies to develop the nation's renewable energy resources. In 2007, the 'Medium and Long-term Development Program for Renewable Energy' set two goals within each class of renewable energy: biomass, wind, solar, hydro and geothermal, with the intention to reach the first goal by 2010 and the second by 2020 [34].

In accordance with these goals, China has begun developing liquid renewable fuels. By 2005, production capacity of grain-based bioethanol reached 1 million tons per year, with the intent of reaching 2.0 million tons per year by 2010, increasing to 10.0 million tons per year by 2020 [34]. However, because of issues with food security, it is necessary to meet these goals using feedstocks that do not compete with the production of food, either through demand for the same feedstocks or competition for arable land. While there are options

being pursued in China using starch or sugar-based ethanol production, of these options only cassava doesn't compete with food crops because it is not a staple food and can be grown on marginal land [35]. This leaves bioethanol produced from lignocellulosic materials as the best option for meeting China's goals for renewable liquid fuel. Two of the best options for lignocellulosic ethanol feedstocks are crop straw residues left following grain harvest and dedicated energy crops or mixed-species feedstocks that can be grown on abandoned or degraded lands. Because feedstock supply to the biorefinery is one of the largest issues facing commercialization of cellulosic ethanol in China [36], the location of the biorefinery will be a critical decision. It is necessary to locate a biorefinery where there is a stable and accessible supply of feedstock, especially for straws that are not dense and can have high storage and transportation costs [36, 37]. As an alternative to liquid fuel production, straw can also be burned to provide electricity, displacing coal and some of its negative impacts [38]. However, just as for the biorefinery, feedstock supply is also an important issue for a power plant.

Most of the research on crop residue production in China has been at the national or provincial scale, as that data is more readily available and easier to analyze. Our goal was to determine the production of crop residues at the next administrative level down, the prefecture level, in an attempt to improve the spatial resolution and better inform on potential locations for biofuel production in Mainland China. We also evaluated the effect of residue-to-grain ratios on the spatial distribution of crop residues. In order to estimate the distribution of usable crop residues in Mainland China, we estimated the amount of crop residues in each province used for pulp and paper production and animal feed, returned to the field as fertilizer, and burned on-field.

Figure 2.1: Map of Mainland China with provinces, autonomous regions, and municipalities.

2.2. Materials and methods

2.2.1. Land area and population data

A labeled map of China is provided for ease of reference (**Figure 2.1**). Data on population and total and specific crop sown area for each city prefecture, autonomous prefecture, was determined from the individual province, autonomous region, and municipality yearbooks [39-69]. Data on population, total sown land area, and total cultivated land area for the provinces, autonomous regions, and municipalities were obtained from the China Statistical Yearbook [70]. For simplicity, from this point forward city prefectures and

autonomous prefectures will both be referred to as prefectures, and autonomous regions and municipalities will also be referred to as provinces. Prefecture total land area and cultivated land area data (where available) were obtained from the China Statistical Yearbook for Regional Economy, and the provincial values were calculated by adding the prefecture values [71]. Prefecture values for the 2006 Fujian total land area; and the Liaoning, Shanghai, Shanxi, Tianjin, and Yunnan city prefecture cultivated land areas were obtained from the China Data Online database [72]. Data for Fujian cultivated area and Shenyang prefecture in Liaoning, were unavailable for 2006, so 2005 data were used for these values [72].

2.2.2. Crop production data

Due to limitations on available prefecture-level data for cereal crop production in all of the provinces, the most recent year that could be modeled was 2006. Crop yield data were collected for each province and prefecture from a variety of sources for the following crops: all cereals including rice, wheat, corn, millet, sorghum, and miscellaneous cereals; legumes and soybeans; tubers and potatoes; cotton; oilseeds including canola, peanut, sesame, sunflower, flaxseed, and miscellaneous oilseeds [39-69, 71]. According to the Chinese classification system, legumes, tubers, and cereals are all considered grain crops and any reference in this paper will include these categories. Because in many cases there was not enough prefecture level data available to estimate the yield of the sugar crops (sugarcane and sugar beets) and tobacco, it was decided to not include them in this analysis.

In some cases data on the miscellaneous cereals or miscellaneous oilseeds were not provided for a given prefecture. This value was then determined by taking the difference of

the total value minus the provided subcategories. When the value of the total cereals was not given, the value of the miscellaneous cereals was determined by subtracting all known cereals, legumes and tubers from the total grain value. In cases where it was necessary to estimate the yields of important crops based on limitations in available data, the provincial crop yield per hectare values [73], sown area, and yield values for each unknown crop type were used to derive the unknown values based on the total grain yield and sown area or total oilseed yield and sown area for each prefecture within the province. Yields were simultaneously calculated for all unknowns within the category (grains or oilseeds) by solving to minimize the differences between the sown areas and total yields for all crop residues being estimated and the actual values for the both the province and the prefectures. In some cases where the yields were not provided and they could not be estimated, those crops with yields of less than 100,000 tons for a given province were neglected from the analysis. Of the crops analyzed that were not provided and had yields > 100,000 tons for the entire province, only prefecture values for corn and wheat in Zhejiang and rice in Inner Mongolia could not be estimated. These were reported at the prefecture scale as part of the miscellaneous cereals.

2.2.3. Crop residue yield calculations and mapping

Crop residue yields were calculated using residue-to-grain ratios reported in literature [74, 75]. For miscellaneous cereal and miscellaneous oilseed yields that did not have a residue-to-grain ratio, we used what seemed to be common values among those reported (**Table 2.1**), namely, 1.5 for cereals and 2.0 for oilseeds. Because there is a large range in values for reported residue-to-grain ratios (**Table 2.1**) we also compared our results to the

Table 2.1: Residue-to-grain ratios of common crops from various sources. All data are specific to Mainland China except those from Kim and Dale [76], which are based on U.S. values and are provided for reference.

Residue- to-Grain Ratios of Various Crops Provided in Literature										
Source	[77]	[78]	[79]	[80]	[74, 75]	[81, 82][a]		[36]	[83]	[76]
Year	1990	1995	1999	2007	2006-2010	Used	Avg.	-	-	-
Rice	1.32	0.623	0.97	0.68	1.00	1.0	1.0	1	1.32	1.4
Wheat	1.72	1.366	1.03	0.73	1.17	1.1	1.4	1	1.72	1.3
Corn	1.37	2.0	1.37	1.25	1.04	2.0	1.6	2	1.27	1.0
Millet	1.61		1.51			1.6	1.5		1.61	
Sorghum	1.59		1.44			2.0	1.6		1.59	1.3
Misc. Cereals		1.0	1.60			1.6	1.5	1.5		1.3
Legumes						1.6	1.5	1.5	1.30	
Soybeans	1.30	1.5			1.50	1.6	1.5			
Tubers		0.5	0.61		0.55[c]	0.5	0.5	1		
Potatoes						0.5	0.5		0.40	
Cotton	1.62	3.0	3.00	5.51	2.91	3.0	2.5	3		
Oilseeds		2.0				2.0	2.0	2	1.35	
Canola	2.99		3.00	1.01	2.87	3.0	2.3	2	2.94	
Peanut	1.35		1.52		1.14	1.5	1.4	2	5.88	
Sesame	5.88		0.64		2.01	3.0	2.6	2		
Sunflower			0.6			3.0	2.0			
Flaxseed						2.0	2.0			
Sugarcane[b]		0.1	0.25			0.1	0.2	0.1	0.8	
Sugar beet						0.1	0.2	0.1	0.45	
Fiber crops	1.81		1.7			1.7	1.9			
Tobacco	1.06				0.71	1.0	1.1			
Cited by:		[84-88]		[89]						

[a] Gao et al. reported a large number of harvest indices for various crops and then chose the most cited value to perform their analysis of crop residue production. We also report the average of the values they cited.

[b] Sugarcane residue only consists of the leaf and doesn't take into account any bagasse remaining following processing.

[c] Average value of those reported for potatoes and sweet potatoes.

most commonly cited dataset from a joint Chinese Ministry of Agriculture and U.S. Department of Energy project [78]. The total residue yields for each prefecture or province equaled:

$$R = \sum_{i=1}^{n} r_i \cdot C_i \quad (2.1)$$

where R = the total residue yield (tons), r_i = the residue ratio for the i-th crop, C_i = the crop yield for the i-th crop (tons), and n = the total number of crops. Province crop residue yields were calculated in the same way based on provincial crop production data rather than summing the prefecture-level data for each province. Residue densities were calculated with respect to cultivated land area as opposed to crop sown area, as sown area is counted twice when double crops are cultivated, once for each crop. By calculating based on sown area, a deceptively low crop residue density (kg/ha) would be generated for a given location if there is a large amount of double- or triple-cropping. Crop density in terms of total prefecture or province area is also presented for comparison.

Maps were generated using DIVA-GIS 7.4 mapping software and shapefiles of the prefecture-level and province-level administrative regions in China [90].

2.2.4. Straw used for pulp and paper production

The amount of straw used for pulp and paper production in each province in 2006 was calculated using data from the China Paper Association Paper Industry Reports [91-93]. Total paper and paperboard production were reported in the industry reports for provinces with greater than 1.0 million tons of production (Hebei, Shandong, Jiangsu, Zhejiang, Fujian,

Guangdong, Henan, Hubei, Hunan, Anhui, Guangxi, Jiangxi, and Sichuan). The production values for the other provinces were estimated from a figure in the 2005 report and assumed to be roughly equal to the production in 2006 (**Table A.1**). Assumptions are as follows: 1) provinces were assigned to regions (east, central, and west) and the sum of provincial values in a given region were required to equal the total for the region, as provided in literature; 2) there was no production of paperboard in Hainan, Qinghai, and Tibet; 3) the amount produced in Shanxi, Inner Mongolia, Yunnan, Xinjiang, Chongqing, and Tianjin were equal; 4) the amount produced in Shaanxi and Ningxia were equal; 6) the amount produced in Liaoning and Heilongjiang were equal and slightly greater than that produced in Shaanxi and Ningxia; 6) values for the other provinces were chosen in order to satisfy the initial assumption and based on their relationship to production in other provinces . An additional assumption was that the production of pulp and paper was performed in the province from which the straw was harvested. For pulp production this is a reasonable assumption as straw is not dense and therefore costly to transport long distances. However, this may be an oversimplification for paper production as pulp could feasibly be transported across province lines.

Total pulp consumption in 2006 was 59.92 million tons, and 1.085 tons of paper were produced for every ton of pulp consumed. Of this, 12.9 million tons of pulp were produced from non-wood sources, equal to 21.5% of the total pulp consumed. The amount of straw-based pulp (rice and wheat) was estimated as 60% of the non-wood pulp based on the decreasing trend in this proportion from 1995 (81%) to 2000 (69%) [94]. It is estimated that 2.25 to 2.5 tons of crop straw are needed to produce one ton of pulp [84, 94]. We assume a conservative estimate of 2.5 tons of straw per ton of pulp. It was assumed that these ratios

were constant for every province. These values were then used to calculate the tons of crop straw consumed for paper and paperboard production in each province using the following equation:

$$S_i = P_i \cdot p \cdot n \cdot s \cdot c \quad (2.2)$$

where S_i is the total straw consumed for pulp and paper in the *i*th province, P_i is the total pulp and paperboard production for the *i*th province, p is the tons of pulp consumed per ton of paper produced (0.922), n is the proportion of pulp from non-wood sources (0.215), s is the proportion of non-wood pulp from rice and wheat straw (0.6), and c is the tons of straw input per ton of pulp output (2.5).

2.2.5. Straw used for animal feed

A previous estimate on the amount of straw needed for animal feed production took into account only the number of cattle in each province and also assumed that provinces with a large area of pastureland would not require the use of crop straw as an animal feed in support of cattle production [78]. There are two issues with these assumptions. First, as all ruminants can consume straw, sheep and goats should also be taken into account in an estimate of the amount of crop straw needed for animal feed. Second, many of the provinces with significant amounts of range and pastureland also have issues with overgrazing, which has led to loss of vegetation, erosion, and dust storms. So it should also not be automatically assumed that rangeland in these provinces can or should support the amount of ruminant animals that are currently being produced. For our estimates we calculated the number of ruminant animals (cattle, buffalo, sheep, and goats) that could be sustainably produced on

rangeland based on the carrying capacity. Any animals unable to be supported by pasture were assumed to be fed a daily recommended amount of crop straw.

Data for the number of ruminant animals and the area of grazing and pastureland for each province in 2006 are from the China Statistical Yearbook [70] (**Table A.2**). Only provinces with over 1 million ha of pastureland were taken into consideration for animal grazing. Dryland carrying capacity (sheep per ha) for Xinjiang, Inner Mongolia, Ningxia, Gansu, Qinghai, and Shaanxi were taken from Shen [95]. The carrying capacities for Jilin and Heilongjiang were assumed to be equal to Inner Mongolia. The average of the carrying capacities of Xinjiang, Ningxia, Gansu, and Qinghai was used for Tibet, Sichuan, and Guizhou. We estimated the total number of ruminants in terms of "animal units", where a cow or buffalo equals 1.0 animal unit and a sheep or goat equals 0.2 animal units. The carrying capacity for each province was converted to animal units by dividing by 5 (**Table A.2**). The number of ruminants that could be supported by the pastureland was calculated by multiplying the carrying capacity by the area of pastureland. This value was subtracted from the total number of ruminants to give the number of ruminants fed on crop straw in each province (**Table A.2**).

There is a maximum amount of straw that should be fed to ruminant animals to maintain their health. Li et al. report a daily recommended amount of 3.49 kg of ammoniated straw per cow per day [78], which equals 1.27 tons of straw consumed per cow (or animal unit) per year. Multiplying this value by the number of ruminant animals that are not pasture-fed gives an estimate of the amount of crop straw that would be needed in each province to support them, assuming that the number of ruminants in each province throughout the year is roughly equal to the number of ruminants at year end as reported in literature, that

logistics will allow continuous feeding of crop straw throughout the year, and that crop straws would be preferentially fed to animals over feed and grain, up to the maximum amount.

2.2.6. Straw returned to the field

For the estimate of crop residues that should to be returned to the field in each province, we used values reported by Cui et al. for different regions in China: 2.25 t/ha for northeast China (Heilongjiang, Jilin, and Liaoning) and the Qinghai-Tibet region (Qinghai and Tibet); and 3.0 t/ha for all other regions [80]. However estimating the amount that should be left behind becomes complicated due to the large amount of double- and triple-cropping that occurs, particularly in central and southern China. By some estimates, multi-cropping systems account for over 1/3 of China's total cropland [96]. Because sown area counts each crop separately though sown on the same land area, if the value of sown land area was used to estimate the amount of straw that should be returned per hectare, this would result in double- or triple-counting multi-cropped land, resulting in excessive return of straw to fields that are multi-cropped. So for our study we attempted to account for multi-cropped land for the crops of interest. First, the provincial cultivated land area for the crops of interest (cereals, legumes, tubers, oilseeds and cotton) was calculated by subtracting the sown area of fiber crops, sugar crops, and tobacco, which were all assumed to be all single-cropping systems, from the total province cultivated land area. Because vegetables are commonly multi-cropped with the crops of interest [96], the vegetable sown area could not directly subtracted. As a result the proportion of vegetable sown land area that was solely planted with vegetables for each province (**Table A.3**) was estimated based on the regions defined by Qiu et al. [96].

Using our assumed proportions, we estimated that 31.3% of the national vegetable sown area was single- or triple-cropped, which is similar to 31.1% that was estimated by another study [96]. Assuming that the remaining vegetable sown area overlapped with our crops of interest, the estimate of single- and triple-cropped vegetable sown area was subtracted from the total province cultivated land area, in addition to the other crop types mentioned previously. This resulted in an estimate of the cultivated land area for our crops of interest for each province. For provinces where the reported sown land area of the crops of interest was less than the calculated cultivated land area, the excess cultivated land was assumed to be either in fallow rotation or a cropping system that was not accounted for. In this case, the sown area of the crops of interest (as reported in literature) was used to calculate the amount of crop straw that should be left on the field. For provinces where the sown area was greater than the calculated cultivated land area for the crops of interest, the difference was assumed to be due to multi-cropping and the cultivated land area was used to determine the amount of crop straw that should be left on the field. The estimated cultivated land area for the crops of interest are listed in **Table A.4**. Sown area of individual farm crops for each province and total cultivated land area for each province in 2006 were taken from the China Statistical Yearbook [70].

2.2.7. Provincial distribution of usable crop residues

The amount of residues able to be used for energy in each province was calculated by subtracting the amount of residues required for pulp and paper production, animal feed, and return to the field, from the total amount of residues produced. It is difficult to estimate the

amount of straw used for rural energy in Mainland China. While the government reports statistics for the amount of rural energy provided by straw combustion for each province, these values are generally believed to overestimate the use of crop residues as a fuel source [97]. Many of the other estimates on the proportion of crop residues used for rural energy are based on values from 2000 [97-99]. We chose not to use these values to estimate the amount of biomass used for rural energy in 2006, as the proportion of biomass used for rural fuel in each province has likely decreased with the increased availability of coal and commercial electricity. Using these values would overestimate the amount of biomass needed for rural energy. As a result our value for usable residues includes the amount currently being used for rural energy. Alternatively, one could use estimates of on-field combustion of crop residues as a measure of the amount of usable residues in each province. We compare values from Wang and Zhang [100] to a scenario where sufficient crop residues are left on the field to maintain soil health and where land is not overgrazed (**Table A.5**).

2.3. Results and discussion

2.3.1. Total crop residue production

Various studies have estimated crop residue production within Mainland China (**Table 2.2**). These amounts range from the most conservative estimate, 433.0 million metric tons in 2006, to the most liberal estimate, 939.3 million metric tons in 1999. Our study value for the total crop residue production in China is at the lower end of the range, with an estimate of 593.5 million dry metric tons produced in 2006. The reason for the differences in crop estimates is at least partly due to the types of crop residues that were included in the analysis

Table 2.2: Estimates on total crop residue yields and usable amounts of crop residues in Mainland China from various sources. The usable residue values for [89, 101] only take into account the amount of collectable residues and not competing uses.

Total Crop Residues (10^6 Mg)	Usable Residues (10^6 Mg)	Year of Estimate	Reference
433.0	175.9	2006	[80]
533.0	452.8	2007	[89]
557.5	-	1995	[86]
593.5	124.7	2006	This work
604.0	254.1	1995	[78]
620.3	-	2002	[87]
627.3	-	1994-2004	[84]
636.2	-	2006	[86]
754.7	114.7	2007	[36]
774.0	-	2008	[88]
788.6	-	-	[102]
841.8	686.0	2005	[101]
939.3	551.4	1999	[85]

Estimates with the lowest numbers either examined a more limited range of feedstocks [78, 80], or they tended to use more conservative estimates for the residue-to-grain ratios [80, 89]. Our value is low for both of these reasons.

Our estimate of total usable residues is also at the lower end of those reported with only 124.7 million dry metric tons usable for fuel. One reason for this is that a number of the other estimates only take into consideration the amount of residues that are able to be harvested based on a collection coefficient and do not consider competing uses [89, 101]. Of the other five studies that report a usable amount of residues, only the study by Liao et al. [85]

is significantly higher than the rest. They assumed that the proportion of usable residues was the same as was determined in the MOA/DOE joint report [78], however their estimate of crop residue production is so large as to make their estimate of usable residues seem unlikely. When we recalculated the total yield of crop residues based on their data, we find that there is an error in their calculations. When calculated directly from their reported data, the total amount of crop residues is 584.1 million metric tons, over 350 million tons less than their reported value of 939.3 million metric tons [85]. Their other calculations are equally suspect based on the inability to match their reported values (such as the amount of unused residue) with our calculations based on their numbers.

The distribution of total yields across Mainland China is shown in **Figure 2.2**. On a mass basis, most of the crop residues are localized in the Huang-Huai-Hai plain and Yangtze River region (Henan, Shandong, Anhui, Jiangsu), and in Heilongjiang in the Northeast. This is a similar finding to what has been reported previously [78, 85, 86]. When examined at the prefecture scale, it is easier to see patches of high crop residue production such as in Chongqing, southern Henan and Hebei, northern Anhui and Jiangsu, and western Heilongjiang and Jilin. The provincial scale also doesn't tell the whole story, particularly for the large provinces. For example, Inner Mongolia in northern China has patches of high crop residue production, particularly in the east where it connects with Heilongjiang and Jilin. However, on the provincial scale its overall yield is fairly low.

Crop residue density, when expressed in terms of the total prefecture or province land area, is unsurprisingly biased toward locations with a high crop residue yield and smaller total land area, such as Shandong, Henan, Anhui, and Jiangsu (**Figure 2.2 - C, D**). Reporting

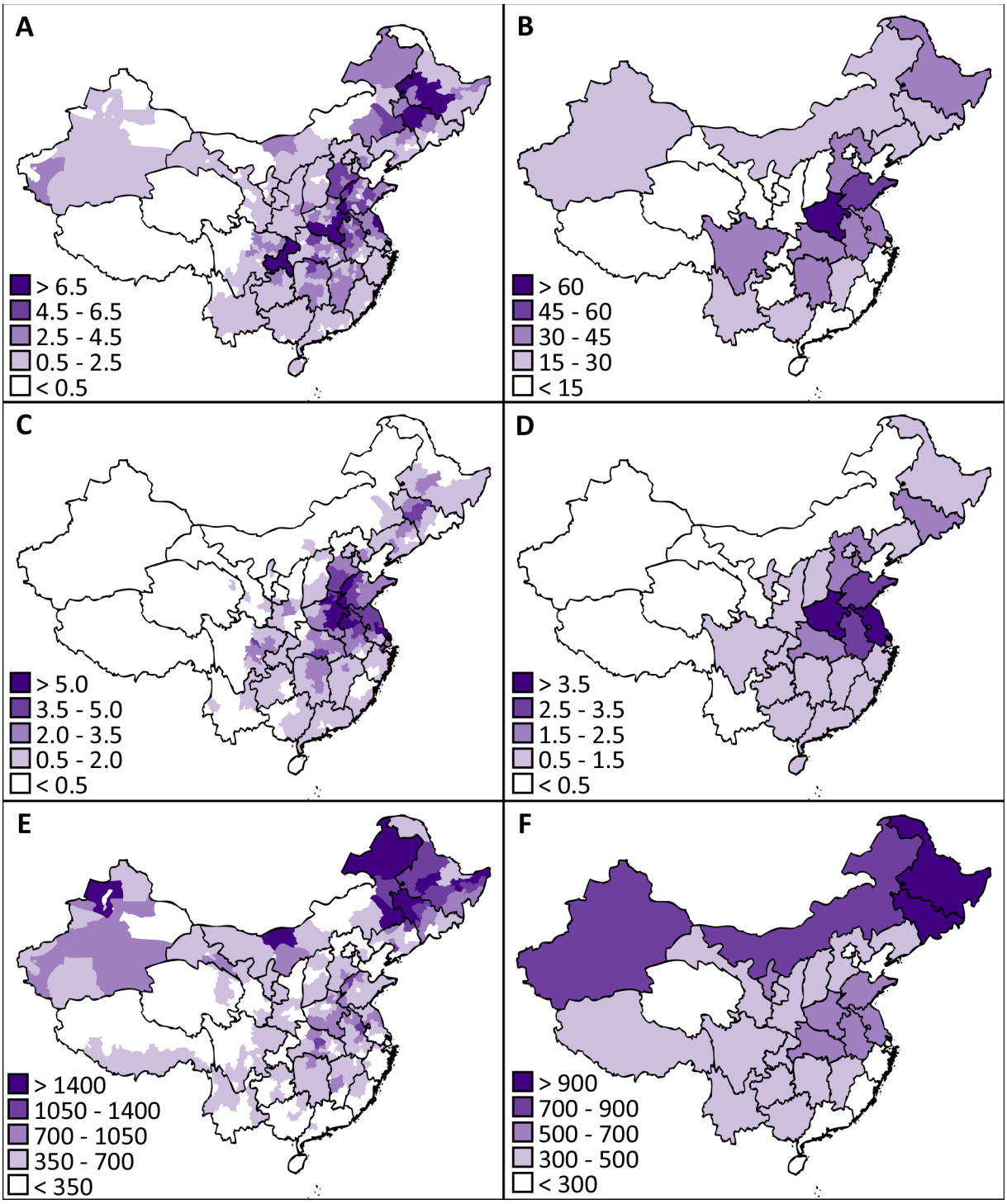

Figure 2.2: Estimated crop residue yields in China in 2006: (A, B) Total yields for each prefecture and province (10^6 Mg); (C, D) Residue yield per total land area (Mg/ha); (E, F) Per capita crop residue yield (kg/person). Residues included cereals, legumes, tubers, cotton, and oilseed crops, and the amounts were estimated using the average harvest indices for the respective crops reported by Xie and colleagues [74, 75].

crop residue production in terms of the total land area probably doesn't have much practical value as the locations of crop production are likely to be more localized and regions with significant amounts of forest, mountains, and deserts will be negatively impacted in the assessments. Expressing crop residue production per capita (**Figure 2.2 - E, F)** tends to bias towards locations that have high crop production and a small population, such as Heilongjiang, Jilin, Inner Mongolia, and Xinjiang [80, 89]; locations with more large-scale mechanization [70]. Per capita yield of crop residues is important if it is desirable for reasons of transportation costs and logistics to keep all stages of bioenergy production through end use at a local scale. This would probably have greater impact when crop residues are used as a feedstock for individual or community biodigesters or home heating and cooking units as opposed to a power plant or a biorefinery which would require significantly larger amounts of crop residues.

2.3.2. Distribution of crops within Mainland China

The different crops have decidedly different crop residue distributions within Mainland China. In terms of total mass yields, wheat production is focused in the Huang-Huai-Hai region (Shandong, Hebei, Henan), with some production in Xinjiang (**Figure 2.3 A**). The largest amount of rice production is located in the Southern region, largely in Hunan, Jiangxi, and Anhui; however, there is also some production in northeast China (**Figure 2.3 B**). Corn production is largely in northern and northeast China (**Figure 2.3 C**). Cotton production is almost entirely located in Xinjiang, with some smaller scale cultivation in the eastern region (**Figure 2.3 D**). Oilseed production, primarily canola, is located in a broad band, stretching

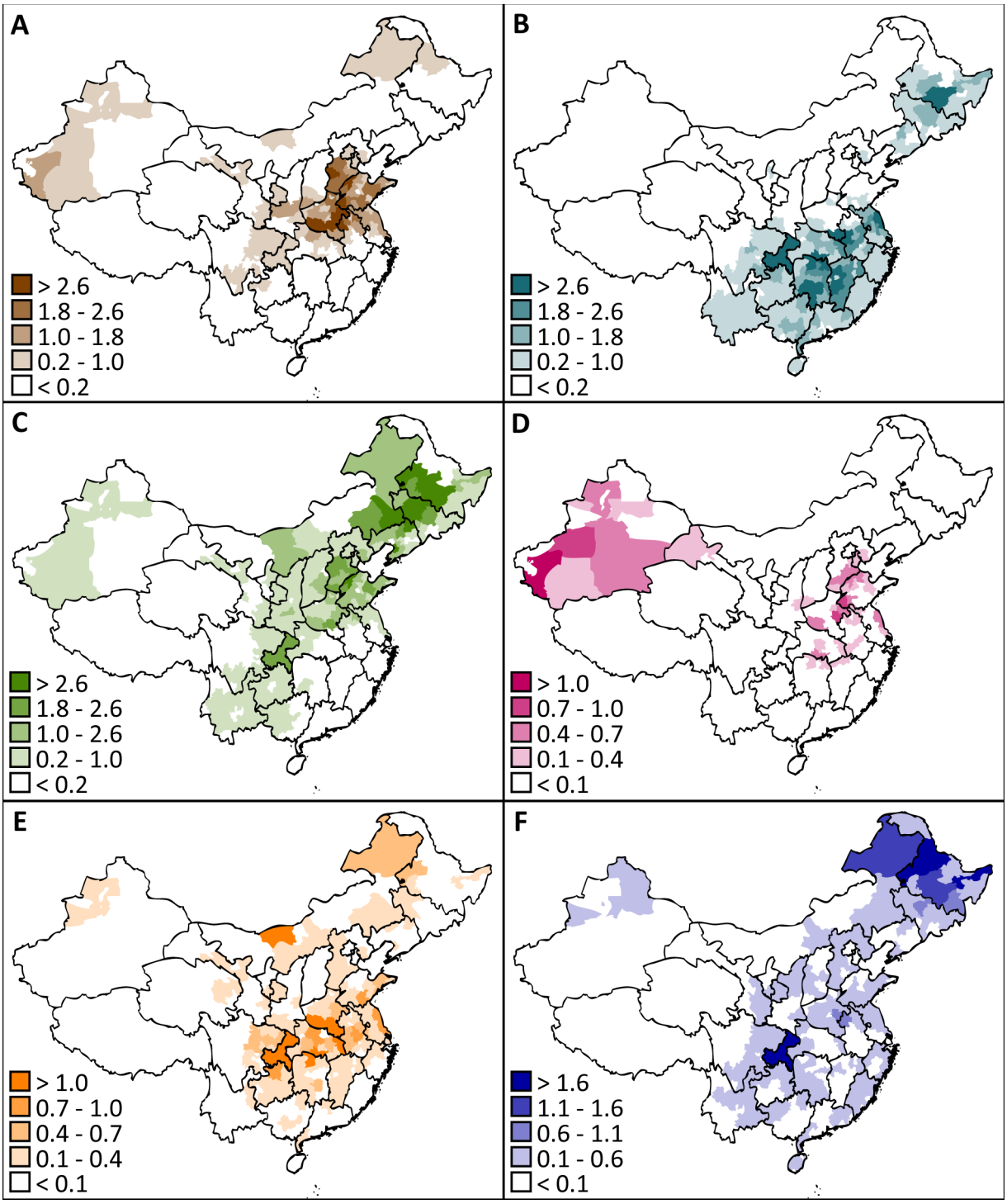

Figure 2.3: Spatial distribution of total residue yields (10^6 Mg) for different types of crops in 2006: (A) wheat, (B) rice, (C) corn, (D) cotton, (E) oilseeds, and (F) legumes and tubers.

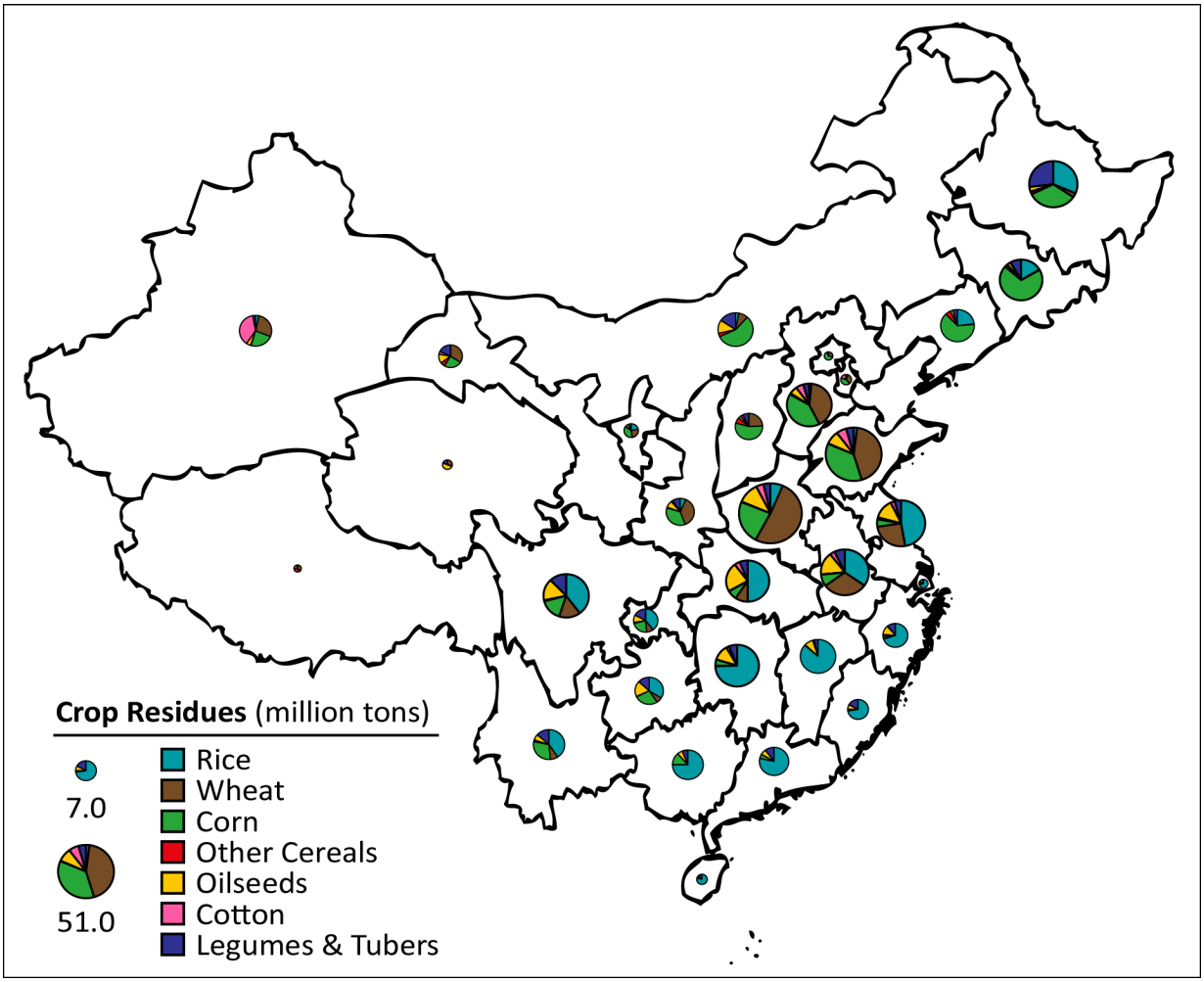

Figure 2.4: Amount and proportion of the different types of crop residues for each province in Mainland China.

across central China (**Figure 2.3 E**), and legumes and tubers are primarily grown in the far north in Heilongjiang and Inner Mongolia (**Figure 2.3 F**). The residue density maps (Mg/ha) for each crop type are provided in the supplemental information (**Figure A.1**).

The relative proportions of the different crop types grown in each province are shown in **Figure 2.4.** The regions with the largest production of crop residues are unsurprisingly areas with significant amounts of double- and triple-cropping [96]. Most of these provinces

produce large amounts of either wheat straw and corn stover (Hebei, Shandong, and Henan) or wheat and rice straw (Jiangsu, Anhui, Hubei, and Hunan). Heilongjiang and Jilin in the northeast also have large amounts of residues from corn, rice, legumes, and tubers, and Sichuan in the west has a large amount of residues that evenly distributed among the different categories.

Within China the variation in the distribution of different crop residues could have an impact on certain bioenergy production scenarios. The type of crop straw can be a very important consideration as different straws can have very different properties. For example, rice straw has a very high ash content, particularly silica, and practical issues that would need to be considered if using this feedstock include rapid equipment wear due to abrasion, significant fouling of combustion boilers unless the minerals are removed by leaching [103], and ultimately some form of waste disposal or end use [104]. In order to avoid these issues, it may be desirable to construct a facility in a location that is dominated by a less problematic feedstock. To give another example, in Chapter 3, it was found that herbaceous dicots and monocots respond differently to AFEX^{TM} pretreatment. Cotton, oilseed straws, legumes and tubers, as they are all dicot species would all likely respond less amenably to AFEX^{TM} pretreatment compared to the cereal crops, which are monocots. If placing an AFEX^{TM} pretreatment technology, it would be more desirable to focus on the major cereal producing regions such as the rice-producing regions of Hunan, Jiangxi, Hubei and Anhui; or the wheat and corn producing provinces of southern Hebei, Shandong, and Henan. Chongqing could be a good place to locate a biorefinery as they have a fairly high population [60], all petroleum and

diesel fuel is imported to the municipality [105], and there is a high mass yield of all three cereal residues (**Figure 2.3 – A, B, C**). However, the residue density is low (**Figure A.1**) which may indicate that feedstock collection and transportation could be an issue. While it may not be an appropriate location for a large biorefinery, it might be better suited for a distributed biomass processing system that is organized at a more local scale [10].

2.3.3. Influence of residue-to-grain ratio on spatial distribution

As very little data is presently available on a large scale for the generation of crop residues, values are typically estimated using from crop yields using a factor, either the harvest index (HI) which is the ratio of the yield of the economic product (seed, tuber, etc…) to the total plant weight, or the residue-to-grain ratio, which is the yield of the crop residue to the yield of economic product. By multiplying the economic crop yield by a residue-to-grain ratio it is possible to estimate the residue yield. The values for the residue-to-grain ratio used by various sources for estimating crop residue amounts in China varies significantly (**Table 2.1**), however many papers base their values on those published in a China Ministry of Agriculture (MOA) and U.S. Department of Energy (DOE) joint report from 1998 [78]. The main issue with using these numbers is that there is no indication as to where they were derived from, whether from literature or experiments. Additionally it is possible that some of the values given in this document are erroneous, particularly the value for rice which is lower and corn which is higher than values reported elsewhere. In the 15 years since the original document was published, advancements in crop production, either through improved varieties or improved production practices have improved wheat and corn yields per hectare

[70]. As crop yields increase per hectare, generally the harvest index, decreases, with the limit being around 0.4 - 0.5 [106], which would be a residue-to-grain ratio of around 0.6 – 0.8. This is most likely true of corn, where the residue-to-grain ratio is reported was 2.0, but in developed countries, the residue-to-grain ratio is typically 0.8 – 1.0 [106]. As crop production and crop varieties in China become more similar to those in the West, the residue-to-grain ratio of corn would be expected to approach these values.

For our data we used the recent values from two papers by Xie et al. that surveyed the literature to determine the residue-to-grain ratios for different crops in different parts of China [74, 75]. These values are fairly conservative but, particularly for the rice straw and corn stover values, they seem more similar to those that have been set forth by others (**Table 2.1**). When we compared results based on our chosen residue-to-grain ratios to those from the MOA study, apart from a general reduction in yields, there was generally little apparent difference in spatial distribution for total crop residue yields either at the prefecture or provincial level. The biggest differences in spatial distribution were for the yields per cultivated land area (**Figure 2.5**). The MOA study, which had a comparatively higher corn residue-to-grain ratio and a lower rice residue-to-grain ratio has an obvious northerly shift in crop residue density compared to our study, with comparatively higher crop residue densities in northern China and lower densities in southern and central China. The choice of residue-to-grain ratio could lead to very different conclusions, particularly if looking at the coarser provincial scale (**Figure 2.5**). From the MOA results it would seem that the highest yields are in the northeast, northern, and central regions. However, for our results the highest yields are in eastern and central China. These results seem more logical than those based on the MOA

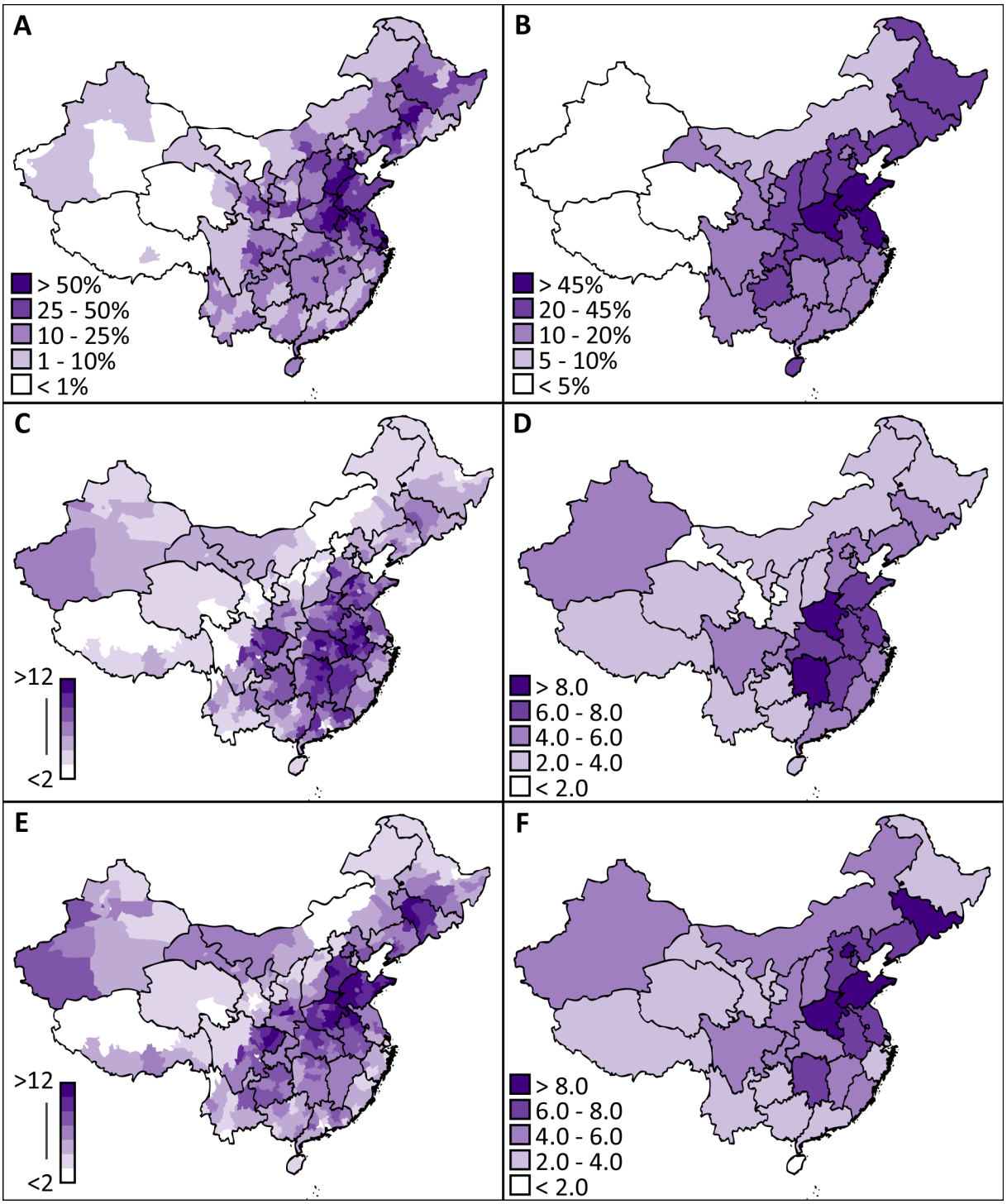

Figure 2.5: Cultivated land as the proportion of total land area in a prefecture or province, and estimated 2006 crop residue yields per prefecture or province cultivated land area (Mg/ha) as affected by the chosen harvest indices. (A,B) Proportion of total land area as cultivated land; (C, D) Harvest indices from Xie et al. [74, 75]; and (E, F) Harvest indices from the Ministry of Agriculture report [78].

study. The eastern and central regions of China have a high prevalence of double- and triple-cropping [96], which would result in greater production of crop residues per cultivated land area compared to single-cropped land, such as is largely present in northeast China.

2.3.4. Usable amount of crop residues

Traditionally in Mainland China, crop straws have been used for rural energy and for animal feed. However, increases large-scale animal production and availability of cheap commercial energy have reduced some of the needs for these materials [107, 108]. This is especially true in eastern China, which has developed the most rapidly. Straw is also used at a smaller scale as a raw material for pulp and paper production and as a substrate for mushroom cultivation [80]. Additionally, a portion of the crop straw should be left on the field in order to maintain soil organic carbon levels, moderate soil temperatures during the summer growing season, limit evaporation, and prevent erosion. However it is possible to leave too much crop residue on the field [80], particularly in no-till and multi-cropped systems. For example, the presence of excess crop residues from one crop rotation can interfere with planting and seedling germination of the subsequent rotation [109, 110] and can increase the prevalence of pests and pathogens. Also, due to the high prevalence of multi-cropping systems, there is often an excess of crop residues that are too abundant to be left on the field. Often there is either insufficient time to clear the field before the next planting or there is no alternative use to make clearing the field worthwhile, so much of these residues are simply burned on field, despite the fact that this practice has been officially banned by the government [111]. Besides wasting a valuable resource, on-field combustion of crop residues

Table 2.3: Estimated use of crop residues in Mainland China from various sources.

	Estimates of Residue Use in Mainland China							
Reference	This Work		Li et al. [78]		Cui et al. [80]		Zhang et al. [36]	
Year	2006		1995		2006		2007	
	millon tons	*%*	*millon tons*	*%*	*millon tons*	*%*	*millon tons*	*%*
Total	594	-	604	-	433	-	755	-
Rural Energy	-	-	355	58	108	25	299	40
Returned to Field	297	50	91	15	130	30	113	15
Animal Feed	154	26	145	24	79	18	208	28
Pulp & Paper	19	3	14	2	20	5	20	3
Mushroom	-	-	-	-	10	2	-	-
Burned	103	17	-	-	86	20	115	15

has a variety of negative impacts including reducing soil organic carbon, harming beneficial soil microorganisms, increasing air pollution, and in a number of cases, reducing visibility in nearby cities to the point of grounding air travel [107, 111-113].

Compared to other reported values for the amount of crop residues returned to the field [36, 78, 80] and the amount of residues used for animal feed [80] our numbers are higher (**Table 2.3**). This is expected as we assumed an ideal scenario where soil health is adequately protected and overgrazing does not occur on pasture and rangeland. It is almost certain that these values are higher than the actual situation in 2006. Li et al. [78] and Zhang et al. [36] both determined the amount of crop residues that should be returned to the field using a set percentage of the total residues (15%). However this amount may not be adequate for maintaining soil health in all locations, under all tillage conditions, and for all cropping systems [114, 115]. For some situations it may be necessary to retain as much as 70

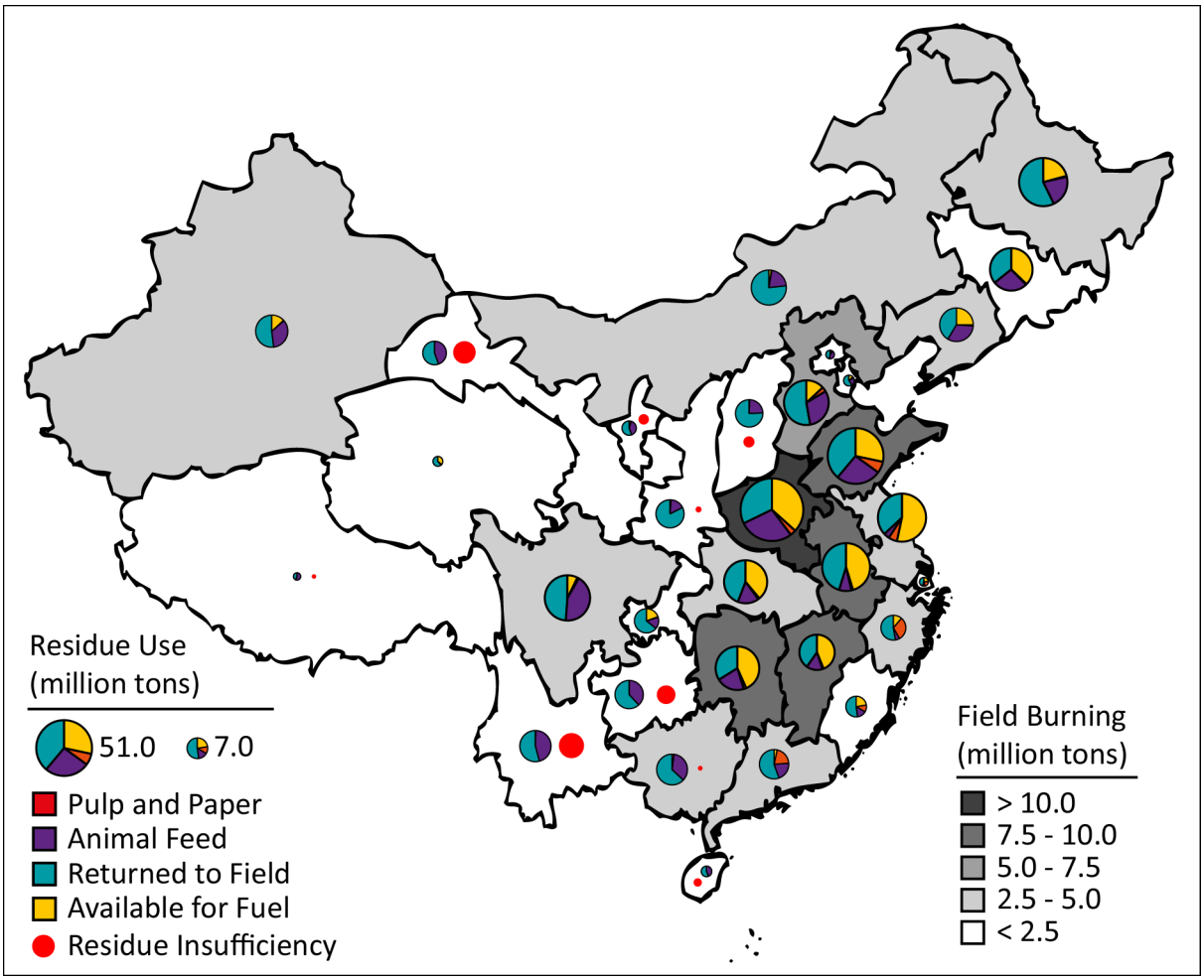

Figure 2.6: Estimated ideal use of crop residues in each province in Mainland China in 2006. Values for the amount of residues used as animal feed and returned to the field are based on ideal scenarios without overgrazing or adversely effects on soil health. Red circles (at the same scale as the pie graphs) are used to show the amount of additional residues needed in provinces that are do not produce the amount required for the ideal scenario. Data on field burning of crop residue are from [100]. A table listing all the values for residue use and on field burning is provided in the supplemental information (**Table A.5**).

– 80% of the residue on the field in order to maintain soil carbon levels, prevent erosion, etc. [114-116]. Our values represent an assumption of a constant amount of crop residue returned per ha of cultivated land. This estimates that, nationally, 50% of the residues should be returned to the field. However, the actual amount that should be retained for a given

location is going to be highly dependent on climate, topography, cropping systems, and management (tillage, irrigation, fertilization). Given a more intensive modeling effort that incorporates these factors, the amount of residue that should be returned to the field across the country would be improved.

A number of provinces did not generate a sufficient quantity of crop residues to sustain livestock production, agriculture, and industry (**Figure 2.6**). Additionally, all of these provinces, except for Guangxi that had the smallest insufficiency, had very little on-field burning of crop residues, most likely due to the high demand for these materials for other applications (**Figure 2.6**). The insufficiency in these locations is not surprising as a number of these provinces are known to have issues with overgrazing and deterioration of agricultural land [95]. However, while these locations are obviously not options for bioenergy production due to the insufficient quantities of crop residues, this is unfortunate because they also account for the poorest farming populations in the country [117] and would most benefit from local bioenergy development.

We were unable to determine the provincial values of the amount of crop straw used for rural energy because, although this is reported in national statistics, it is generally believed to be significantly overestimated [97]. It is also difficult to determine the amount used in each province from other literature. As a result, the amount of usable residues in each province includes the amount of straw needed for rural energy (**Figure 2.6)**. If the straw was combusted at a power plant or converted to biogas, this would directly replace the household combustion of straw for fuel. As the efficiency of these conversion processes are higher [118], more energy would be produced for the same amount of straw without negative impacts of

pollution due to indoor burning of straw for fuel [119-121]. If this material was used for liquid fuel production, although there is extra electricity produced by the process and sent to the grid [12], it may not be sufficient to replace the rural energy. Ideally if it is not sufficient, the difference would be replaced by some other renewable source of electricity and not by kerosene or coal, however coal is the most likely replacement.

One possible solution to the demands for energy and animal feed is ammonia fiber expansion (AFEX^{TM}) pretreatment, a pressurized ammonia treatment of plant materials. In addition to significantly improving the enzymatic digestibility of a variety of plant materials for the ethanol conversion process, this method has also been shown to improve their quality as an animal feed. AFEX^{TM}-treated plant materials have increased digestibility and non-protein nitrogen content and have been shown to stimulate milk production in ruminants [122, 123]. AFEX^{TM} technology could reduce the projected dependence on animal feed imports by improving grasses and crop residues for animal production, thereby increasing the potential animal feed base within the country. The north region of China (Beijing, Tianjin, Hebei, Shanxi, Shandong and Henan) is predicted to have the highest future production of ruminant animals (for meat and milk) and the highest demand for animal feed [124]. By our estimates Shandong, Henan, and Sichuan have the largest number of non-pastured ruminants (**Table A.2**). Henan and Shandong are also the largest producers of crop residues, together contributing over 19% of the total national production of crop straw (**Table A.5, Figure 2.2**). Both of these provinces are also among the largest potential sources of usable fuel residues and the largest amount of on-field crop residue combustion. Rural development is also

important to consider when considering bioenergy applications. Of the provinces with the largest amount of residues available for energy generation, farmers in Henan, Anhui, and Hunan are comparatively less well off and would perhaps benefit more from the development of a rural bioenergy compared to Shandong, Jiangsu, and Jilin [117]. Given all of the different considerations, Henan in particular, and the central, eastern, and northeastern regions of China in general, seem like the best locations for generation of bioenergy from crop residues.

2.4. Conclusion

Total crop residue production from the main crops in Mainland China in 2006 was estimated at 593.5 million dry metric tons. The largest total amounts of crop straw were produced in Henan, Shandong, Heilongjiang, Jiangsu, and Anhui, while the densest production (Mg/ha cultivated land) was in the central and eastern regions where there is intensive multi-cropping. When different residue-to-straw ratios were used which were based on the Chinese Ministry of Agriculture evaluation, the regions of highest density straw production shifted north in conjunction with the relatively higher corn and lower rice residue-to-straw ratios. The yields of all the crop straws were largely distributed in different areas of the country, and this distribution and the type of crop straw available at a given location could impact the choice of location for a bioenergy facility. The amount of usable residues for bioenergy was estimated to be 124.7 million metric tons. Given the location of usable residues, the potential for rural development, and, if using AFEX^{TM}, the production of animal feed as a co-product, Henan in particular, and central, eastern, and northeastern regions of China in general, appear to be the best locations for the deployment of bioenergy systems.

CHAPTER 3 :
CLASSIFICATION SCALE: INFLUENCE OF VARIABLE SPECIES COMPOSITION ON THE SACCHARIFICATION OF AFEX™ PRETREATED BIOMASS FROM UNMANAGED FIELDS IN COMPARISON TO CORN STOVER

3.1. Introduction

Feedstock cost is predicted to be the largest contributor to the overall cost of cellulosic ethanol production [12], and the relative importance of the feedstock will only increase as the liquid biofuel industry matures. The success of the cellulosic biofuel industry will be highly dependent on the availability of diverse sources of inexpensive, highly digestible plant materials. Potential biofuel feedstocks can be categorized in terms of their energy and chemical inputs, and diversity, ranging from high-input, low-diversity (conventional monoculture crops *e.g.* corn & soybeans) to low-input, high-diversity (native prairie / mixed-species grasslands) [125]. In addition to native prairie, old fields are another type of low-input natural mixed species ecosystem. Old fields are defined as agricultural fields that have been abandoned and no longer undergo reseeding and maintenance. First year production from these abandoned fields is comprised primarily of mixed-species annual weeds, which in later years typically succeeds into perennial grasses, composites and legumes, and eventually into shrubs and trees [126]. Mixed-species ecosystems such as native prairie, and to some extent old fields, provide higher value ecosystem services compared to conventional monocultures, including wildlife habitat and pollination services [127-129], water quality maintenance [130], nitrogen-fixation in fields containing legumes [131, 132], improved soil carbon fixation/lower carbon debt [4, 132, 133] and decreased global warming potential and release of fine particulate matter [133-135]. However, from the perspective of the biorefinery the inherent

heterogeneity of polycultures increases the apparent risk associated with these materials. Because the processing characteristics, potential yields, and digestibility cannot currently be predicted or controlled, this could intensify the challenges associated with determining feedstock value and appropriate processing conditions compared to monoculture feedstocks. Additionally, because of high harvest costs associated with low predicted biomass yields, at the farm scale mixed-species fields are not considered to be economically competitive with other bioenergy cropping systems [136]. As a result, polycultures are often believed to be undesirable feedstocks.

But in spite of these issues, energy generation from mixed-species feedstocks has been examined experimentally using a number of different methods including biogas production [137], supercritical gasification [138], liquefaction [139] and co-combustion with coal [140]. To date there has been no experimental research on ethanol production via pretreatment and saccharification of mixed-species feedstocks, although two studies have reported theoretical ethanol yields [141, 142]. Tilman et al. [141], in their paper on low-input high-diversity grasslands, used a generic ethanol yield (0.255 L/kg DM) which was based on a reported value for corn stover [143]. Adler et al. [142] estimated ethanol yields from conservation grasslands based on composition data with a set conversion rate, and determined fermentability using in vitro gas production. However, predicting ethanol yields solely from composition data and then drawing comparisons between feedstocks gives no indication of potential differences in digestibility, which can also be affected by differences in organization of components within the cell wall, the presence of inhibitory compounds, etc. For example, woody materials often have higher structural sugar contents than grasses and

based solely on composition data they might be expected to perform better, but actually they are typically less digestible and give lower sugar yields [14].

Because enzymatic sugar yields directly impact subsequent ethanol yields, it is important to determine whether there are general characteristics of mixed-species feedstocks that impact yields and subsequent profitability for the biorefinery. One characteristic that is unique to mixed-species feedstocks is the combination of species from different botanical classifications. The simplest classification that is often used for ecology and forage research includes the grasses and relatives (graminoids) and forbs (herbaceous non-graminoids) [144]. There is already evidence that there are distinct differences in effectiveness of chemical pretreatments and saccharification efficiency between species from these two groups [13, 145]. By comparing feedstocks that contain varying distributions of the different plant classifications, it should be possible to observe whether there are classification effects on pretreatment efficiency and saccharification yields. This information might then be used to better manage a mixed-species ecosystem to maintain most of the ecological benefits of an unmanaged system while preserving most of the downstream economic benefits of an intensively managed grass monoculture.

For this study, we evaluated the sugar yields following ammonia fiber expansion (AFEX^{TM}) pretreatment and enzymatic hydrolysis of biomass harvested from five newly abandoned alfalfa fields (e.g. early successional old fields), each of which varied in its mixture of annual forb and grass species. This study was then broadened to include a single mixed-species sample that was prepared by mixing biomass harvested from three fields that had been abandoned for at least 45 years (e.g. late successional old fields) and, as a control, a

separate sample of corn stover. Samples were pretreated using AFEX™ and sugar yields were measured following enzymatic saccharification. As animal feed is one possible co-product for a biorefinery, the AFEX™-treated samples were also evaluated for their forage quality as measured by a standard *in vitro* digestibility assay.

3.2. Materials and methods

3.2.1. Sample harvest and preparation

Corn stover including cobs (CS) was provided by the Great Lakes Bioenergy Research Center (GLBRC) at the University of Wisconsin – Madison. This material was harvested on September 3rd, 2008. The corn stover was milled through a 2 mm screen using a Retsch centrifugal mill prior to composition analysis and enzymatic hydrolysis. Old field samples were provided from the five replicates (R1 – R5, treatment G9) of the GLBRC intensive experiment site at W.K. Kellogg Biological Station (KBS) of Michigan State University (MSU). During the previous year (2007) this site had been planted with alfalfa. Individual plots were 40 x 28 m and had received no maintenance or inputs in 2008 other than initial disking in May. Three quadrats of 2.0 x 0.5 m were harvested within each plot on August 20-21st, 2008. A sixth sample from the Long-Term Ecological Research (LTER) project was also provided from KBS and consisted of a mixed sample from three late-successional old fields (SF1 – SF3) that had been abandoned and unmanaged since 1964, 1948 and 1963 respectively [146]. Five quadrats of 2.0 x 0.5 m were harvested on August 13-18th, 2008. For the GLBRC and the LTER

plots, all plants rooted in each quadrat were clipped at ground level, bagged and dried at 60°C for a minimum of 48 hr. The dry weights were determined for each species, following which the quadrats for each plot were combined and milled through a 2 mm screen. The species composition of these plots is listed in the supporting information in **Table B.1** (GLBRC) and **Table B.2** (LTER). The yield (g/m^2) for each plot was calculated from the combined quadrat yields (GLBRC: g/3 m^2 or LTER: g/5 m^2).

3.2.2. Composition analysis

Biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). The dry matter (DM) composition of each sample (extractives, ash, lignin, glucan and xylan content) was determined based on the NREL standard protocols [147]. The acid insoluble lignin analysis method was modified to use 47 mm, 0.22 µm pore-size, mixed-cellulose ester filter discs (Millipore Corp.; Bedford, MA) during the filtration step instead of fritted crucibles. Due to problems with burning, these discs with the filtered lignin residue were dried overnight in a desiccator prior to weighing rather than in a vacuum oven. The nitrogen content of the extracted and unextracted samples were determined via the combustion method for nitrogen determination [148] using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). Nitrogen values were multiplied by 6.25 to determine the crude protein content. This conversion factor assumes that 16% of the protein is nitrogen and that there is negligible non-protein nitrogen present in the biomass. This factor varies with different types of plant samples due to differences in protein structure,

however accurate determination of this factor requires a complete amino acid analysis [149]. As protein content does not directly impact our results and conclusions, we assume that the standard factor of 6.25 allows for a reasonable approximation. The protein that was removed during extraction steps was subtracted from the total extractives content.

3.2.3. Effect of pretreatment conditions on hydrolysis yields from early successional samples

AFEX^{TM} pretreatment of the early successional old field samples was conducted using 3.0 g DM of sample in 22 mL reaction vessels as outlined by Bals et al. [150]. AFEX^{TM} conditions for the pretreatment optimization experiments were chosen using a three level Box-Behnken statistical design and the parameters ranged from 0.5 to 2.0 g NH_3:g DM, 0.5 to 2.0 g H_2O:g DM, 90 to 180°C, and 5 to 30 min residence time. The central design point was conducted in triplicate to give a total of 24 different conditions and 27 experiments for each sample. In an attempt to improve the fit of the statistical models, three additional experimental points were tested (**Table B.3**). Enzymatic hydrolysis was then conducted on the samples as described in Section 3.2.5. and the enzymatic hydrolysis total sugar conversions (g oligomeric and monomeric glucose and xylose released per g sugar theoretically present in the untreated, dry biomass) were used as the metric of pretreatment efficacy.

The following polynomial quadratic equation of the AFEX^{TM} pretreatment conditions was fitted to the enzymatic hydrolysis total sugar yield data (combined monomeric and oligomeric

glucose and xylose in terms of the theoretical maximum) using Minitab15 Statistical Software (2006 Minitab Inc, Pennsylvania, USA):

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} a_{ij} x_i x_j \quad (3.1)$$

where Y is the sugar yield; a_0 is the regression constant; a_i is the linear regression coefficient for the *i*th parameter; a_{ii} is the quadratic regression coefficient for the *i*th parameter; a_{ij} is the interaction coefficient for the *i*th and *j*th parameters; x_i and x_j are the values of the *ith* and *j*th parameters; and n is the number of factors, which in this case is 4. Coefficients with $p > 0.1$ were removed stepwise from the model, beginning with the largest p-value. The resulting coefficients and adjusted and predictive R^2 values for each sample are reported in **Table B.4.**

An attempt was made to determine the optimum pretreatment conditions for each early successional sample using the polynomial models. However, the predictive capability of the models was very poor, as indicated by the low predictive R^2 values (**Table B.4**) and we were unable to determine the optimum pretreatment conditions. So for the later experiments, the same pretreatment condition was chosen for all of the feedstocks: 2.0 g NH_3:g DM; 0.5 g H_2O:g DM; 90°C; 30 min. This set of conditions was chosen as it resulted in comparatively high sugar yields for four of the five feedstocks (the highest sugar yields for E7 and E87 and among the top five highest yields for E60 and E83) (**Table B.3, Figure B.1**). As the

last sample, E21, performed better when pretreated at slightly higher temperatures, we raised the selected temperature slightly to 100°C.

3.2.4. AFEX™ pretreatment for comparison of early successional old field replicates with late successional old field corn stover samples

AFEX™ was performed on the early successional samples, the late successional sample (LSF) and corn stover (CS) in a 300 mL stainless steel (#316) Parr reactor according to the method detailed in Bals et al. [123]. The conditions were chosen because of the relatively high hydrolysis yields for the early successional feedstocks as determined in the previous section: 2.0 g NH_3:g DM, 0.5 g H_2O:g DM, 100°C, 30 min.

3.2.5. Enzymatic hydrolysis

Samples for pretreatment characterization were hydrolyzed in 20 mL screw-cap vials at 1.5% total sugar (glucan + xylan) loading and a total volume of 15 mL. For the feedstock comparison experiments, samples were hydrolyzed in 20 mL screw-cap vials at 3% solids loading and a total volume of 10 mL. Samples were adjusted to a pH of 4.8 by 1M citrate buffer solution. To prevent fungal and bacterial contamination during enzymatic hydrolysis, cycloheximide and tetracycline were loaded at a final concentration of 30 μg/mL and 40 μg/mL, respectively.

Accelerase®1000, Multifect® Xylanase, and Multifect® Pectinase (Genencor Division of Danisco US, Inc.) were used for all of the hydrolysis experiments. The protein content of each of the enzymes are as follows: Accelerase®1000 (84 mg protein/mL), Multifect® Xylanase (32 mg protein/mL), and Multifect® Pectinase (61 mg protein/mL). Enzyme protein content was determined from total N analysis using the Dumas method for combustion of nitrogen to NOx [148] following trichloroacetic acid (TCA) precipitation to remove non-protein nitrogen [151].

For the pretreatment characterization experiments, Accelerase®1000 was added at 26.8 mg protein/g glucan in the untreated biomass, and Multifect® Xylanase and Multifect® Pectinase were each added at 7.5 mg protein/g xylan in the untreated biomass. For the feedstock comparison experiments, enzymes were loaded at 6.0 mg protein/g DM sample and in the following relative percentages by protein mass, which were similar to those found effective for Alamo switchgrass (unpublished data): Accelerase®1000 (42%), Multifect® Xylanase (24%), and Multifect® Pectinase (34%).

Enzymatic hydrolysis for all experiments was conducted in a New Brunswick Scientific (Edison, NJ) shaking incubator at 50°C and 200 rpm. Samples were taken at 72 hours of enzymatic hydrolysis for both monomeric and oligomeric sugar analysis, as detailed below. Samples taken for monomeric sugar analysis were heated at 100°C for 15-20 min, cooled in the freezer, and then centrifuged at 15,000 × g for 5 minutes. The supernatant was filtered

into HPLC shell vials using a 25 mm, 0.2 µm polyethersulfone syringe filter (Whatman Inc. Florham Park, NJ), then stored at -20°C until further sugar analysis.

3.2.6. Oligomeric sugar analysis

Oligomeric sugar analysis of the pre-wash liquid and hydrolysate was conducted using a scaled-down version of the standard NREL method for oligomeric sugar determination of liquid streams [152]. The modified method was identical except that it was scaled down to use 2 mL of sample and run in duplicate in 10 mL screw-cap culture tubes. Instead of being autoclaved, the tubes were incubated in a 121°C bench-top hot plate for one hour, cooled on ice, and the liquid was filtered into HPLC vials. The oligomeric sugar concentration was determined by subtracting the monomeric sugar concentration of the non-hydrolyzed samples from the total sugar concentration of the acid hydrolyzed samples.

3.2.7. HPLC analysis

Sugar contents of all composition analysis, wash liquid, and hydrolysate samples were determined using a Bio-Rad (Hercules, California, USA) Aminex HPX-87H column equipped with appropriate guard columns. Degassed 5 mM aqueous H_2SO_4 was used as the mobile phase and the column temperature was held at 60°C. The reported total sugar (glucose or xylose) concentration was recalculated as the sum of the average monomeric and the average oligomeric sugar concentration for each sample. Because of the presence of soluble sugars in

the biomass, the glucose percent conversions were calculated using the following equation, where 0.9 corrects for addition of the water molecule upon hydrolysis of glucan to glucose:

$$Glucose\ Conversion\ (\%) = \frac{Glu_{HY}}{Gln/0.9 + Glu + Suc*(180.2/342.3)} \quad (3.2)$$

Glu_{HY} = hydrolysate glucose mass yield (g/kg dry biomass)

Gln = biomass glucan content (g/kg dry biomass)

Glu = biomass soluble glucose content (g/kg dry biomass)

Suc = biomass soluble sucrose content (g/kg dry biomass)

180.2/342.3 = correction for glucose contribution by sucrose (molecular weight of glucose/molecular weight of sucrose)

3.2.8. In vitro *rumen digestibility and neutral detergent fiber determination*

In vitro rumen digestibility was performed in triplicate based on the method reported in Tilley and Terry [153] using rumen fluid obtained from a fistulated dairy cow. Neutral detergent fiber (NDF) concentration (without amylase digestion) was determined for each sample (treated and untreated) after 0 h and 48 h of incubation. The amount of NDF digested after 48 h was determined as the difference between these two values.

3.2.9. Statistical analysis

All statistical analyses, except for Pearson's correlation coefficients, which were calculated using Excel (Microsoft® Office Excel® 2007), were performed using Minitab15 Statistical Software (2006 Minitab Inc, Pennsylvania, USA). This included the response surface

optimization (as described previously in Section 3.2.3.), linear regressions, and Tukey's pairwise comparisons.

3.3. Results

3.3.1. Feedstock characteristics and plot yields

The species mass composition of the old field replicates (E7, E21, E60, E83, E87) consisted almost entirely of annual forbs and grasses (**Table B.1**). These samples have been relabeled for ease of analysis in terms of their % grass content on a mass basis. There were 7 to 14 species in each replicate (**Table 3.1**), however five main species contributed > 95% of the total mass for all five replicates. The late successional old field sample (LSF) was composed of three different replicate plots with 20 to 45 species in each replicate (**Table 3.1)**. Seven of these species comprised 86% of the combined sample mass (**Table B.2**). There was no distinct trend between plant classification and biomass yield (g/m^2) for the five early successional replicates. The late successional replicate that contained mostly grass had a much higher biomass yield than the other two samples that were composed predominantly of woody species.

The dry matter composition of each sample is shown in **Table 3.2**. The early successional replicates that had lower grass contents (E7 and E21) also had lower structural sugar contents compared to the samples that had higher grass contents (E60, E83, E87, and LSF). Corn stover had a significantly higher structural sugar content compared to all other samples (65.5% of the total dry mass). The Klason lignin content of the different samples ranged from 13.6% - 18.3% of the total dry biomass and was inversely correlated with the

Table 3.1: Species composition, biomass yield, and distribution for the GLBRC old-field and LTER replicates.

			# of Species				Biomass Yield (g/m^2)				Mass Distribution (%)		
	Expt.	Field	Grass	Forb	Wood	*Total*	Grass	Forb	Wood	*Total*	Grass	Forb	Wood
Early Successional Old Field Treatment Replicates													
	E7	R1	4	10	0	*14*	54	673	-	*727*	7%	93%	-
	E21	R4	1	6	0	*7*	210	799	-	*1009*	21%	79%	-
	E60	R5	4	4	0	*8*	516	347	-	*863*	60%	40%	-
	E83	R3	3	8	0	*11*	753	158	-	*911*	83%	17%	-
	E87	R2	4	4	0	*8*	576	88	-	*664*	87%	13%	-
Late Successional Old Field Replicates													
		SF1	6	14	0	*20*	213	47	-	*260*	82%	18%	-
	LSF	SF2	5	10	13	*28*	7	1	23	*31*	21%	4%	75%
		SF3	3	25	17	*45*	<1	17	47	*64*	0%	27%	73%

Table 3.2: Composition analysis data as % of total dry matter (DM). The standard error is reported in parenthesis and represents three replicates. LSF = late successional old field sample.

Feedstock		Structural Carbohydrates*							Water Extractives[h]					
		Gln	Xyl	Ara	Total	Klason Lignin	Ash	Crude Protein[g]	Acetyl	Glu	Suc	Other	Ethanol Ext.[h]	Total Mass
Early Successional Old Field	E7	23.0[d] *(0.1)*	13.9[f] *(0.1)*	2.1[c] *(0.04)*	39.0[e] *(0.2)*	18.3[a] *(0.1)*	9.7[a] *(0.2)*	11.2[a] *(0.1)*	1.9[b] *(0.1)*	1.8[c] *(0.2)*	1.8[d] *(0.4)*	14.2 *(0.6)*	3.4 *(0.01)*	101.3 *(0.8)*
	E21	26.4[e] *(0.7)*	15.1[e] *(0.1)*	2.1[c] *(0.1)*	43.7[d] *(0.7)*	16.3[b] *(0.3)*	8.8[bc] *(0.04)*	5.5[d] *(0.1)*	2.0[b] *(0.1)*	3.2[a] *(0.3)*	3.3[c] *(0.7)*	11.8 *(1.0)*	4.8 *(0.3)*	99.8 *(1.5)*
	E60	29.1[b] *(0.8)*	17.2[d] *(0.6)*	2.5[b] *(0.2)*	48.9[bc] *(1.0)*	16.9[b] *(0.3)*	9.0[bc] *(0.1)*	4.6[f] *(0.1)*	1.7[c] *(0.1)*	2.5[b] *(0.1)*	3.1[c] *(0.1)*	13.5 *(0.3)*	2.8 *(0.2)*	103.1 *(1.1)*
	E83	29.6[b] *(0.9)*	18.7[b] *(0.5)*	2.1[c] *(0.2)*	50.5[b] *(1.0)*	14.0[d] *(0.1)*	8.5[c] *(0.1)*	4.9[e] *(0.02)*	1.8[bc] *(0.1)*	2.4[b] *(0.05)*	5.8[a] *(0.1)*	11.2 *(0.5)*	2.9 *(0.2)*	102.0 *(1.2)*
	E87	30.7[b] *(0.7)*	17.8[cd] *(1.4)*	2.5[b] *(0.1)*	50.9[b] *(1.6)*	13.6[d] *(0.5)*	9.2[ab] *(0.1)*	6.2[c] *(0.2)*	1.6[cd] *(0.1)*	2.2[bc] *(0.05)*	4.9[b] *(0.2)*	13.3 *(2.0)*	3.6 *(1.4)*	105.5 *(2.9)*
	LSF	26.5[c] *(0.7)*	18.2[bc] *(0.4)*	2.6[b] *(0.1)*	47.3[c] *(0.8)*	14.9[c] *(0.1)*	5.7[d] *(0.1)*	8.5[b] *(0.1)*	1.5[d] *(0.1)*	1.2[d] *(0.1)*	0.2[e] *(0.03)*	21.6 *(0.4)*	ND	100.9 *(0.9)*
	Corn Stover	36.8[a] *(0.5)*	25.0[a] *(0.2)*	3.5[a] *(0.1)*	65.5[a] *(0.6)*	14.9[c] *(0.1)*	4.6[e] *(0.5)*	NM	2.5[a] *(0.1)*	0.4[e] *(0.02)*	0.3[e] *(0.04)*	8.0 *(0.3)*	2.6 *(0.1)*	98.6 *(0.8)*

*Gln= glucan; Xyl = xylan; Ara = arabinan; Glu = glucose; Suc = sucrose; Ext. = extractives

[a-f] Means with different superscripts in each column are significantly different at the 95% confidence level using Tukey's pairwise comparison.

[g] Crude protein content was not determined for the corn stover sample

[h] The ethanol extraction was performed, but not quantified for the SF replicates sample. "Other Water Extractives" also includes the amount of ethanol extractives.

sample grass content (r = -0.95, p = 0.01, n = 7) when measured on a cell wall basis (generalized as the sum of the structural carbohydrates and Klason lignin). Ash content andsoluble glucose and sucrose content were higher for the early successional samples compared to the late successional and corn stover samples.

3.3.2. Relationship of AFEX™ pretreatment conditions to hydrolysis yields from early successional samples

Each of the five early successional replicates was pretreated with AFEX™ using various ammonia and water loadings, temperatures and residence times. Each sample was tested using 30 different sets of pretreatment conditions, except for E87 where one result was omitted from the analysis due to a high residual value that corresponded to abnormally low sugar yields.) Information on the specific conditions examined and the resulting total sugar yields are reported in the supplemental information (**Table B.3**). The sugar conversion varied significantly for each feedstock across the pretreatment conditions (**Figure 3.1**) and trends appeared to be associated in part with forb vs. grass-dominated samples. E7 had the lowest conversions of monomeric and oligomeric sugars (glucose: 46-67%; xylose: 46-82%), followed by E21 (glucose: 54-80%; xylose: 57-80%). E60, E83, and E87 all had similar ranges for xylose release (E60: 69-102%; E83: 67-98%; E87: 65-102%). The yields that are slightly over 100% are likely due to small errors that occurred during compositional analysis. Glucose release tended to increase from E87 to E83 to E60 and is more evident from **Figure 3.1** than from the range of glucose yields (E60: 65-91%; E83: 59-84%; E87: 56-82%). Pretreatment improved sugar yields in all cases

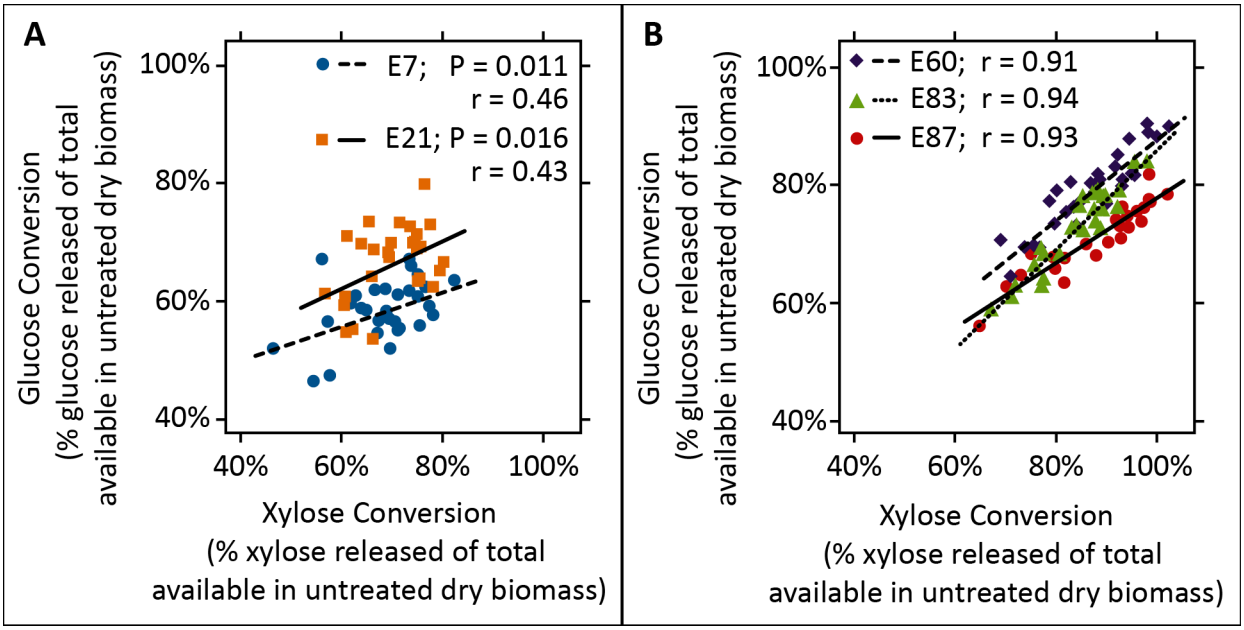

Figure 3.1: Relationship between enzymatic hydrolysis glucose and xylose yields for the GLBRC old field replicates. (A) Forb-dominated samples (E7 and E21). (B) Grass-dominated samples (E60, E83, and E87). Yields are calculated as the total monomeric and oligomeric sugar solubilized based on the total sugar theoretically available in the untreated dry biomass. For the E60, E83, and E87 regressions, ($p = 0.000$). Each data point represents one of 30 different pretreatment conditions (except for E87, which only has data for 29 conditions due to one significant outlier).

compared to the untreated feedstock, for which sugar yields ranged from 32.2% for E7 to 46.3% for E83 (data not shown).

Each feedstock showed a significant linear correlation ($p < 0.05$) between glucose yield and xylose yield (**Figure 3.1**). However, there was a much larger spread for the data points for the two feedstocks that had the lowest grass content (< 20% grass), E7 and E21 ($r \sim 0.45$). The correlation between glucose and xylose release was both highly linear ($r > 0.90$) and highly significant ($p < 0.001$) for the feedstocks with the higher grass content (> 60% grass), E60, E83, and E87.

Due to high error associated with the constructed response surface models (**Table B.4**), the optimum pretreatment conditions could not be accurately determined for the feedstocks. Because of this, the raw data were used to select a pretreatment condition that gave reasonably high yields for most of the feedstocks and could be used for further experiments. The pretreatment condition chosen was 2.0 g NH_3:g DM, 0.5 g H_2O:g DM; 100°C and 30 min residence time. This condition was chosen as a similar pretreatment condition at 90°C resulted in the highest sugar yields for E7 and E87 and among the top five sugar yields for E60 and E83 (**Table B.3, Figure B.1**). Of those materials examined, only E21 did not obtain high yields when operated at these conditions (lower than the optimum condition by 50 g sugars released per kg untreated dry biomass), and it tended to require slightly higher temperatures compared to the other feedstocks. This difference appears unrelated to the differences between forbs and grasses. For example, sample E7, the other forb dominated feedstock, has the same optimum as E87. The difference may instead be related to the specific species that were present in the mixture. E21 contained a much larger percentage of *Amaranthus retroflexus* L. (redroot pigweed), and it may be that this forb is more indigestible than the forb species present in the E7 sample.

3.3.3. In vitro digestibility

The sale of pretreated biomass as an animal feed co-product has the potential to improve the process economics in a biorefinery [10], so the i*n vitro* rumen digestibility was determined for the untreated and the pretreated old field replicates (**Table 3.3**). In all cases,

Table 3.3: ***In vitro*** **rumen digestibility of untreated and AFEX™-treated early successional old field samples.**

Initial NDF (g NDF/kg DM)						
	Untreated		AFEX™-Treated			
	Average	SEM[a]	Average	SEM[a]	Difference[b]	% Increase[c]
E7	528	6.6	442	4.5	86	-
E21	574	3.0	475	4.0	99	-
E60	586	1.9	526	5.9	60	-
E83	597	4.9	509	5.8	87	-
E87	595	2.7	491	0.9	104	-
Digested (g NDF/kg DM) [d]						
	Untreated		AFEX™-Treated			
	Average	SEM[a]	Average	SEM[a]	Difference[b]	% Increase[c]
E7	130	9.7	133	11.8	3	2%
E21	205	7.9	212	4.6	7	4%
E60	260	2.4	327	6.5	67	26%
E83	290	15.2	313	8.2	23	8%
E87	313	6.6	323	2.8	10	3%
Total Digested (g NDF/kg DM) [e]						
	Average	SEM[a]	Difference[b]	% Increase[c]	% Digested[f]	
E7	218	12.7	89	68%	41%	
E21	311	3.7	107	52%	54%	
E60	387	3.3	127	49%	66%	
E83	400	7.6	111	38%	67%	
E87	427	3.8	114	37%	72%	

[a] Standard error of the mean based on three replicates

[b] Difference between AFEX™-treated and untreated samples

[c] Percent Increase in treated sample over untreated sample

[d] NDF digested during 48 h *in vitro* rumen digestion

[e] Total NDF removed for AFEX™-treated samples, including both the amount removed due to AFEX™ and the amount digested during *in vitro* rumen digestion

[f] Total amount of NDF removed due to AFEX™ and during *in vitro* rumen digestion as a percentage of untreated NDF

AFEX™ treatment decreased neutral detergent fiber (NDF) content compared to untreated controls, a difference of 60 – 104 g NDF/kg DM, indicating an increase in digestibility of pretreated materials. Except for the E60 sample, where almost 26% more was digested for the AFEX™-treated sample, there was very little difference between the untreated and AFEX™-treated samples in the amount of NDF digested by the rumen microbes. The percent of digested NDF for the AFEX™-treated samples, from largest to smallest was E87 > E83 > E60 > E21 > E7, which corresponded with decreasing grass content in the samples. In all cases, AFEX™ improved overall NDF digestion compared to untreated samples. The materials with the lowest grass content had the largest improvement in digestibility due to AFEX™ pretreatment. One issue with NDF determination is that pectins, which are present in much larger amounts in forbs, are digested by the NDF process, resulting in a lower estimate of NDF value than is actually the case [154]. As pectins can be utilized by ruminants, this may result in underestimating the value of the forb-dominated samples as an animal feed.

3.3.4. Comparison of early successional old field replicates to late successional old field (LSF) and corn stover (CS) samples

The glucose, xylose and total sugar hydrolysis yields (g/kg dry biomass) are reported in **Figure 3.2**. All of the total sugar yields were significantly different except for E60, E83, and E87, and can be arranged in decreasing order from CS > (E87, E83, E60) > LSF > E21 > E7. Both LSF and CS had a large amount of xylo-oligomers remaining, indicating that for these samples the

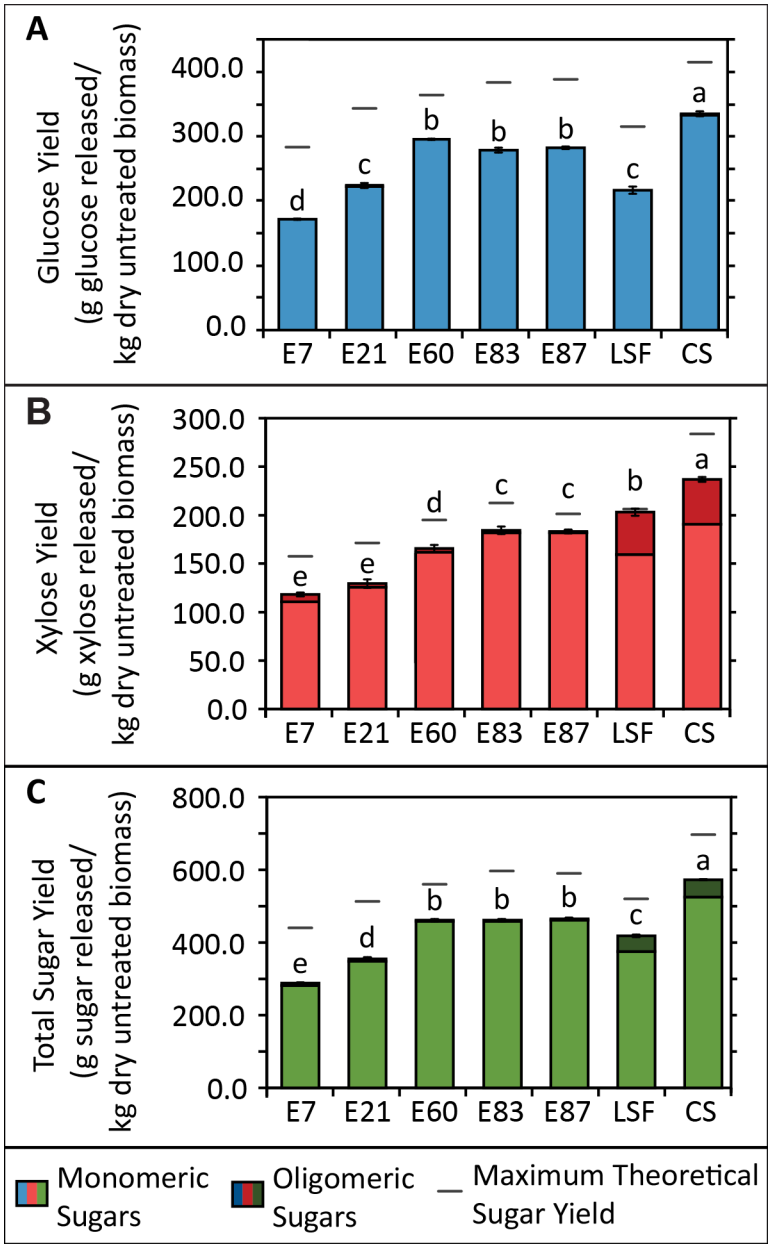

Figure 3.2: Comparative monomeric and oligomeric sugar yields. (A) Glucose yields. (B) Xylose yields. (C) Total sugar (glucose + xylose) yields. Oligomeric sugars are reported in monomeric equivalents. The maximum theoretical sugar yield is the maximum amount of glucose, xylose or total sugars that could be released from the untreated dry biomass. Total (monomeric and oligomeric) sugar yields with different letters are statistically different based on Tukey's test ($p < 0.05$). E7, E21, E60, E83, E87 = early successional old field replicates from the GLBRC intensive site; LSF = LTER late-successional old field combined sample; CS = corn stover. Each sample was subjected to the same pretreatment and enzymatic hydrolysis conditions.

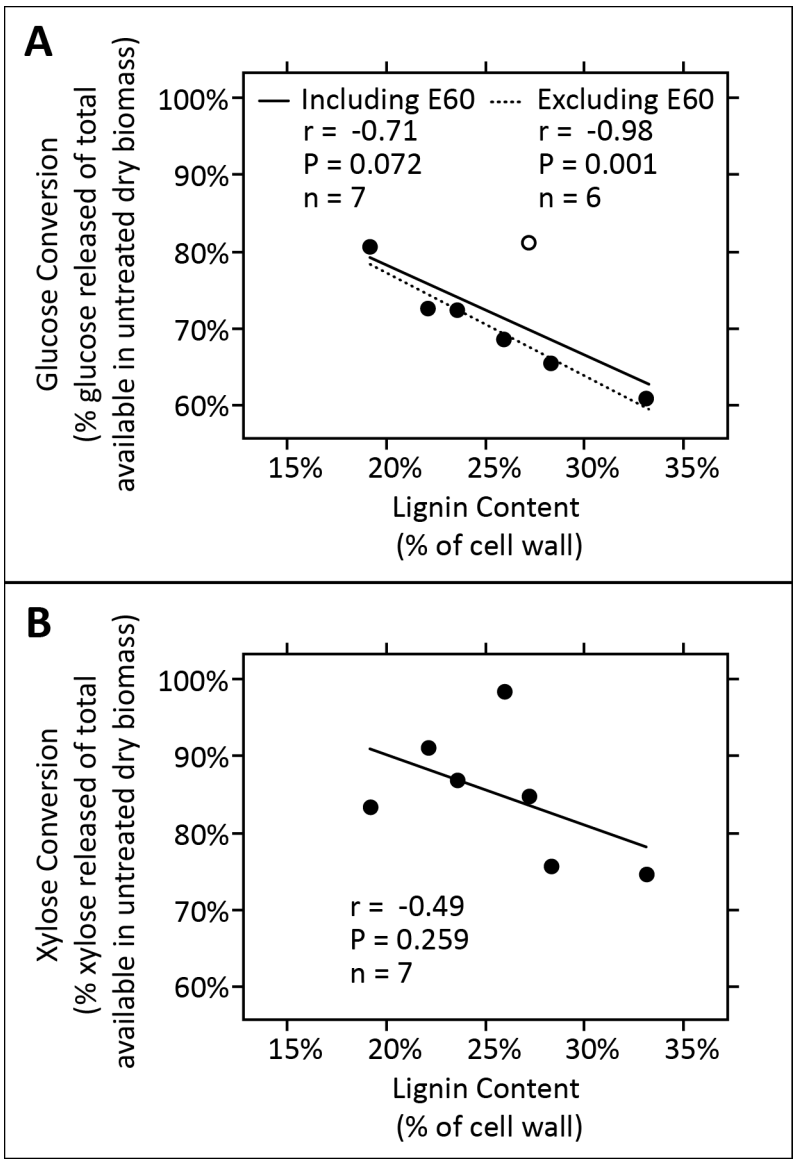

Figure 3.3: Correlation of glucose (A) and xylose (B) percent conversion (g sugar released/g theoretically available in untreated dry biomass) to Klason lignin content on a cell wall basis for the early successional old field replicates, late-successional old field sample, and corn stover. The solid line represents the correlation when the old field replicate E60 (represented by an open circle) is included in the analysis and the dashed line indicates the correlation when sample E60 is removed from the analysis.

enzyme combination used was not adequate to convert all of the xylo-oligomers to xylose. None of the samples reached the theoretical maximum sugar yield, except for the LSF xylose yield. The grass-enriched samples (E60, E83, and E87) had higher sugar mas yields and conversion efficiencies compared to the forb-enriched samples (E7 and E21). Based on Tukey's test ($p < 0.05$), the different feedstocks can be divided into two statistically different categories based on the total sugar yields on a percent basis, the low grass content samples (< 20% grass): E7 and E21; and the corn stover and high grass content samples (> 60% grass): E60, E83, E87, LSF and CS.

When the total (monomeric + oligomeric) sugar yields were plotted against the lignin content on a cell wall basis, there was some correlation between xylose release and lignin content **(Figure 3.3**), but it was not statistically significant. However, there was a stronger and more significant correlation between glucose release and lignin content, which increased considerably when sample E60 was removed from the analysis.

3.4. Discussion

Old field mixed-species samples are considered unsuitable for biofuel production due to low predicted yields per hectare that cause the cost to the farmer to become prohibitively expensive [136]. Additionally, as harvestable yield per unit area decreases, the collection radius for the biorefinery increases, leading to significantly higher transportation costs. The large-scale biomass yields from the old field samples can be extrapolated from the small-scale sample data to estimate the biomass yields for a larger harvested area; however these numbers should be viewed with caution due to the potential for over- or under-estimation of yields. The early

successional old field replicates had rather high extrapolated yields (6.6-10.1 Mg/ha) that are promising but would probably not be sustainable over multiple years without additional inputs. Additionally, the yields may be high because the GLBRC intensive plot, while unfertilized, was previously planted to alfalfa, and there may have been residual nitrogen remaining in the soil due to nitrogen fixation. The key species that grew in the old field plots, particularly *Amaranthus retroflexus* L. (redroot pigweed) and *Chenopodium album* L. (common lambsquarters) are extremely responsive to soil nitrogen levels and are highly efficient in nitrogen uptake [155]. Any fixed nitrogen remaining from the alfalfa would have been readily utilized by these plants, increasing production of biomass, assuming the presence of adequate amounts of other potentially limiting nutrients. The late successional (SF) replicates, harvested from sites which have been abandoned and unmanaged for ~50 years, may give a more realistic estimate of long term yields from fields which contain very few nitrogen-fixing species (0.3-2.6 Mg/ha, *extrapolated*). The biomass yields from all three late successional (SF) replicates have continually decreased since 1993, particularly SF2 and SF3 which have become dominated by woody species (>70% of the total biomass) [146].

For mixed-species feedstocks, the relationship between glucose and xylose yields for different pretreatment conditions, the sugar conversion efficiencies (a measure of feedstock digestibility), and the *in vitro* digestibility were related to the grass content the sample. Based on the results shown, the old field replicates can be categorized into either the grass-dominated samples (≥ 60% grass species on a mass basis): E60, E83, and E87; or the forb-dominated samples (≤ 20% grass species on a mass basis): E7 and E21. The differences observed between the two groups could be explained by the fact that grasses and their relatives

(commelinids) and forbs (non-commelinids) have very different cell wall chemistries, including the type, structure, and localization of hemicellulose as well as lignin monomer content and composition [17, 18, 27, 156-158]. Release of xylose from the cell wall of grasses [150] or grass-dominated samples is strongly correlated to increases in glucose yields. However, the relationship was not as strong for the forb-dominated samples. This may indicate that compared to the grass cell wall, the release of glucose from the forb cell wall depends more on other factors than on the presence or absence of xylan. This is supported by research on dilute acid pretreatment, which selectively removes xylan from the cell wall [159]. Dien et al. [13] observed that a dilute acid pretreated forb (alfalfa stems) had a lower glucan conversion efficiency that was not dependent on the release of non-glucan sugars, compared to two pretreated grasses. They hypothesized that the stem cellulose that was not digested may be more closely associated with lignin, which unlike for commelinids, is not evenly distributed within the non-commelinid cell wall [160, 161].

It is well documented that total lignin content is negatively correlated with both rumen degradability [162, 163] and enzymatic saccharification [164, 165]. However, it is important to note that lignin content, while correlated with digestibility, is in itself not a sufficient indicator for biomass digestibility, particularly if one is interested in total sugars. Based on our results, the cell wall lignin content was strongly negatively correlated with glucose digestibility, but not xylose digestibility, and the correlation was stronger when E60 was not included in the analysis. This is interesting because there are indications that there is something different about E60 compared to the other samples that were tested. E60, which is the most digestible sample and contains nearly 60% grass, and E21, which is significantly less digestible and contains only 21%

grass, have statistically identical Klason lignin contents (**Table 3.1**). The large difference in digestibility between these samples could be attributed to differences between forbs and grasses, such as lignin distribution within the cell wall, or there might be some unique characteristic about E60 that makes it more responsive to pretreatment compared to the other samples. E60 had the largest increase in glucose release between the untreated material and the optimally pretreated materials (45% increase versus 34-39% for the other old field replicates). E60 is also the only sample that experienced a large increase in rumen digestibility because of AFEX^{TM} pretreatment (**Table 3.3**). Another possibility is that there are different types and quantities of covalent linkages between cellulose and lignin in the different plants. Recent evidence indicates the presence of oxygen-containing linkages (ether or ester) between lignin and cellulose in corn leaves [166]. Ester linkages are known to be more readily cleaved under alkaline conditions [19], so if more of these were present linking the cellulose and lignin in the E60 sample, then this might partially explain an increase in digestibility.

At the same pretreatment conditions and enzyme loading, the high grass content samples, CS, E87, E83, E60, and LSF, had statistically identical sugar conversions (g sugar released/g sugar theoretically available), approximately 80% of the total sugars. Woody species have previously been shown to result in lower yields compared to cereal residues [14], but their presence in the LSF sample did not reduce the digestibility. This was largely because a high xylose yield compensated for a lower glucose yield (**Figure 3.2**). Glucose yields for the mixed-species samples ranged from 170 – 300 g/kg biomass and total sugars ranged from 290 – 470 g/kg biomass. This is in comparison to the glucose and total sugars released from corn stover: 340 and 580 g/kg biomass, respectively. While the corn stover had the highest sugar yields, this

was not because it was significantly more digestible than the other materials, but rather because it had a significantly higher cell wall sugar content. This may be partly related to the relative maturity of the samples. Even though they were harvested around the same time of the year (albeit in different locations), the corn stover may have been more mature than the old field replicates, because the cell wall characteristics (high glucan, xylan and lignin with low ash and soluble sugars) were typical of a more mature plant [158]. If the old field samples had been allowed to mature further and harvested in October or November, it is possible that the structural sugar content and subsequent sugar mass yields would have been higher.

AFEX^{TM} pretreatment has been examined as a possible treatment for forages due to its similarity to the ammonia treatment that has been used by farmers for decades to increase digestibility of ruminant feeds [123]. The increase in digestibility we observed due to AFEX^{TM} pretreatment of the mixed species feedstocks is quite similar to that observed by Bals et al. for the more readily digestible forages [123]. As they concluded, it seems unlikely that the increase in digestibility observed for these types of materials is large enough to warrant the use of pretreatment. It may be that if the mixed species feedstocks we examined were allowed to mature further and harvested in late October or November, the recalcitrance would increase and there would be more of an effect by pretreatment on digestibility.

From the biorefinery standpoint, grass-dominated feedstocks will likely be more profitable because of their higher mass sugar yields that are directly related to a higher cell wall sugar content and greater digestibility. C4 grasses have previously been predicted to produce the greatest amount of ethanol per hectare [142]. It might be possible to manage mixed-

species sites to have a majority of grass species, which could have a beneficial impact on the biomass yield, but this would likely come with some cost to the species diversity [142]. Additionally, although a farmer may not wish to replace their corn crop with a mixed-species feedstock based on the economic considerations, it may be worthwhile to harvest one of their abandoned fields or convert it to a mixed-species stand, such as native prairie or mixed native grasses, for use as a biofuel feedstock. The addition of a small amount of fertilizer to the fields and/or the incorporation of legumes in the species mixture may also increase yields to the extent that the fields become more profitable for the farmer, while still limiting the cost associated with inputs and fertilizer-related environmental impacts.

Mixed-species feedstocks have been considered unsuitable for biofuel production because of their inherently heterogeneous nature. However, no lignocellulosic feedstock will be homogenous, even monocultures of intensively selected varieties. This is because the feedstock characteristics and digestibility are highly dependent on the fraction of the plant considered (leaf, stem, etc.), maturity, location, and environmental conditions experienced during growth [29, 150, 167]. Large biorefineries, because of transportation costs and supply limitations, will need to accept a wide variety of feedstocks and have a method to quickly determine their value. However, it may be feasible to process most feedstocks at similar conditions to obtain the highest yields. Four of the five feedstocks gave fairly high yields at the chosen operating condition. It is very likely, given the example of E21, that there will be some feedstocks that do not process as well at the chosen condition. The operator will need to decide which is more cost effective: changing operating conditions for different materials to maximize yields, operating at the same conditions for all feedstocks and potentially losing yields for some of

them, or perhaps blending feedstocks to make up for deficiencies in low-yielding materials and improve overall process stability.

While dedicated, managed monocultures will likely give higher yields and be more reliable once established, polycultures can be equally digestible, and could be used as a supplemental feedstock. By implementing this approach, the biorefinery can diversify their feedstock supply and increase source security, while simultaneously decreasing the collection area and reducing transportation costs. At the same time, mixed-species feedstocks provide more valuable ecological services compared to monocultures and have much to offer the lignocellulosic biorefinery and surrounding communities and landscapes.

CHAPTER 4 :
SPECIES SCALE: OPTIMIZATION OF AFEX™ PRETREATMENT CONDITIONS AND ENZYME MIXTURES TO MAXIMIZE SUGAR RELEASE FROM UPLAND AND LOWLAND SWITCHGRASS

4.1. Introduction

Switchgrass (*Panicum virgatum* L.) is a perennial C4 grass, native to the Great Plains of North America. Experimental research on switchgrass breeding has been conducted since the 1970s, with most currently available cultivars developed for improved forage qualities [168]. After evaluating 35 potential herbaceous crops, the US Department of Energy (DOE) chose switchgrass as one of the promising species for bioenergy production because of its potential for high yields, wide range of distribution, and beneficial environmental characteristics [169, 170]. Switchgrass cultivars can be broadly classified according to their cytotype (upland vs. lowland) and latitude-of-origin (southern vs. northern). Lowland varieties are tetraploid and tend to be taller, coarser, and have thicker stems and wider leaves compared to upland varieties, which are either tetraploid or octaploid [168, 171]. Typically, upland cultivars have higher biomass yields and survivability at northern latitudes, while lowland cultivars perform better at more southern latitudes [172, 173]. Cytotype, latitude-of-origin, and location significantly impact biomass yield, survivability, cell wall composition, and *in vitro* digestibility of different switchgrass varieties [172, 174].

One method to convert switchgrass to a usable transportation fuel is through biochemical conversion of the biomass structural sugars to a liquid fuel, such as ethanol. This process uses a mechanical or thermochemical pretreatment to disrupt biomass structure, followed by enzymatic hydrolysis of the structural carbohydrates to fermentable sugars. While there have been many recent publications covering a range of pretreatment options for

biochemical conversion of switchgrass, only three papers have compared multiple switchgrass cultivars and/or cytotypes [150, 175, 176]. It is possible that different cultivars could have different optimal pretreatment parameters and enzyme combinations for hydrolysis. However, because of the strong effect of location [172] and harvest timing [177] on switchgrass phenotype, it is necessary to grow and harvest the different varieties under as similar conditions as possible to accurately determine which differences are specifically tied to the cultivar, and not differences in environment or harvest timing.

One way to improve the optimization of biofuel processing parameters is by using statistical design of experiments. The benefit of statistical methods compared to the one-factor-at-a-time approach is that, for a comparable number of experiments, statistical methods provide more detailed information including the interactions between process variables. Examples of statistical methods include factorial designs, which are best suited for determining which factors significantly impact the process output; response surface optimization, which is useful for predicting the optimum process design points given a target endpoint; and mixture optimization, which is useful for optimizing the ratio of components in a mixture given a desired output [178]. Statistical optimization methods have been used to characterize various steps of the bioconversion process including optimization of pretreatment parameters [179, 180], enzyme combinations [181, 182], enzymatic hydrolysis and fermentation parameters [183], and fermentation media formulations [184].

Our objective for this project was to use a response surface optimization method to determine and compare the optimal ammonia fiber expansion (AFEX^{TM}) pretreatment parameters for two switchgrass varieties, one from each cytotype, Alamo (lowland) and

Shawnee (upland) that had the same harvest timing (December) in the same region (central Oklahoma). The optimally pretreated switchgrass was then used to determine the optimum combination of commercial enzymes (Spezyme® CP, Novozyme® 188, Multifect® Xylanase and Multifect® Pectinase) for each variety using a mixture optimization design.

4.2. Materials and methods

4.2.1. Feedstock

Two switchgrass varieties, Alamo, an upland ecotype, and Shawnee, a lowland ecotype, were provided by Ceres, Inc. (Thousand Oaks, CA). Both varieties were planted in June 2005; the Alamo switchgrass in Ardmore, OK (34°N, Elev. 870 ft.) and the Shawnee in Stillwater, OK (36°N, Elev. 960 ft.), and both were harvested in December 2006. Following harvest, the switchgrass was air-dried to less than 10% moisture and then milled through a 2 mm screen using a standard Wiley mill (Thomas Scientific, Swedesboro, NJ). Samples were stored at room temperature until composition analysis, pre-washing, or pretreatment were performed.

4.2.2. Pre-wash

A pre-wash step was performed to remove any soluble sugars that could mask the solubilization of cell wall sugars. 100 g of switchgrass was soaked in 1 L of 80-90°C distilled water for 10-15 min. The switchgrass slurry was vacuum-filtered through Whatman No. 1 filter paper (Whatman Ltd.). This process was repeated three times and after each wash step a

portion of the filtrate was retained for oligomeric sugar analysis. The washed solids were dried in a 45°C oven. The extracted weight loss of the switchgrass was determined by subtracting the dry weight of the washed switchgrass and the dry mass loss to the filter paper from the initial dry weight. Washed switchgrass was used for the hydrolysis rate determination and enzyme mixture experiments, but not for the pretreatment response surface experiments.

4.2.3. Composition analysis

Biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). The composition of each sample (extractives, ash, lignin, glucan, and xylan) was determined according to the standard National Renewable Energy Laboratory (NREL) protocol that uses a two-stage extraction followed by two-step acid hydrolysis [147]. The acid-insoluble lignin analysis method was modified to use 47 mm, 0.22 µm pore-size mixed-cellulose ester filter disks (Millipore Corp., Bedford, MA) during the filtration step instead of fritted crucibles. The filtered lignin residues were dried overnight in a desiccator prior to weighing. The nitrogen content of the extracted and unextracted samples were determined via the combustion method for nitrogen determination [148] using a Skalar Primacs SN Total Nitrogen Analyzer (Breda, The Netherlands). Nitrogen values were multiplied by 6.25 to determine the crude protein content. This conversion factor assumes that 16% of the protein is nitrogen and that there is negligible non-protein nitrogen present in the biomass. This factor varies with different types of plant samples due to differences in protein structure, however accurate determination of this factor requires a complete amino acid analysis [149]. As protein content does not directly impact our results, we assume that the standard factor of 6.25 allows for a

reasonable approximation. Protein that was removed during the extraction steps was subtracted from the total extractives content. The composition data for unwashed and washed Alamo and Shawnee switchgrass are listed in **Table 4.1**.

Table 4.1: Composition analysis data for untreated Alamo and Shawnee switchgrass (% of total dry biomass). Washed switchgrass samples had been sequentially washed three times with 80-90°C water in order to remove the majority of the soluble sugars. Values with different superscripts in each row were statistically different based on Tukey's pair-wise comparisons with $\alpha = 0.05$.

		Unwashed		Washed	
		Alamo	Shawnee	Alamo	Shawnee
	Glucan	$27.3^{d} \pm 0.5$	$30.2^{c} \pm 0.5$	$34.8^{b} \pm 0.3$	$37.1^{a} \pm 1.2$
	Xylan	$21.2^{b} \pm 0.3$	$21.3^{b} \pm 0.7$	$26.3^{a} \pm 0.3$	$25.0^{a} \pm 0.8$
	Arabinan	$3.2^{c} \pm 0.1$	$3.1^{c} \pm 0.1$	$4.6^{a} \pm 0.1$	$4.2^{b} \pm 0.1$
	Klason Lignin	$15.4^{d} \pm 0.1$	$17.4^{c} \pm 0.1$	$18.9^{b} \pm 0.3$	$20.8^{a} \pm 0.3$
	Total Ash	$5.3^{a} \pm 0.1$	$5.3^{a} \pm 0.1$	n.d.	n.d.
	Protein	$6.5^{a} \pm 0.2$	$6.4^{a} \pm 0.2$	$6.0^{b} \pm 0.1$	$4.7^{c} \pm 0.1$
Extractives	**Soluble Glucose**	$2.6^{b} \pm 0.2$	$3.3^{a} \pm 0.2$	0.7 ± 0.0*	0.8 ± 0.0*
	Sucrose	$5.2^{a} \pm 0.4$	$1.9^{b} \pm 0.2$	n.d.	n.d
	Acetyl	$2.0^{b} \pm 0.1$	$2.0^{b} \pm 0.0$	$2.2^{a} \pm 0.1$	$2.0^{b} \pm 0.0$
	Other Extractives	15.5 ± 0.9	13.0 ± 0.4	0	0
	Total	104.2	103.9	93.1	93.9

n.d. = not determined; *Soluble monomeric glucose in the acid-hydrolyzed water extract – includes both soluble glucose and glucose contributed by sucrose.

4.2.4. Design of experiments

4.2.4.1. Response surface optimization of pretreatment conditions

AFEX™ conditions for the pretreatment optimization experiments were chosen using a Box-Behnken statistical design and the parameters ranged from 0.5 to 2.0 g NH_3:g dry matter (DM), 0.5 to 2.0 g H_2O:g DM, 90 to 180°C and 5 to 30 min residence time. The central design point was conducted in triplicate to give a total of 27 experiments for both the Alamo and Shawnee. In an attempt to improve the fit of the statistical models, five additional experiment points were tested for Alamo and three for Shawnee (**Table C.1**). Enzymatic hydrolysis was then conducted on the samples as described in Section 4.2.6. The enzymatic hydrolysis sugar yields (g sugar released per g sugar theoretically present in the untreated, dry biomass) were used as the metric of pretreatment efficacy.

The following polynomial quadratic equation of the AFEX™ pretreatment conditions was fitted to the enzymatic hydrolysis total sugar yield data (combined monomeric and oligomeric glucose and xylose in terms of the theoretical maximum) using Minitab15 Statistical Software (2006 Minitab Inc., Pennsylvania, USA):

$$Y = a_0 + \sum_{i=1}^{n} a_i x_i + \sum_{i=1}^{n} a_{ii} x_i^2 + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} a_{ij} x_i x_j \qquad (4.1)$$

where Y is the sugar yield; a_0 is the regression constant; a_i is the linear regression coefficient for the *i*th parameter; a_{ii} is the quadratic regression coefficient for the *i*th parameter; a_{ij} is the

interaction coefficient for the *i*th and *j*th parameters; x_i and x_j are the values of the *ith* and *j*th parameters; and n is the number of factors, which in this case is 4. Coefficients with $p > 0.1$ were removed stepwise from the model, beginning with the largest p-value. The model was then optimized for the AFEX™ conditions that gave the highest composite desirability for both glucose and xylose (monomeric + oligomeric) sugar yields following enzymatic hydrolysis. Contour plots were generated that show the effect of pairs of pretreatment parameters on glucose and xylose yields, with the remaining two parameters in each figure held at their optimum levels.

4.2.4.2. Mixture optimization of hydrolysis enzymes

Minitab was also used to create and analyze a mixture optimization experiment to determine the optimal combination of β-glucosidase (Novozyme® 188, Novozymes Corp., Bagsværd, Denmark), Spezyme® CP, Multifect® Xylanase, and Multifect® Pectinase (Genencor Division of Danisco US Inc., New York, USA) for release of sugars from optimally pretreated Alamo and Shawnee switchgrass. For this experiment, an extreme vertices design with a design degree of three was generated, which included the four enzymes and two enzyme loadings (15 and 30 mg total protein per g glucan). The constraints on the relative enzyme proportions in terms of total protein were: Spezyme® CP ≥ 20%, Novozyme® 188 ≤ 50%, Multifect® Xylanase ≤ 80%, and Multifect® Pectinase ≤ 80%. For this design, only vertices (type 1) and edge midpoints (type 2) were replicated, resulting in 84 total design points, including replicates. A

regression model was generated for each sugar yield (monomeric and total glucose, xylose, and glucose + xylose) in Minitab from the hydrolysis yield data:

$$Y = \sum_{i=1}^{n} a_i x_i + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} a_{ij} x_i x_j + \sum_{\substack{i,j,k=1 \\ i \neq j \neq k}}^{n} a_{ijk} x_i x_j x_k + z\left(b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{\substack{i,j=1 \\ i \neq j}}^{n} b_{ij} x_i x_j + \sum_{\substack{i,j,k=1 \\ i \neq j \neq k}}^{n} b_{ijk} x_i x_j x_k \right) \quad (4.2)$$

where Y is the sugar yield; a_i is the linear regression coefficient for the *i*th component; a_{ij} is the quadratic interaction coefficient for the *i*th and *j*th components; a_{ijk} is the cubic interaction coefficient for the *i*th, *j*th, and *k*th components; x_i, x_j, and x_k are the values of the *i*th, *j*th and *k*th parameters; n is the number of components, in this case 4; z is the enzyme loading; b_0 is the enzyme loading coefficient; and b_i, b_{ij}, b_{ijk} are the linear, quadratic, and cubic enzyme loading interaction regression coefficients, respectively. The coefficients in the regression models were selected stepwise, beginning with the four linear enzyme terms and sequentially adding terms with $\alpha < 0.05$ and removing terms with $\alpha > 0.05$. The regression model was then used to predict the optimum mixture composition for each switchgrass variety and generate contour plots showing the effect of enzyme combinations on sugar yields.

4.2.5. AFEX™ pretreatment

AFEX™ pretreatment of the Alamo and Shawnee switchgrass for the pretreatment optimization experiments was conducted in 22 mL reactors as outlined by Bals et al. [150]. Conditions used for these experiments are detailed earlier in the design of experiments section and listed in the supplemental information (**Table C.1**). For the enzyme mixture optimization and hydrolysis rate determination experiments, AFEX™ was performed on Alamo and Shawnee switchgrass in a 300 mL reactor as detailed by Kim et al. [175]. The pretreatment conditions used for each switchgrass variety were chosen based on the results of the pretreatment response surface optimization: Alamo: 1.5 g NH_3:g DM, 2.0 g H_2O:g DM, 140°C, 20 min; Shawnee: 1.5 g NH_3:g DM, 2.0 g H_2O:g DM, 150°C, 30 min.

4.2.6. Enzymatic hydrolysis

Samples for pretreatment optimization and enzyme mixture optimization were hydrolyzed in 20 mL screw-cap vials at 1% glucan loading and a total volume of 15 mL. For the rate determination experiments, samples were hydrolyzed in 250 mL Erlenmeyer flasks at 1% glucan loading and a total volume of 100 mL. All samples were adjusted to a pH of 4.8 using 1 M citrate buffer solution. To prevent fungal and bacterial contamination during enzymatic hydrolysis, cycloheximide and tetracycline were loaded at a final concentration of 30 μg/mL and 40 μg/mL, respectively.

For the pretreatment optimization experiments, the standard enzyme loading was used: 15 filter paper units (FPU) Spezyme® CP and 30 cellobiase units (CBU) β-glucosidase per g glucan in the untreated biomass, or 27.33 mg total protein per g glucan. For the enzyme mixture optimization, all four enzymes were loaded in combinations as determined by the experimental design at two different protein loadings (15 mg protein per g glucan and 30 mg protein per g glucan). For the rate determination experiments, two different enzyme mixtures were compared: 15 FPU Spezyme® CP and 30 CBU Novozyme® 188 per g glucan in the untreated substrate, and the optimal enzyme loading determined from enzyme mixture optimization loaded at 27 mg protein per g glucan. The protein content and enzyme activity for each of the enzymes are as follows, where known: Spezyme® CP (82 mg protein/mL, 50 FPU/mL), Novozyme® 188 (67 mg protein/mL, 735 CBU/mL), Multifect® Xylanase (27 mg protein/mL) and Multifect® Pectinase (52 mg protein/mL). Protein content was determined from total N analysis using the Dumas method for combustion of nitrogen to NOx [148] following trichloroacetic acid (TCA) precipitation to remove non-protein nitrogen [151].

Enzymatic hydrolysis for all experiments was conducted in a shaking incubator at 50°C and 200 rpm. For the optimization experiments, samples were taken at 72 hours of enzymatic hydrolysis for both monomeric and oligomeric sugar analysis, as detailed below. For the rate determination experiments, samples were taken at 1 h, 24 h, and 168 h for monomeric sugar analysis. Samples taken for monomeric sugar analysis were heated at 100°C for 15-20 min, cooled in the freezer, and then centrifuged at 15,000g for 5 min. The supernatant was filtered

into HPLC shell vials using a 25 mm, 0.2 μm polyethersulfone syringe filter (Whatman Inc. Florham Park, NJ) then stored at -20°C until further sugar analysis.

4.2.7. Soluble total and oligomeric sugar analysis

Oligomeric sugar analysis of the pre-wash liquid and hydrolysate was conducted using a scaled-down version of the standard NREL method for oligomeric sugar determination of liquid streams [152]. The modified method was identical except that it was scaled down to use 2 mL of sample, which were run in duplicate in 10 mL screw-cap culture tubes. Instead of being autoclaved, the tubes were incubated in a 121°C bench-top hot plate for one hour, cooled on ice, and the liquid was filtered into HPLC vials. The oligomeric sugar concentration was determined by subtracting the monomeric sugar concentration of the non-hydrolyzed samples from the total sugar concentration of the acid hydrolyzed samples.

4.2.8. HPLC analysis

Sugar contents of all composition analysis, wash liquid, and hydrolysate samples were determined using a Bio-Rad (Hercules, California, USA) Aminex HPX-87H column equipped with appropriate guard columns. Degassed 5 mM H_2SO_4 was used as the mobile phase and the column temperature was held at 60°C. Glucose, xylose (plus galactose and mannose), and arabinose concentrations were determined for each liquid stream. Because the xylose, galactose, and mannose peaks cannot be separated using the HPX-87H column [185], any results reported for xylose also includes mannose and galactose. For grasses the galactose and

mannose contents tend to be very low – in sum less than 1.5% of the total biomass [186]. Sugar yields were calculated as reported previously [30], taking into account soluble glucose and sucrose present in the untreated switchgrass.

4.3. Results and discussion

4.3.1. Switchgrass characteristics

The Alamo and Shawnee switchgrass used for these experiments did not exhibit large differences in composition (**Table 4.1**). Shawnee had a statistically higher glucan content and higher lignin content compared to the Alamo, and a statistically lower total extractives content (data not shown), which may indicate that it is a slightly more mature sample [158]. However xylan, ash, and protein were all statistically identical, so this conclusion is not certain. Following washing of the switchgrass to remove potentially interfering soluble sugars, the overall cell wall content increased as expected, and the composition data corresponded to expected values based on mass balance calculations around the washing step (data not shown). The relative differences in the cell wall content between Alamo and Shawnee did not change to any large extent.

A previous study on AFEX^{TM} pretreatment of switchgrass cultivars used lowland switchgrass (Alamo) that was harvested in Alabama, and upland switchgrass (Cave-in-Rock) that was harvested in Michigan [150]. This difference in latitude made it difficult to accurately determine whether the differences in the results were due to the difference in cytotype, harvest timing, or location. For our experiments, Alamo and Shawnee were both planted and harvested at the same time in the same year and within 2° latitude, which reduces the

environmental impacts on digestibility compared to the previous work [150]. Other research has shown that when grown at the same latitude in the south central U.S., upland varieties tended to have lower cellulose content and consistently produced lower biomass yields compared to the lowland varieties [174]. The 2° higher latitude of the Shawnee switchgrass could be one reason for Shawnee's higher glucan content compared to the Alamo.

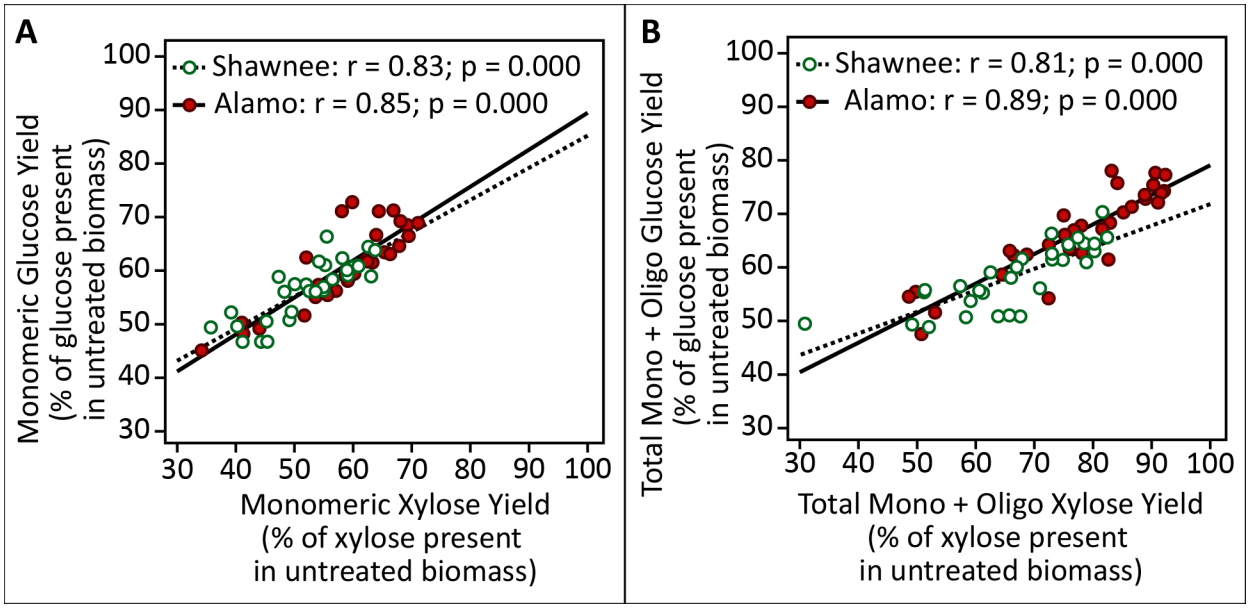

Figure 4.1: Relationship between enzymatic hydrolysis glucose and xylose yields for Alamo and Shawnee switchgrass. (A) Monomeric sugar yields. (B) Total monomeric + oligomeric sugar yields. Yields are calculated as the total sugar solubilized based on the total sugar theoretically available in the untreated dry biomass. Each data point represents one of 30 (Shawnee) or 32 (Alamo) different pretreatment experiments.

4.3.2. Pretreatment parameter optimization

The monomeric and total monomeric + oligomeric sugars for each of the pretreatment experiments are shown graphically in **Figure 4.1**. Total monomeric + oligomeric glucose and xylose yields from untreated Shawnee were 23.5% and 19.8%, respectively, and from untreated

Alamo were 25.3% and 25.4%, respectively. In all cases, pretreatment increased sugar yields compared to the untreated samples. In general Alamo tended to have higher sugar yields (% sugar released of sugar present in untreated biomass) compared to the Shawnee switchgrass, indicating greater digestibility. In some cases, oligomeric xylose accounted for as much as 30-35% of the total xylose solubilized from the pretreated biomass during enzymatic hydrolysis. For AFEX^{TM} pretreated grasses, often there is a statistically significant linear relationship between glucose yields and xylose yields. As increasing amounts of xylose and xylo-oligomers are released from the biomass, increasing amounts of glucose are also released [150]. For other pretreatments that selectively remove xylan from the plant cell wall, such as dilute acid, glucan conversion in grasses can also be dependent on release of hemicellulose sugars [13].

For the pretreatment optimization, regression models of total monomeric and oligomeric glucose and xylose yields were evaluated in terms of the pretreatment parameters and only coefficients with $\alpha < 0.10$ were included in the final model (**Table 4.2**). This value was chosen because using a lower value such as 0.05 resulted in the removal of too many terms from the regression models and extremely poor representation of the data. Of the terms in the models, moisture content for Alamo switchgrass, the interaction between ammonia and residence time for Shawnee, and the residence time quadratic term for Shawnee were less significant with $\alpha > 0.05$. The models adequately describe the data with adjusted R^2-values of around 85% for both regressions. Unlike the standard R^2 value, adjusted R^2-values take into account the number of terms in the model and may decrease compared to the standard R^2-value if there are more terms than necessary to describe the data. The deviation of values

predicted by the model from the actual yield values for each of the pretreatment experiments is shown in **Figure 4.2**. The histograms show the number of experiments that deviated by a certain percent yield. Negative values indicate that the model underpredicted the sugar yields and values greater than one indicate that the model overpredicted the sugar yields. In general the Alamo model tended to underpredict, while the Shawnee model tended to overpredict yields, however both curves were fairly normal in distribution with a couple of outliers. For

Table 4.2: Response surface optimization of pretreatment parameters in terms of total monomeric and oligomeric glucose and xylose release following enzymatic hydrolysis.

A = ammonia loading (g NH_3:g DM), B = water loading (g H_2O:g DM), C = temperature (°C), D = residence time (min).

	Alamo				Shawnee			
Term	**Coef.[a]**	**SE[b]**	**T**	**P**	**Coef.[a]**	**SE[b]**	**T**	**P**
Constant	-88.7919	19.517	-4.550	0.000	-54.1452	12.787	-4.234	0.000
A	26.5272	6.081	4.362	0.000	18.5639	4.549	4.081	0.001
B	-13.6733	7.568	-1.807	0.085	3.3716	1.053	3.201	0.004
C	1.6561	0.211	7.865	0.000	1.1577	0.160	7.230	0.000
D	3.6793	0.578	6.363	0.000	1.2562	0.321	3.919	0.001
A^2	-4.4631	1.540	-2.897	0.009	-3.2350	1.139	-2.842	0.010
C^2	-0.0057	0.001	-7.443	0.000	-0.0039	0.001	-6.729	0.000
D^2	-0.0270	0.010	-2.699	0.013	-0.0130	0.007	-1.751	0.094
AD	-0.4064	0.180	-2.253	0.035	-0.2468	0.140	-1.763	0.092
BC	0.1239	0.054	2.290	0.032	-	-	-	-
CD	-0.0132	0.004	-3.753	0.001	-	-	-	-
R^2	89.22%				90.29%			
R^2 (adj)[c]	84.08%				86.59%			
R^2 (pred)[d]	68.97%				78.65%			

[a]Coef. = regression model coefficient; [b]SE = standard error; [c]R^2 (adj) = adjusted R^2-value; [d]R^2 (pred) = predictive R^2-value

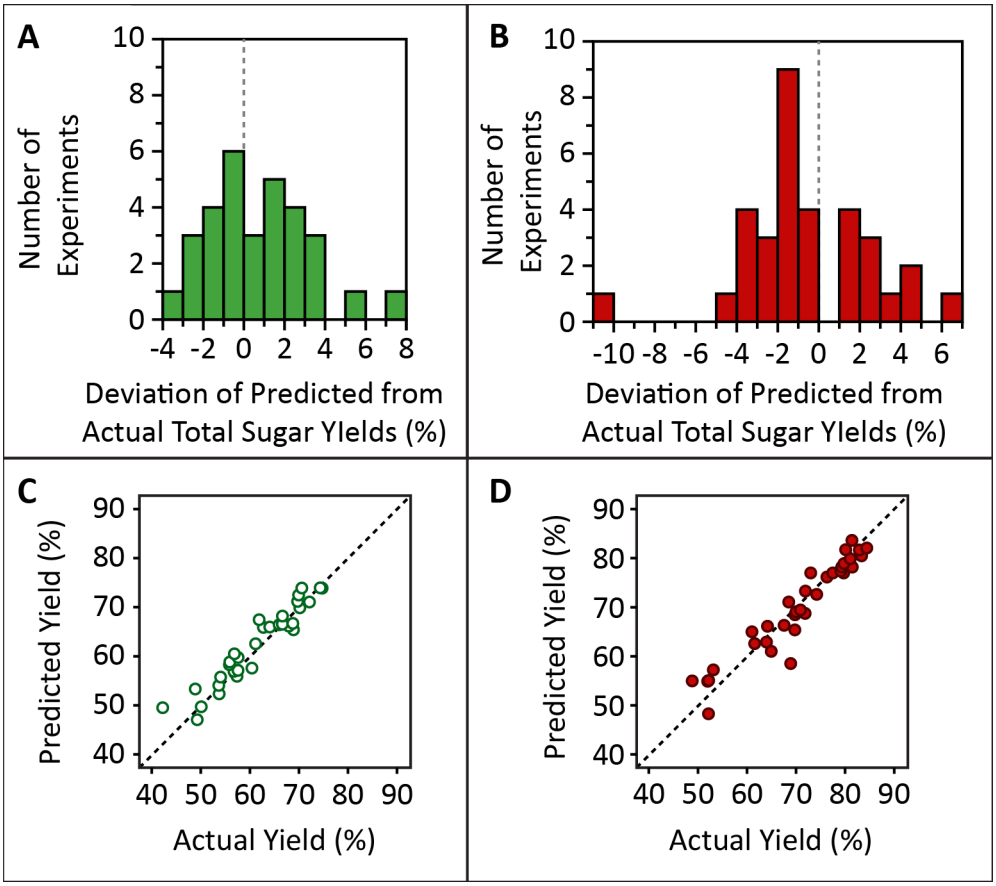

Figure 4.2: Histograms and scatter plots showing deviation of predicted sugar yields from actual sugar yields for the pretreatment regression models. (A) Shawnee histogram. (B) Alamo histogram. (C) Shawnee scatter plot. (D) Alamo scatter plot.

both switchgrass samples, over 50% of the predicted values were within ±2% of the actual value and 94% of the predicted yields were within ±4% and ±5% of the actual sugar yields from Shawnee and Alamo, respectively.

The regression models were used to determine the optimum pretreatment conditions for both Shawnee and Alamo switchgrass using the Minitab response surface optimizer for maximizing total monomeric + oligomeric yields from both glucose and xylose. The optimum condition for Shawnee was initially determined to be 1.75 g NH_3:g DM, 2.0 g H_2O:g DM, 146°C

and 30 min residence time and for Alamo was 2.49 g NH_3:g DM, 2.0 g H_2O:g DM, 152°C and 12.3 min residence time. However, due to constraints on the 300 mL reactor operation, the optimal conditions were constrained to 1.5 g NH_3:g DM and the other parameters were adjusted to obtain yields as close as possible to the original optimum (**Table 4.3**). The optimal pretreatment conditions for Alamo and Shawnee were fairly similar, with Alamo's optimum at a slightly lower temperature and shorter residence time, while simultaneously obtaining higher sugar yields. Compared to previously determined optima for AFEX™ pretreatment of switchgrass, the values determined here seem closest to those proposed by Bals et al. [150] for Alamo switchgrass. The optimal values found by Alizadeh et al. [187] were quite different from our findings, and while the information on the harvest date and variety for those experiments were not provided, the optimum pretreatment conditions were similar to the mild optimum pretreatment conditions for early harvest Cave-in-Rock [150]. This may indicate that the initial work on AFEX™-treated switchgrass also used a fairly immature sample. Dacotah switchgrass, which was used for the CAFI III project [30, 175, 188], was harvested in South Dakota in May following over-wintering on the field. This sample had consistently lower glucose yields compared to Alamo and Shawnee for all pretreatment methods tested [175].

Contour plots of monomeric glucose and xylose yields (**Figure C.1 and Figure C.2**) were nearly identical to the contour plots of total monomeric + oligomeric glucose and xylose yields (**Figure 4.3 and Figure 4.4**), and have been included in the online supplemental information for reference. The contour plots show that the optimum pretreatment conditions for total xylose release corresponded roughly with the optimum values for total glucose release. The optimum

Table 4.3: Comparison of literature on optimal AFEX™ pretreatment conditions and sugar yields from switchgrass. Predicted yields are sugar yields as predicted by the response surface regression model. Values in parenthesis represent monomeric + oligomeric sugars. Pred. = predicted

	Switchgrass				AFEX™ Pretreatment Conditions				Sugar Yields			
									Glucose		Xylose	
Ref.	Variety	Cytotype	Location	Harvest Timing	NH_3 (g: g DM)	H_2O (g: g DM)	Temp (°C)	Time (min)	Pred. (%)	Actual (%)	Pred. (%)	Actual (%)
This Study	Alamo	Lowland	Oklahoma	Dec.	1.5	2.0	140	20	68.0 (75.4)	69.3 (75.3)	68.7 (93.3)	67.7 (97.9)
	Shawnee	Upland	Oklahoma	Dec.	1.5	2.0	150	30	63.9 (67.3)	62.0 (66.7)	63.0 (82.1)	59.5 (79.1)
[188]	Dacotah	Upland	South Dakota	May	1.5	2.0	150	30	-	52.7 (54.5)	-	58.0 (79.9)
[150]	Cave-in-Rock	Upland	Michigan	Jul.	0.9	0.4	80	20	-	75.2	-	44.5
				Oct.	2.0	0.4	130	30	-	44.6	-	35.5
	Alamo	Lowland	Alabama	Jul.	1.6	2.0	160	30	-	51.8	-	34.4
				Oct.	2.0	2.0	150	25	-	57.3	-	38.3
[187]	-	-	-	-	1.0	0.8	100	5[d]	-	~80	-	~65

[a] Same enzymatic hydrolysis conditions as this paper.

[b] Same enzymatic hydrolysis conditions except biomass loaded at 3% solids and 3.2 FPU Accelerase per g DM (~10 FPU per g glucan).

[c] Enzymatic hydrolysis using 15 FPU Spezyme CP per g glucan and 40 CBU Sigma β-glucosidase per g glucan.

[d] Included a 30 minute heat-up.

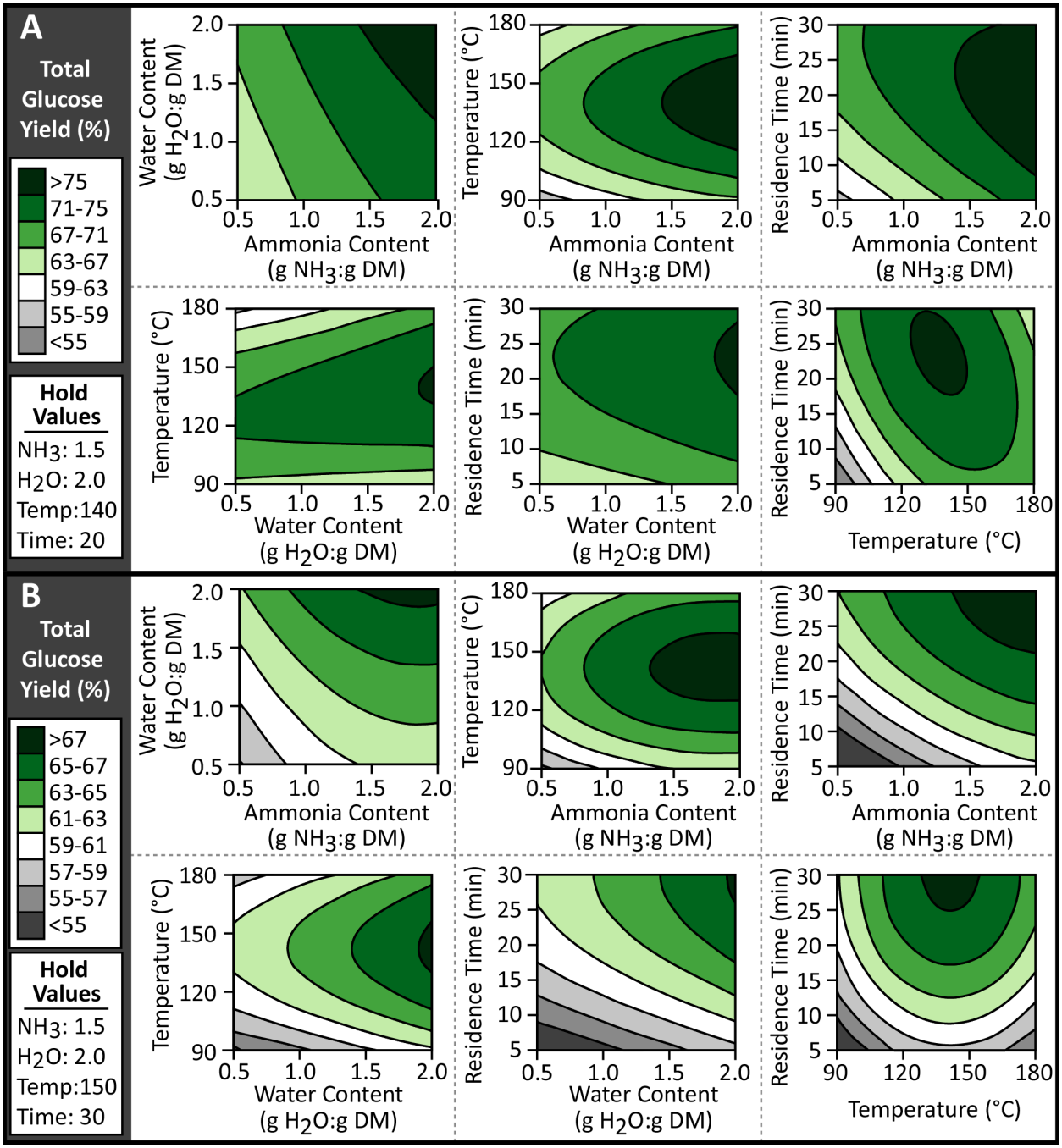

Figure 4.3: Contour plots showing the interactive effect of pairs of AFEX™ pretreatment parameters on monomeric + oligomeric glucose yields from (A) Alamo and (B) Shawnee switchgrass. The two pretreatment parameters not shown in each sub-figure were held at the optimal level. Hydrolysis was conducted at 50°C, 200 rpm, and 1% glucan loading using 30 FPU Spezyme® CP and 15 CBU Novozyme® 188 per g glucan, with 72 h sampling.

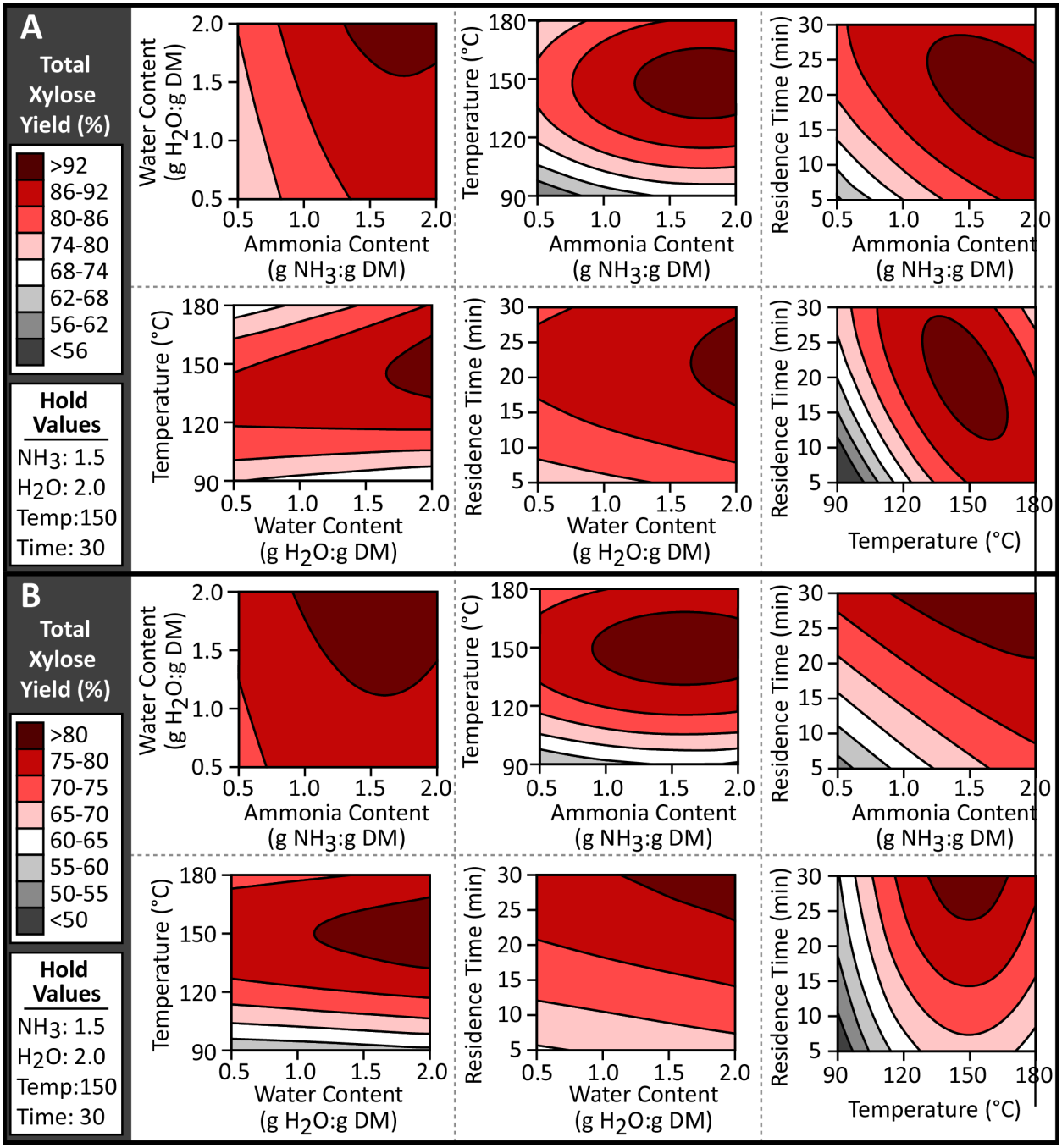

Figure 4.4: Contour plots showing the interactive effect of pairs of AFEX™ pretreatment parameters on monomeric + oligomeric xylose yields from (A) Alamo and (B) Shawnee switchgrass. The two pretreatment parameters not shown in each sub-figure were held at the optimal level. Hydrolysis was conducted at 50°C, 200 rpm, and 1% glucan loading using 30 FPU Spezyme® CP and 15 CBU Novozyme® 188 per g glucan, with 72 h sampling.

ammonia loading tended to be slightly lower for xylose release compared to glucose release, which in most cases was outside the charted range. Of the pretreatment parameters, water loading had the smallest impact on both Alamo glucose and xylose yields and Shawnee xylose yields, although all pretreatments showed a strong interaction between water loading and temperature, with the highest yields at moderate temperatures and high water loading. A moderate residence time (~20-25 min) resulted in higher glucose and xylose release from Alamo switchgrass; however, the optimum for Shawnee may actually be higher than 30 min, which was the limit of the parameters tested. However, it may not be economically desirable to operate for a longer residence time, as this increases the capital cost associated with the pretreatment reactor for the same amount of throughput and can significantly increase the minimum ethanol selling price (MESP) [189].

While it is apparent that solubilization of hemicellulose is important for increasing glucose yields from AFEX^{TM} treated switchgrass, even when operated at optimal conditions, the pretreatment was still insufficient to solubilize 100% of the hemicellulose or obtain greater than 75% glucose yields from both varieties of switchgrass. While hemicellulose and lignin are known to be extracted from the biomass and redeposited on the cell wall surface during AFEX^{TM} pretreatment [188, 190], much of the lignin remains insoluble even following enzymatic hydrolysis [30]. It seems likely that this lignin will still have a portion of the hemicellulose associated with it following pretreatment, which would be rendered inaccessible to enzymes. This lignin may also impede access to the cellulose. In addition to lignin content, reduction in cellulose degree of polymerization is also important for improving cellulose

conversion. As AFEX™ is known to not influence this parameter significantly [191], the lack of cellulose reducing ends may also hinder enzymatic hydrolysis. One possibility that is currently being explored is to pretreat biomass using liquid ammonia with very little water, which has been shown to generate the cellulose III_I crystalline allomorph [192]. This crystalline structure is more readily converted by enzymes compared to native cellulose I [193], and using this method it may be possible to further increase glucan conversion from switchgrass.

4.3.3. Commercial enzyme mixture optimization

Sugar yield data from the mixture optimization experiments was fitted to a regression model for both Alamo (**Table 4.4**) and Shawnee (**Table 4.5**) switchgrass. The only terms initially included in the model were the four base commercial enzyme mixtures: Spezyme® CP (S), Novozyme® 188 (B), Multifect® Xylanase (X) and Multifect® Pectinase (P). New terms were sequentially added ($\alpha < 0.05$) or removed from the model ($\alpha > 0.05$). The models adequately describe the data with adjusted R^2-values of around 95% for all sugar yields except monomeric + oligomeric xylose for which adjusted R^2-values were around 80%. The low R^2-value for the monomeric + oligomeric xylose yields is likely due to the smaller spread in the total xylose sugar yields between all the experiments (~12-14%) compared to the other sugars (~30-35%).

In addition to the base enzymes, the amount of enzyme was also significant for all sugar yields for both switchgrass varieties. Other terms which were in all eight models include the interactions between: Spezyme x Xylanase, Spezyme x Pectinase, and Spezyme x Xylanase x Pectinase. β-glucosidase had a significant impact on sugar yields, however in all cases the

highest sugar yields were obtained when this enzyme was not included in the enzyme mixture (**Table 4.6**). Both Multifect® Pectinase, and to a lesser extent, Spezyme® CP contain

Table 4.4: Mixture regression of enzymes and total protein loading in terms of sugar release following enzymatic hydrolysis of Alamo switchgrass. S = Spezyme® CP; B = Novozyme® 188 (β-glucosidase); X = Multifect® Xylanase; P = Multifect® Pectinase; Amt = Enzyme loading in terms of total protein.

	Monomeric Glucose		Monomeric Xylose		Monomeric + Oligomeric Glucose		Monomeric + Oligomeric Xylose	
Term	**Coef.[a]**	**P**	**Coef.[a]**	**P**	**Coef.[a]**	**P**	**Coef.[a]**	**P**
S	57.57	*	50.14	*	63.79	*	85.26	*
B	7.85	*	26.42	*	23.39	*	81.43	*
X	43.12	*	55.43	*	44.11	*	83.77	*
P	40.19	*	61.92	*	41.26	*	80.52	*
SB	101.69	0.000	49.96	0.000	60.70	0.000	-	-
SX	39.55	0.000	27.77	0.000	42.26	0.000	14.15	0.001
SP	65.63	0.000	55.62	0.000	50.29	0.000	11.80	0.008
BX	112.7	0.000	78.54	0.000	81.56	0.000	-	-
BP	-	-	36.02	0.032	-	-	-	-
SBP	129.66	0.001	109.33	0.030	100.50	0.007	-	-
SXP	268.50	0.000	201.82	0.000	247.00	0.000	44.48	0.009
BXP	-	-	-	-	-	-	97.16	0.008
Amt	13.93	0.000	7.28	0.000	13.92	0.000	4.02	0.000
SB*Amt	-	-	16.37	0.001	-	-	-	-
SX*Amt	18.44	0.006	-	-	-	-	-	-
BX*Amt	-	-	-	-	34.17	0.005	-	-
SBP*Amt	-	-	-77.19	0.010	-	-	-	-
R^2	95.73%		97.19%		95.80%		81.61%	
R^2 (adj)[b]	95.08%		96.67%		95.16%		79.59%	
R^2 (pred)[c]	93.95%		96.10%		94.05%		76.25%	

[a]Coef. = regression model coefficient; [b]R^2 (adj) = adjusted R^2-value; [c]R^2 (pred) = predictive R^2-value

Table 4.5: Mixture regression of enzymes and total protein loading in terms of sugar release following enzymatic hydrolysis of Shawnee switchgrass. S = Spezyme® CP; B = Novozyme® 188 (β-glucosidase); X = Multifect® Xylanase; P = Multifect® Pectinase; Amt = Enzyme loading in terms of total protein.

	Monomeric Glucose		Monomeric Xylose		Monomeric + Oligomeric Glucose		Monomeric + Oligomeric Xylose	
Term	**Coef.[a]**	**P**	**Coef.[a]**	**P**	**Coef.[a]**	**P**	**Coef.[a]**	**P**
S	52.56	*	50.94	*	59.68	*	84.73	*
B	8.36	*	38.46	*	52.49	*	81.37	*
X	40.36	*	60.37	*	43.24	*	83.12	*
P	32.41	*	60.98	*	34.33	*	77.85	*
SB	96.12	0.000	31.22	0.004	-	-	-	-
SX	31.29	0.000	13.52	0.010	26.30	0.000	8.00	0.038
SP	67.68	0.000	50.15	0.000	49.97	0.000	16.74	0.000
BX	94.58	0.000	-	-	-	-	-	-
BP	41.20	0.000	-	-	-	-	-	-
SBX	-	-	190.75	0.000	125.95	0.000	40.34	0.018
SBP	-	-	144.95	0.000	-	-	-	-
SXP	202.77	0.000	175.80	0.000	185.18	0.000	95.82	0.000
BXP	-	-	129.67	0.008	-	-	79.06	0.020
Amt	12.58	0.000	7.50	0.000	11.68	0.000	3.74	0.000
P*Amt	-	-	-3.03	0.032	-	-	-	-
BX*Amt	-	-	-	-	21.93	0.021	-	-
R^2	96.34%		95.34%		96.24%		84.52%	
R^2 (adj)[b]	95.84%		94.57%		95.79%		82.59%	
R^2 (pred)[c]	95.41%		93.82%		95.32%		79.87%	

[a]Coef. = regression model coefficient; [b]R^2 (adj) = adjusted R^2-value; [c]R^2 (pred) = predictive R^2-value

β-glucosidase activity [194]. Between these two enzymes, there was sufficient activity to eliminate the need for Novozyme® 188 in the enzyme mixture. In fact, the optimal mixture, when loaded at the same total protein loading, had higher estimated β-glucosidase activity

compared to the standard enzyme loading of 15 FPU Spezyme® CP and 30 CBU Novozyme® 188 per g glucan (**Table 4.6**). Other research on AFEX™ and dilute acid pretreatment has found that when Multifect® Xylanase and Multifect® Pectinase are included in the enzyme mixture, Novozyme® 188 is not necessary for optimal sugar yields [150, 181]. In terms of the regression models, this indicates that those terms that include β-glucosidase can be neglected as insignificant and the model can be thought of as a ternary mixture of the other three enzymes. Once the β-glucosidase terms are neglected from the model, the amount of enzyme has little significant interaction with the specific enzymes used, only with Pectinase for Shawnee monomeric xylose yields and with Spezyme x Xylanase for Alamo monomeric glucose yields. Even so, when contour plots of the two enzyme loadings (15 mg protein per g glucan and 30 mg protein per g glucan) were compared; there was almost no difference in the shape of the curves (data not shown). At the two enzyme loadings, glucose release is impacted more significantly with the increase in enzymes compared to the xylose release. This may indicate either that the xylo-oligomers and hemicellulose are competing substrates for the cellulase enzymes, or that the small amount of hemicellulases is limiting the capacity to break down hemicelluloses that block enzyme access to the cellulose.

When comparing the models between the two switchgrass varieties, most of the coefficients for the same sugar yield are very similar. This indicates that the response curves of the two varieties should also be very similar, which is indeed the case (**Figure 4.5**). (The total glucose contour plot is almost identical to the monomeric glucose contour plot and so was not included.) As can be seen, the monomeric glucose and xylose yield contour plots are nearly

Table 4.6: Base case and optimized commercial enzyme mixtures for monomeric and monomeric + oligomeric (total) sugar yields (both glucose + xylose) from Alamo and Shawnee switchgrass. Enzyme proportions are expressed as a percentage of total protein in the enzyme mixture. Sugar yields are presented as % of sugars theoretically available in untreated, dry biomass. Sugar yields in parentheses represent total monomeric + oligomeric sugars, and those not in parentheses represent monomeric sugar yields. Enzyme activities for each enzyme mixture are estimated based on activities per mL reported by Dien et al. [194] for each of the commercial enzyme preparations.

	Alamo			Shawnee		
	Base Case	Maximize		Base Case	Maximize	
		Monomers	Total		Monomers	Total
Enzyme Mixture (% of total protein)						
Spezyme CP	90%	46%	54%	90%	47%	57%
β-Glucosidase	10%	-	-	10%	-	-
Xylanase	-	20%	23%	-	20%	20%
Pectinase	-	34%	23%	-	33%	23%
Predicted Sugar Yields (g/g sugar theoretically present in untreated biomass)						
15 mg/g glucan						
Glucose	54.8 (58.2)	63.3 (64.7)	62.9 (65.7)	50.5 (53.1)	56.9 (58.2)	57.0 (59.1)
Xylose	47.9 (82.9)	69.1 (85.9)	66.5 (86.4)	48.8 (82.5)	67.4 (86.6)	64.5 (86.6)
30 mg/g glucan						
Glucose	68.7 (72.2)	79.0 (78.6)	79.1 (79.6)	63.1 (64.8)	69.5 (69.8)	69.5 (70.8)
Xylose	56.6 (86.9)	76.4 (89.7)	73.8 (90.4)	56.3 (86.3)	73.9 (90.3)	71.3 (90.3)
Estimated Activity of the Mixture Shown Above (activity per 10 mg protein in mixture)						
Cellulase (FPU[a])	6.5	3.6	4.1	6.5	3.7	4.3
β-Glucosidase	24	32	27	24	32	27
Xylanase (OSX[b])	290	2100	2400	290	2100	2100
α-Arabinofuranosidase	3	120	85	3	120	85
β-Xylosidase	1	14	11	1	14	10
α-Galactosidase	1.8	2.3	1.6	1.8	2.2	1.6
Feruloyl esterase	0.0	0.6	0.4	0.0	0.6	0.4

[a]FPU = Filter paper units; [b]OSX = Oat spelt xylan.

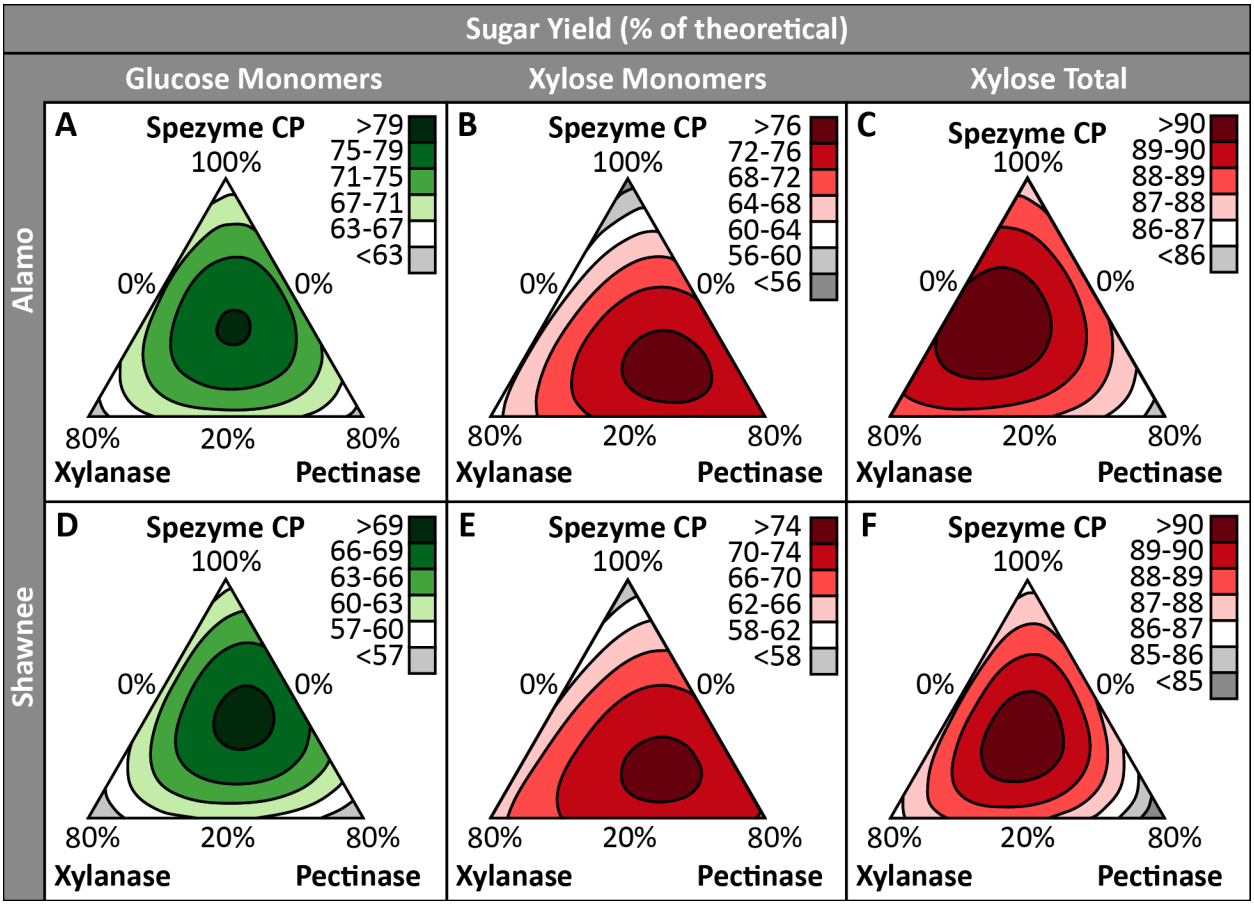

Figure 4.5: Ternary plots based on the enzyme mixture regression model showing the interactive effect of Spezyme® CP, Multifect® Xylanase and Multifect® Pectinase on sugar yields from AFEX™ pretreated Alamo (A-C) and Shawnee (D-F) switchgrass. (A, D) monomeric glucose; (B, E) monomeric xylose; (C, F) monomeric + oligomeric xylose. Percentages of each enzyme are with respect to total protein loading, which was held at 30 mg per g glucan for these figures. β-glucosidase was held at 0% in all plots. Enzyme loadings were constrained to Spezyme CP > 20%, Multifect Xylanase < 80% and Multifect Pectinase < 80% of total enzyme protein added to hydrolysis.

identical between the two varieties and the optimal enzyme mixtures are also almost identical (**Table 4.6**). There is a more obvious difference in the shape of the total xylose contour plots for the two varieties, with Alamo shifted toward a higher xylanase content compared to the Shawnee. However, the range in total xylose yields for these figures are very small (~4-5% for

the entire plot), and any number of points within the center of these diagrams should give almost identical yields. Even the base case of Spezyme® CP and Novozyme® 188 only has about 3-4% less total monomeric + oligomeric xylose release compared to the optimized enzyme mixtures (**Table 4.6**). What this indicates is that pretreatment has the greatest impact on effective removal and solubilization of hemicellulose from the cell wall, and most of the impact by the xylanase and pectinase is on conversion of glucan and soluble hemicellulose oligomers to monomers. Accessory enzymes are known to be necessary to increase xylose yields from AFEX™ treated grasses [195]. Other work on AFEX™-treated switchgrass also found that the optimal enzyme mixture contained both xylanase and pectinase [150]. Xylanase is necessary for its high xylanase content that breaks apart the xylan backbone, while pectinase is necessary for its high accessory enzyme activity (including β-glucosidase and β-xylosidase) [194]. If it is possible to directly use the oligomeric sugars, perhaps by employing a microorganism that can consume or ferment oligosaccharides, then the proportion of pectinase in the optimized mixture could be decreased.

While the optimized enzyme mixtures were theoretically expected to increase monomeric glucose release compared to the base case (**Table 4.6**), this was not observed experimentally (**Figure 4.6**), with only a slight increase in glucose yields due to mixture optimization. Alamo had higher glucose yields compared to Shawnee, although in terms of total sugar release per kg biomass, the yields were almost identical. The primary benefit of the optimized enzyme mixture is in increasing the initial rate of xylose release through more rapid conversion of the soluble oligomers. For both switchgrass varieties the initial rate of xylose

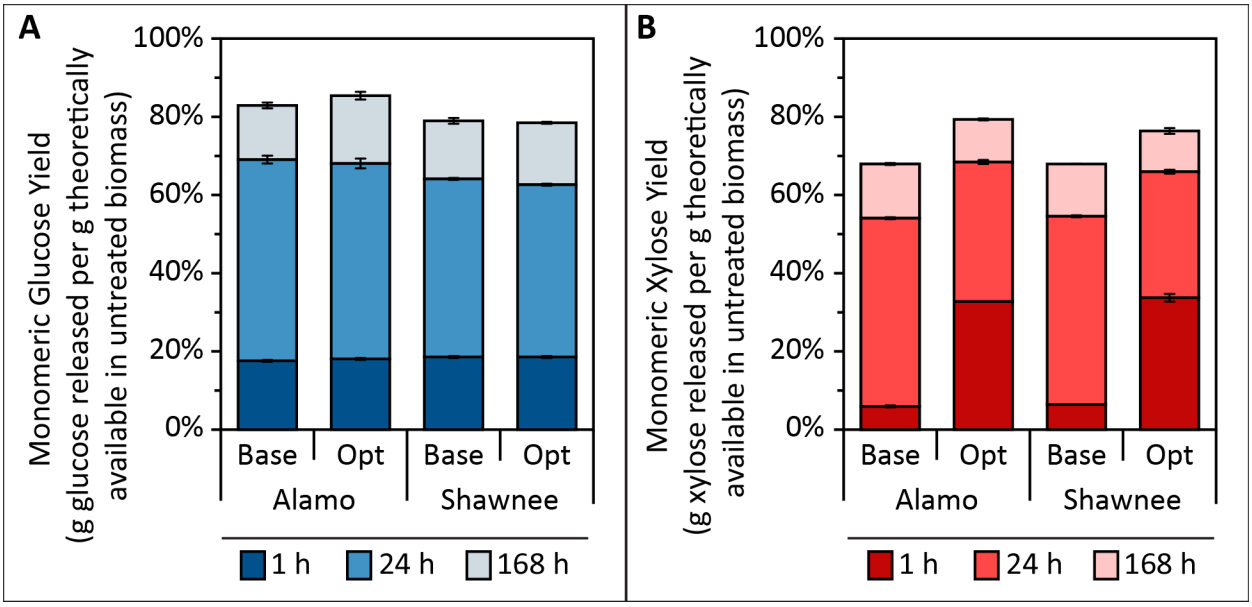

Figure 4.6: Monomeric glucose (A) and xylose (B) release from optimally pretreated Alamo and Shawnee switchgrass hydrolyzed with base enzyme loading (15 FPU Spezyme® CP and 30 CBU Novozyme® 188 per g glucan) compared to with our optimized enzyme mixture for each variety containing Spezyme® CP, Multifect® Xylanase and Multifect® Pectinase. Enzyme loading was 27 mg protein per g glucan and samples were taken at 1 h, 24 h and 168 h.

hydrolysis at 1 h increases significantly for the optimized mixture compared to the base case. This again indicates that much of what is hindering xylose yields is lack of appropriate xylanases and accessory enzymes in the standard enzyme cocktail. Interestingly, the optimum enzyme cocktail is able to achieve identical glucose yields compared to the base case, although the base case mixture contains nearly double the cellulase activity (**Table 4.6**). As stated earlier, this may indicate that the xylanase and pectinase enzymes either improve access to the cellulose or remove a competing substrate.

The cost of enzymes, either purchased or produced in-house, is one of the major contributors to the total costs for the production of cellulosic ethanol. A recent study by the National Renewable Energy Laboratory (NREL, Golden, Colorado) found that the cost of

enzymes produced in-house accounted for almost 16% of the minimum ethanol selling price (MESP), nearly half of the cost of the feedstock [196]. This increased to 20% of the MESP if the enzymes were purchased from external sources. One way to reduce the costs of producing cellulosic ethanol is to make improvements to enzyme efficiency that allow for reductions in either the enzyme loading required to maintain a set level of conversion (leading to reduced enzyme costs), and/or the residence time required for a set level of conversion (leading to reduced capital and operating costs). For our work, the optimized mixture gave higher xylose yields but lower glucose yields at the lower enzyme loading (15 mg/g glucan) compared to the base case at the higher enzyme loading (30 mg/g glucan) (**Table 4.6**). By optimizing the enzyme mixture, it was not possible to maintain glucan-to-glucose conversion while reducing the total protein loading by 50%, a finding that has been observed in other studies [181]. However, xylan-to-xylose conversions were increased and as a result, the total monomeric sugar conversion was slightly higher for the optimized enzyme loading at 15 mg/g glucan compared to the base case at 30 mg/g glucan for both switchgrass varieties.

One final point to note is that while this lab-scale analysis provides a baseline for optimum pretreatment conditions and enzymatic hydrolysis enzyme combinations, it is entirely possible that the optimum parameters and combinations would change due to reactor scale-up and increased enzymatic hydrolysis solids loading. Further work would need to be conducted to determine these new optima, however the results presented here provide a starting point for further analysis.

4.4. Conclusions

When grown in similar locations and harvested at the same time of the year, there was little difference in optimal pretreatment conditions and optimal enzyme loadings required for conversion of Alamo (lowland) and Shawnee (upland) switchgrass. Additionally, the total sugar yields on a mass basis were almost identical for both varieties. Inclusion of hemicellulases in the enzyme mixture primarily functioned to increase monomeric xylose release and allowed for a 50% reduction in enzyme loading while maintaining total sugar yields. The biorefinery should be able to effectively process both switchgrass varieties using the same pretreatment conditions and obtain relatively high yields.

CHAPTER 5 :
COMPONENT SCALE: OPTIMIZING HARVEST OF CORN STOVER FRACTIONS BASED ON OVERALL SUGAR YIELDS FOLLOWING AFEX™ PRETREATMENT AND ENZYMATIC HYDROLYSIS

5.1. Introduction

Corn stover, the aboveground, vegetative portion of maize (*Zea mays* L.), makes up roughly 80% of all agricultural residues produced in the USA [116]. Data on annual corn stover production in the USA are not readily available; so various sources have independently estimated that anywhere from 200 to 250 million dry tons of corn stover are produced per year [114, 116, 197, 198]. Sustainably harvested corn stover could be used as a feedstock for a variety of applications, including lignocellulosic ethanol production. It has been estimated that 38.4 billion liters of ethanol per year could be produced from North American corn stover, assuming that 40% of the stover is collected [76]. It is widely acknowledged that a percentage of the produced corn stover should be retained on the field following harvest in order to prevent soil erosion and maintain soil organic carbon (SOC) levels. The amount that can be sustainably harvested is highly debated and depends heavily on cropping practices, climate, topography, and soil type [114, 199-201]. Estimates on the amount of corn stover that can be sustainably harvested vary widely because of these factors, anywhere from 20-80% [76, 116, 199].

Lignocellulosic feedstocks, such as corn stover, derive their name from the three primary components of the plant cell wall: cellulose, hemicellulose and lignin. The complex polysaccharides, cellulose and hemicellulose, must be broken down into monomeric form (primarily glucose and xylose) prior to microbial fermentation into ethanol or other valuable products. High sugar yields require a two-step process: generally a chemical and/or physical

pretreatment step followed by enzymatic hydrolysis of the polysaccharides. Previous work has shown that ammonia fiber expansion (AFEX™) is a promising pretreatment that can be used in the process of converting corn stover polysaccharides into ethanol as a liquid fuel source [202-205]. AFEX™ pretreatment uses concentrated ammonia-water mixtures under moderate temperatures (60-180°C) and high pressures (200-1000 psi) to disrupt the cellular structure of the plant material by decrystallizing the cellulose; partially depolymerizing and solubilizing the hemicellulose; and altering the form, location, and structure of lignin [203, 204].

The structure and composition of the plant cell wall depends on a number of factors including: developmental stage at harvest, geographical origin, type of tissue, and other external factors including season of harvest and environmental conditions experienced during growth [206]. Corn stover, like most grasses, experiences considerable compositional changes throughout the yearly growth period, as well as significant variation between the various fractions of the plant (that is, leaf versus stem) [158, 207, 208]. Largely because of these differences in composition, stover fractions have been shown to respond differently to pretreatment and enzymatic hydrolysis, resulting in different sugar yields [209-211]. It is reasonable to assume that differences in composition, due largely to differences in morphology and cell and tissue organization, could cause different stover fractions to have different optimal pretreatment conditions for maximizing sugar yields. For example, wheat straw leaves, when treated with dilute NaOH, required less severe pretreatment conditions to optimize glucan yields than stem internodes and nodes [212]. The same might be true for corn stover pretreated with ammonia (or AFEX™). Maximum sugar yields from individual fractions would

be one criterion for determining which fractions should be left on the field following harvest. Assuming that there are no other constraining factors, it would be most logical to harvest the least recalcitrant biomass and leave the remainder for erosion control and soil organic carbon maintenance [213]. Crofcheck and Montross recommended, based on glucose yields from fractionated corn stover, a roughly 30% corn stover harvest scenario where the selectively harvested corn stover (SHCS) was composed of all of the available cobs and 74% of the leaves and husks, leaving the most recalcitrant stalks on the field [209].

For our experiment, AFEX^{TM} followed by enzymatic hydrolysis was performed on four corn stover fractions (stem, leaf, husk, and cob) from September (early) and November (late) harvests. The objectives of this project were: (1) to determine whether individual stover fractions have different optimal AFEX^{TM} conditions and whether this is different from previously optimized values for homogeneously milled corn stover [203, 204]; (2) to discover which fractions give the highest glucose and xylose yields at optimal pretreatment conditions; and (3) to model optimal harvest scenarios, assuming 30% and 70% collection of total available dry corn stover, based on the maximum monomeric glucose and xylose yields from each fraction.

5.2. Materials and methods

5.2.1. Harvest and milling

Corn stover, from a variety intended for grain production, was manually harvested from the Michigan State University Agronomy Center in East Lansing, Michigan, USA in September

(early harvest) and November (late harvest) of 2006. The early and late stover harvests were hand-sorted into four individual fractions: stems, leaves with leaf sheaths, cobs, and husks. The early husk and early cob fractions were not used due to spoilage of the material prior to use. All other fractions were air-dried, with stems split lengthwise in order to increase the drying rate. Fractions were then milled using a Fitzpatrick JT-6 Homoloid mill (Continental Process Systems, Inc., Westmont, Illinois, USA), with leaf, husk, and cob fractions passing through a 4.763 mm (3/16 in) mesh screen, and stem fractions passing through a 3.175 mm (1/8 in) mesh screen.

5.2.2. Composition analysis

Biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; California, USA). The composition of each corn stover fraction (ash, lignin, glucan and xylan content) was determined using the National Renewable Energy Laboratory (NREL, Colorado, USA) standard protocols for ash analysis, removal of extractives, and structural carbohydrates and lignin [214-216]. The acid insoluble lignin analysis method was modified to use 47 mm, 0.22 µm pore-size, mixed-cellulose ester filter discs (Millipore Corp, Massachusetts, USA) during the filtration step instead of fritted crucibles. Due to problems with burning, these discs, with their filtered lignin residue, could not be dried in the vacuum oven and were therefore dried overnight in a desiccator prior to weighing. Soluble sugars could not be quantified after extraction due to difficulties in resolving distinct peaks using high-performance liquid chromatography (HPLC) and were therefore not included in the composition.

5.2.3. AFEX™ treatment

A small-scale bench top reactor system, consisting of four separate 22 mL stainless steel (No. 316) reaction vessels, was used for the pretreatment process. Prior to its loading, the biomass was adjusted to the appropriate moisture content with deionized water, after which 3.0 g dry weight (dwb) of biomass was added to each reaction vessel. A metal screen was placed over the biomass inside each vessel, to prevent escape of biomass during venting. The loaded reactor units were weighed and attached to the reactor manifold, and any air within the reactor vessels was then removed using a rotary vacuum pump. Liquid anhydrous ammonia was dispensed into the manifold via Swagelok screw valves (Swagelok Co, Ohio, USA) and then added to the reactor vessels. The reactors were weighed in order to determine the amount of ammonia added and they were then vented slightly to reach the appropriate ammonia loading. A heating mantle was used to raise the reactors to the desired temperature and maintain it for the set residence time. On completion of the residence time, the reactor pressure was released and the reactor was simultaneously cooled. The pretreated biomass was removed from the vessel and left in the fume hood overnight to allow the residual ammonia to evaporate.

5.2.4. Enzymatic hydrolysis

NREL protocol (LAP 009) [152] was followed for the enzymatic hydrolysis of pretreated and untreated (control) samples. All samples were hydrolyzed in 20mL screw-cap vials at 1% glucan loading and a total volume of 15mL. Samples were adjusted to a pH of 4.8 by 1M citrate buffer solution. Spezyme® CP (Genencor Division of Danisco US, Inc., New York, USA) cellulase

Table 5.1: Enzymatic hydrolysis xylanase loading in terms of xylan content of each fraction.

Corn stover fraction		Xylanase loading mg xylanase g^{-1} xylan	Xylanase activity OSX* g^{-1} xylan
Early	**Leaves**	4.78	2891
	Stem	5.73	3456
Late	**Leaves**	5.03	3030
	Stem	4.97	2997
	Husk	4.57	2754
	Cob	2.64	1593

*Oat spelt xylan, based on activity numbers from Dien et al. [194]

at 15 FPU g^{-1} glucan (31.3 mg protein g^{-1} glucan) and β-glucosidase (Novozyme® 188, Novozymes Corp., Bagsværd, Denmark) at 64 p-NPGU g^{-1} glucan (41.3 mg protein g^{-1} glucan) were added to each vial with a total protein content of 72.6 mg protein g^{-1} glucan. In addition, certain samples were also hydrolyzed using xylanase (Multifect® Xylanase, Genencor Division of Danisco US Inc.) at 10% of total cellulase protein (1871 OSX (oat spelt xylan) g^{-1} glucan or 3.1 mg protein g^{-1} glucan), giving a total protein content of 75.7 mg protein g^{-1} glucan. The data for the xylanase activity are based on the activity per mL provided by Dien et al. [194] and the activity, in terms of the xylan content of each sample, is included in **Table 5.1**. Enzyme loading throughout the paper is referred to in terms of protein loading, as opposed to activity, because of the probable relationship between protein and enzyme cost to the biorefinery [217]. Samples were placed in a New Brunswick Scientific (New Jersey, USA) incubator shaker and hydrolyzed at 50°C and 150 rpm for 72 h. The hydrolysates were sampled at 24 h and 72 h,

following which samples were heated at 90°C for 15 min, cooled and centrifuged at 15K for 5 min. The supernatant was filtered into HPLC shell vials using a 25 mm, 0.2 µm polyethersulfone syringe filter (Whatman Inc., New Jersey, USA) after which samples were stored at -20°C until further sugar analysis.

5.2.5. Sugar analysis

An HPLC system was used to determine the sample monomeric glucose and xylose concentrations following enzymatic hydrolysis. The HPLC system consisted of a Waters (Massachusetts, USA) pump, auto-sampler and Waters 410 refractive index detector, equipped with a Bio-Rad (Hercules, California, USA) Aminex HPX-87P carbohydrate analysis column with attached deashing guard column. Degassed HPLC grade water was used as the mobile phase, at 0.6 mL min^{-1}, with the column temperature set at 85°C. Injection volume was 10 µL with a run time of 20 min per sample. Mixed sugar standards were used to quantify the amount of monomeric glucose and xylose in each hydrolysate sample. All sugar yields are from the enzymatic hydrolysate and are reported in terms of the untreated dry biomass.

5.2.6. Statistical analysis

Monomeric glucose and xylose yields following enzymatic hydrolysis were analyzed using MANOVA in Minitab15 Statistical Software (2006 Minitab Inc., Pennsylvania, USA). The interactive effects plot which compares the harvest period and the stover fraction with each

other, the four AFEX™ pretreatment parameters (moisture content, ammonia loading, temperature and residence time) and the xylanase addition was also constructed using Minitab.

5.2.7. Empirical modeling of harvest scenarios

For this analysis, the sugar yields used were from the 72 h hydrolysis of AFEX™ -treated late-harvest corn stover. The option of an early harvest was not analyzed because of the lack of data for husk and cob fractions. Scenarios were analyzed with regard to the effect of increasing ammonia loading from 1.0 to 1.5 (g NH_3 g^{-1} biomass) and for the maximized sugar yield, either glucose or xylose. This gave four potential scenarios (1.0 + glucose, 1.5 + glucose, 1.0 + xylose and 1.5 + xylose). All other AFEX™ and hydrolysis conditions were held constant (60% dwb moisture, 90°C, 5 min residence time + 10% xylanase addition). As the glucose yields were consistently higher than the xylose yields, the harvest conditions used to obtain maximum glucose yields for all of the scenarios also corresponded with the maximum total sugar yields.

5.3. Results

5.3.1. Composition analysis

The composition of each of the corn stover fractions from each harvest is listed in **Table 5.2**. The value of the 'other' column was determined by the difference of the total of the other columns from 100%. The standard deviation is representative of three replicates. Statistically, the early and late stem and the late leaves and husk had the highest glucan content, while the

Table 5.2: Corn stover composition for early and late harvest stover fractions. Values with different superscripts in a column were statistically different using Tukey's pairwise comparison with $\alpha = 0.05$. The 'other' column determined by difference from 100%.

Corn stover fraction		Corn stover fraction composition (% dry biomass)				
		Glucan	Xylan	Acid-insoluble lignin	Ash	Other
Early	Leaves	$27.5^{b} \pm 3.2$	$17.8^{e} \pm 1.7$	$13.2^{bc} \pm 0.7$	$7.3^{a} \pm 0.13$	34.2
	Stem	$35.1^{a} \pm 2.6$	$19.0^{de} \pm 1.1$	$14.9^{bc} \pm 0.2$	$3.4^{c} \pm 0.10$	27.6
Late	Leaves	$35.3^{a} \pm 1.2$	$21.8^{cd} \pm 0.6$	$13.6^{bc} \pm 1.7$	$6.0^{b} \pm 0.25$	23.3
	Stem	$37.8^{a} \pm 0.9$	$23.6^{bc} \pm 0.4$	$16.9^{b} \pm 0.5$	$2.4^{d} \pm 0.08$	19.3
	Husk	$39.0^{a} \pm 2.2$	$26.5^{b} \pm 1.5$	$11.6^{c} \pm 0.3$	$2.1^{e} \pm 0.11$	20.8
	Cob	$27.5^{b} \pm 1.1$	$32.3^{a} \pm 1.3$	$25.8^{a} \pm 2.6$	$1.1^{f} \pm 0.02$	13.3

early leaves and late cob had the lowest glucan content. The xylan content of the late fractions was significantly higher than their early counterparts and tended to decrease from late cob > late husk > late stem > late leaves > early stem > early leaves. The acid-insoluble lignin content was similar for all fractions, except for the cob, which had the highest lignin content, and the late husk, which had statistically less lignin than the late stem. The ash content of all fractions were statistically different and decreased from early leaves > late leaves > early stem > late stem > late husk > late cob.

5.3.2. AFEX™ pretreatment and hydrolysis

Pretreatment conditions for AFEX™-treated corn stover have been previously optimized at 1.0 (g NH_3 g^{-1} dry biomass), 60% moisture content (dry-weight basis; dwb), 90°C and 5 min residence time [203, 204]. These conditions were treated as the 'base case' for the

analysis of pretreatment conditions. The effect of pretreatment conditions on monomeric glucose and xylose yields following hydrolysis was tested by varying one process parameter (temperature, ammonia loading, moisture content or residence time) at a time (for example, raising the temperature from 90°C to 100°C). Once the preliminary data had been gathered, the untreated control, base case and best case were supplemented with xylanase during hydrolysis to observe the effect on sugar yields.

Figure 5.1 shows the monomeric glucose and xylose yields for a variety of conditions with particular comparisons between untreated and AFEX™-treated materials at a range of ammonia loadings. The effect of xylanase addition to the enzyme cocktail can also be observed in **Figure 5.1**. Error bars in all figures represent the mean ±1 standard deviation. From **Figure 5.1**, it can be seen that AFEX™ substantially improves both glucose and xylose monomeric sugar yields for all harvest periods and corn stover fractions when compared to untreated materials.

The increase in ammonia loading from 0.5 to 1.5 (g NH_3 g^{-1} biomass) had different effects on early harvest and late harvest corn stover fractions. For the early harvest stover without xylanase addition, glucose yields peak at 1.0 (g NH_3 g^{-1} biomass). This optimum is similar to what has been seen previously with AFEX™-treated corn stover [203, 204], which may indicate that that material was from an earlier harvest. The xylose yields are relatively unaffected by any further increase above 1.0 (g NH_3 g^{-1} biomass). However, when performing

the same experiment with the late harvest corn stover, there is an increase in both glucose and xylose yields for all fractions when increasing from 1.0 to 1.5 (g NH_3 g^{-1} biomass).

Xylanase addition had little effect on the increase of either glucose or xylose sugar yields

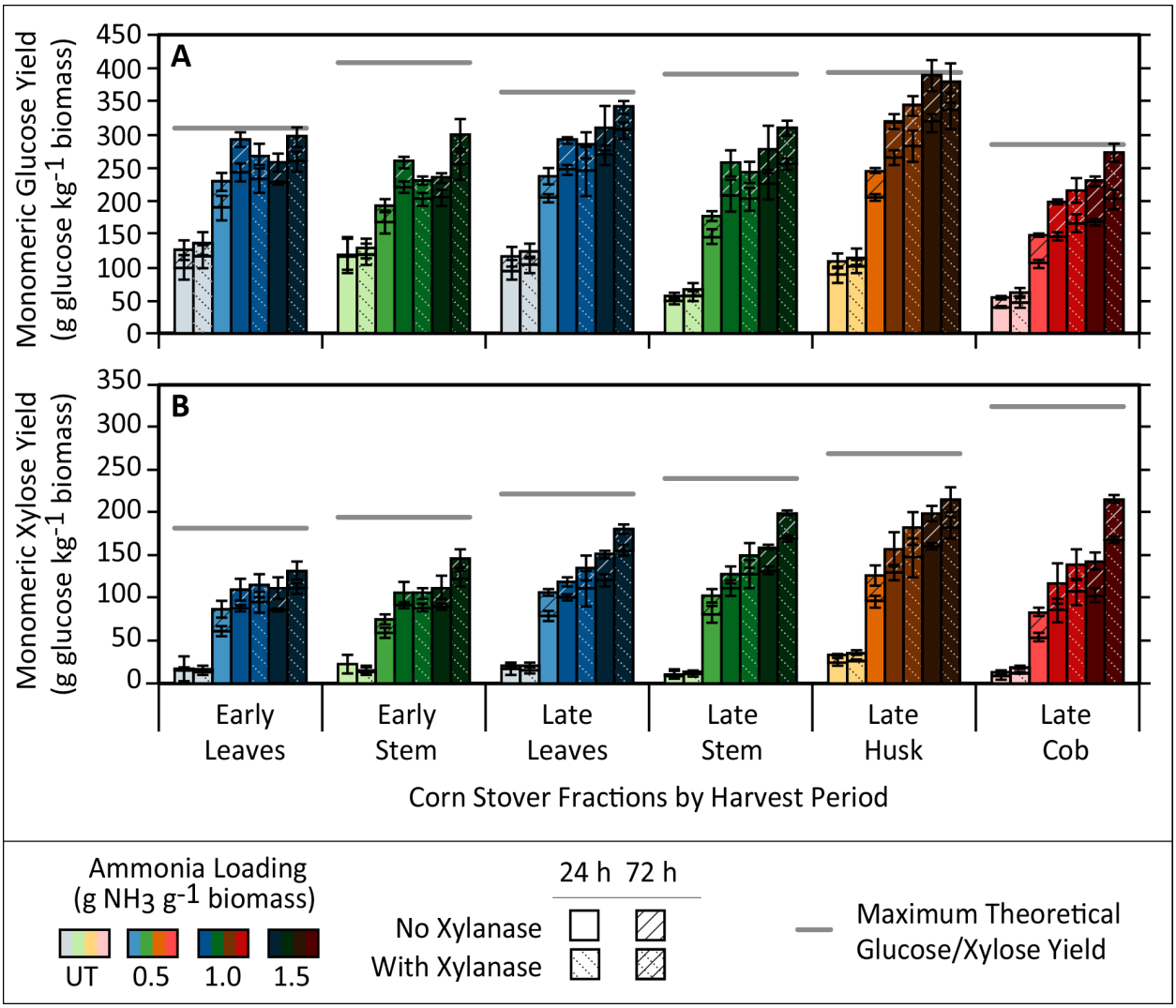

Figure 5.1: Effect of ammonia fiber expansion (AFEX™) pretreatment ammonia loading and xylanase addition on enzymatic hydrolysis monomeric sugar yields. Glucose yields are reported in part A and xylose yields are in part B. All AFEX™ runs were kept at constant moisture content (60% dry-weight basis), temperature (90°C) and residence time (5 min). Yields are in terms of sugar available in untreated dry biomass.

in untreated corn stover fractions. For AFEX™-treated early harvest fractions, the addition of xylanase at 1.0 (g NH_3 g^{-1} biomass) had no effect on monomeric xylose yields and it slightly lowered glucose yields. At 1.5 (g NH_3 g^{-1} biomass), all fractions and harvests experienced an increase in both the monomeric xylose and glucose yields with the addition of xylanase.

The leaf and stem, for both early and late harvests, have similar glucose yields at 1.5 (g NH_3 g^{-1} biomass) ammonia loading. However, the leaf glucan is more digestible, as seen by the greater yield (percent of maximum theoretical glucan available). The late harvest husk

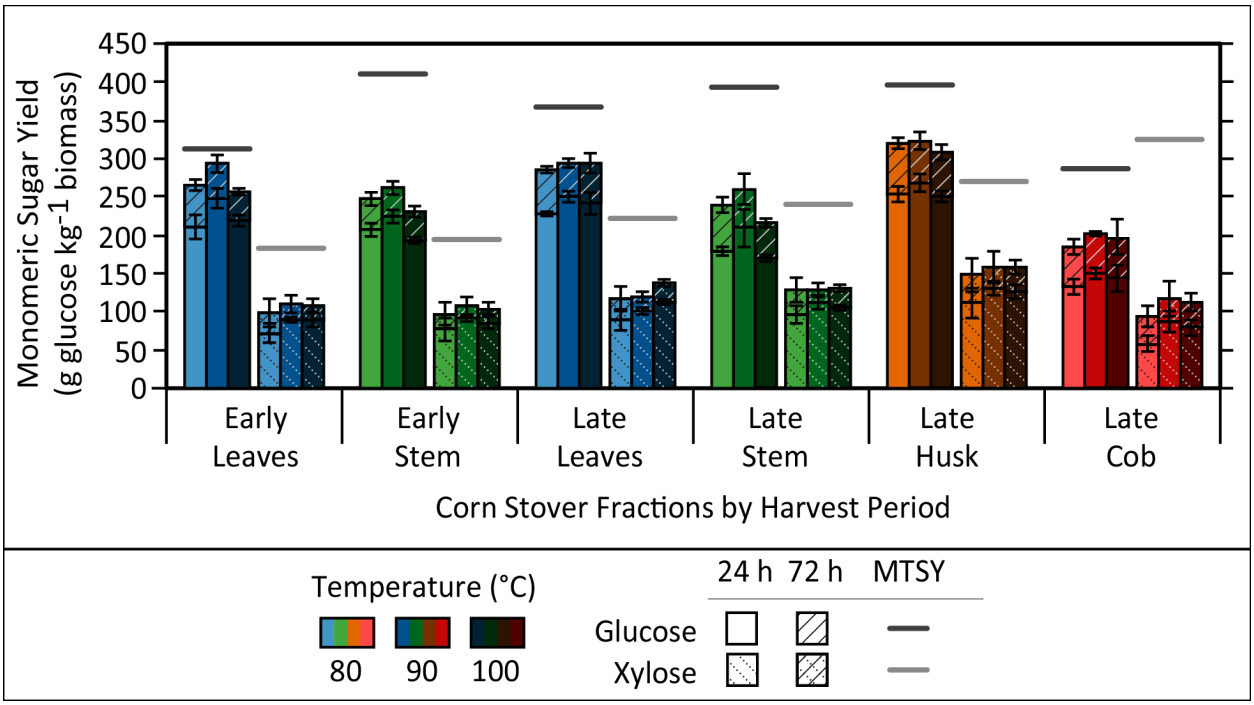

Figure 5.2: Effect of ammonia fiber expansion (AFEX™) pretreatment temperature on enzymatic hydrolysis monomeric sugar yields. All AFEX™ runs were kept at a constant moisture content (60% dry-weight basis), ammonia loading (1.0 g NH_3 g^{-1} dry biomass) and residence time (5 min). Yields are in terms of sugar available in untreated dry biomass. Glu = glucose, Xyl = xylose, MTSY = maximum theoretical sugar yield.

approaches theoretical glucose yields at the optimal condition of 1.5 (g NH_3 g^{-1} biomass). As a result of this, the addition of xylanase for this pretreatment condition increases husk xylose yields slightly but not the glucose yields, as is seen in the other fractions. With the addition of xylanase at 1.5 (g NH_3 g^{-1} biomass), the cob and leaf also approach theoretical glucose yields.

Figure 5.2 shows the effect of pretreatment temperature on glucose and xylose yields from corn stover fractions. Altering the temperature by 10°C from the base case had little effect on glucose and xylose yields. There is a definite peak in glucose yields at 90°C for the

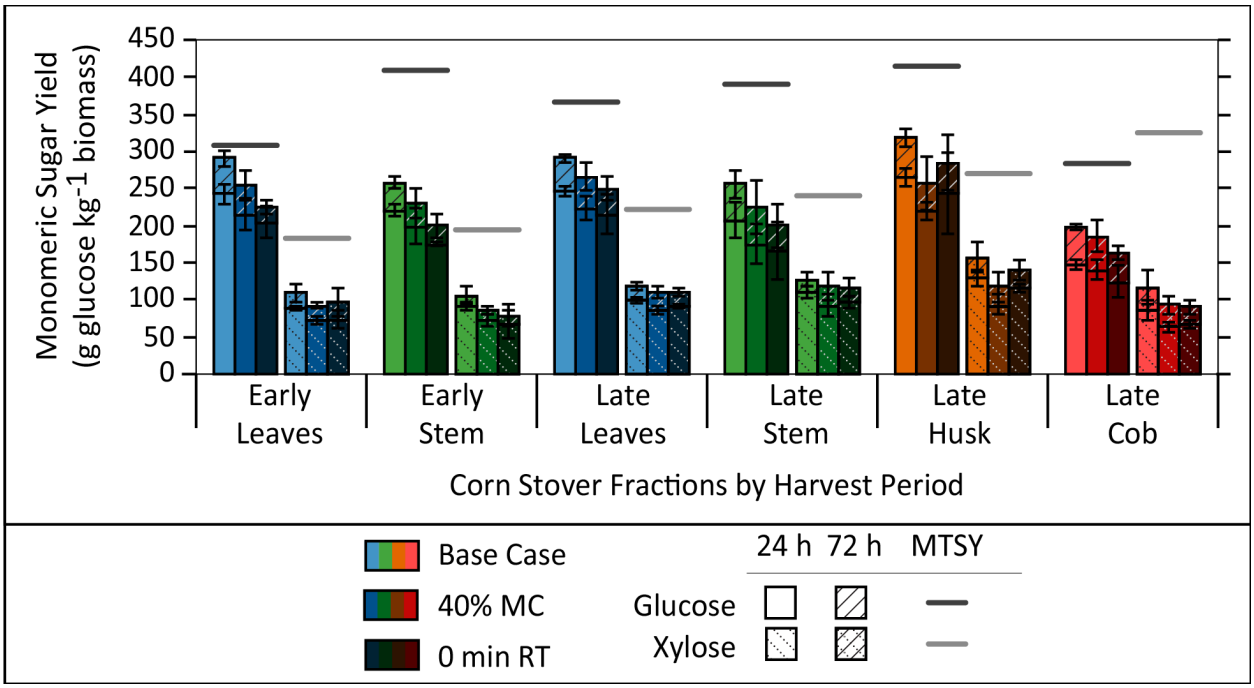

Figure 5.3: Effect of ammonia fiber expansion (AFEX™) moisture content and residence time on enzymatic hydrolysis monomeric sugar yields. Base AFEX™ conditions: moisture content (60% dry-weight basis), ammonia loading (1.0 g NH_3 g^{-1} dry biomass), temperature (90°C) and residence time (5 min). Yields are in terms of sugar available in untreated dry biomass. MC = moisture content, RT = residence time, Glu = glucose, Xyl = xylose, MTSY = maximum theoretical sugar yield.

early harvest but the late harvest has no apparent difference in yields for 80°, 90° or 100°C. In a previous work [203], raising the temperature above 90°C had a negative impact on ethanol yields from simultaneous saccharification and fermentation.

Decreasing the moisture content to 40% (dwb) and eliminating the residence time (the time for which the reactor was held at the set temperature following heat-up) each had a negative impact on glucose and xylose yields for all fractions (**Figure 5.3**). For all stover fractions, except the late husk, it was more detrimental in terms of sugar yields to decrease the residence time rather than the moisture content.

Table 5.3: Analysis of variance for factors influencing sugar yields.

	***p*-value**			
Factor	**24 h Glucose**	**72 h Glucose**	**24 h Xylose**	**72 h Xylose**
Harvest date	0.775	0.437	0.000*	0.000*
Corn stover fraction	0.000*	0.006*	0.526	0.528
Ammonia loading	0.000*	0.000*	0.000*	0.000*
Temperature	0.082	0.161	0.000*	0.022*
Moisture content	0.018*	0.002*	0.000*	0.000*
Residence time	0.001*	0.000*	0.003*	0.000*
Xylanase addition	0.001*	0.002*	0.000*	0.000*
Harvest x ammonia	0.007*	0.001*	0.001*	0.002*
Harvest x temperature	0.918	0.932	0.824	0.392
Harvest x moisture	0.687	0.762	0.943	0.424
Harvest x residence time	0.829	0.719	0.377	0.317
Harvest x xylanase	0.919	0.760	0.111	0.063
Fraction x ammonia	0.288	0.080	0.416	0.152
Fraction x temperature	0.746	0.684	0.588	0.400
Fraction x moisture	0.278	0.075	0.163	0.109
Fraction x residence time	0.916	0.859	0.715	0.542
Fraction x xylanase	0.711	0.300	0.008*	0.030*

*Significant at $\alpha = 0.05$.

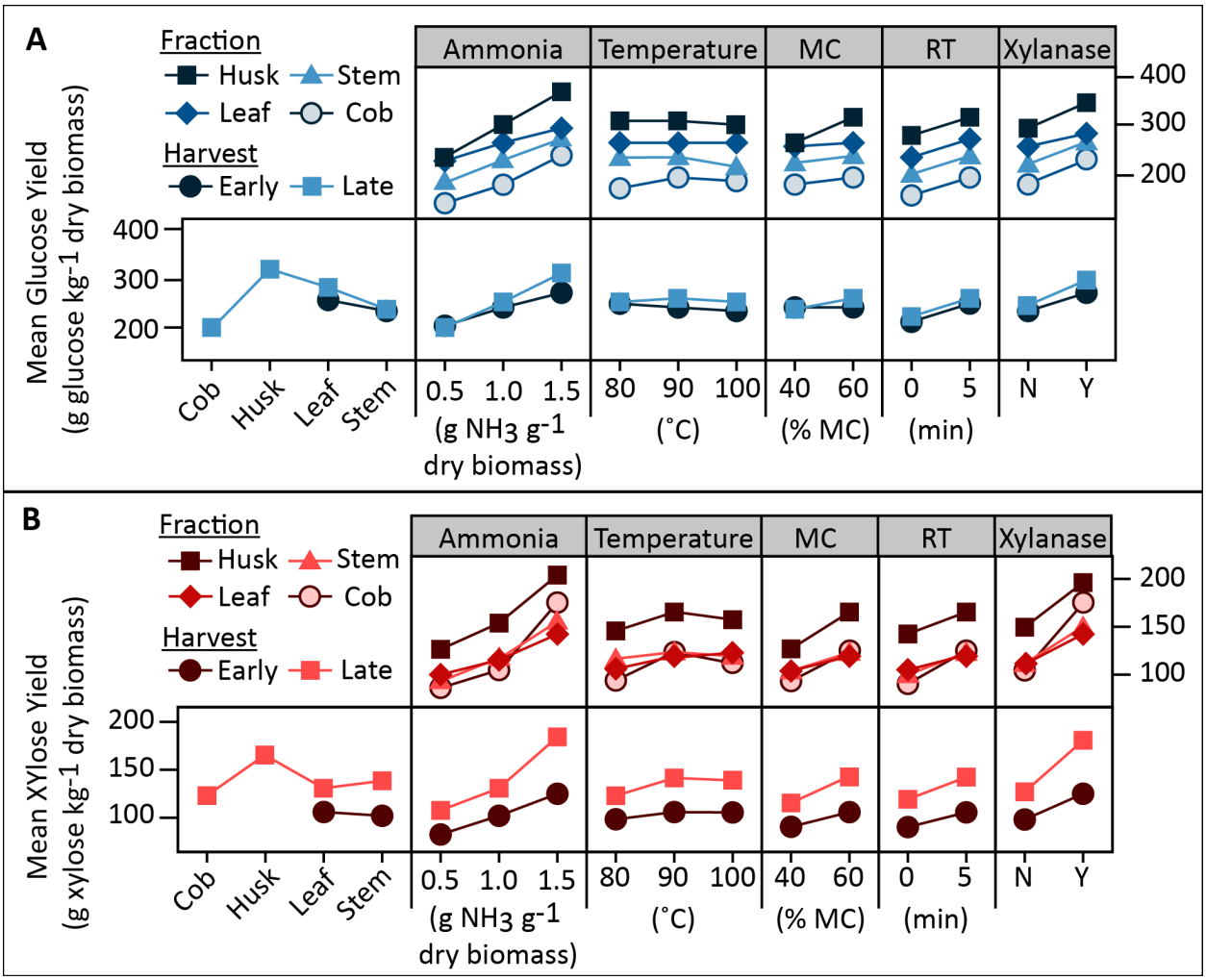

Figure 5.4: Interaction effect plot of AFEX™ parameters, stover fraction and harvest period on monomeric (A) glucose and (B) xylose yields (g sugar per kg untreated dry biomass) following 72 h enzymatic hydrolysis. MC = moisture content, RT = residence time, DWB = dry-weight basis, N = no xylanase added, Y = xylanase added (10% of total cellulase protein).

5.3.3. Statistical analysis

Multivariate analysis of variance (MANOVA) was conducted in order to determine the significance of harvest date, corn stover fraction, AFEX™ parameters and xylanase addition on both the 24 hour and 72 hour monomeric glucose and xylose yields. Interactive effects were also examined between harvest date and stover fraction and each of the other parameters. As

the conclusions regarding significance were the same for 24 hour and 72 hour yields for both glucose and xylose (**Table 5.3**), only the 72 hour yields were used for the interactive effects plot (**Figure 5.4**). Glucose yields were significantly affected by three of the AFEX^{TM} pretreatment conditions: ammonia loading, moisture content and residence time, but not by temperature. Glucose yields were also dependent on the corn stover fraction and whether xylanase was added to the hydrolysis cocktail. Of the interactive effects analyzed, only harvest date x ammonia loading had any significant affect on monomeric glucose yields. If the α-value is increased to 0.1, the fraction x ammonia and fraction x moisture also significantly affect 72 h glucose yields. However, compared to the majority of the other significant parameters (except the moisture content and harvest x ammonia effect on 24-hour glucose yields), which are significant at $\alpha < 0.005$, the effect of these two interactions on the glucose yield seems minimal.

Xylose yields were significantly affected by all four AFEX^{TM} pretreatment conditions, including temperature. Unlike the case for glucose yields, xylose yields were not significantly affected by corn stover fraction, but they were affected by both the harvest date and the addition of xylanase to the hydrolysis cocktail. There were also interactive effects on xylose yields from harvest date x ammonia loading and corn stover fraction x xylanase addition.

When analyzing the interactive effects plot (**Figure 5.4**), significant interactive effects will have very different slopes for the different lines in that portion of the graph. For example, when observing the interactive effect of harvest x ammonia on xylose yields, the slope of the early and late harvest lines are roughly the same when the ammonia loading is increased from 0.5 to 1.0 (g NH_3 g^{-1} biomass). However, when the ammonia loading is increased from 1.0 to 1.5

(g NH_3 g^{-1} biomass), the slope of the late harvest line is significantly steeper than the slope of the early harvest line. This difference in slope signifies that most of the impact of ammonia loading on this interaction is due to the second, not the first increase. This implies that the higher ammonia loading has a greater effect on the late harvest than the early harvest.

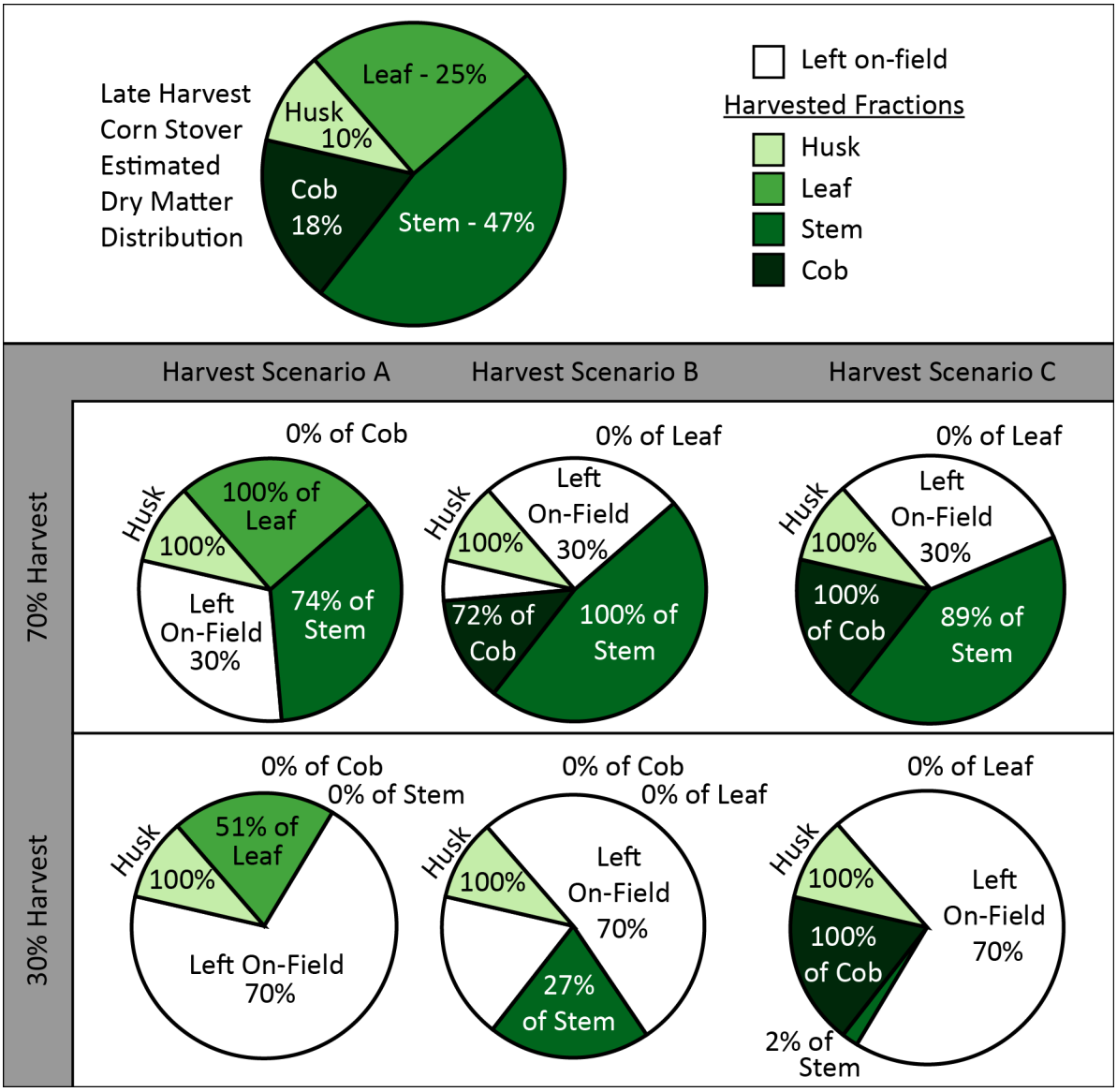

Figure 5.5: Estimated dry matter distribution for 70% and 30% (dry-weight basis) harvest of late harvest corn stover. Percentages of the individual fractions harvested are based on the total amount of each fraction available.

Table 5.4: Optimized harvest scenarios based on desired sugar and ammonia fiber expansion ammonia loading.

Harvest scenario	A	B	C
Optimized sugar	**Glucose/total**	**Xylose**	**Xylose**
Ammonia loading ($g\ NH_3\ g^{-1}$ dry SHCS)	**1.0, 1.5**	**1.0**	**1.5**
Best fraction	Husk	Husk	Cob
	Leaf	Stem	Husk
	Stem	Cob	Stem
Worst fraction	Cob	Leaf	Leaf

5.3.4. Optimization of harvest scenarios

The conditions selected resulted in three scenarios for selectively harvesting corn stover (**Table 5.4**) because the harvest scenario to maximize glucose yields was the same for both ammonia loadings. The relative amounts of harvested fractions for each scenario are represented in **Figure 5.5** for both the 70% and 30% harvests. A comparison of **Table 5.5** and **Table 5.6** reveals that the amount of corn stover harvested has the largest impact on theoretical ethanol yield per hectare. Decreasing stover collection from 70% of available material to 30%, with the same harvest scenario, decreased theoretical ethanol yields by 852 – 1139 L ha^{-1}. Decreasing the ammonia loading from 1.5 to 1.0 (g ammonia g^{-1} biomass) for the same harvest scenario caused a decrease in the theoretical ethanol yield of 150 – 462 L ha^{-1}, while switching desired sugars from glucose to xylose (that is, changing harvest scenarios but keeping stover collection and AFEX^{TM} and enzymatic hydrolysis conditions constant) caused a decrease in the theoretical ethanol yield of 29 – 64 L ha^{-1}. In order to determine the sensitivity

Table 5.5: Estimated yields for 70% collection of selectively harvested corn stover (SHCS) following AFEX™, enzymatic hydrolysis and fermentation.

Yield		1.0 g NH_3 g^{-1} dry SHCS			1.5 g NH_3 g^{-1} dry SHCS		
		Scenario A	Scenario B	Worst Case	Scenario A	Scenario C	Worst case
g sugar kg^{-1} SHCS	**Glucose**	273.7	254.2	240.9	331.5	310.8	303.1
	Xylose	150.0	153.1	146.6	195.7	206.8	203.2
	Total	423.6	407.3	387.5	527.2	517.5	506.3
L kg^{-1} SHCS	**Theoretical ethanol**	0.274	0.263	0.250	0.341	0.335	0.327
L ha^{-1}	**Theoretical ethanol**	1648	1585	1508	2051	2014	1970

Table 5.6: Estimated yields for 30% collection of selectively harvested corn stover (SHCS) following AFEX™, enzymatic hydrolysis and fermentation.

Yield		1.0 g NH_3 g^{-1} dry SHCS			1.5 g NH_3 g^{-1} dry SHCS		
		Scenario A	Scenario B	Worst Case	Scenario A	Scenario C	Worst case
g sugar kg^{-1} SHCS	**Glucose**	305.1	278.2	228.7	354.4	311.3	288.2
	Xylose	151.8	161.3	144.0	192.7	215.4	210.2
	Total	456.9	439.5	372.6	547.1	526.7	498.3
L kg^{-1} SHCS	**Theoretical ethanol**	0.295	0.284	0.241	0.354	0.340	0.322
L ha^{-1}	**Theoretical ethanol**	762	733	621	912	878	831

of changing the harvest scenario, the model was also run for a worst case scenario, where the biomass was harvested in a manner that would give the worst possible sugar yields. The worst case scenario led to a decrease in the theoretical ethanol yields per hectare ranging from 81 –

141 L ha^{-1}. As expected, when comparing untreated corn stover to the AFEX^{TM}-treated cases (data not shown), the theoretical ethanol yield was substantially lower for the untreated cases: a decrease of 1234 – 1695 L ha^{-1} for the 70% harvest and 527 – 719 L ha^{-1} for the 30% harvest.

5.4. Discussion

5.4.1. Composition analysis

Fractions from the late harvest tended to have a slightly higher percentage of cell wall components and slightly lower percentage of ash compared to their early harvest counterparts. For corn stover, the increase in lignin and cellulose and the decrease in ash have been observed elsewhere [218, 219]. There is also a general increase in all cell wall components with a decrease in soluble solids and non-structural carbohydrates and an increase in lignin and xylan with increasing maturity [207, 220]. This observed increase in the cellulose (glucan), hemicellulose (glucan and xylan) and lignin content is due to the secondary thickening of the plant cell wall that continues to occur for as long as the plant matures. During this time there is also a decrease in ash content [158]. However, while there is a continual change in the dry matter composition until late in the season, there tend to be very small changes during the grain harvest period [197, 207], the time during which our samples were harvested.

5.4.2. AFEX^{TM} pretreatment

Based on the final total sugar yields, the optimal AFEX^{TM} pretreatment conditions were observed to be consistent for all fractions, for both early and late harvest corn stover: 1.5:1 (g

NH_3 g^{-1} biomass), 60% moisture content (dwb), 90°C, 5 min residence time and 10% xylanase addition (mg xylanase protein mg^{-1} cellulase protein), in addition to the standard enzyme mixture used during enzymatic hydrolysis. For AFEX™-treated early harvest fractions, the addition of xylanase at 1.0 (g NH_3 g^{-1} biomass) had no effect on monomeric xylose yields and slightly lowered the glucose yields. This drop in glucose yields could be due to the competition for binding sites on the cellulose chains between enzymes in the xylanase and cellulase mixtures. The fact that there is no increase in xylose yields with the addition of xylanase supports this conclusion. If the xylanase, which has a much lower cellulase activity [194], is competitively binding to the cellulose instead of the xylan, this could result in a decrease in glucose yields with no significant change in xylose yields.

The higher optimal ammonia loading for the late harvest fractions compared with the early harvest could be due to a number of reasons. AFEX™, by the ammoniation of the active methoxyl sites of lignin [221], may be preventing the lignin from binding to the hydrolysis enzymes. This may be one of the main reasons for the increase of 0.5 to 1.0 (g NH_3 g^{-1} biomass). However, if this were the reason for the difference in optimum ammonia loading between the early and late harvests, then the lignin content of the later harvest should be greater. This is not the case however, as statistically the lignin contents of the early and late fractions are identical. Another possibility is that, although they have identical lignin contents, the later harvest may contain a greater quantity of methoxyl sites compared to the early harvest, which may make the later harvest lignin more reactive with ammonia. In both hardwoods and grasses there has

been an observed increase in the amount of syringyl residues and/or the S:G ratio as they mature [162, 222] which would increase the relative methoxyl content of the lignin (**Figure 1.4**). It is also possible that the difference in optimal ammonia loading could be due to the increase in xylan content and possibly increased cross-linking between hemicellulose and lignin from the early to late harvest. Ferulate cross-linking occurs between lignin and arabinoxylan in the plant cell wall, with the ferulates ether-linked to lignin and ester-linked to the arabinoxylan [20]. Ammonolysis of the ferulate ester linkages to arabinoxylan side-chains is believed to be one major reaction occurring during the AFEX^{TM} process [223]. These mechanisms may be opening up the cell wall ultrastructure more effectively at the higher ammonia loading, allowing the enzymes greater access to cellulose. Also, by increasing access to the substrate, the xylanase enzymes would have more potential xylan binding sites and therefore be less likely to bind competitively to the cellulose chains. This could also explain the increase in glucose yields with the addition of xylanase for 1.5 (g NH_3 g^{-1} biomass).

These hypotheses are supported by the fact that the husk, the material with the lowest lignin content, while having the second-highest xylan content, is least affected by the combination of increased ammonia loading and xylanase addition. At 1.5 (g NH_3 g^{-1} biomass), the xylose yield only increases by 6.1% with the addition of xylanase to the hydrolysis cocktail. The late cob, which has a significantly higher lignin and xylan content than all of the other materials, experiences the largest impact on xylose yields due to the combination of increased ammonia loading and addition of xylanase - a 22.5% increase. The higher ammonia loading would cleave more linkages between the hemicellulose and lignin, solubilizing more oligomeric

and monomeric xylose and, perhaps, some lignin as well. These exposed, solubilized sugars would be much easier to hydrolyze with the xylanase. It might be possible, given the very high xylan content of the cob, that more xylanase would be needed to achieve near complete monomeric xylose yields. As the xylanase loading was based on a percentage of the cellulase loading (and therefore the glucan content), and because the glucan-to-xylan ratio for the cob (0.85 g glucan g^{-1} xylan) is much lower than the other fractions (1.47 – 1.85 g glucan g^{-1} xylan), the xylanase loading in terms of the xylan content (mg xylanase g^{-1} xylan) is much lower for the cob fraction (**Table 5.1**). This may be one reason for the much lower xylose yield relative to the maximum theoretical xylose yield of the late cob fraction.

5.4.3. Statistical analysis

All AFEX^{TM} parameters had significant impacts on sugar yields, except for temperature, which had no significant effect on glucose yields. Based on least squares means analysis (data not shown), the temperature effect on xylose yield is likely due to a greater yield increase as the temperature is raised from 80° to 90°C rather than the decrease in yield when temperature is raised from 90° to 100°C. For the range of conditions tested, optimizing the ammonia loading, moisture content, and residence time are more important for maximizing sugar yields from corn stover. However, this conclusion may change for a different range of temperatures and should not be extrapolated to other conditions.

Harvest date had a significant impact on xylose yields but not on glucose yields. This is largely due to the fact that the xylan content of the late fractions was greater than the xylan content of the early fractions, whereas the glucan content was not significantly different between harvests. The corn stover fractions tested had a significant effect on glucose yields but not on xylose yields. The late stem, husk, leaf, and early stem fractions had no significant statistical difference in their glucan contents, so their relative recalcitrance, in terms of glucose yields, can be inferred from **Figure 5.4**. As the husk has the highest glucose yield, it can be considered the least recalcitrant, followed by the leaf and then the stem. Inferences cannot be made regarding the cob because its glucan content is statistically lower than the other three fractions. However, because the cob approaches theoretical glucan yields at optimal conditions while the stem does not (**Figure 5.1**), it may be less recalcitrant in terms of glucan conversion.

For interactive effects, only two were significant: harvest date X ammonia loading and corn stover fraction x xylanase addition. The harvest x ammonia interaction was significant for both glucose and xylose yields. The increase in ammonia loading from 1.0 to 1.5 (g NH_3 g^{-1} biomass) appears to have a greater effect on the late harvest than the early harvest, but this may be due to the lack of data for the early harvest cob. As the cob is the fraction most affected by the increase in ammonia loading, particularly for xylose yield, the lack of early cob data may lead to an apparent difference in effects that is not actually present between harvests. None of the other AFEX™ parameters show this relationship with either harvest date or corn stover fraction, which indicates that the same pretreatment conditions (moisture, temperature and

residence time) can be used to maximize glucose release, regardless of the fractional composition of the corn stover or the harvest date.

The second significant interactive effect was for corn stover fraction x xylanase addition, but only for xylose yields. The main reason for this effect, as can be observed from **Figure 5.4**, is due to the cob fraction that was much more strongly affected by the addition of xylanase than all of the other fractions, whose responses were fairly similar. This conclusion is supported by the fact that when the data for the late cob was removed from the analysis, the fraction x xylanase interaction became non-significant (data not shown). Taken together, these results indicate that of all the corn stover components, the cob reacts more differently during enzymatic hydrolysis. As mentioned previously, the cob may require a much higher xylanase loading than the other fractions in order to release xylose remaining in the biomass or to convert the AFEX^{TM} solubilized xylo-oligomers.

5.4.4. Empirical modeling of harvest scenarios

As a result of the wide range of opinions on how much corn stover can be sustainably harvested and because the amount will likely change for a given field depending on environmental conditions and agricultural practices [114, 199-201], we have modeled a number of corn stover harvest scenarios for both a liberal harvest estimate (70% of available corn stover) and a conservative harvest estimate (30% of available corn stover). The goal was to determine which combination of fractions provides the most benefit to the biorefinery in terms of sugar yields, and to determine the preferential order in which fractions should harvested.

Crofcheck and Montross [209] found that the weighted sum of the glucose yields from individual pretreated fractions was not statistically different from the glucose yield from whole pretreated corn stover. This means that glucose yields for SHCS could be predicted using glucose yields from individual fractions. Our estimate of the late harvest dry matter distribution of corn stover (**Figure 5.5**), was based on published data from four sources [207-209, 218], and is similar to standard estimates of corn stover dry matter distribution near harvest [224]. Corn stover dry matter yields, particularly of the husk and leaf, tend to decrease rapidly due to weathering over the course of the harvest season [207, 208, 213, 218-220, 225]. This estimate attempts to account for both the effects of the late harvest date as well as our inclusion of the leaf sheath with the leaf fraction instead of the stem fraction, as is often the case [207, 208].

The estimated whole corn stover dry matter distribution was used to predict monomeric glucose and xylose yields from the three different harvest scenarios and the worst case scenario (where the least digestible fractions were harvested) for both a 70% (**Table 5.5**) and a 30% (**Table 5.6**) harvest of on-field corn stover using weighted averaging of individual fraction sugar yields. It is important to note that values given in these tables do not attempt to take into account the ability or inability to harvest the specific fractions or any losses due to inefficiencies in harvest, transport and storage of corn stover, which can be significant depending on the methods used. A recent study found that the maximum amount of corn stover was available at grain physiological maturity (15.6 t ha^{-1}) and steadily decreased over the harvest period to a minimum of 8.6 t ha^{-1} [225]. As this value takes into account the late season of harvest, and because it is within the range of most estimates of corn stover yields reported in the published

literature (7.8 – 8.8 t ha^{-1}) [116, 197, 226], 8.6 t ha^{-1} of available corn stover was chosen to estimate the total sugars that could be produced per hectare for the given harvest scenario. The standard value of 0.51 (theoretical g ethanol produced g^{-1} sugar consumed) was used to determine the theoretical ethanol production from both a kilogram of SHCS and a hectare of harvested SHCS and does not take into account inefficiencies of fermentation.

Harvest scenario A, which selectively harvests the husk followed by the leaf, stem and, lastly, the cob, obtained the highest sugar and ethanol yields of all the scenarios and, as a result, was chosen as the optimal harvest scenario for AFEX^{TM}-treated corn stover. Harvest scenario A was also preferable to scenarios B and C for a number of other reasons. First, optimizing the collection for maximum glucose yields is preferable because most current and relevant microbial strains selectively utilize hexoses over pentoses as a carbon source during ethanolic fermentation [205, 227]. Second, harvest scenario A selectively leaves behind the more lignified fractions on the field which may prove more valuable for improving SOC levels due to the longer half-life of lignin compared to cellulose and hemicellulose [114, 228]. Lastly, harvest scenario A seems to be the most feasible option from a technical viewpoint.

Selective harvesting of corn stover fractions will involve either returning the cob and/or husk to the field following the removal of the grain from the ear, and/or raising the header on the combine to increase the stover cut height [197, 229, 230]. As a result of the association of the leaves with the stem, at higher cut heights it would be almost impossible to remove all of the leaves while leaving the entire stem behind. Taking these factors into consideration, of all of the scenarios, the most feasible from a technical aspect would be: scenario A (70% harvest),

where all of the cob and a portion of the lower stem is returned to the field; and scenario C (30% harvest), where only the stover associated with the ear (husk and cob) is retained. Scenario A (30% harvest) could also be feasible if we replaced the percentage associated with the leaf material with a mixture of the upper-most portion of the corn plant (leaf and stem). This might be a reasonable option, because the upper portion of the stem tends to be more easily digestible than the lower portion of the stem and also has a higher sugar content than the leaf [221, 229]. So, harvesting the upper portion of the corn plant could hypothetically give higher yields than harvesting the leaf alone. Unfortunately, this cannot be modeled because for this study, only the entire, homogenized corn stem was tested.

Crofcheck and Montross [209] recommended, based on glucose yields from fractionated corn stover, a roughly 30% corn stover harvest scenario where the SHCS was composed of all of the available cobs and 74% of the leaves and husks, leaving the most recalcitrant stalks on the field. The difference between their optimal harvest scenario and ours is most probably due to their experimental methods for pretreatment and the subsequent analysis. Pretreatment of lignocellulosic biomass using dilute sodium hydroxide solubilizes much of the lignin and some of the hemicellulose into the liquid pretreatment stream [231, 232]. It is therefore unlikely that glucan content of the pretreated corn stover corresponds to glucan content of the untreated corn stover. For similar pretreatment conditions of corn stover, Varga et al. found a 41.9% mass loss from the untreated dry corn stover to the pretreated solids and the composition of the pretreated material shifted in favor of a higher glucan content [232]. The cob has a significantly higher xylan and lignin content than the other fractions of the corn plant and, therefore, it is reasonable to assume that it will lose a greater proportion of its mass following dilute alkali

pretreatment. As this mass loss was not taken into account [209], the amount of glucan that could be obtained on a mass basis from the untreated fractions was over-exaggerated, particularly from the xylan- and lignin-rich cob. If the mass loss had been taken into account, it is likely that their choice of optimal fractions for harvest would have been different. As AFEX™ is a dry-to-dry process with insignificant mass loss during pretreatment, the glucan content of the pretreated material can be assumed to be the same as the glucan content of the untreated material [204]. It is feasible because of differences in reaction chemistries, that other pretreatment methods would give different results for the selective harvest ratio of corn stover fractions compared to those for AFEX™. However, because Crofcheck and Montross did not take into account the mass losses which occurred during their pretreatment and as we therefore do not know their sugar yields based on the untreated stover fractions, we cannot attribute the difference between our results and theirs to differences between the pretreatment methods. Rather the difference may be due to errors in their analysis.

Shinners et al. [229] analyzed the effect of cut height of corn stover (a harvest scenario that leaves a portion of the lower stem and leaves behind) on predicted ethanol yields and found that the amount of ethanol produced was only ~3% greater (L Mg^{-1} DM) for the low cut compared to the high cut. If one were to assume that the amount of material harvested per hectare was constant, focusing only on the composition differences in the harvested material, this result would indicate that the fraction harvested has little impact on the theoretical ethanol production, which is similar to our results. However, when they analyzed their results based on the ethanol yield per hectare, the increase in total dry matter harvested with the lower cut

height increased the predicted ethanol yield by 52% compared to the higher cut [229], which indicates that the amount of material harvested has a significant impact on theoretical ethanol yields and corresponds to our findings.

Based on these results, optimizing the fractions collected during harvest has a much smaller impact on potential yields than optimizing pretreatment and hydrolysis conditions, even if the worst case scenario occurs and the least digestible materials are preferentially harvested. However, the amount of stover harvested has the greatest impact on theoretical ethanol production per hectare. It will be very important, in terms of maximizing ethanol production, to develop methods to efficiently maximize harvest of corn stover, while still maintaining soil productivity and preventing erosion.

5.5. Conclusions

Based on monomeric glucose and xylose yields, the optimal AFEX^{TM} conditions, for all stover fractions (leaf, stem, husk and cob) regardless of harvest period, were found to be 1.5 (g NH_3 g^{-1} biomass), 60% moisture content (dwb), 90°C and 5 min residence time; with enzyme loading during hydrolysis of 31.3 mg of cellulase (Spezyme® CP), 41.3 mg of β-glucosidase (Novozyme® 188) and 3.1 mg xylanase, g^{-1} glucan. These conditions are different from those presented in previous analyses [203, 204] largely due to the inclusion of xylanase in the hydrolysis cocktail. The addition of xylanase was necessary in order to achieve high xylose yields

at moderate cellulase loadings and moderate AFEX™ conditions, particularly with respect to the more recalcitrant cob and stem fractions.

The optimal harvest scenario for the collection of SHCS would harvest the husk followed by the leaves, then the stem, and lastly, the cob. This harvest scenario was independent of ammonia loading during AFEX™ pretreatment and maximized glucose and ethanol yield from SHCS. This scenario, combined with the optimal AFEX™ pretreatment conditions for SHCS, gave a theoretical ethanol yield of 2051 L ha^{-1} for the 70% dry matter harvest and 912 L ha^{-1} for the 30% dry matter harvest. Decreasing the stover collection from 70% to 30% dropped the ethanol yield by 852 – 1139 L ha^{-1}, depending on harvest scenario and pretreatment conditions. Maximizing stover collection while protecting soil health will be the most important factor for maximizing ethanol yields from corn stover.

Optimizing the collection of corn stover fractions has little impact on the theoretical ethanol yield (29 – 141 L ha^{-1}), especially compared to optimizing pretreatment and hydrolysis conditions (150– 462 L ha^{-1}). The dry matter distribution of collected corn stover fractions is less important than the optimization of the ethanol production process. However, it is still something that needs to be taken into account because harvesting the worst fractions can still decrease ethanol yields, especially when a smaller percentage of the stover is collected.

CHAPTER 6 :
MOLECULAR SCALE: AFEX™ PRETREATMENT OF POPLAR MODIFIED FOR LIGNIN CONTENT AND COMPOSITION

6.1. Introduction

Lignin is one of the three main components present in the higher plant cell wall, comprising between 12 – 30% of the total mass, depending on the type of plant [18, 233]. Lignin is important for plant growth and survival as it provides strength and rigidity to the plant structure that is necessary for hydraulic transport, as well as passive defense against pest and pathogen attack. Lignin is also of interest to biofuel researchers as it represents both a significant hindrance to biological processing of plant structural carbohydrates to liquid fuels [233-235], and also, by providing carbon-neutral energy and steam for biorefinery operations, an economic and environmental asset [12, 235].

With advances in plant transgenic techniques, it has become possible to tweak individual cell wall components by altering genes that encode enzymes in molecular pathways. The total lignin content and monomer composition can be altered by either up- or down-regulating or knocking-out different enzymes within the synthesis pathway (**Figure 1.4**). A number of reviews have been published comparing the different lignin transgenics and mutants that have been researched in recent years and the corresponding changes in lignin content, structure and composition due to these modifications [22, 25, 27, 236, 237]. By comparing modified plant materials with their unmodified counterparts, it is possible to examine changes in interactions due to altering a single parameter in the plant biochemistry. It is also possible to determine which modifications are most significant in terms of improving plant digestibility and

reducing process costs either by allowing use of a milder pretreatment or reducing the enzyme loading required during enzymatic hydrolysis. Previous experiments on AFEX™ pretreatment of hardwoods (poplar and black locust) found that high ammonia and water loadings, temperatures (180°C), and enzyme loadings during hydrolysis are necessary to achieve significant saccharification yields [14, 31]. At these conditions, it is unlikely that an AFEX™-based biorefinery could profitably use hardwoods. However, by genetically modifying the plant lignin content or composition, it may be possible to increase yields sufficiently to be profitable.

It is well-known that reductions in total lignin content can improve enzymatic saccharification [164, 234, 238, 239], however there is no clear evidence of the effect of alterations to monomer composition [21, 164, 234]. One study which tested the saccharification potential of cinnamyl alcohol dehydrogenase (CAD) down-regulated switchgrass with altered lignin content and composition found a negative correlation between the syringyl:guaiacyl (S:G) ratio and total sugar release, however, the authors concluded that the total lignin content of the samples was the determining factor for increased saccharification efficiency [239]. Another study on caffeic acid o-methyltransferase (*comt)* downregulated alfalfa with significantly reduced S-lignin content showed an increase in sugar yields following dilute-ammonia pretreatment compared to the control [240]. In contrast to these two studies, Studer et al. [241] found for a large group of naturally occurring poplar, samples with S:G greater than 2.0 released more sugar compared to those with S:G less than 2.0. And finally, some studies find no relationship between the lignin composition and sugar yields. When model plant cell walls were constructed with varying ratios of lignin subunits, there was no

subunit effect on degradation of these materials by fungal enzymes, or extraction of lignin by NaOH [242], which led the authors to conclude that improvements in digestibility that were previously related to changes in lignin subunits were actually the result of other changes in cell wall chemistry or architecture. However, as the materials they used were model primary plant cell walls, it is uncertain how well they represent naturally occurring plant materials, which are more complex systems.

For this project we examined the interaction of three different lignin modifications in poplar: up-regulated ferulate-5-hydroxylase (F5H), down-regulated 4-coumarate:CoA ligase (4CL), and down-regulated cinnamoyl-CoA reductase (CCR)), with AFEX™ pretreatment conditions and the subsequent effect on sugar yields. We also assessed whether modifications to lignin content (CCR-downregulation) or composition and structure (C4H::F5H up-regulation) would allow for reductions in the severity of pretreatment, by testing these materials at three different sets of AFEX™ conditions: low temperature – long time, moderate temperature – moderate time, high temperature – short time. We also used an AFEX™-pretreated poplar hybrid, NM-6 (*Populus maximowiczii x nigra)*, to determine an optimum combination of commercial enzymes for hydrolysis of AFEX™-treated hardwoods consisting of Accellerase® 1000, Accellerase® XY, and Multifect® Pectinase.

6.2. Materials and methods

6.2.1. Feedstock

One control and two genetically engineered lines of poplar that had been down-regulated for cinnamoyl-CoA reductase expression (ΔCCR 5-2-3 and ΔCCR 5-2-40) were grown in a greenhouse at VIB-Ghent. Three different progeny from each line were examined separately, for a total of nine samples. One control and one genetically engineered line of hybrid poplar (*Populus tremula x Populus alba*) that had been up-regulated for ferulate-5-hydroxylase (F5H) expression (C4H::F5H construct) were grown in a greenhouse at the University of British Columbia [243]. One control and nine genetically engineered lines of poplar that had been down-regulated for 4-coumarate:CoA ligase (4CL) expression (35s::antisense Pt4CL1a construct) were grown in the greenhouse at Michigan State University. The lines were selected for level of suppression of protein expression: weak (1, 2 and 3), medium (4, 22, and 32), and strong (7, 12, and 16); and three progeny from each control and transgenic line were selected and tested. For the enzyme mixture optimization experiments, year-old coppiced hybrid poplar *Populus maximowiczii x nigra* (NM-6) was harvested from the Michigan State University Tree Research Center. All of the samples were manually debarked, after which the CCR and F5H poplar samples were milled twice through a 5.56 mm (7/32”) screen and 4CL and NM-6 samples were milled once through a 6.35 mm (1/4”) screen using a Fitzpatrick JT-6 Homoloid mill (Continental Process Systems, Inc, Westmont, Illinois, USA). All samples were then milled through a 2 mm screen using a FOSS Cyclotec Mill (FOSS, Hillerød, Denmark) and stored at room temperature.

6.2.2. Composition analysis

Biomass composition was determined by the Great Lakes Bioenergy Research Center (GLBRC) cell wall analytical platform. Samples were initially ground into a powder by robot and then sequentially treated with 70% ethanol followed by an extraction using 1:1 (v/v) chloroform/methanol solution, in order to remove soluble materials [244]. Any starch in the sample was then removed via amylase treatment. The resulting cell wall material was then analyzed for hemicellulose sugars, crystalline cellulose, acetyl bromide lignin [245] and lignin composition via thioacidolysis [246]. The hemicellulose sugar composition was determined by treating the extracted samples with trifluoroacetic acid and then deriviatizing the solubilized monosaccharides into alditol-acetates, which were then separated and quantified by GC-MS [247]. Crystalline cellulose was isolated from the cell wall residue using Updegraff reagent and then hydrolyzed with sulfuric acid to generate glucose that was quantified using an anthrone colorimetric assay [248]. The composition of the NM-6, CCR, F5H, and 4CL samples are reported in the supplemental information (**Table D.1**).

6.2.3. Acidic and alkaline digestibility assay

Alkaline and acidic pretreatment of the F5H and control poplar, followed by enzymatic digestibility assays, were conducted by the GLBRC cell wall digestibility platform using their micro-scale method for analyzing biomass digestibility [249]. Untreated samples were compared to those that had been pretreated using 2% H_2SO_4 at 120°C; 2.0%, 0.2%, and 0.02%

NaOH at 90°C; and hot water at 90°C. All samples were hydrolyzed using Accellerase® 1000. Glucose release was determined using an enzymatic assay kit (K-GLUC, Megazyme, Ireland).

6.2.4. AFEX™ pretreatment

AFEX™ pretreatment was conducted in 22 mL reactors as outlined by Bals et al. [150]. Pretreatment conditions were chosen for their similarity to previously determined optima for AFEX™ pretreatment of hardwoods [14, 31], however water loading was reduced in order to minimize potential soluble mass losses during the venting and unloading steps. Four batches of NM-6 poplar and two batches of each 4CL sample were pretreated at 1.0 g NH_3:g DM, 1.0 g H_2O:g DM, 180°C, and 20 min residence time. The batches for each sample were then combined to homogenize batch differences prior to enzymatic hydrolysis. Two batches of each CCR and F5H sample were pretreated using 1.0 g NH_3:g DM, 1.0 g H_2O:g DM, and because of the significant impact of temperature on yields from hardwoods when operated at the same residence time [14, 31], we chose to test three different temperature and residence time combinations: 180°C for 20 min, 120°C for 60 min, and 60°C for 240 min. These conditions were chosen to compare the optimum high-temperature, short-time to a low-temperature, long-time pretreatment that may be suitable for a regional processing center [250].

Untreated and pretreated biomass moisture content was determined using a moisture analyzer (A&D, Model MF-50; San Jose, CA). Prior to microplate enzymatic hydrolysis, untreated and pretreated samples were milled through a 0.5 mm screen using the FOSS Cyclotec Mill.

6.2.5. Enzyme optimization - enzymatic hydrolysis and sugar analysis

Enzyme protein content was determined using trichloroacetic acid (TCA) precipitation to remove non-protein nitrogen [151] follwed by total N analysis using the Dumas method for combustion of nitrogen to NOx [148]. Enzyme protein contents were: Accellerase® 1500 (67 mg protein/mL), Accellerase® XY (29 mg protein/mL), and Multifect® Pectinase (72 mg protein/mL) (Genencor Division of Danisco US, Inc., New York, USA).

The pretreated NM-6 poplar was used to optimize the enzyme cocktail that was then used for the transgenic poplar experiments. Enzymatic hydrolysis was conducted using the microplate technique [251]. Samples were loaded at 0.2% glucan loading with one of two different enzyme loadings: 15 mg total protein per g glucan or 30 mg total protein per g glucan. Four replicates were run for each enzyme combination. Glucose and xylose release were both analyzed using bio-enzymatic assay kits (glucose: R-Biopharm, Inc., Marshall, MI; xylose: K-XYLOSE, Megazyme, Ireland), however due to extremely high error associated with the xylose readings, those results were inconclusive. It is unclear why the xylose error was large for the enzyme optimization experiments, but not for the poplar comparison experiments. It seems most likely that it was either due to the specific test kits that were used or to operator error.

6.2.6. Poplar comparison - enzymatic hydrolysis and sugar analysis

Samples were hydrolyzed in 20 mL screw-cap vials at 0.0125 g cell wall sugars (cellulose, glucan, xylan, arabinan, mannan and galactan) per mL (1.25% total sugar loading) and 15 mL final hydrolysis volume. The pH was adjusted to 4.8 using 1 M citrate buffer, and to limit microbial contamination, cycloheximide and tetracycline were loaded at final concentrations of 30 and 40 μg/mL, respectively. Enzymes were loaded at 24.00 mg total protein per g cell wall sugars and in the optimum proportions by mass protein as were determined for the NM-6 poplar: Accellerase® 1500 (80%), Accellerase® XY (10%), and Multifect® Pectinase (10%). Vials were placed in a New Brunswick Scientific (Edison, NJ) incubator shaker and hydrolyzed at 50°C and 200 rpm for 168 h. Samples were then taken from each hydrolysate at 24 h and 168 h, filtered in microplates, and the glucose and xylose contents were determined using microplate enzyme assay kits (glucose: R-Biopharm, Inc., Marshall, MI; and xylose: K-XYLOSE, Megazyme, Ireland) [249, 251].

6.2.7. Statistical analysis

Except for the 95% confidence intervals on the S:G ratio that were conducted in Excel, all other statistical analyses including Pearson correlation coefficients and p-values, box plots, fully-nested ANOVAs, and Tukey's pairwise comparisons (95% CI) were conducted using Minitab16 Statistical Software (2010 Minitab Inc, Pennsylvania, USA). Pearson coefficients and p-values were determined for the correlations between different cell wall components in the untreated samples and for the correlations between cell wall components and sugar yields

from untreated and pretreated samples. For the 4CL samples, the fully nested ANOVAs and general linear models for Tukey's comparisons were conducted by nesting sample pool within the line, and the line within the strength of downregulation. For the CCR samples, the pool was nested within the parent line. For Tukey's comparisons on sugar yields from the CCR and F5H samples, the interactions between pretreatment x line and pretreatment x pool were also included in the general linear model. Letters were used to indicate statistically different sugar yields and cell wall component values based on Tukey's pairwise comparisons ($\alpha < 0.05$).

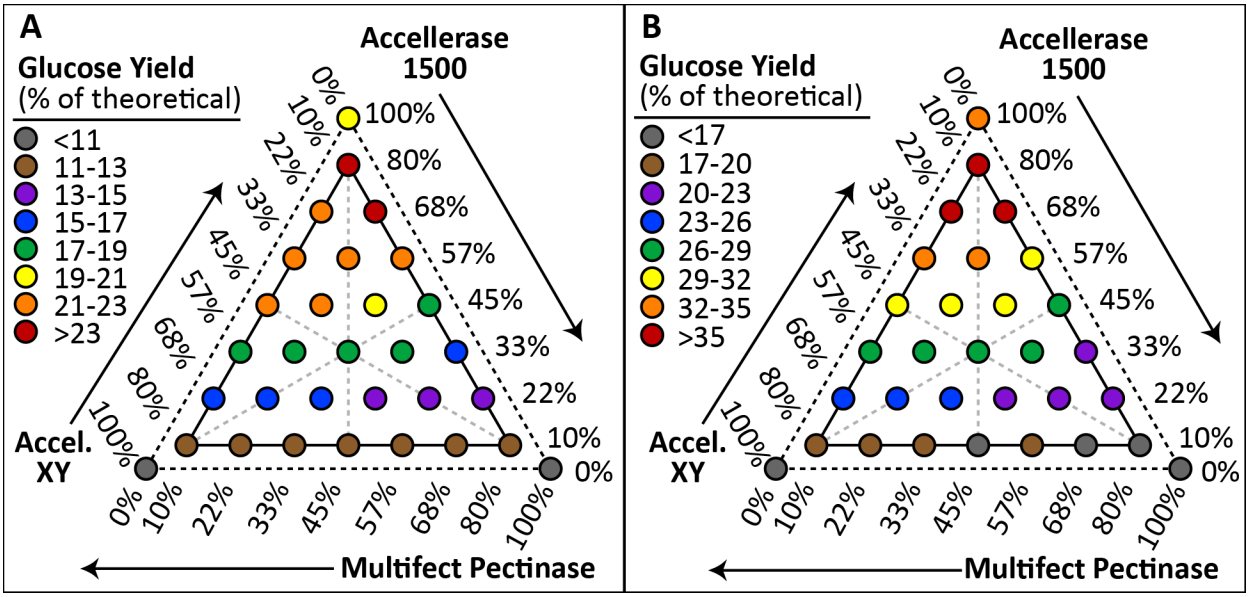

Figure 6.1: Ternary diagrams for enzyme optimization experiments on AFEX™-treated poplar (NM-6) using either (A) 15 or (B) 30 mg total protein per g glucan. Hydrolysis performed was in microplates at 0.2% glucan loading with quadruplicates of each enzyme combination. Glucose yields are reported as the percentage of the theoretically available glucan (hemicellulose glucan and cellulose) in untreated, dry biomass that was released.

6.3. Results and discussion

6.3.1. Enzyme optimization

For previous work where AFEX™-treated poplar was hydrolyzed at a constant cellulase loading, supplementation with xylanase significantly increased glucan and xylan conversion, which is expected given that AFEX™-pretreated poplar retains all of the xylan going into enzymatic hydrolysis [252]. However, no work was done to determine an optimum enzyme mixture for a constant enzyme loading, which is what we attempted to do for our experiments using NM-6 hybrid poplar. Glucose yields for the various enzyme combinations are reported in **Figure 6.1**. Xylose yields were also measured using an enzyme-based assay, however the replicate error in most cases was greater than the difference between enzyme combinations, and no conclusions could be made. For our experiments, the trends in yields were similar regardless of the enzyme loading used (15 mg total protein per g glucan or 30 mg total protein per g glucan). This similarity in optimum enzyme combinations for different enzyme loadings was also observed for AFEX™-treated switchgrass (refer to Chapter 4**)**. In all cases, the xylanase (Accellerase® XY) and pectinase (Multifect® Pectinase) by themselves gave the lowest yields; however, some supplementation with the accessory enzymes was necessary to give the highest yields. Compared to the optimum enzyme combination determined for switchgrass, around 50% cellulase, 20% xylanase and 30% pectinase (refer to Chapter 4); the optimal enzyme mixture for poplar required a much higher cellulase content, as much as 80% of the enzyme mixture. Poplar glucan tends to be much more indigestible compared to grass glucan [14] and this higher cellulase loading may be necessary to compensate. For further experiments, the

optimal enzyme mixture was set at 80% Accellerase® 1500, 10% Accellerase® XY, and 10% Multifect® Pectinase.

6.3.2. Influence of 4CL downregulation on cell wall composition

4-coumarate:CoA ligase (4CL) occurs fairly early in the lignin synthesis pathway (**Figure 1.4**) and catalyzes the conversion of *p*-coumaric acid to the thioester, *p*-coumaryl-Co-A. 4CL downregulation in plants typically results in decreased total lignin content [253-256]. This was observed for our samples, with all but line 1 containing less lignin than the control line (**Figure 6.2**). There was also a decreasing trend in lignin content as the strength of downregulation increased (**Figure 6.3**). However there was no statistical difference in lignin content between the medium and strong downregulation. This is in contrast to findings by Voelker et al. [257]; although thioacidolysis results indicated a reduced total lignin content for the 4CL transgenic lines compared to the control, there was very little reduction in acetyl bromide lignin content (~10%). They attributed this to the presence of flavonoids that were unable to be extracted by the sample preparation methods and interfered with the UV absorbance readings. There is the possibility that this may have occurred with our samples, which would result in overestimates of the transgenics' lignin content, and mean an even stronger decrease in lignin content for these samples compared to the control.

In some 4CL downregulated plants, the decrease in total lignin content is sometimes concurrent with an increase in the percentage of cellulose [253-255], however it is unclear from reported data whether the apparent increase is due to the plant producing more cellulose or less biomass. In other studies there is no significant change in structural sugars [256]. For our

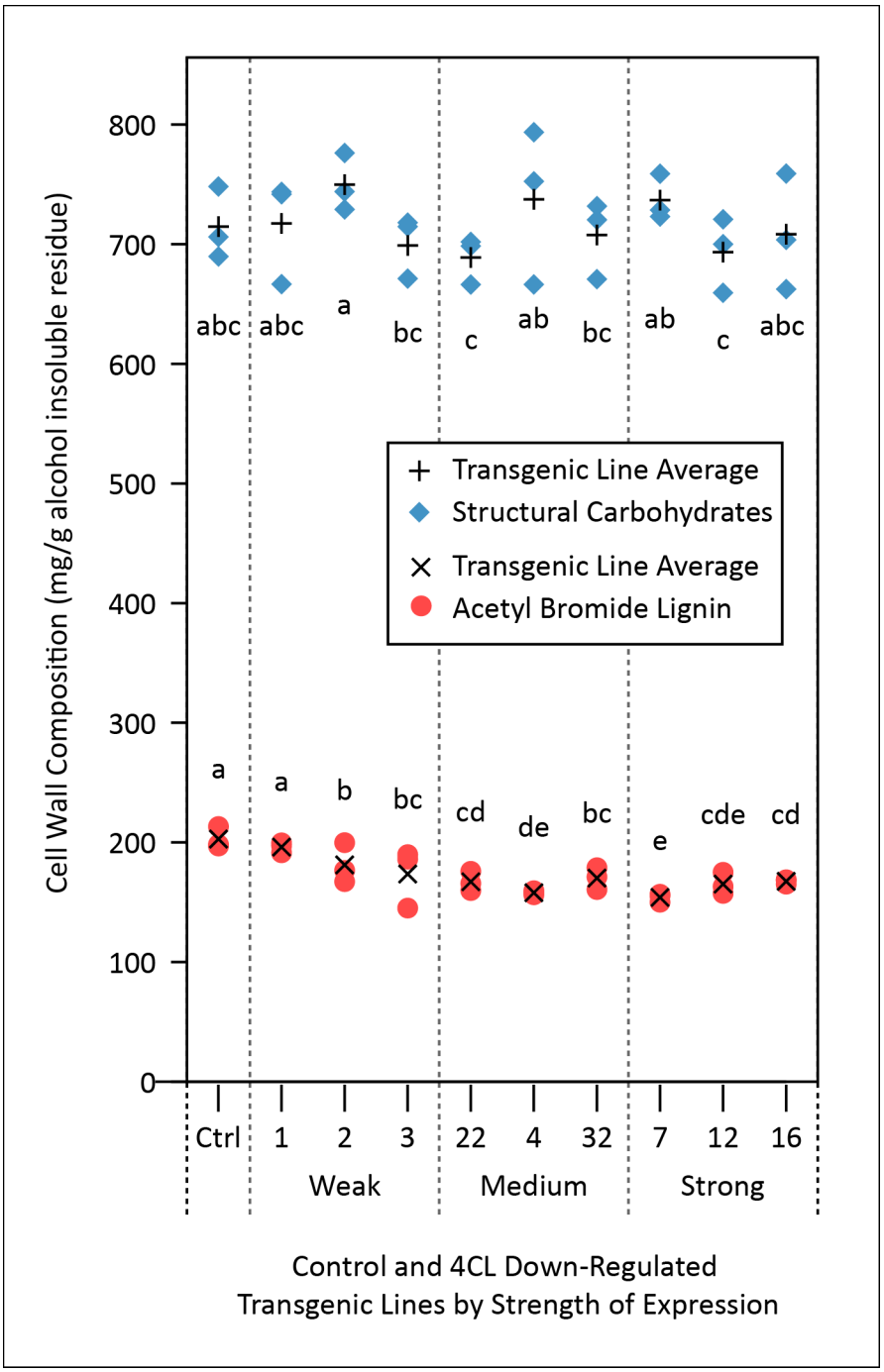

Figure 6.2: Structural carbohydrate and acetyl bromide lignin content of the control and 4CL downregulated transgenics, arranged by strength of downregulation (weak, medium, or strong) and parent line. Each data point represents one pool. The (**+**) symbols represent the average structural carbohydrate content across pools for each transgenic line, and the (**x**) symbol represents the same for acetyl-bromide lignin. Values with different letters for the average line structural carbohydrates or lignin are statistically different based on Tukey's pairwise comparisons (95% CI) ($p < 0.05$).

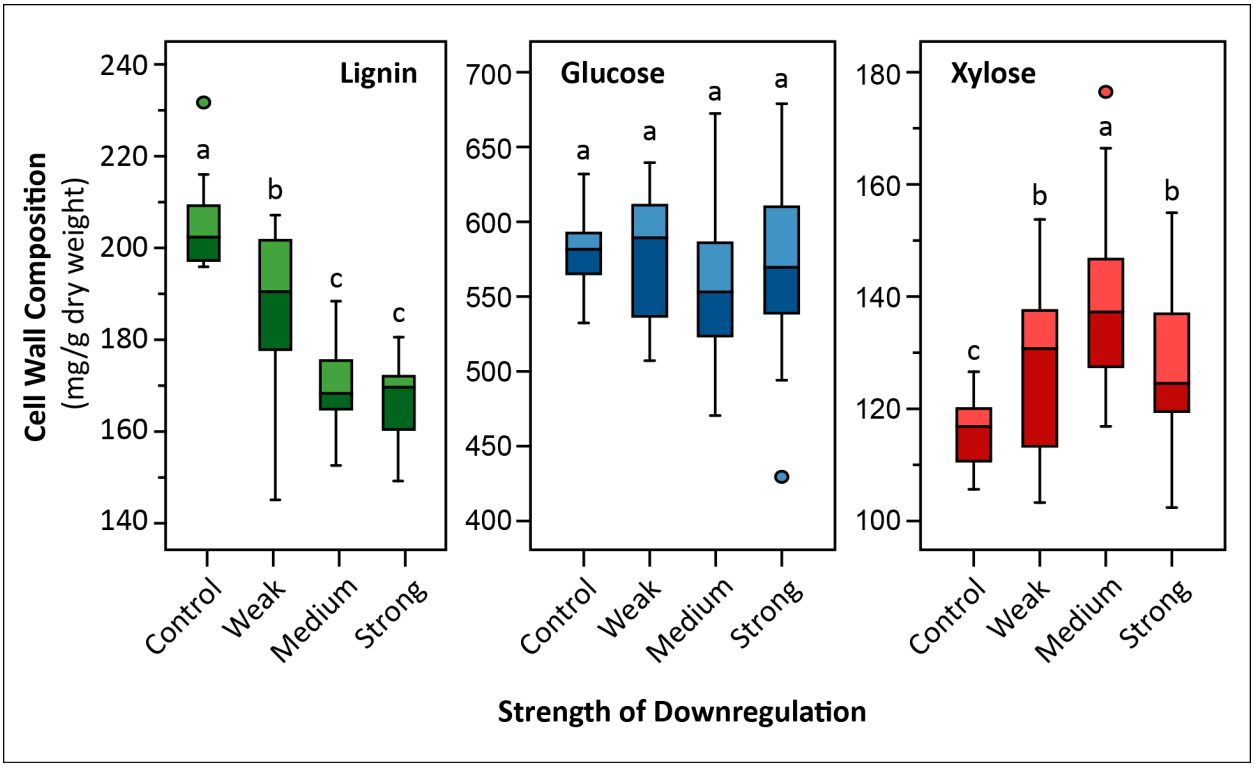

Figure 6.3: Boxplot of lignin, total glucose (from hemicellulose glucan and crystalline cellulose), and xylose within the cell wall for the different strengths of 4CL downregulation compared to the control. Boxplots with different letters within each component are statistically different based on Tukey's pairwise comparisons (95% CI) ($P < 0.05$).

samples there was very little statistical difference in total sugar content between the lines (**Figure 6.2**). Of the parent lines, line 2 had the highest structural carbohydrate content, and lines 22 and 12 had the lowest, and none of the lines were statistically different from the control. Total glucose contents for the different strengths of downregulation were statistically identical to the control (**Figure 6.3**), but xylose contents were statistically greater. While this could indicate that some of the lines responded to the downregulation by increasing xylan deposition within the cell wall, there was no statistical correlation between the lignin content

and xylose content for the samples (**Table 6.1**). This indicates that a reduction in lignin content in a specific sample did not necessarily correspond to an increase in xylan.

The acetyl bromide lignin content was most influenced by the strength of the downregulation, which explained 52% of the between sample variance (**Table D.2**). For the structural sugar content, the biggest influence on differences between samples was from between pool effects, as opposed to differences between the parent lines or the strength of downregulation. This indicates that the downregulation itself did not contribute the most significant effects to differences in sugar content between the different samples, but differences were mostly due to natural variability. Three of the sugars, mannose, glucose, and crystalline cellulose were most influenced by error in the method. Glucomannans, particularly those with low galactose substitution such as are present in hardwoods, can be tightly associated with the cellulose microfibril [258]. It is likely that small errors in sample preparation may not solubilize the most tightly associated glucomannans. There is a small set of samples with decidedly lower glucose and mannose hemicellulose sugar contents, so It seems likely that for these samples the glucomannans were not effectively solubilized during composition processing (**Figure D.1**). The hemicellulose glucose, and perhaps the mannose, would be measured as cellulose in the colorimetric assay used for cellulose quantification, and we do see that those samples with an abnormally low glucose content also had an abnormally high cellulose content. This error should not have strongly impacted the enzymatic hydrolysis, as samples were loaded on total cell wall sugars basis rather than a cellulose basis, and mannose content for the samples were comparatively low.

Table 6.1: Pearson coefficients for 4CL poplar sample cell wall components. Data were analyzed as the combined wildtype and transgenic samples.

	Ara	Xyl	Man	Gal	Glc	Cry	Total Sugars
Xyl	0.50[a]						
Man	-0.20	-0.04					
Gal	0.19	-0.19	0.68[a]				
Glc	-0.23[c]	0.04	0.96[a]	0.57[a]			
Cry	-0.11	-0.48[a]	-0.14	0.23[c]	-0.25[c]		
Lignin	0.05	-0.04	0.25[c]	0.22[c]	0.18	-0.04	-0.001

Influence was statistically significant with [a]$p = 0.000$, [b]$p < 0.01$, [c]$p < 0.05$.
Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; Cry = crystalline cellulose;

Of the cell wall sugars, there was a high level of positive correlation ($p = 0.00$) between xylose and arabinose ($R = 0.50$); mannose, galactose and glucose ($R \sim 0.57$-0.96); and a strong negative correlation between crystalline cellulose and xylose content ($R = -0.48$), which is more difficult to interpret (**Table 6.1**). The strong correlation between glucose and mannose ($R = 0.96$) relates to the sample glucomannan content, the second most abundant hardwood hemicellulose after 4-*O*-methyl-glucuronoxylan [18, 259, 260]. The small amount of arabinose and galactose, and their weaker correlations to xylose and mannose/glucose, respectively ($R \sim 0.5 – 0.7$) could be related to glucuronoarabinoxylan and galactoglucomannan which can be present in very small amounts in dicot cell walls [15]. However, most of the rhamnose, arabinose and galactose are likely derived from pectins, which are a major component of the dicot primary cell wall [16]. Of the sugars, mannose and galactose have a slight positive correlation to lignin content ($p < 0.05$, $R = 0.25$). In work examining lignin-carbohydrate linkages

it was found that galactose and xylose residues are the most common residues linked to beech residual lignin, and mannose and galactose residues are most commonly linked to spruce residual lignin [261]. The observed correlation may indicate that there is a tendency for reduced linkages between the hemicellulose sugars and lignin with decreased lignin content.

The S:G ratio for all the 4CL samples were between 1.8 and 2.2 (except for one sample at 2.4), which is a similar range to what has been reported previously [253, 257]. There are conflicting reports on the effect of 4CL downregulation on the ratio of syringyl to guaiacyl monomers (S:G ratio) in angiosperms, in some cases showing no apparent change compared to the control [253, 254], and in others a slight increase for some of the lines [257]. Six out of the

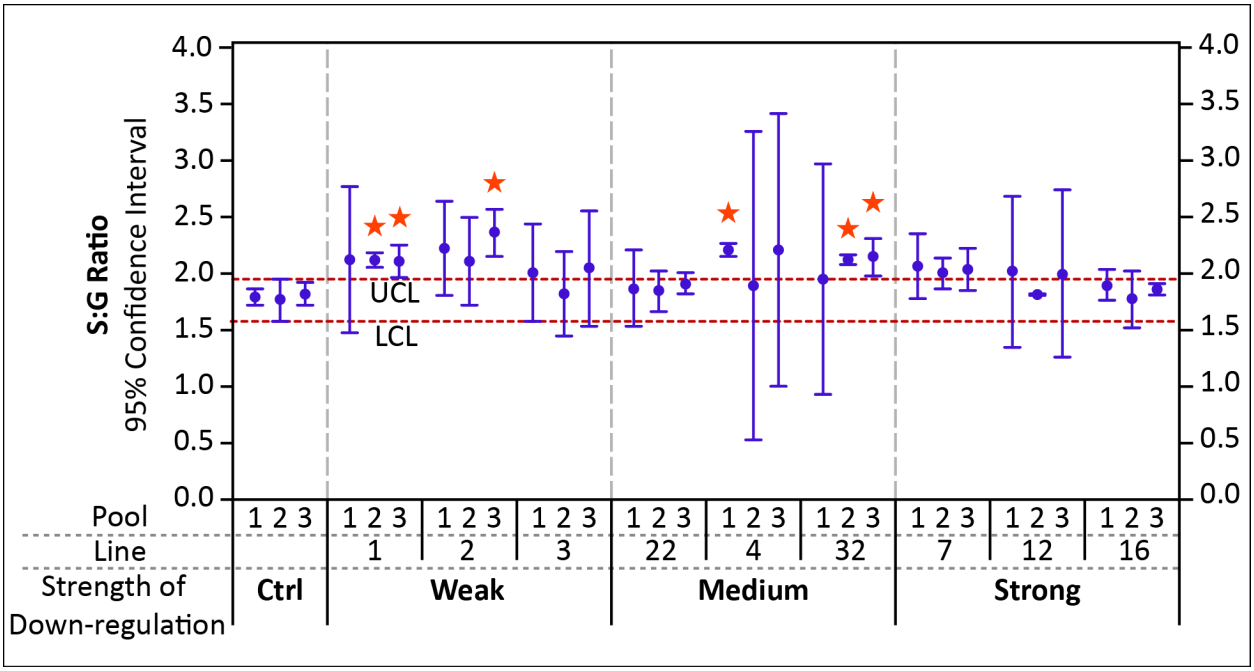

Figure 6.4: 95% confidence intervals around the mean S:G ratio for each 4CL control and transgenic line. UCL and LCL lines represent the upper and lower confidence limit for all the control samples. Stars represent lines that were statistically different from all the control samples. Ctrl = control.

27 transgenic lines showed statistically higher S:G ratios compared to the control (**Figure 6.4**), however, there appeared to be no relationship with either strength of downregulation or acetyl-bromide lignin content. In a population of 1,100 *Populus trichocarpa* samples, the average S:G ratio was 2.0, but ranged from 1.0 to 3.0 [241]. It is entirely likely that the range in S:G ratios observed for our samples fall within the natural variation for the population.

6.3.3. Influence of CCR downregulation on cell wall composition

Cinnamoyl-CoA reductase (CCR) occurs later in the lignin synthesis pathway than 4CL and catalyzes the transformation of feruloyl and *p*-coumaryl thioesters to their respective aldehydes (**Figure 1.4**). Like 4CL downregulation, CCR downregulation typically results in decreased total lignin content [238, 262-266]. For our samples, the control line had the highest lignin content, followed by 5-2-40, and then 5-2-3 (**Table D.1**). Of the samples, only 5-2-40 pool 3 had statistically identical lignin content to the controls. The lignin contents of all other transgenic lines were statistically identical to each other and lower than the controls. Like 4CL downregulation, there are also conflicting reports on the effect of CCR downregulation on structural carbohydrates. One experiment saw an increase in proportion of structural carbohydrates following CCR downregulation [265]. Another found an increase in cellulose content, but a decrease in hemicellulose sugars [266]. Arabinose was statistically lower in the transgenics; galactose was higher in 5-2-3; and xylose, mannose, hemicellulose glucose and crystalline cellulose were statistically identical for all the lines (**Table D.1**). Because glucose and xylose contents are statistically identical, when the enzymatic hydrolysis xylose and glucose sugar yields (% of theoretical) are compared between samples, they should show a similar

Table 6.2: Pearson coefficients for CCR poplar sample cell wall components. Data were analyzed as the combined wildtype and transgenic samples.

	Ara	Xyl	Man	Gal	Glc	Cry	Total Sugars	Lignin	S	G
Xyl	0.44[c]									
Man	-0.17	-0.42[c]								
Gal	0.15	0.48[c]	0.23							
Glc	-0.50[b]	-0.56[b]	0.90[a]	0.13						
Cry	-0.07	0.51[b]	0.01	0.25	-0.01					
Lignin	0.73[a]	0.13	0.07	-0.24	-0.21	-0.02	0.03			
S:G	-0.40[c]	0.05	-0.17	-0.01	0.09	0.26	0.21	-0.58[b]		
S	-0.27	-0.04	-0.09	-0.08	0.13	0.19	0.13	-0.43[c]		
G	0.46[c]	-0.09	0.19	-0.01	-0.08	-0.29	-0.25	0.64[a]	-0.91[a]	
H	-0.23	0.26	-0.15	0.22	-0.15	0.09	0.16	-0.20	-0.63[a]	0.25

Influence was statistically significant with [a]p = 0.000, [b]p < 0.01, [c]p <0.05.
Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; Cry = crystalline cellulose; S = syringyl lignin; G = guaiacyl lignin; H = *p*-hydroxyphenyl lignin

relationship to the mass sugar yields. As with the 4CL samples, there was a strong positive correlation between hemicellulose glucose and mannose contents (R = 0.90, p = 0.000) (**Table 6.2**) indicative of the presence of glucomannan in the samples.

The largest influence on differences in structural sugar contents between the CCR samples was due to between pool effects (**Table D.2**). This indicates that the downregulation did not contribute the most significant effects to differences in sugar content between the different samples. Conversely the lignin content was most strongly influenced by the transgenic modification. Like the sugars, the variability in the syringyl and guaiacyl lignin monomer content, was mostly explained by differences between pools (natural variation). For CCR down-regulaed angiosperms, generally there is a decrease in total monomer yield and an increase in the S:G

ratio [238, 263-265], though in some cases there is no significant difference [266]. In our case, the S:G ratio was negatively correlated with lignin content ($R = -0.58$, $p < 0.01$) (**Table 6.2**) and three of the six transgenic lines showed a statistically significant increase in the S:G ratio compared to the control samples (**Figure 6.5**). Because thioacidolysis selectively cleaves the most reactive β-O-4 ether linkages, the increase in the thioacidolysis S:G ratio from CCR downregulated materials has been attributed to a reduction in β-O-4 linked G units [263, 264].

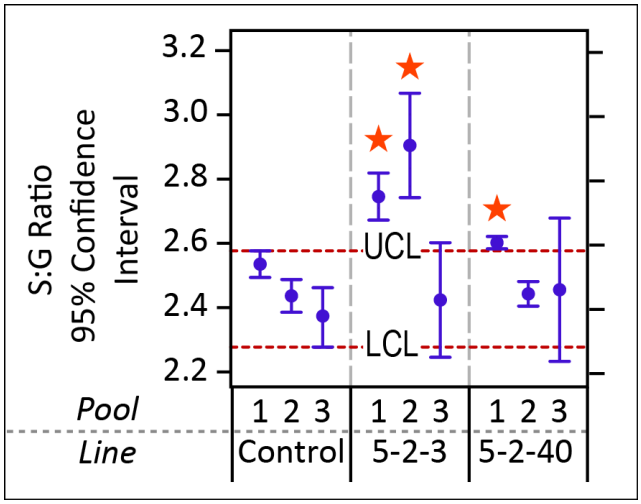

Figure 6.5: 95% confidence intervals around the mean S:G ratio for each CCR control and transgenic line. UCL and LCL lines represent the upper and lower confidence limit for all the control samples. Stars represent lines that were statistically different from all the control samples.

6.3.4. Influence of composition and pretreatment on enzymatic digestibility – F5H upregulation

Deposition of syringyl and guaiacyl lignin in the plant cell wall can be controlled by manipulating the ferulate-5-hydroxylase (F5H) enzyme, which controls the flux of

coniferaldehyde as a precursor to syringyl lignin (**Figure 1.4**). By knocking out the gene it is possible to produce plants with mostly guaiacyl residues, while up-regulating using a cinnamate-4-hydroxylase promoter (C4H) results in mostly syringyl residues [243, 267, 268]. The lignin chains resulting from such up-regulations are highly linear and lower in molecular weight compared to the control [243], and it is believed that these structural changes increase the ability to extract lignin polymers from the plant cell wall [243, 268]. Samples of C4H::F5H upregulated and control materials were initially examined for structural carbohydrate and total lignin content (**Table D.1**). Statistically there was no difference in any of the major cell wall carbohydrates between the control and transgenic samples ($p < 0.05$). Total acetyl bromide

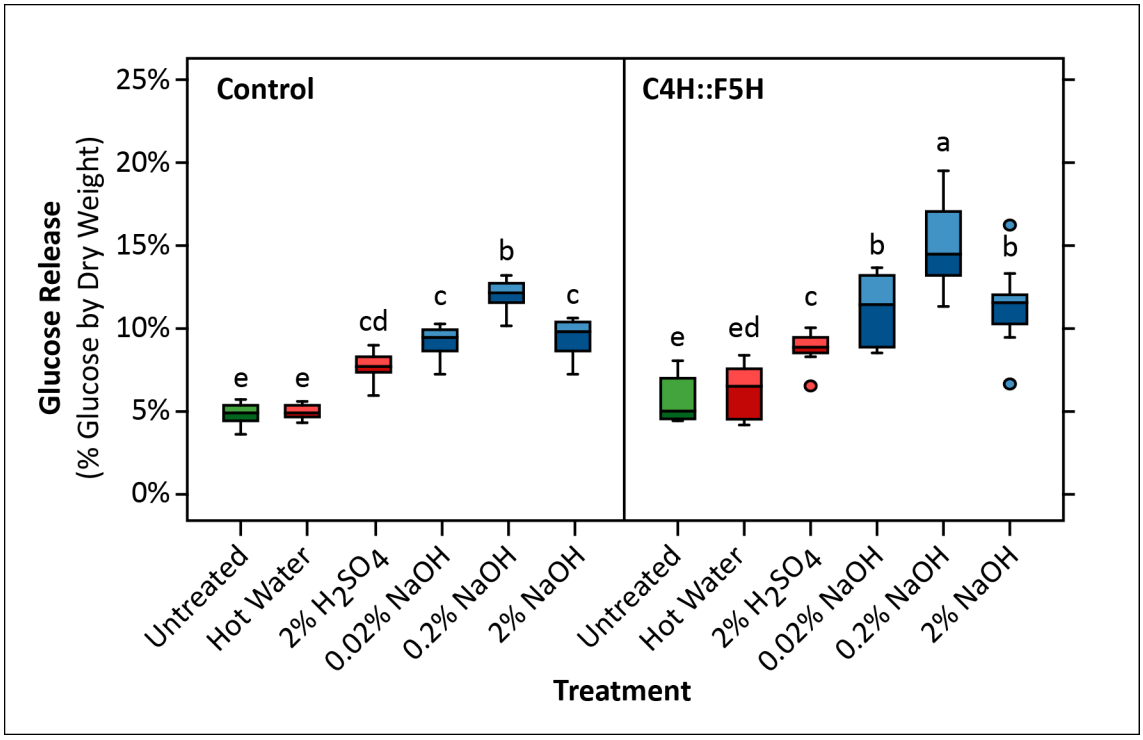

Figure 6.6: Pretreatment and enzymatic digestibility assay for control and C4H::F5H poplar samples. Green boxplots represent no pretreatment, red represents acidic, and blue represents alkaline. Boxplots with different letters have statistically different glucose release based on Tukey's pairwise comparisons (95% CI), ($p < 0.05$).

lignin content was higher for the control compared to the transgenic, although the statistical significance could not be determined. The lignin contents are similar to those reported for acid insoluble lignin of these materials by Stewart et al. [243]. In their case, the total amount of acid insoluble plus acid soluble lignin was the same for both samples.

When the samples were screened under a variety of different pretreatments followed by enzymatic hydrolysis (**Figure 6.6**), there was no difference in glucose release between the control and C4H::F5H transgenic for the untreated and acidic pretreatments, although in both cases the dilute acid pretreatment was more effective than both the untreated and hot water pretreatments. In contrast, the C4H::F5H transgenic released statistically greater quantities of sugars compared to the control under alkaline pretreatment conditions ($p < 0.05$). Sodium hydroxide, unlike sulfuric acid or other acidic pretreatments, selectively removes lignin from the plant cell wall [231, 269]. This supports the hypothesis that the C4H::F5H lignin is more extractable than control lignin under alkaline conditions, increasing the susceptibility of the poplar carbohydrates to enzymatic conversion. As the samples for the digestibility assays were loaded on a solids basis, there is the potential for differences in digestibility to be observed due to differences in total glucan content of the biomass. However, because the structural carbohydrate contents of the two samples were statistically identical, the observed increase in digestibility for the transgenic sample should be due primarily to the difference in lignin structure.

The same materials were also pretreated using AFEX^{TM} pretreatment at three different conditions: low-temperature, long-time; moderate-temperature moderate-time; and high-

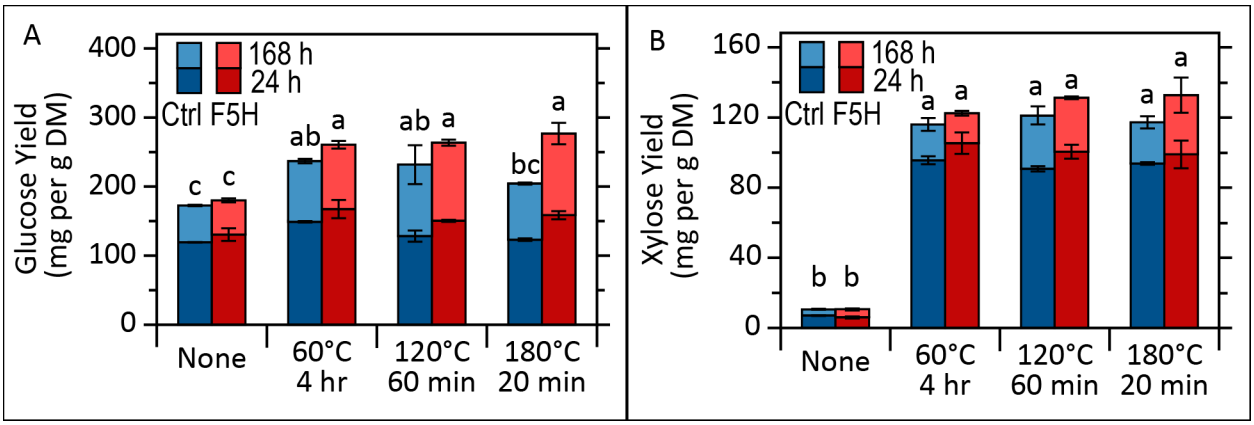

Figure 6.7: (A) Glucose and (B) xylose yields from control and C4H::F5H poplar samples for different AFEX™ pretreatment conditions. Sugar yields (168 h) with different letters within each subplot are statistically different based on Tukey's pairwise comparisons (95% CI), ($p < 0.05$). All samples were pretreated using 1:1 g NH_3:g DM and 1:1 g H_2O:g DM. Enzymatic hydrolysis was conducted at 200 rpm and 1.25% total sugar loading with 24 mg total protein per g cell wall sugars (80% Accellerase® 1500, 10% Accellerase® XY, 10% Multifect® Pectinase). Ctrl = Control; F5H = C4H::F5H.

temperature, short-time. The glucose and xylose yields from the untreated transgenic samples were identical to the control (**Figure 6.7**), as was observed for the digestibility assay. The xylose yields for the pretreated samples from both the control and the transgenic were statistically identical at all pretreatment conditions. Also, there is no statistical effect of pretreatment condition on xylose yields from either sample. For the control there was a lower glucose release for the 180°C, 20 min pretreatment that was not observed for the C4H::F5H sample. The glass transition temperature in lignin is often reported as being between 130-150°C [270] and it is possible that the lower molecular weight lignin in the C4H::F5H becomes more fluidic at the higher temperatures compared to the control, facilitating its removal from the cell wall. Interestingly there is not a large difference in yields across pretreatment conditions. This

indicates that it may be possible to operate at a lower temperature for a longer residence time and obtain similar yields from AFEX™-treated poplar. While there wasn't a significant improvement in yields for these novel transgenic materials, conventional AFEX™ also does not remove lignin as effectively as other alkaline pretreatments [30], this is at least partly due to the low liquid to solid ratio of the pretreatment method. For AFEX™-treated hardwoods there also seems to be limitations on accessibility to the cellulose [31]. More interesting results may be obtained by using a pretreatment method currently under development which uses liquid ammonia to simultaneously delignify the biomass [271] and generate the more readily digestible cellulose III_I allomorph [272].

6.3.5. Influence of composition and pretreatment on enzymatic digestibility – 4CL and CCR downregulation

Two studies have looked at 4CL and CCR downregulated transgenics for biofuel production. CCR down-regulated alfalfa showed improved *in vitro* dry matter digestibility and saccharification efficiency following dilute acid pretreatment, which was related to the amount of total lignin reduction [238]. In the study by Voelker et al. on poplar [257], except for the most severely suppressed lines which had reduced yields, 4CL downregulated transgenics showed no difference in total sugar released (glucose + xylose) following hot water pretreatment and enzymatic hydrolysis compared to the control (around 0.55 g sugar per g DM). However they also loaded 72.5 mg of enzyme per g of biomass. For their samples this was equivalent to around 150 mg of enzyme per g glucan, which is roughly 4.5 to 5 times the

amount of enzyme used in this study and most likely significantly overloading the amount required for conversion. This is particularly true for the small particle size used for their microplate hydrolysis, where enzyme mass transfer limitations are less significant. Saturating with enzymes can mask differences in sugar yields between feedstocks.

The actual sugar yields from untreated and pretreated control and transgenic 4CL and CCR poplar for each line are reported in the supporting information (**Figure D.2 - Figure D.4**). For the pretreated 4CL materials, there was no difference in 24 h glucose yields between lines compared to the control. Except for line 22 (medium downregulation), all other samples had higher 24 h xylose yields than the control, with the highest from lines 7, 32 and 3. Only lines 22 and 3 had higher 168 h glucose yields compared to the control, and lines 7, 32, 3, 2, and 12 had the highest xylose 168 h xylose yields, all higher than the control. For the pretreated CCR samples, 5-2-3 consistently had higher glucose and xylose yields compared to 5-2-40 and the control. While xylose yields from 5-2-40 were lower than the control. Glucose yields from untreated poplar were between 20-40% for all samples. Studer et al. also observed a high yield for untreated poplar [241]. In some cases their glucose release was as high as 0.36 g per g dry biomass. If their samples contained 500 mg glucose per g dry biomass, then this would equal a 70% glucose yield. Quite a few of their samples yielded between 0.1 and 0.2 g glucose per g dry biomass, which would be a 20-40% yield given the hypothetical glucose content.

Glucose release from the 4CL untreated poplar samples was negatively correlated with xylose content ($R = -0.54$, $p = 0.000$) and was not correlated with lignin content (**Table 6.3**, **Figure 6.8**). Once pretreated, the correlation with xylose content disappears. As the pretreatment yields indicate, AFEX^{TM} pretreatment is very effective at solubilizing and

releasing all of the 4CL poplar xylan (**Figure 6.8, Figure D.3**) and this is particularly interesting given the low release of xylose from untreated poplar (**Figure 6.8, Figure D.2**), even given the inclusion of xylanases in the enzyme cocktail. Removal of the interfering xylan from the cell wall improves access to the cellulose and removes the negative relationship between xylan content and glucan conversion. There is no negative relationship between xylose content and glucose yields from the untreated CCR poplar samples (**Table 6.3**), which, although they have a higher xylose content, have less difference in xylose content compared to the 4CL samples. In contrast

Table 6.3: Pearson coefficients for 24 h and 168 h enzymatic hydrolysis sugar yields from untreated and pretreated 4CL poplar samples. Data were analyzed as the conglomerate of all control and transgenic samples. AFEX^{TM}-pretreatment conditions were 1:1 g NH_3:g DM; 1:1 g H_2O:g DM; 180°C and 20 min.

	Cell Wall Composition					Sugar Yields		
	Total Sugars	Total Glucose	Xylose	Lignin	S %	24 Glc	24 Xyl	168 Glc
Untreated								
24 Glc	-0.06	0.10	-0.53[a]	0.13	-0.15			
24 Xyl	-0.20	-0.16	-0.01	-0.49[a]	-0.21	-0.30[c]		
168 Glc	-0.08	0.08	-0.54[a]	0.08	-0.16	0.97[a]	-0.23	
168 Xyl	-0.29[c]	-0.24	0.03	-0.38[b]	-0.18	-0.22	0.86[a]	-0.10
Pretreated								
24 Glc	-0.57[a]	-0.59[a]	0.38[b]	-0.15	-0.28[c]			
24 Xyl	0.29[c]	0.29[c]	-0.13	-0.43[b]	0.30[c]	0.20		
168 Glc	-0.60[a]	-0.64[a]	0.46[a]	-0.17	-0.25[c]	0.75[a]	0.02	
168 Xyl	0.18	0.14	0.08	-0.37[b]	0.25	0.21	0.69[a]	0.41[b]

Influence was statistically significant with [a]$p = 0.000$, [b]$p < 0.01$, [c]$p < 0.05$.
Glc = glucose; Xyl = xylose; S % = percentage of lignin monomers as syringyl lignin;

to the glucose yields, xylose yields from untreated 4CL samples were negatively correlated with lignin content. However, the yields are so low that there is not much difference between samples. CCR untreated poplar samples show different relationships compared to the 4CL samples in that both glucose and xylose yields were negatively correlated with lignin content (**Table 6.3**), though the impact is slight (**Figure 6.8**).

Although the transgenic modifications effectively altered lignin content in many of the samples compared to the controls, the modification did not have any impact on glucose yields (except for the untreated and 120°C/60 min pretreated CCR (**Table 6.4**)), which is somewhat surprising. Many other studies, including our own on AFEX™-treated mixed-species feedstocks (refer to Chapter 3) have observed a strong impact of lignin content on glucose yields from a variety of different materials [164, 234, 238, 239, 241], so it is unclear why there is no relationship in this case. Completely opposite to our earlier findings we find a negative correlation between lignin content and xylose yields for both CCR and 4CL materials (**Table 6.3**, **Table 6.4, Figure 6.8**). When the CCR transgenics are grouped with the control samples, there is no apparent relationship between lignin content and xylose release (**Figure 6.8 - K and L, dashed line**). However, when they are grouped separately (**Figure 6.8 - K and L, solid red lines**), a definite effect of lignin content on xylose release from the transgenics is observed.

Studer et al. [241] reported that poplar S:G ratios greater than 2.0 gave higher sugar release (glucose and xylose) compared to samples with S:G ratios less than 2.0. They state that at S:G ratios > 2.0 there is no impact by overall lignin content on sugar yields (glucose & xylose). We found very little impact of S:G ratio on sugar yields, perhaps because of the much smaller

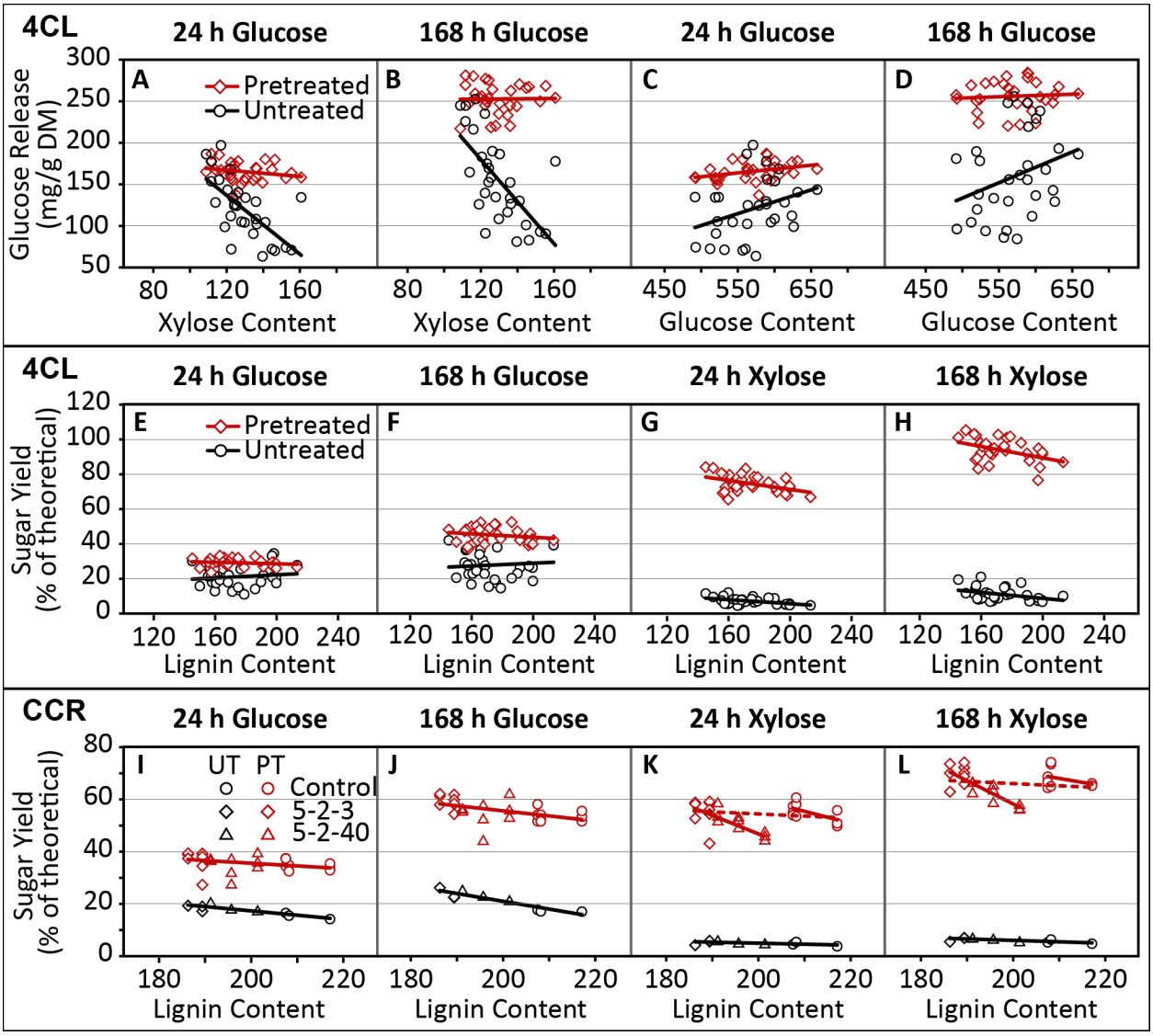

Figure 6.8: Correlations between sugar yields and cell wall composition (g/g DM) for 4CL (A-H) and CCR (I-L) poplar samples across all pretreatment conditions. The top row shows correlation of sample xylose content (A-B) and glucose content (C-D) on glucose release from 4CL poplar. The effect of lignin content on glucose yields (E-F, I-J) and xylose yields (G-H, K-L) is shown for 4CL and CCR poplar. Untreated poplar samples are shown in black and pretreated samples are shown in red. Sugar yields are expressed in terms of the total sugar theoretically present in the untreated dry biomass. Lines represent the linear regression for the respective parameters. The dashed lines in (K) and (L) represent all of the pretreated samples. The two solid lines represent the separate regression for transgenic samples and control samples.

Table 6.4: Pearson coefficients for 24 h and 168 h enzymatic hydrolysis sugar yields from untreated and pretreated CCR poplar samples. Data were analyzed as the conglomerate of all control and transgenic samples. AFEX^{TM}-pretreatment conditions were 1:1 g NH_3:g DM; 1:1 g H_2O:g DM for all pretreated samples.

	Cell Wall Composition					Sugar Yields		
	Total Sugars	Total Glucose	Xylose	Lignin	S %	24 Glc	24 Xyl	168 Glc
Untreated								
24 Glc	-0.04	-0.01	-0.06	-0.83[a]	0.12			
24 Xyl	-0.38	-0.26	-0.35	-0.50[c]	-0.26	0.56[c]		
168 Glc	0.24	0.30	0.05	-0.89[a]	0.35	0.91[a]	0.38	
168 Xyl	-0.24	-0.02	-0.42	-0.66[b]	-0.05	0.56[c]	0.88[a]	0.49[c]
60°C – 240 min								
24 Glc	-0.11	-0.37	0.31	-0.43	0.23			
24 Xyl	-0.61[b]	-0.50[c]	-0.46	-0.28	0.23	0.64[b]		
168 Glc	0.06	-0.16	0.34	-0.53[c]	0.41	0.86[a]	0.51[c]	
168 Xyl	-0.51[c]	-0.38	-0.44	-0.19	0.36	0.44	0.83[a]	0.57[c]
120°C – 60 min								
24 Glc	0.01	-0.04	0.08	-0.55[c]	0.60			
24 Xyl	-0.54[c]	-0.27	-0.65[b]	-0.31	0.53[c]	0.63[b]		
168 Glc	0.16	0.24	0.01	-0.61[b]	0.76[a]	0.87[a]	0.56[c]	
168 Xyl	-0.47	-0.18	-0.66[b]	-0.26	0.60[c]	0.51[c]	0.89[a]	0.58[c]
180°C – 20 min								
24 Glc	0.56[c]	0.41	0.51[c]	0.03	0.27			
24 Xyl	-0.05	0.18	-0.33	0.11	0.54[c]	0.51[c]		
168 Glc	0.63[b]	0.35	0.71[b]	-0.34	0.01	0.30	-0.45	
168 Xyl	-0.42	-0.08	-0.71[b]	-0.03	0.15	0.62[b]	0.10	-0.36

Influence was statistically significant with [a]P = 0.000, [b]P < 0.01, [c]P <0.05.

Glc = glucose; Xyl = xylose; S % = percentage of lignin monomers as syringyl lignin;

range of S:G ratios present in our samples. Glucose yields from pretreated 4CL had a slight negative correlation to the percentage of S-lignin in the samples, and the xylose content had a slight positive correlation. Of the CCR treatments, only the 120°C and 180°C treatments showed any relationship to monomer composition. The samples pretreated at 120°C had a positive correlation between the percent S-lignin and 24 h and 168 h xylose, and 168 h glucose. There may be some impact of the S:G ratio on sugar yields, but the correlations observed were not consistent across the different transgenics, nor strong enough to make any definite statements.

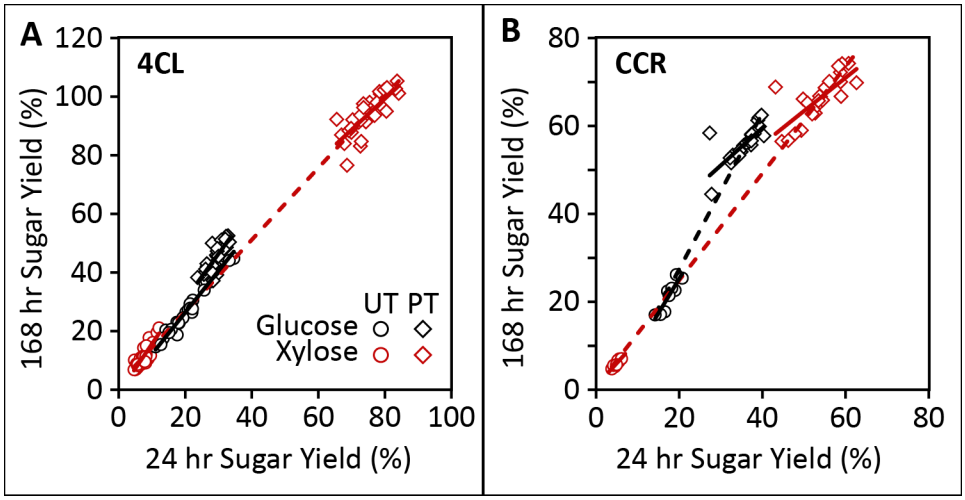

Figure 6.9: Influence of intial rate of hydrolysis at 24 h on 168 hr glucose and xylose yields from the control and transgenic (A) 4CL and (B) CCR control poplar. Xylose yields are shown in red and glucose yields in black. Solid ines represent regressions on the given sugar yields for separate untreated and pretreated groupings. Dashed lines in each figure represent the linear regression for untreated and pretreated samples as one group.

Of all the correlations, whether 4CL or CCR, untreated or pretreated (except the 180°C condition), the 24 h glucose yields had the largest, most significant relationship with 168 h glucose yields, and the same for 24 h xylose yields with 168 h xylose yields ($R \geq 0.83$ (except pretreated 4CL), $p = 0.000$) (**Table 6.3, Table 6.4**, **Figure 6.9**). While it may be a stretch to call 24 h sugar release an initial rate, the sugar release at this point is a deciding factor for the amount of sugars released from the biomass over longer periods of time. It also appears to have a greater impact on 168 h sugar yields than most of the other factors examined. It is difficult to release glucose from AFEX™-treated hardwoods, particularly at larger particle sizes [31]. One issue that may be hindering enzymatic breakdown of poplar samples, even more so than lignin content, is cellulose crystallinity. Although poplar and corn stover they have similar crystallinity indices, poplar crystallinity is much less affected by AFEX™. Cellulose crystallinity is also a major factor determining the initial rate of hydrolysis [273-275]. The increase in initial rate for ionic liquid pretreatment compared to AFEX™ is likely one reason for the comparatively higher yields from this method [276]. After a certain amount of cellulose decrystallization occurs, the cellulose crystallinity plays a less important role in limiting hydrolysis [274]. By 168 h, xylose should no longer be a major hindrance to cellulose conversion, as most of the xylan has been solubilized. However, it is possible that even after 168 h of conversion, hardwood cellulose crystallinity may still be a major limiter of yields. Zhu et al. also found that for long hydrolysis periods crystallinity is more important for yields when lignin content is higher [275]. As AFEX™ does little to remove lignin, it is likely that the issue of

poplar crystallinity is exacerbated. The addition of more enzymes can compensate for the hindrance of cellulose crystallinity [273, 275] however this is not a practical solution from an industrial perspective. Another possibility that might be effective is to completely alter the cellulose crystallinity by using an extractive ammonia pretreatment, such as is currently being developed in our laboratory and was mentioned previously [272].

In addition to the cellulose crystallinity, another factor that may be hindering glucose yields is the presence of hemicellulose still retained within the biomass. Although xylans are effectively released from AFEX™ treated hardwoods in the presence of hemicellulases, it is unknown what is happening with the other compounds, particularly glucomannnans. Glucomannans can be tightly associated with cellulose [258, 277], and it is unknown how they are affected by AFEX™ pretreatment. If the glucomannans are still associated with the cellulose microfibrils following pretreatment, and if sufficient mannan degrading enzymes are not present in the enzyme cocktail, these compounds could present a significant hindrance to conversion of hardwood cell walls, and even more so for softwoods which have a higher glucomannan content.

6.4. Conclusions

The optimum commercial enzyme cocktail for conversion of AFEX™ treated hardwoods contained primarily cellulase (Accellerase® 1500 – 80%) with supplemental amounts of hemicellulases (Accellerase® XY – 10% and Multifect® Pectinase – 10%), indicating that when

compared to the grass optimal enzyme mixture that contains around 50% cellulase, AFEX™-treated hardwood cellulose is comparatively more difficult to break down.

C4H::F5H poplar is a promising feedstock for biofuel production and performs better with alkaline pretreatments than acidic due to its more readily extractible lignin. For AFEX™ – pretreated materials however, there was very little difference between the transgenic and the control, indicative of the small amount of lignin AFEX™ typically removes from the biomass. A pretreatment with a higher liquid:solid ratio may perform better, improving extraction of biomass components. 4CL and CCR downregulations both resulted in transgenic lines with reduced lignin contents, and in some cases, increased S:G ratios. However, the reduction in lignin content did not serve to improve glucose yields, for which there was no relationship with overall lignin content. Instead reductions in lignin content were positively correlated to xylose yields both untreated and pretreated poplar samples. However, the general impact was still fairly low, less than 20% increase in yields for reductions in lignin content of 60 mg per g dry biomass. The impact of S:G ratio on sugar yields was inconclusive though in some cases there was some evidence for improvements in sugar yields due to an increased S:G ratio. Of the lines examined, some had higher yields compared to the control, but the differences were not very pronounced, particularly with respect to glucose yields. In general there was little difference in the AFEX™ pretreatment conditions tested, indicating that high temperature-short time pretreatments and low temperature-long time pretreatments may be interchangeable in terms of biomass conversion efficiencies.

AFEX™ is most successful at solubilizing xylan from hardwood materials, achieving 60-70% xylan yields within 24 hours. In most cases AFEX™ improved release of glucose, however in almost all cases, 168 hr hydrolysis yields were less than 60%. Two factors may be limiting conversion of hardwood cellulose: cellulose crystallinity, which is relatively unchanged by AFEX™ pretreatment, and the potential association of residual glucomannan with cellulose microfibrils, hindering enzyme access.

CHAPTER 7 :
CONCLUSIONS AND RECOMMENDATIONS

7.1. Conclusions

The characteristics of a given feedstock and its interactions with bioenergy conversion processes influence the decisions that are made with respect to bioenergy conversion at a variety of different scales. At the landscape scale, the distribution and availability of the feedstock is one factor that impacts the decision on where to locate a new biorefinery. In Mainland China, the majority of crop residues are produced in the central, eastern and northeastern regions, with the highest yields in areas with a high incidence of multi-cropping. Of all the provinces, Henan appears to be the location most suited for construction of a biorefinery due to the large amount of crop residues available for bioenergy, the potential for rural development, and a potential market for an animal feed co-product if using an AFEX^{TM} platform for ethanol production. The central, eastern, and northeastern regions of China all appear to be potential locations for a biorefinery due to the large amount of crop residues that are available for bioenergy.

Of the scales examined for interactions between feedstock and biomass processing, the classification scale and the component scale showed the largest differences between different materials. Comparatively, there was little difference in optimal pretreatment and hydrolysis conditions and resulting yields at the species scale for two different varieties of switchgrass. Across the scales, Klason lignin content was a major inhibitor of glucose yields. This was observed for both the mixed-species feedstocks and the corn stover fractions. Interestingly this was not observed for the poplar samples that had been modified for reduced lignin content, as

this alteration resulted in no significant improvement to glucose yields following AFEX™ pretreatment. In contrast, extreme changes to lignin composition showed some increase in sugar yields. The C4H::F5H transgenic poplar sample that had a high proportion of syringyl units was slightly more digestible than the control sample. This lignin is more extractable than the control poplar lignin, however because conventional AFEX™ does little to extract cell wall components, the difference in yields between the two samples was not very large. More distinct results may be obtained using a pretreatment that is able to more effectively solubilize and remove lignin from the plant cell wall.

Mixed-species feedstocks are an ecologically desirable bioenergy feedstock, but due to their inherent heterogeneity, they are often viewed negatively from a processing perspective. However, this seems to be an unfair assessment as mixed-species with a high proportion of grass species were as digestible as the conventional biofuel feedstock, corn stover. It may be possible to increase yields of both biomass and of soluble sugars following processing by managing mixed-species stands for a higher grass content. While this may counteract some of the environmental benefits of these systems, with some management they could still be more environmentally beneficial than a corn monoculture, and have better production and processing characteristics than a naturally occurring system.

Within the respective experiments on mixed-species feedstocks, switchgrass varieties, and corn stover fractions, it was found that similar processing conditions could be used to obtain high sugar yields from most of the materials. It may be possible to process samples harvested from a similar location and at the same maturity using the same pretreatment and

hydrolysis conditions. But because there will be some samples where the chosen processing condition is far from the optimum, it will be up to the operator to determine whether it is preferable to maintain set operating conditions and sacrifice some of the potential yields, alter operating conditions to maximize the yields, or blend feedstocks in order to make-up for deficiencies in low-yielding materials and improve process stability.

When harvesting plant materials, without considering other factors, it is most desirable to harvest the portions of the plant that give the highest yields. For corn stover, this is both technically feasible and the best option for increasing soil organic carbon levels. The optimum order of selective harvest was also the order of decreasing lignin content: husk > leaf > stem > cob. This order of harvest can be readily accomplished by raising the header on the combine and by ejecting the cob back onto the field following removal of the grain. Additionally, because of the longer half-life of lignin compared to the other cell wall components, by leaving behind the fractions with higher lignin content, soil organic carbon levels are more likely to increase. However, this selective harvest of corn stover may not provide sufficient ground cover to prevent wind and water erosion and would need to be examined in greater detail.

7.2. Recommendations for future research

There are broad opportunities to expand on the research that was presented here. In the chapter on distribution of crop residues within Mainland China, potential locations for a biorefinery were determined based on the available feedstock supply. This information provides an opportunity for future case studies that are based in one or more specific locations and that investigate the feasibility and the impacts of constructing bioenergy facilities,

potentially looking at the farm-scale economics, biorefinery economics, environmental impacts, and rural development opportunities. Additionally it would be worthwhile to compare the feasibility and impacts of constructing and operating a single centralized biorefinery versus a decentralized system with a single biorefinery and local biomass processing depots. The decentralized system holds a great deal of promise because farming in China, unlike the U.S., is still largely operated at the small-scale.

Another area of potential future research could be in development of crop models that examine the impacts of climate, topography, and management practices on cropland, the environment, and biomass yields. These types of models would allow better estimates of the amount of crop residues that should be left on field. As farming in China tends to be more intensive than in the U.S., models and assumptions that apply here may not be applicable within a Chinese context and a China-specific model would be a very valuable tool for furthering the biofuel industry.

At the classification scale there is a need for research that compares the differences between representative materials and potential biofuel feedstocks from each of the different biofuel classes. Additionally, although AFEX^{TM} pretreatment facilitates the removal of xylans from the cell walls of grasses, herbaceous dicots, and hardwoods, it is not well known how AFEX^{TM} pretreatment impacts the other types of hemicelluloses, particularly mannans that could be more strongly associated with cellulose within the plant cell wall. Future research could compare the impact of AFEX^{TM} on solubilization and redistribution of the different classes of hemicelluloses within the cell walls of different classes of plant materials. It is also

likely that the necessary mannases are either not present or are not present in sufficient quantities in the enzyme cocktails during enzymatic hydrolysis. Future work could also examine the impact of adding these classes of enzymes to enzymatic hydrolysis of hardwoods and softwoods to determine whether the recalcitrance of these materials is entirely due to the ineffectiveness of AFEX™, or whether the addition of appropriate enzymes is sufficient to alleviate this recalcitrance.

Another area of research that would benefit from further research is the nature cellulose recalcitrance within the hardwood cell wall. It will be very important to test these materials using the extractive AFEX™ pretreatment that is currently under development. It may be that the combination of lignin removal and the conversion of cellulose I to cellulose III is sufficient to increase digestibility of these materials.

Little difference was found between the processing conditions for the two varieties of switchgrass that were tested. However, it is important to test a larger number of switchgrass varieties in order to determine whether this is a generalization that can be made across the board. It is also important to determine the impact of harvest date on processing yields from switchgrass. There are indications that harvest date has a much more significant effect on yields than the differences between varieties, and this should be examined in greater depth, particularly with respect to the impact of maturation and over-wintering on the biomass structure and the subsequent impact on processing yields.

APPENDICES

APPENDIX A : SUPPLEMENTARY INFORMATION FOR CHAPTER 2

Table A.1: Paper and paperboard production (million metric tons) in 2006. Values for provinces with greater than 1 million metric tons of production (and Jiangxi) are from [93]. All other values are estimated from [92].

Paper and Paperboard Production (*million metric tons*)					
East	48.80	Central	12.96	West	3.24
Shandong	11.66	Henan	6.23	Sichuan	1.25
Zhejiang	10.44	Hunan	1.80	Ningxia	0.51
Guangdong	9.69	Anhui	1.20	Shaanxi	0.51
Jiangsu	7.58	Guangxi	1.15	Xinjiang	0.26
Hebei	3.70	Hubei	1.13	Yunnan	0.26
Fujian	2.54	Jiangxi	0.91	Chongqing	0.26
Shanghai	0.96	Inner Mongolia	0.26	Gansu	0.15
Heilongjiang	0.68	Shanxi	0.26	Guizhou	0.05
Liaoning	0.68	Hainan	0.00	Tibet	0.00
Jilin	0.48			Qinghai	0.00
Tianjin	0.26				
Beijing	0.15				

Table A.2: Total and non-pastured ruminant animals (million head), grazing and pastureland area (million ha), and carrying capacity per province in 2006. Data on ruminant production and pastureland area are from [70], and carrying capacity data are from [95], except for Heilongjiang, Jilin, Sichuan, Guizhou and Gansu, which were estimated.

	Cattle and Buffalo	Sheep and Goats	Grazing and Pasture Area	Land Carrying Capacity	Non-pastured Ruminants
	million head		*million ha*	*animal unit per ha*	*million animal units*
National	105.9	285.6	261.9	-	120.5
Beijing	0.2	0.8	0.0	-	0.4
Tianjin	0.3	0.4	0.0	-	0.3
Hebei	4.8	15.8	0.8	-	7.9
Shanxi	1.1	7.5	0.7	-	2.6
Inner Mongolia	6.1	50.6	65.6	0.198	3.3
Liaoning	3.3	6.8	0.3	-	4.7
Jilin	5.4	4.6	1.0	0.198	6.1
Heilongjiang	5.2	8.2	2.2	0.198	6.4
Shanghai	0.1	0.1	0.0	-	0.1
Jiangsu	0.3	4.0	0.0	-	1.1
Zhejiang	0.2	1.1	0.0	-	0.4
Anhui	1.4	5.4	0.0	-	2.5
Fujian	0.6	0.8	0.0	-	0.8
Jiangxi	2.2	0.6	0.0	-	2.3
Shandong	5.7	23.4	0.0	-	10.4
Henan	10.3	19.4	0.0	-	14.2
Hubei	3.1	3.0	0.0	-	3.7
Hunan	4.1	5.0	0.1	-	5.1
Guangdong	2.2	0.4	0.0	-	2.3
Guangxi	4.0	1.6	0.7	-	4.3
Hainan	0.8	0.6	0.0	-	0.9
Chongqing	0.9	1.2	0.2	-	1.2
Sichuan	9.9	17.1	13.7	0.143	11.3
Guizhou	5.1	2.2	1.6	0.143	5.3
Yunnan	7.3	8.3	0.8	-	8.9
Tibet	6.2	17.1	64.4	0.143	0.5
Shaanxi	1.7	6.7	3.1	0.416	1.7
Gansu	4.2	15.9	12.6	0.138	5.6
Qinghai	4.5	15.0	40.4	0.184	0.0
Ningxia	1.0	3.9	2.3	0.114	1.5
Xinjiang	3.8	38.4	51.1	0.134	4.6

Table A.3: Estimated amount of vegetable sown area cropped only with vegetables, either single-cropped or triple-cropped (not multi-cropped with other types of crops) for different regions in Mainland China. Regions defined based on Qiu et al. [96].

Region	Provinces	Percentage of Vegetable Sown Area Cropped Only with Vegetables
Northeast/North	Heilongjiang, Inner Mongolia, Jilin, Liaoning	90%
Northwest	Gansu, Xinjiang, Ningxia, Qinghai, Shaanxi, Tibet	70%
North China Plain	Beijing, Hebei, Henan, Shandong, Shanxi, Tianjin	50%
West	Chongqing, Guizhou, Sichuan, Yunnan	30%
Middle & Lower Yangtze	Hubei, Hunan, Jiangsu, Jiangxi, Shanghai, Zhejiang	10%
South	Fujian, Guangdong, Guangxi, Hainan	5%

Table A.4: National and provincial sown, cultivated, and fallow land area of cereals, legumes, tubers, oilseeds and cotton crops in Mainland China in 2006.

	Land Area *(million ha)*			
	Reported Sown Land Area	**Calculated Cultivated Land Area**	**Estimated Fallow Land**	**Estimated Cultivated Land Area of Crops of Interest**
National	124.63	112.62	9.60	103.02
Beijing	0.23	0.19	-	0.19
Tianjin	0.38	0.38	-	0.38
Hebei	7.36	5.73	-	5.73
Shanxi	3.46	3.93	0.47	3.46
Inner Mongolia	5.12	6.86	1.73	5.12
Liaoning	3.32	3.75	0.43	3.32
Jilin	4.61	5.31	0.70	4.61
Heilongjiang	9.45	11.35	1.89	9.45
Shanghai	0.19	0.24	0.05	0.19
Jiangsu	6.15	4.64	-	4.64
Zhejiang	1.78	1.83	0.05	1.78
Anhui	8.04	5.63	-	5.63
Fujian	1.52	1.22	-	1.22
Jiangxi	4.19	2.73	-	2.73
Shandong	8.60	6.60	-	6.60
Henan	11.65	6.90	-	6.90
Hubei	5.82	4.47	-	4.47
Hunan	5.89	3.51	-	3.51
Guangdong	3.08	2.59	-	2.59
Guangxi	3.65	3.29	-	3.29
Hainan	0.49	0.65	0.16	0.49
Chongqing	2.75	2.05	-	2.05
Sichuan	7.68	5.44	-	5.44
Guizhou	3.67	4.13	0.45	3.67
Yunnan	4.50	5.22	0.73	4.50
Tibet	0.20	0.35	0.15	0.20
Shaanxi	3.65	3.77	0.12	3.65
Gansu	3.00	4.41	1.41	3.00
Qinghai	0.42	0.52	0.10	0.42
Ningxia	0.88	1.06	0.18	0.88
Xinjiang	2.92	3.87	0.95	2.92

Table A.5: Crop residue production and use by province. The amount that is usable as fuel includes the amount used for rural energy and assumes this amount is either replaced by combustion of crop straw for electricity or that this energy is replaced by some other means. Values for the amount of crop residues burned on-field are from Wang and Zhang [100].

	Crop Residues (*million metric tons*)					
	Total	**Pulp and Paper**	**Animal Feed**	**Returned to Field**	**Usable as Fuel**	**Burned On-Field**
National	593.53	19.35	153.52	296.45	124.69	103.34
Beijing	1.20	0.04	0.50	0.57	0.09	0.25
Tianjin	1.90	0.08	0.44	1.14	0.25	0.34
Hebei	32.70	1.10	10.09	17.19	4.32	5.84
Shanxi	12.25	0.08	3.31	10.37	-1.51	2.26
Inner Mongolia	20.08	0.08	4.15	15.37	0.48	2.69
Liaoning	18.44	0.20	5.95	7.63	4.66	2.73
Jilin	29.64	0.14	7.77	10.61	11.11	4.53
Heilongjiang	38.11	0.20	8.20	21.74	7.96	4.56
Shanghai	1.28	0.29	0.12	0.57	0.30	0.31
Jiangsu	37.86	2.26	1.46	13.93	20.22	9.53
Zhejiang	10.13	3.11	0.55	5.34	1.14	2.61
Anhui	37.46	0.36	3.19	16.88	17.03	9.74
Fujian	6.99	0.76	1.01	3.65	1.58	1.99
Jiangxi	20.38	0.27	2.96	8.20	8.95	1.76
Shandong	50.97	3.47	13.24	19.81	14.45	9.04
Henan	64.36	1.85	18.08	20.70	23.73	10.04
Hubei	30.34	0.34	4.77	13.40	11.83	2.65
Hunan	31.07	0.54	6.47	10.52	13.55	8.53
Guangdong	14.06	2.88	2.91	7.77	0.49	4.25
Guangxi	15.49	0.34	5.45	9.87	-0.18	4.76
Hainan	1.82	0.00	1.14	1.46	-0.77	0.55
Chongqing	9.62	0.08	1.51	6.16	1.88	1.03
Sichuan	33.54	0.37	14.42	16.32	2.43	3.29
Guizhou	13.11	0.01	6.81	11.02	-4.74	1.41
Yunnan	15.97	0.08	11.35	13.49	-8.95	1.76
Tibet	0.87	0.00	0.58	0.45	-0.16	0.06
Shaanxi	12.94	0.15	2.19	10.94	-0.34	2.17
Gansu	9.11	0.04	7.13	9.00	-7.07	1.37
Qinghai	1.58	0.00	0.05	0.97	0.57	0.18
Ningxia	3.35	0.15	1.88	2.63	-1.32	0.56
Xinjiang	16.91	0.08	5.84	8.75	2.24	2.56

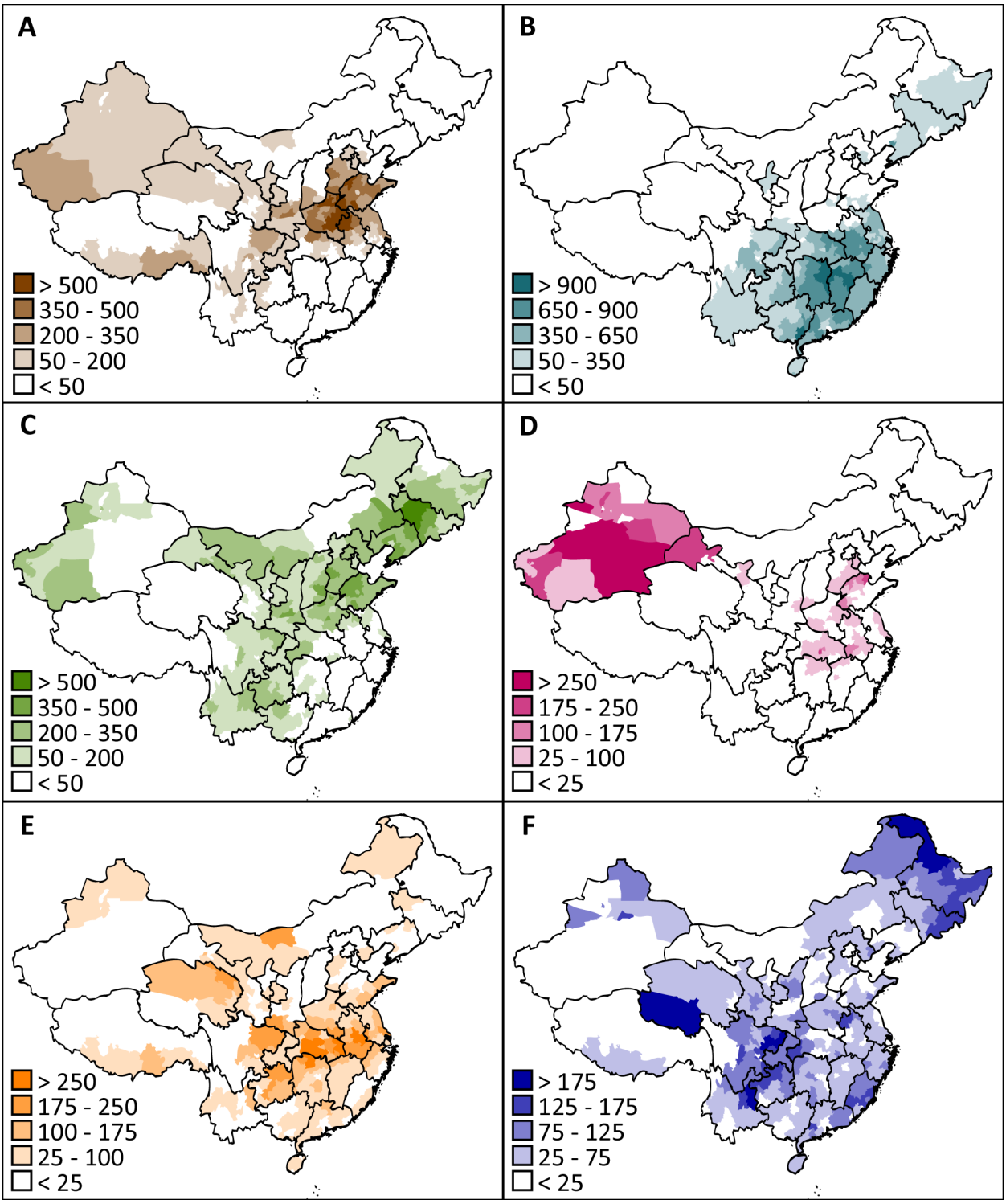

Figure A.1: Residue density based on prefecture cultivated area (Mg/ha) for different crops in 2006: (A) wheat, (B) rice, (C) corn, (D) cotton, (E) oilseeds, and (F) legumes and tubers.

APPENDIX B : SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Table B.1: Species composition of GLBRC early successional old field replicates.

Scientific Name	Common Name	Growth Habit*	ANPP (g/3m^2)**				
			R1 (E7)	R2 (E87)	R3 (E83)	R4 (E21)	R5 (E60)
Grasses							
Digitaria sanguinalis (L.) Scop.	Hairy Crabgrass	A	128.1	1040.5	540.0	630.7	1515.8
Echinochloa crus-galli (L.) Beauv.	Barnyardgrass	A	35.0	683.4	1699.9	-	0.99
Panicum dichotomiflorum Michx.	Fall Panicgrass	A	-	0.8	-	-	-
Poa compressa L.	Canada Bluegrass	P	0.0	-	-	-	-
Setaria faberi Herrm.	Giant Foxtail	A	-	-	-	-	18.35
Setaria viridis (L.) Beauv.	Green Foxtail	A	-	1.9	19.8	-	14.2
Forbs							
Abutilon theophrasti Medikus	Velvetleaf	A	38.9	46.7	27.1	40.9	-
Amaranthus retroflexus L.	Redroot Pigweed	A	248.7	-	60.5	1219.5	319.8
Capsella bursa-pastoris (L.) Medicus	Shepherd's Purse	A	285.3	5.6	4.1	0.5	0.1
Chenopodium album L.	Lambsquarters	A	1378.7	207.8	379.5	1134.5	720.5
Lamium purpureum L.	Purple Deadnettle	A	0.1	-	-	-	-
Phytolacca americana L.	American Pokeweed	P	2.3	-	1.6	-	-
Silene alba (Mill.) E.H.L.Krause	White Campion	B/P	-	-	-	1.1	-
Stellaria media (L.) Vill.	Common Chickweed	A/P	60.8	-	0.1	0.1	-
Taraxacum officinale Weber	Common Dandelion	P	2.0	-	-	-	-
Trifolium pratense L.	Red Clover	B/P	-	-	-	-	0.0
Trifolium repens L.	White Clover	P	0.7	-	-	-	-
Veronica sp.	Speedwell	A/P	-	3.5	0.3	-	-
Unknown Dicots	-	-	0.1	-	0.0	-	-

*A = Annual, B = Biennial, P = Perennial; **ANPP = annual net primary productivity

Table B.2 : Species composition of LTER late successional old field replicates.

Scientific Name	Common Name	Growth Habit*	ANPP (g/5m^2)**		
			SF1	SF2	SF3
Grasses					
Arrhenatherum elatius (L.) Beauv. ex J. & C. Presl	Tall Oatgrass	P	421.4	-	-
Dactylis glomerata L.	Orchardgrass	P	5.9	-	-
Danthonia spicata (L.) Beauv. ex R. & S.	Poverty Oatgrass	P	-	0.9	-
Elytrigia repens (L.) Nevski	Quackgrass	P	286.9	-	-
Panicum sp.	-	P	-	1.5	-
Phleum pratense L.	Timothy	P	209.6	0.7	-
Poa compressa L.	Canada Bluegrass	P	0.6	29.3	0.2
Poa pratensis L.	Kentucky Bluegrass	P	142.8	-	0.1
Unknown Grasses	-	-	-	0.0	0.0
Forbs					
Achillea millefolium L.	Common Yarrow	P	17.7	-	0.0
Alliaria petiolata (Bieb.) Cavara & Grande	Garlic Mustard	A/B	-	2.9	12.7
Antennaria neglecta Greene	Field Pussytoes	P	-	0.5	-
Apocynum cannabinum L.	Indianhemp	P	-	0.3	*
Asplenium platyneuron (L.) Oakes	Ebony Spleenwort	P(F)	0.3	-	3.5
Aster pilosus Willd.	Hairy White Oldfield Aster	P	3.3	-	0.2
Barbarea vulgaris R. Br.	Garden Yellowrocket	B	0.0	-	*
Circaea lutetiana L.	Broadleaf Enchanter's Nightshade	P	-	-	2.5
Daucus carota L.	Queen Anne's Lace	B	7.3	-	-
Dianthus armeria L.	Deptford Pink	A/B	0.2	0.3	0.1
Desmodium sp.	Ticktrefoil	P	-	-	1.4
Erigeron annuus (L.) Pers.	Eastern Daisy Fleabane	A	-	-	0.1

*A = Annual, B = Biennial, P = Perennial, P(F) = Perennial Fern; **ANPP = annual net primary productivity

Table B.2 (cont'd): Species composition of LTER late successional old field replicates.

Scientific Name	Common Name	Growth Habit*	ANPP (g/5m^2)**		
			SF1	SF2	SF3
Forbs					
Euphorbia corollata L.	Flowering Spurge	P	-	-	2.0
Euthamia graminifolia (L.) Nutt.	Flat-Top Goldenrod	P	0.3	-	-
Geum laciniatum Murray	Rough Avens	P	-	-	4.9
Geum sp.	Avens	P	-	0.5	34.6
Hieracium sp.	Hawkweed	P	-	0.2	-
Lactuca canadensis L.	Canada Lettuce	A/B	-	-	0.8
Oxalis stricta L.	Common Yellow Oxalis	P	-	-	0.1
Parthenocissus quinquefolia (L.) Planch.	Virginia Creeper	P	-	-	5.7
Phytolacca americana L.	American Pokeweed	P	-	-	0.4
Polygonum convolvulus L.	Black Bindweed	A	0.0	-	-
Potentilla recta L.	Sulphur Cinquefoil	P	0.4	-	-
Rumex acetosella L.	Common Sheep Sorrel	P	3.7	1.6	-
Rumex obtusifolius L.	Bitter Dock	P	-	-	0.0
Silene alba (Mill.) E.H.L.Krause	White Campion	B/P	0.1	-	-
Solidago canadensis L.	Canada Goldenrod	P	200.9	-	-
Solidago nemoralis Ait.	Gray Goldenrod	P	-	0.5	-
Taraxacum officinale F.H. Wigg.	Common Dandelion	P	-	-	0.0
Torilis japonica (Houtt.) DC.	Erect Hedgeparsley	A	-	-	6.0
Trifolium pratense L.	Red Clover	B/P	-	-	0.9
Trifolium repens L.	White Clover	P	0.1	-	-
Verbena urticifolia L.	White Vervain	P	-	-	2.5
Veronica chamaedrys L.	Germander Speedwell	P	-	-	1.8
Unknown Dicots	-	-	0.1	0.2	7.0

*A = Annual, B = Biennial, P = Perennial, P(F) = Perennial Fern; **ANPP = annual net primary productivity

Table B.2 (cont'd): Species composition of LTER late successional old field replicates.

Scientific Name	Common Name	Growth Habit*	ANPP ($g/5m^2$)**		
			SF1	SF2	SF3
Woody					
Acer spp.	Maple	P	-	0.3	4.3
Celastrus orbiculatus Thunb.	Oriental Bittersweet	P	-	31.3	169.6
Crataegus spp.	Hawthorn	P	-	8.6	2.7
Elaeagnus umbellata Thunb.	Autumn Olive	P	-	0.3	8.3
Lonicera spp.	Honeysuckle	P	-	-	9.5
Populus sp.	Cottonwood/Poplar	P	-	-	1.0
Prunus serotina Ehrh.	Black Cherry	P	-	0.8	0.7
Quercus spp.	Oak	P	-	0.3	0.3
Rhamnus cathartica L.	Common Buckthorn	P	-	0.8	8.2
Rhamnus frangula L.	Glossy Buckthorn	P	-	61.5	1.5
Rosa sp.	Rose	P	-	-	8.1
Rubus allegheniensis T.C. Porter	Allegheny Blackberry	P	-	10.3	12.3
Rubus occidentalis L.	Black Raspberry	P	-	-	1.7
Rubus sp.	Blackberry	P	-	0.1	-
Sassafras albidum (Nutt.) Nees	Sassafras	P	-	2.5	-
Toxicodendron radicans (L.) Ktze.	Eastern Poison Ivy	P	-	0.0	1.7
Unknown Woody	-	-	-	0.3	3.9

*A = Annual, B = Biennial, P = Perennial, P(F) = Perennial Fern; **ANPP = annual net primary productivity

Table B.3 : Pretreatment conditions and total sugar yields for each design point for the early successional samples. Three extra design points were included in an attempt to improve the model fit. The center design point (red) was replicated three times. The pretreatment condition chosen for further experiments is highlighted in teal. The top five sugar yields for each sample and the highest total sugar yield for each sample are highlighted in orange and yellow, respectively. One sample of E87 (gray) was excluded from the analysis as a statistical outlier.

Pretreatment Conditions				Total Mono & Oligomeric Sugar Yields (g glucose + xylose released/ g untreated, dry biomass)				
Ammonia g:g DM	**Moisture** g:g DM	**Temp** °C	**Time** min	**E7**	**E21**	**E60**	**E83**	**E87**
0.5	0.5	135	17.5	267.8	344.4	432.0	482.7	441.8
0.5	1.25	90	17.5	250.7	358.3	436.5	457.4	416.9
0.5	1.25	135	30	267.2	302.6	468.8	395.5	480.7
0.5	1.25	135	5	260.3	367.7	403.2	431.1	446.0
0.5	1.25	180	17.5	217.5	287.7	374.8	493.1	399.5
0.5	2.0	135	17.5	268.3	346.0	518.9	482.4	471.2
1.25	0.5	90	17.5	284.3	353.8	457.0	471.5	471.5
1.25	0.5	135	30	266.8	368.6	464.1	527.1	482.3
1.25	0.5	135	5	271.6	307.9	404.1	460.9	411.6
1.25	0.5	180	17.5	225.2	291.8	398.4	532.5	349.4
1.25	1.25	90	5	303.4	342.7	424.7	479.0	511.3
1.25	1.25	90	30	271.8	359.5	492.3	488.7	428.2
1.25	1.25	135	17.5	285.2	369.7	469.9	490.2	482.9
1.25	1.25	135	17.5	297.6	377.6	460.2	386.9	500.4
1.25	1.25	135	17.5	289.7	362.6	483.8	491.4	484.0
1.25	1.25	180	30	220.7	345.3	393.6	492.8	424.4
1.25	1.25	180	5	280.4	302.7	486.0	460.5	385.8
1.25	2.0	90	17.5	266.3	343.1	447.8	428.0	463.8
1.25	2.0	135	5	271.2	347.7	442.6	490.6	455.7
1.25	2.0	135	30	277.5	343.5	486.1	466.7	501.0
1.25	2.0	180	17.5	278.4	328.4	506.7	475.0	417.2
2.0	0.5	135	17.5	301.4	361.6	472.6	434.8	484.6
2.0	1.25	90	17.5	305.7	398.8	446.3	417.2	474.2
2.0	1.25	135	5	290.8	361.0	457.3	386.6	295.9
2.0	1.25	135	30	256.9	362.0	478.7	412.1	481.7
2.0	1.25	180	17.5	273.6	293.4	436.9	369.7	417.5
2.0	2.0	135	17.5	286.6	342.3	529.9	406.5	493.4
Extra Design Points								
1.25	2.0	135	17.5	269.7	343.6	474.4	481.3	474.2
2.0	0.5	90	30	310.1	341.1	523.1	501.8	517.0
2.0	2.0	90	30	290.8	354.7	518.1	469.2	488.5

Table B.4: Response surface model coefficients for pretreatment optimization of the early successional samples in terms of total monomeric and oligomeric glucose and xylose release following enzymatic hydrolysis. A = ammonia loading (g:g DM), B = water loading (g:g DM), C = temperature (°C), D = residence time (min). Pred. R^2 = predictive R^2 value; Adj. R^2 = adjusted R^2 value.

	Response Surface Model Coefficients				
	E7	**E21**	**E60**	**E83**	**E87**
Constant	126.699	-80.802	153.556	-15.0175	172.089
A	53.740	163.313	25.934	99.8024	18.705
B	-67.235	19.372	-76.597	67.6287	-54.109
C	1.836	4.971	3.255	5.435	4.757
D	5.070	4.454	10.235	0.9736	1.810
A^2	-14.117	-27.168	-	-	-
B^2	-	-36.357	-	-	-
C^2	-0.008	-0.021	-0.012	-0.022	-0.023
D^2	-	-0.096	-	-	-
AB	-	-	-	-47.747	-
BC	0.532	0.623	0.807	-	0.510
CD	-0.037	-	-0.067	-	-
Pred. R^2	60.1%	0.0%	45.5%	66.5%	78.6%
Adj. R^2	72.5%	43.5%	62.4%	72.5%	83.4%

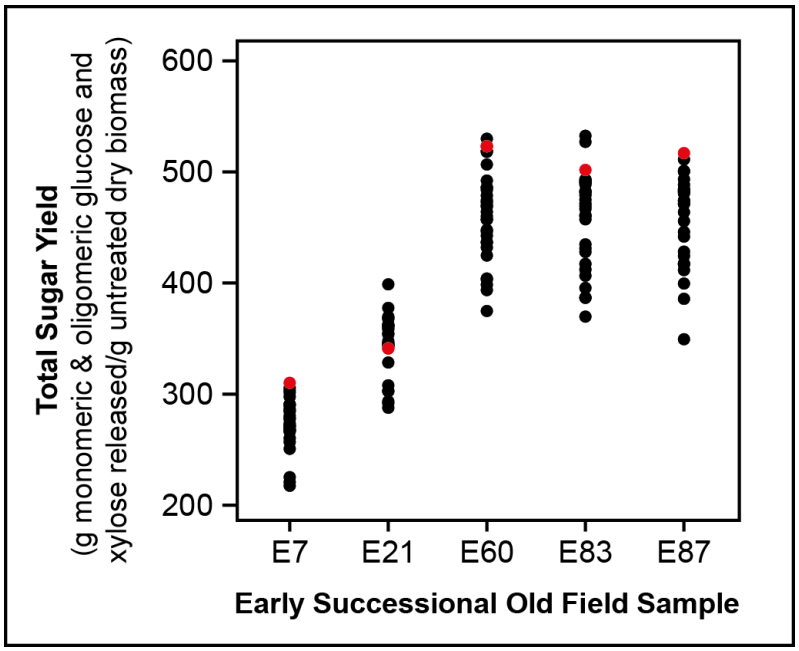

Figure B.1: Range of total sugar yields and location of the chosen pretreatment condition within the range for each early successional old field sample. The red circle represents the location of the pretreatment condition that was chosen as a basis for further experimentation within the range of the sugar yield raw data.

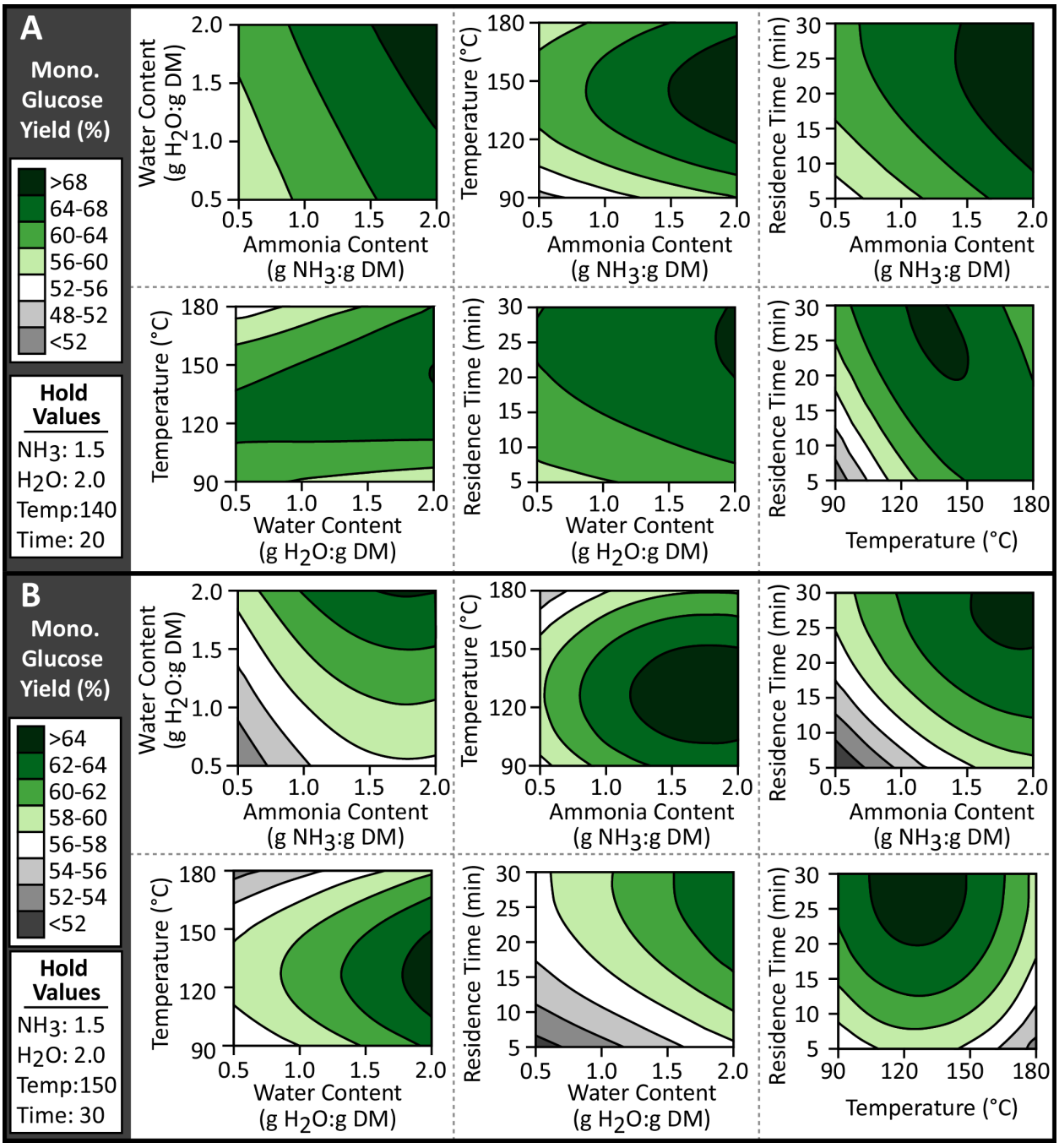

Figure C.1: Contour plots showing the interactive effect of pairs of AFEX™ pretreatment parameters on monomeric glucose yields from (A) Alamo and (B) Shawnee switchgrass. The two pretreatment parameters not shown in each sub-figure were held at the optimal level. Enzymatic hydrolysis was conducted at 50°C, 200 rpm, and 1% glucan loading using 30 FPU Spezyme® CP and 15 CBU Novozyme® 188 per g glucan, with 72 h sampling.

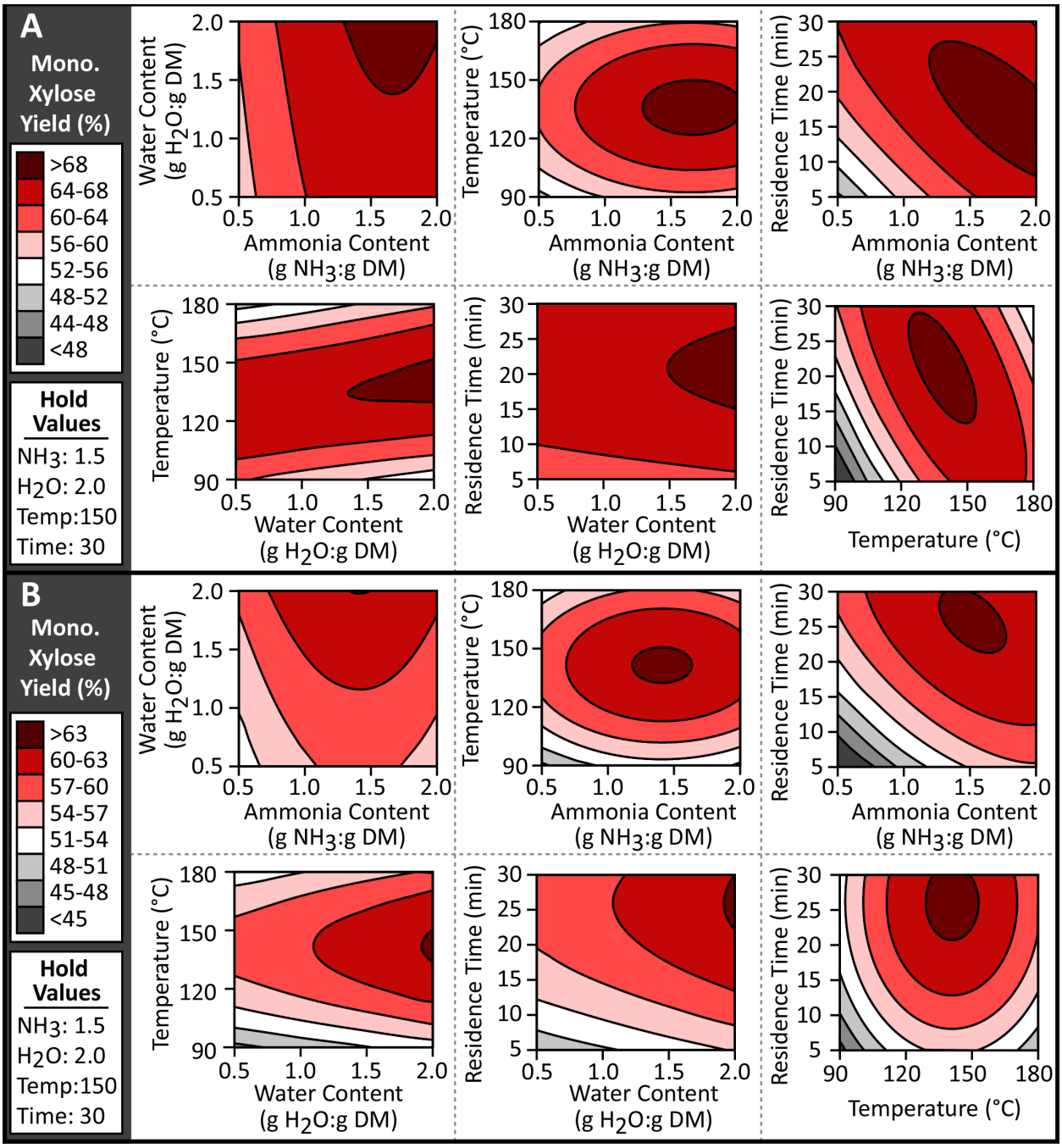

Figure C.2: Contour plots showing the interactive effect of pairs of AFEX™ pretreatment parameters on monomeric xylose yields from (A) Alamo and (B) Shawnee switchgrass. The two pretreatment parameters not shown in each sub-figure were held at the optimal level. Enzymatic hydrolysis was conducted at 50°C, 200 rpm, and 1% glucan loading using 30 FPU Spezyme® CP and 15 CBU Novozyme® 188 per g glucan, with 72 h sampling.

Table C.1: Experimental levels and additional design points for Box-Behnken response surface optimization of AFEX™ pretreatment conditions.

	NH_3 Loading	H_2O Loading	Temp	Time
	(g:g DM)	*(g:g DM)*	*(°C)*	*(min)*
Parameter Levels				
+1	2.0	2.0	180	30
0	1.25	1.25	135	17.5
-1	0.5	0.5	90	5
Additional Design Points				
Alamo	1.5	2.0	150	30
	2.0	1.25	150	15
	2.0	2.0	130	30
	2.0	2.0	150	30
	3.0	2.0	150	15
Shawnee	1.5	2.0	150	30
	2.0	2.0	150	30
	3.0	2.0	150	15

Table C.2: ANOVA of pretreatment optimization regression model for total sugar yields (monomers + oligomers of glucose and xylose) from Alamo and Shawnee switchgrass.

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
Alamo						
Regression	10	2616.21	2616.21	261.62	17.37	0.000
Linear	4	1220.96	1429.43	357.36	23.73	0.000
Square	3	1060.66	853.98	284.66	18.90	0.000
Interaction	3	334.59	334.59	111.53	7.41	0.001
Residual	21	316.25	316.25	15.06		
Total	31	2932.45				
Shawnee						
Regression	8	1625.05	1625.05	203.13	24.41	0.000
Linear	4	1215.21	589.42	147.36	17.71	0.000
Square	3	383.97	402.20	134.07	16.11	0.000
Interaction	1	25.88	25.88	25.88	3.11	0.092
Residual	21	174.77	174.77	8.32		
Total	29	1799.82				

[a]DF = degrees of freedom; [b]Seq SS = sequential sum of squares; [c]Adj SS = adjusted sum of squares; [d]Adj MS = adjusted mean square

Table C.3: ANOVA of Alamo switchgrass enzyme mixture regression. Total glucose and total xylose refer total monomeric + oligomeric sugar yields.

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
Mono Glucose						
Regression	11	6913.44	6913.44	628.49	146.85	0.000
Linear	3	280.72	958.23	319.41	74.63	0.000
Quadratic	4	1158.78	1048.65	262.16	61.25	0.000
Cubic	2	613.66	613.66	306.83	71.69	0.000
Amount	1	4826.39	2111.01	2111.01	493.24	0.000
Comp*Amt Quadratic	1	33.89	33.89	33.89	7.92	0.006
Residual	72	308.15	308.15	4.28		
Lack-of-Fit	42	163.91	163.91	3.90	0.81	0.737
Pure Error	30	144.24	144.24	4.81		
Mono Xylose						
Regression	13	4659.56	4659.56	358.43	183.84	0.000
Linear	3	2334.00	173.74	57.91	29.70	0.000
Quadratic	5	767.27	571.12	114.22	58.59	0.000
Cubic	2	313.52	321.19	160.60	82.37	0.000
Amount	1	1211.47	547.33	547.33	280.73	0.000
Comp*Amt Quadratic	1	19.77	24.70	24.70	12.67	0.001
Comp*Amt Cubic	1	13.53	13.53	13.53	6.94	0.010
Residual	69	134.53	134.53	1.95		
Lack-of-Fit	39	105.76	105.76	2.71	2.83	0.002
Pure Error	30	28.77	28.77	0.96		
Total Glucose						
Regression	11	6649.99	6649.99	604.54	149.21	0.000
Linear	3	738.02	1344.03	448.01	110.57	0.000
Quadratic	4	768.96	675.22	168.80	41.66	0.000
Cubic	2	504.46	504.46	252.23	62.25	0.000
Amount	1	4604.54	2741.82	2741.82	676.72	0.000
Comp*Amt Quadratic	1	34.00	34.00	34.00	8.39	0.005
Residual	72	291.72	291.72	4.05		
Lack-of-Fit	42	161.24	161.24	3.84	0.88	0.651
Pure Error	30	130.48	130.48	4.35		

[a]DF = degrees of freedom; [b]Seq SS = sequential sum of squares; [c]Adj SS = adjusted sum of squares; [d]Adj MS = adjusted mean square

Table C.3 (cont'd): ANOVA of Alamo switchgrass enzyme mixture regression. Total glucose and total xylose refer total monomeric + oligomeric sugar yields.

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
Regression	8	525.11	525.11	65.64	40.48	0.000
Linear	3	98.65	38.80	12.94	7.98	0.000
Quadratic	2	48.31	31.04	15.52	9.57	0.000
Cubic	2	48.45	50.64	25.32	15.62	0.000
Amount	1	329.70	329.70	329.70	203.34	0.000
Residual	73	118.36	118.36	1.62		
Lack-of-Fit	45	99.22	99.22	2.21	3.23	0.001
Pure Error	28	19.14	19.14	0.68		

[a]DF = degrees of freedom; [b]Seq SS = sequential sum of squares; [c]Adj SS = adjusted sum of squares; [d]Adj MS = adjusted mean square

Table C.4: ANOVA of Shawnee switchgrass enzyme mixture regression. Total glucose and total xylose refer to the total monomeric + oligomeric sugar yields.

Source	DF[a]	Seq SS[b]	Adj SS[c]	Adj MS[d]	F	P
Mono Glucose						
Regression	10	4935.22	4935.22	493.52	192.37	0.000
Linear	3	401.09	765.82	255.27	99.50	0.000
Quadratic	5	900.73	787.55	157.51	61.40	0.000
Cubic	1	311.10	311.10	311.10	121.27	0.000
Amount	1	3322.30	3322.30	3322.30	1295.02	0.000
Residual	73	187.28	187.28	2.57		
Lack-of-Fit	43	121.88	121.88	2.83	1.30	0.227
Pure Error	30	65.40	65.40	2.18		

[a]DF = degrees of freedom; [b]Seq SS = sequential sum of squares; [c]Adj SS = adjusted sum of squares; [d]Adj MS = adjusted mean square

Table C.4 (cont'd): ANOVA of Shawnee switchgrass enzyme mixture regression. Total glucose and total xylose refer total monomeric + oligomeric sugar yields.

Mono Xylose						
Regression	12	3642.71	3642.71	303.56	121.43	0.000
Linear	3	1642.84	197.57	65.86	26.35	0.000
Quadratic	3	490.23	273.68	91.23	36.49	0.000
Cubic	4	529.92	529.92	132.48	53.00	0.000
Amount	1	967.77	627.71	627.71	251.11	0.000
Comp*Amt Linear	1	11.95	11.95	11.95	4.78	0.032
Residual	71	177.49	17.49	2.50		
Lack-of-Fit	41	123.34	123.34	3.01	1.67	0.074
Pure Error	30	54.15	54.15	1.81		
Total Glucose						
Regression	9	4754.14	4754.14	528.24	210.74	0.000
Linear	3	803.90	1366.97	455.66	181.78	0.000
Quadratic	2	442.26	304.34	152.17	60.71	0.000
Cubic	2	344.40	344.40	172.20	68.70	0.000
Amount	1	3149.58	1929.27	1929.27	769.67	0.000
Comp*Amt Quadratic	1	14.01	14.01	14.01	5.59	0.021
Residual	74	185.49	185.49	2.51		
Lack-of-Fit	44	121.86	121.86	2.77	1.31	0.223
Pure Error	30	63.63	63.63	2.12		
Total Xylose						
Regression	9	540.98	540.98	60.11	43.69	0.000
Linear	3	83.95	73.91	24.64	17.91	0.000
Quadratic	2	50.78	32.91	16.46	11.96	0.000
Cubic	3	120.01	117.42	39.14	28.45	0.000
Amount	1	286.24	286.24	286.24	208.07	0.000
Residual	72	99.05	99.05	1.38		
Lack-of-Fit	44	63.80	63.80	1.45	1.15	0.351
Pure Error	28	35.25	35.25	1.259		

[a]DF = degrees of freedom; [b]Seq SS = sequential sum of squares; [c]Adj SS = adjusted sum of squares; [d]Adj MS = adjusted mean square

APPENDIX D : SUPPLEMENTARY INFORMATION FOR CHAPTER 6

Table D.1: Composition data for the NM-6 poplar, and F5H, CCR, and 4CL control and transgenic lines.

			Cell Wall Composition (*mg/g alcohol insoluble residue*)								**Lignin Composition (%)***		
			Structural Carbohydrates										
			Hemicellulose Sugars						**Crystalline**	**ABSL**	**Monomers**		
Expt	**Line**	**Pool**	***Rha***	***Ara***	***Xyl***	***Man***	***Gal***	***Glc***	**Cellulose**		**S**	**G**	**H**
NM-6			2.0 (0.3)	2.9 (0.1)	106.6 (5.8)	11.1 (0.8)	5.8 (0.3)	38.6 (4.6)	415.7 (14.3)	n.d.	n.d.	n.d.	n.d.
F5H	**Ctrl**		1.8^{a} (0.1)	1.6^{a} (0.1)	100.7^{a} (3.2)	4.1^{a} (0.2)	4.0^{b} (0.2)	16.8^{b} (1.0)	508.7^{b} (16.9)	191.4	65.5	34.5	-
	C4H::F5H		1.7^{a} (0.1)	1.5^{b} (-)	95.8^{b} (3.8)	4.1^{a} (0.2)	4.3^{a} (0.1)	18.5^{a} (2.1)	527.5^{a} (19.3)	157.0	93.4	6.6	-
CCR	**Ctrl**	**1**	3.4ab (0.3)	3.9a (0.3)	230.3ab (5.7)	7.1ab (0.5)	6.1b (0.4)	43.5cd (3.9)	408.5a (11.6)	207.5ab (5.4)	71.5bc (0.2)	28.2ab (0.2)	0.3c (0.1)
		2	3.4^{abc} (0.5)	3.6^{abc} (0.7)	210.5^{b} (21.6)	7.5^{ab} (0.5)	6.3^{ab} (0.9)	50.7^{ab} (1.7)	393.0^{a} (30.6)	208.2^{ab} (5.6)	70.7^{cd} (0.3)	29.0^{a} (0.2)	0.3^{c} (0.1)
		3	3.6^{a} (0.2)	3.9^{a} (0.3)	237.7^{ab} (12.4)	7.5^{ab} (0.1)	6.7^{ab} (0.5)	48.2^{bc} (1.7)	437.5^{a} (31.0)	217.1^{a} (5.7)	70.1^{cd} (0.5)	29.6^{a} (0.4)	0.3^{c} (0.0)

*S and G values for the F5H poplar samples are thioacidolysis values from Stewart et al. [243].
For the different experiments, values in each column with different superscript letters are statistically different based on Tukey's pairwise comparisons (95% CI), $P < 0.05$. Ctrl: control; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; n.d.: not determined; ABSL: acetyl bromide soluble lignin; S: syringyl lignin; G: guaiacyl lignin; H: *p*-hydroxyphenyl lignin;

Table D.1 cont'd.: Composition data for the NM-6 poplar, and the CCR, 4CL, and F5H control and transgenic lines.

			Cell Wall Composition (*mg/g alcohol insoluble residue*)								**Lignin Composition (%)**		
			Structural Carbohydrates										
			Hemicellulose Sugars						**Crystalline**	**ABSL**	**Monomers**		
Expt	**Line**	**Pool**	***Rha***	***Ara***	***Xyl***	***Man***	***Gal***	***Glc***	**Cellulose**		**S**	**G**	**H**
CCR	**5-2-3**	**1**	3.1^{abc} (0.3)	3.1^{abcd} (0.3)	235.5^{ab} (12.6)	7.3^{ab} (0.4)	6.8^{ab} (0.6)	49.2^{b} (3.0)	441.6^{a} (4.0)	189.4^{d} (2.9)	73.1^{ab} (0.3)	26.6^{cd} (0.3)	0.2^{c} (0.0)
		2	2.8^{bc} (0.1)	2.9^{bcd} (0.1)	233.3^{ab} (13.1)	6.9^{bc} (0.1)	6.9^{ab} (0.3)	48.1^{bc} (0.7)	432.2^{a} (30.7)	186.3^{d} (2.7)	74.2^{a} (0.6)	25.6^{d} (0.6)	0.2^{c} (0.1)
		3	3.1^{abc} (0.2)	3.2^{abcd} (0.1)	222.3^{b} (8.6)	7.7^{ab} (0.1)	7.5^{a} (0.2)	51.5^{ab} (0.7)	406.4^{a} (18.6)	189.4^{d} (2.5)	70.0^{cd} (0.9)	28.9^{a} (0.8)	1.2^{b} (0.1)
	5-2-40	**1**	2.8^{c} (0.1)	2.5^{d} (0.1)	217.4^{b} (15.3)	7.9^{a} (0.2)	6.3^{ab} (0.2)	55.3^{a} (0.8)	452.6^{a} (27.1)	195.7^{cd} (3.7)	71.3^{cd} (0.0)	27.4^{bc} (0.1)	1.4^{b} (0.1)
		2	2.8^{bc} (0.0)	2.8^{cd} (0.1)	225.6^{b} (10.7)	7.6^{ab} (0.1)	6.8^{ab} (0.1)	50.2^{ab} (1.4)	407.4^{a} (43.5)	191.2^{cd} (0.8)	69.9^{cd} (0.3)	28.6^{ab} (0.2)	1.5^{b} (0.2)
		3	3.3^{abc} (0.1)	3.7^{ab} (0.1)	263.2^{a} (8.3)	6.3^{c} (0.2)	6.9^{ab} (0.2)	38.7^{d} (1.2)	435.6^{a} (16.6)	201.4^{bc} (2.5)	69.6^{d} (1.2)	28.4^{ab} (0.9)	2.0^{a} (0.3)
4CL	**Ctrl**	**1**	3.7abc (0.2)	2.7cdefg (0.2)	122.2defg (3.7)	10.2a (0.9)	7.1abcd (0.3)	44.2a (3.7)	561.9abcde (22.0)	213.3a (19.5)	63.9 (0.1)	35.7 (0.1)	0.4 (0.0)
		2	2.9abcd (0.1)	3.1bcdef (0.9)	116.9efg (2.3)	8.2ab (0.3)	7.2abc (0.1)	32.0ab (0.3)	538.8bcdefg (9.6)	198.2abc (3.3)	63.5 (0.3)	36.1 (0.3)	0.4 (0.0)
		3	3.1abcd (0.1)	2.5efg (0.1)	108.8g (4.5)	9.0ab (0.5)	7.4ab (0.4)	33.4ab (0.7)	528.6bcdefg (27.0)	197.0abc (2.8)	64.3 (0.1)	35.3 (0.1)	0.4 (0.0)

For the different experiments, values in each column with different superscript letters are statistically different based on Tukey's pairwise comparisons (95% CI), $P < 0.05$. Ctrl: control; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; n.d.: not determined; ABSL: acetyl bromide soluble lignin; S: syringyl lignin; G: guaiacyl lignin; H: *p*-hydroxyphenyl lignin;

Table D.1 cont'd.: Composition data for the NM-6 poplar, and the CCR, 4CL, and F5H control and transgenic lines.

Expt	Strength	Line	Pool	Cell Wall Composition (mg/g alcohol insoluble residue)								Lignin Composition (%)		
				Structural Carbohydrates								Monomers		
				Hemicellulose Sugars						Crystalline Cellulose	ABSL			
				Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*			S	G	H
4CL	Weak	1	1	3.3^{abcd} (0.1)	2.5^{defg} (0.2)	128.1^{bcdefg} (16.1)	7.9^{ab} (0.5)	6.0^{cdefg} (0.6)	29.9^{ab} (2.3)	492.2^{defg} (3.5)	199.6^{abc} (0.3)	67.7 (0.8)	31.9 (0.8)	0.4 (0.1)
			2	2.4^{d} (0.6)	2.5^{efg} (0.1)	113.9^{fg} (15.6)	5.7^{ab} (3.4)	5.9^{cdefg} (1.1)	18.6^{b} (17.5)	595.2^{abc} (20.8)	197.3^{abc} (6.9)	67.7 (0.1)	32.0 (0.1)	0.3 (0.0)
			3	3.4^{abcd} (0.2)	2.7^{cdefg} (0.1)	136.0^{abcdefg} (2.3)	9.2^{ab} (0.5)	7.0^{abcde} (0.1)	38.2^{ab} (2.5)	550.6^{bcdefg} (7.2)	191.4^{abcd} (10.5)	67.6 (0.2)	32.1 (0.2)	0.3 (0.0)
		2	1	3.0^{abcd} (0.6)	3.0^{bcdef} (0.0)	126.3^{cdefg} (7.9)	7.9^{ab} (3.1)	7.5^{a} (0.8)	28.1^{ab} (14.3)	603.5^{ab} (2.7)	167.2^{defghi} (15.3)	68.8 (0.5)	30.9 (0.5)	0.4 (0.0)
			2	2.7^{abcd} (0.2)	2.4^{fg} (0.1)	111.6^{g} (1.5)	8.1^{ab} (0.5)	6.4^{abcdefg} (0.2)	31.8^{ab} (2.6)	568.8^{abcd} (10.8)	176.6^{bcdefg} (6.2)	67.6 (0.5)	32.0 (0.5)	0.4 (0.0)
			3	3.4^{abcd} (0.4)	2.5^{efg} (0.3)	129.8^{bcdefg} (7.3)	9.3^{ab} (1.0)	7.2^{abc} (0.5)	39.3^{ab} (4.4)	555.9^{abcdef} (10.6)	199.8^{ab} (4.6)	69.9 (0.2)	29.6 (0.2)	0.5 (0.1)
		3	1	3.2^{abcd} (0.3)	2.8^{cdefg} (0.3)	136.4^{abcdefg} (15.6)	6.2^{ab} (0.3)	6.6^{abcdef} (0.2)	27.2^{ab} (1.1)	535.6^{bcdefg} (22.6)	189.8^{abcde} (4.9)	66.3 (0.5)	33.0 (0.6)	0.7 (0.1)
			2	3.6^{abc} (0.1)	2.7^{cdefg} (0.1)	144.7^{abcde} (4.1)	6.4^{ab} (0.4)	5.4^{fg} (0.2)	29.5^{ab} (2.2)	482.6^{efg} (6.7)	186.0^{bcdef} (7.0)	64.2 (0.5)	35.2 (0.5)	0.6 (0.0)
			3	3.0^{abcd} (0.1)	2.3^{fg} (0.1)	111.7^{g} (2.4)	8.0^{ab} (0.3)	7.0^{abcde} (0.0)	34.1^{ab} (1.1)	554.9^{abcdefg} (36.8)	145.1^{i} (7.0)	66.8 (0.6)	32.7 (0.6)	0.5 (0.0)

For the different experiments, values in each column with different superscript letters are statistically different based on Tukey's pairwise comparisons (95% CI), $P < 0.05$. Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; n.d.: not determined; ABSL: acetyl bromide soluble lignin; S: syringyl lignin; G: guaiacyl lignin; H: *p*-hydroxyphenyl lignin;

Table D.1 cont'd.: Composition data for the NM-6 poplar, and the CCR, 4CL, and F5H control and transgenic lines.

Expt	Strength	Line	Pool	Cell Wall Composition (mg/g alcohol insoluble residue)								Lignin Composition (%)		
				Structural Carbohydrates								Monomers		
				Hemicellulose Sugars						Crystalline Cellulose	ABSL			
				Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*			S	G	H
4CL	Medium	22	1	2.9^{abcd} (0.0)	2.2^{g} (0.1)	122.5^{defg} (6.5)	7.4^{ab} (0.0)	6.0^{cdefg} (0.1)	30.7^{ab} (0.6)	529.6^{bcdefg} (13.6)	159.8^{ghi} (4.2)	64.8 (0.4)	34.6 (0.5)	0.6 (0.1)
			2	3.8^{a} (0.2)	3.3^{bcd} (0.1)	160.7^{a} (5.0)	5.6^{ab} (0.1)	5.2^{g} (0.2)	23.7^{ab} (0.4)	468.0^{g} (12.7)	165.8^{efghi} (3.2)	64.4 (0.3)	34.9 (0.3)	0.6 (0.0)
			3	3.5^{abcd} (0.1)	2.7^{cdefg} (0.1)	155.4^{ab} (18.2)	6.7^{ab} (0.5)	5.4^{fg} (0.3)	26.6^{ab} (2.5)	505.0^{defg} (11.2)	175.8^{bcdefg} (8.7)	65.3 (0.1)	34.2 (0.1)	0.5 (0.0)
		4	1	2.6^{bcd} (0.7)	2.6^{cdefg} (0.2)	120.5^{defg} (5.9)	6.3^{ab} (4.0)	6.1^{bcdefg} (0.9)	20.5^{ab} (19.1)	637.7^{a} (26.3)	159.6^{ghi} (3.3)	68.7 (0.1)	31.1 (0.1)	0.3 (0.0)
			2	3.2^{abcd} (0.6)	3.1^{bcde} (0.1)	136.0^{abcdefg} (14.5)	6.6^{ab} (2.5)	6.6^{abcdef} (0.5)	26.4^{ab} (13.8)	573.7^{abcd} (3.5)	158.0^{ghi} (6.5)	65.1 (1.9)	34.4 (1.8)	0.5 (0.1)
			3	3.3^{abcd} (0.2)	3.0^{bcdef} (0.3)	130.3^{bcdefg} (6.7)	7.5^{ab} (0.2)	6.3^{abcdefg} (0.5)	29.7^{ab} (0.7)	489.5^{defg} (44.2)	156.2^{ghi} (8.8)	68.5 (1.4)	31.0 (1.4)	0.5 (0.0)
		32	1	3.8^{ab} (0.8)	3.7^{ab} (0.1)	139.6^{abcdef} (9.6)	6.9^{ab} (2.5)	7.0^{abcde} (0.6)	25.7^{ab} (12.5)	549.0^{bcdefg} (29.6)	178.9^{bcdefg} (7.3)	65.6 (1.4)	33.7 (1.3)	0.7 (0.1)
			2	3.5^{abcd} (0.2)	2.6^{cdefg} (0.0)	134.6^{abcdefg} (0.8)	7.4^{ab} (0.4)	6.2^{abcdefg} (0.1)	30.4^{ab} (2.8)	489.8^{defg} (6.0)	160.6^{ghi} (5.9)	67.7 (0.1)	31.9 (0.1)	0.5 (0.0)
			3	3.7^{abc} (0.6)	4.2^{a} (0.3)	146.3^{abcd} (5.4)	6.3^{ab} (2.2)	7.1^{abcd} (0.4)	22.1^{ab} (10.9)	534.5^{bcdefg} (15.2)	171.1^{defgh} (5.2)	67.8 (0.2)	31.6 (0.2)	0.6 (0.0)

For the different experiments, values in each column with different superscript letters are statistically different based on Tukey's pairwise comparisons (95% CI), $P < 0.05$. Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; n.d.: not determined; ABSL: acetyl bromide soluble lignin; S: syringyl lignin; G: guaiacyl lignin; H: *p*-hydroxyphenyl lignin;

Table D.1 cont'd.: Composition data for the NM-6 poplar, and the CCR, 4CL, and F5H control and transgenic lines.

Expt	Strength	Line	Pool	Cell Wall Composition (mg/g alcohol insoluble residue)								Lignin Composition (%)		
				Structural Carbohydrates								Monomers		
				Hemicellulose Sugars						Crystalline Cellulose	ABSL			
				Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*			S	G	H
4CL	Strong	7	1	3.0abcd (0.1)	2.4efg (0.1)	115.9fg (3.1)	8.0ab (0.3)	6.6abcdef (0.3)	37.3ab (1.1)	552.9abcdefg (22.4)	156.7ghi (12.7)	66.9 (0.4)	32.4 (0.3)	0.7 (0.1)
			2	2.8abcd (0.2)	2.6cdefg (0.1)	124.3defg (5.5)	6.5ab (0.3)	6.1bcdefg (0.2)	29.2ab (0.8)	559.9abcde (17.5)	155.4ghi (6.1)	66.2 (0.2)	33.1 (0.2)	0.7 (0.0)
			3	2.7abcd (0.7)	2.6cdefg (0.0)	119.0defg (18.6)	5.1b (3.0)	5.9cdefg (0.5)	18.5b (16.2)	607.7ab (18.0)	150.0ghi (3.7)	66.7 (0.3)	32.7 (0.2)	0.6 (0.0)
		12	1	2.9^{abcd} (0.1)	2.3^{fg} (0.2)	125.5^{cdefg} (4.6)	7.8^{ab} (0.2)	$6.4^{abcdefg}$ (0.5)	30.2^{ab} (0.7)	548.5^{bcdefg} (7.9)	157.4^{ghi} (12.1)	66.5 (0.8)	33.0 (0.9)	0.5 (0.2)
			2	3.4^{abcd} (0.0)	2.7^{cdefg} (0.1)	141.1^{abcdef} (4.2)	6.9^{ab} (0.2)	$6.4^{abcdefg}$ (0.1)	30.8^{ab} (1.2)	512.0^{cdefg} (4.2)	163.1^{fghi} (3.3)	64.1 (0.0)	35.3 (0.0)	0.6 (0.0)
			3	3.7^{abc} (0.1)	3.3^{bc} (0.1)	152.4^{abc} (2.2)	5.5^{ab} (0.2)	5.8^{defg} (0.1)	21.8^{ab} (0.9)	470.7^{fg} (54.5)	175.0^{cdefg} (1.2)	66.0 (0.9)	33.1 (1.0)	0.9 (0.1)
		16	1	3.1^{abcd} (0.0)	2.4^{efg} (0.2)	123.3^{defg} (7.3)	7.3^{ab} (0.2)	5.6^{fg} (0.0)	32.7^{ab} (1.2)	662.5^{defg} (28.8)	165.1^{fghi} (1.9)	65.1 (0.2)	34.3 (0.2)	0.6 (0.0)
			2	3.2^{abcd} (0.2)	2.3^{g} (0.1)	124.2^{defg} (4.2)	7.4^{ab} (0.4)	$6.2^{abcdefg}$ (0.2)	29.4^{ab} (2.3)	703.6^{bcdefg} (46.8)	168.8^{defghi} (3.9)	63.6 (0.4)	35.9 (0.4)	0.5 (0.0)
			3	2.5^{cd} (0.4)	$2.^{efg}$ (0.3)	122.3^{defg} (2.7)	4.8^{b} (2.3)	5.7^{efg} (0.2)	17.4^{b} (12.6)	759.0^{ab} (77.7)	168.4^{defghi} (7.1)	64.8 (0.1)	34.8 (0.1)	0.5 (0.0)

For the different experiments, values in each column with different superscript letters are statistically different based on Tukey's pairwise comparisons (95% CI), $P < 0.05$. Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; n.d.: not determined; ABSL: acetyl bromide soluble lignin; S: syringyl lignin; G: guaiacyl lignin; H: *p*-hydroxyphenyl lignin;

Table D.2: Variance within the fully nested ANOVA for each line, related to downregulation strength (4CL only), parent line, or sample pool for the 4CL and CCR samples. An ANOVA was unable to be performed for the 4CL lignin monomers as information on the individual data replicates was not provided.

		Hemicellulose Sugars					Cry	Total Glc	Total Sugar	Lignin	Lignin Monomers		
		Ara	Xyl	Man	Gal	Glc					S	G	H
	% of Total Variance												
	Strength	13.10	14.69	20.45	14.12	13.12	0.00	0.00	0.00	52.15	-	-	-
4CL	Line	5.68	10.96	0.00	21.86	0.00	13.27	17.66	6.28	7.97	-	-	-
	Pool	58.71	43.08	13.32	31.86	22.16	60.07	57.66	56.68	25.59	-	-	-
	Error	22.52	31.27	66.23	32.15	64.71	26.65	24.68	37.04	14.29	-	-	-
	% of Total Variance												
CCR	Line	39.23	0.00	0.00	23.70	0.00	0.00	0.00	0.00	81.57	21.81	26.56	75.58
	Pool	34.57	57.97	76.88	14.24	87.87	24.41	26.52	23.51	8.37	66.45	60.46	21.28
	Error	26.20	42.03	23.12	62.07	12.13	75.59	73.48	76.49	10.06	11.74	12.98	3.14

Ara = arabinose; Xyl = xylose; Man = mannose; Gal = galactose; Glc = glucose; Cry = crystalline cellulose; S = syringyl lignin; G = guaiacyl lignin; H = *p*-hydroxyphenyl lignin

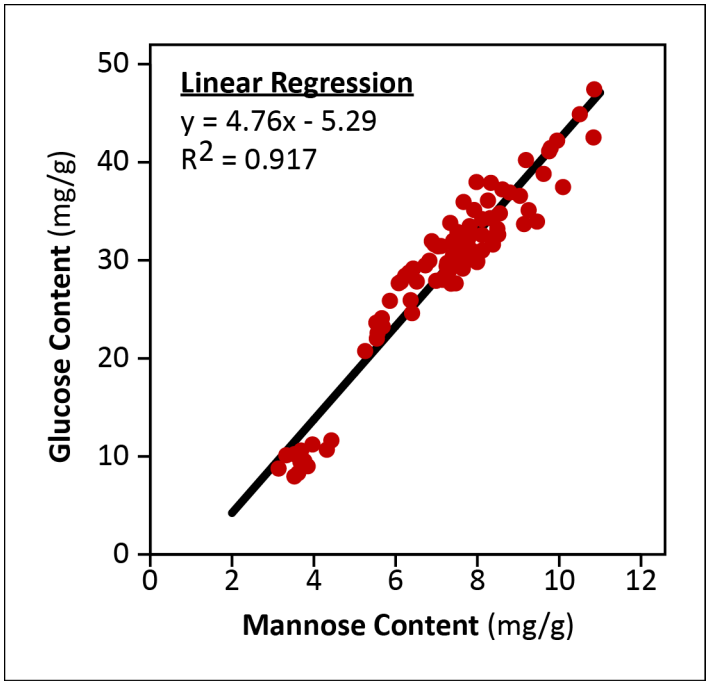

Figure D.1: Hemicellulose glucose vs. mannose content for 4CL poplar samples. The equation represents the linear regression of the data. Glucose is derived from hemicellulose and does not include cellulose-derived or soluble glucose.

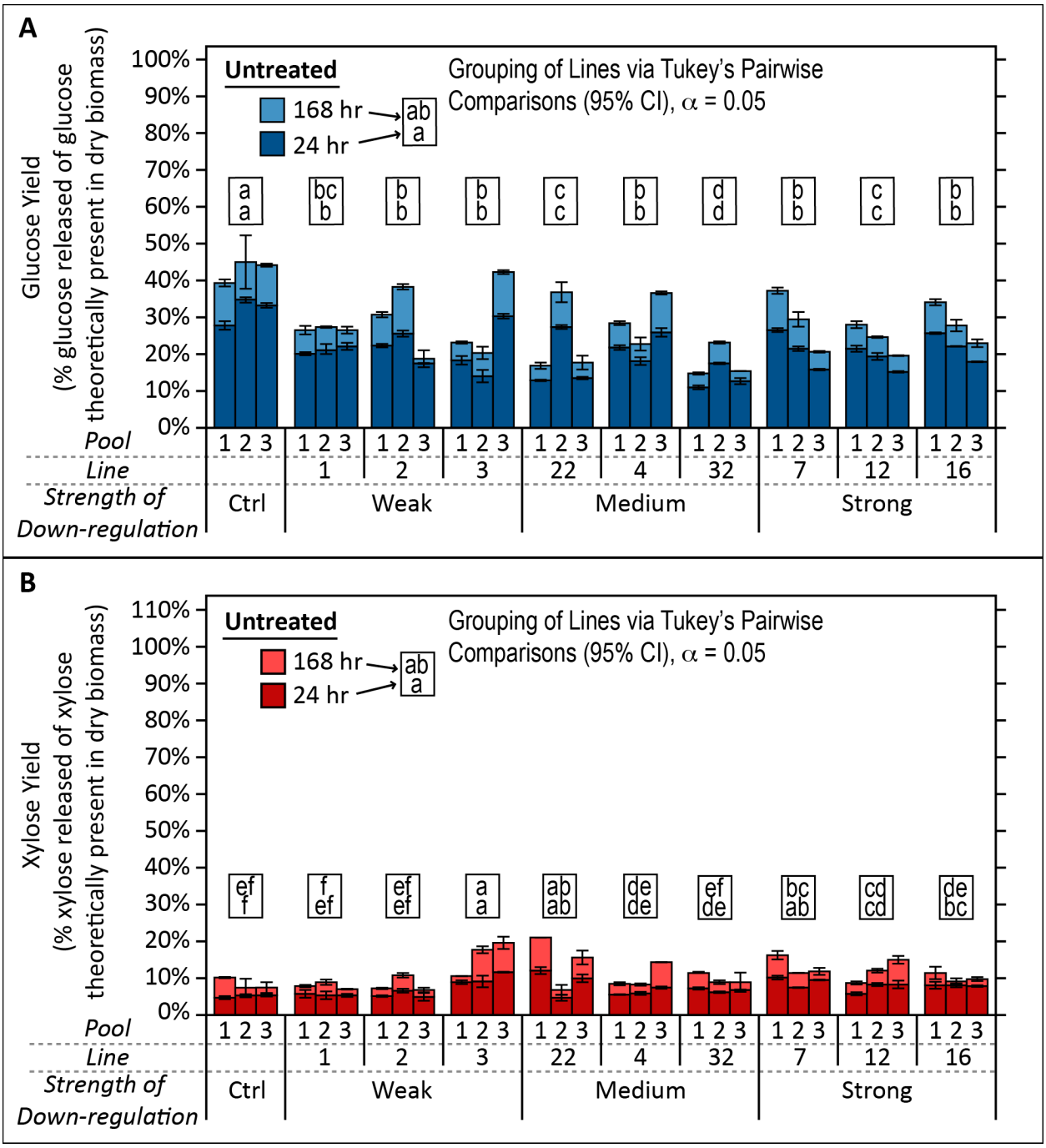

Figure D.2: Untreated wildtype and 4CL downregulated poplar (A) glucose and (B) xylose yields. Sugar yields with different letters within each subplot are statistically different based on Tukey's pairwise comparisons (95% CI), ($p < 0.05$) and are not comparable between 24 h and 168 h data. Enzymatic hydrolysis was conducted at 200 rpm and 1.25% total sugar loading with 24 mg total protein per g cell wall sugars (80% Accellerase® 1500, 10% Accellerase® XY, 10% Multifect® Pectinase).

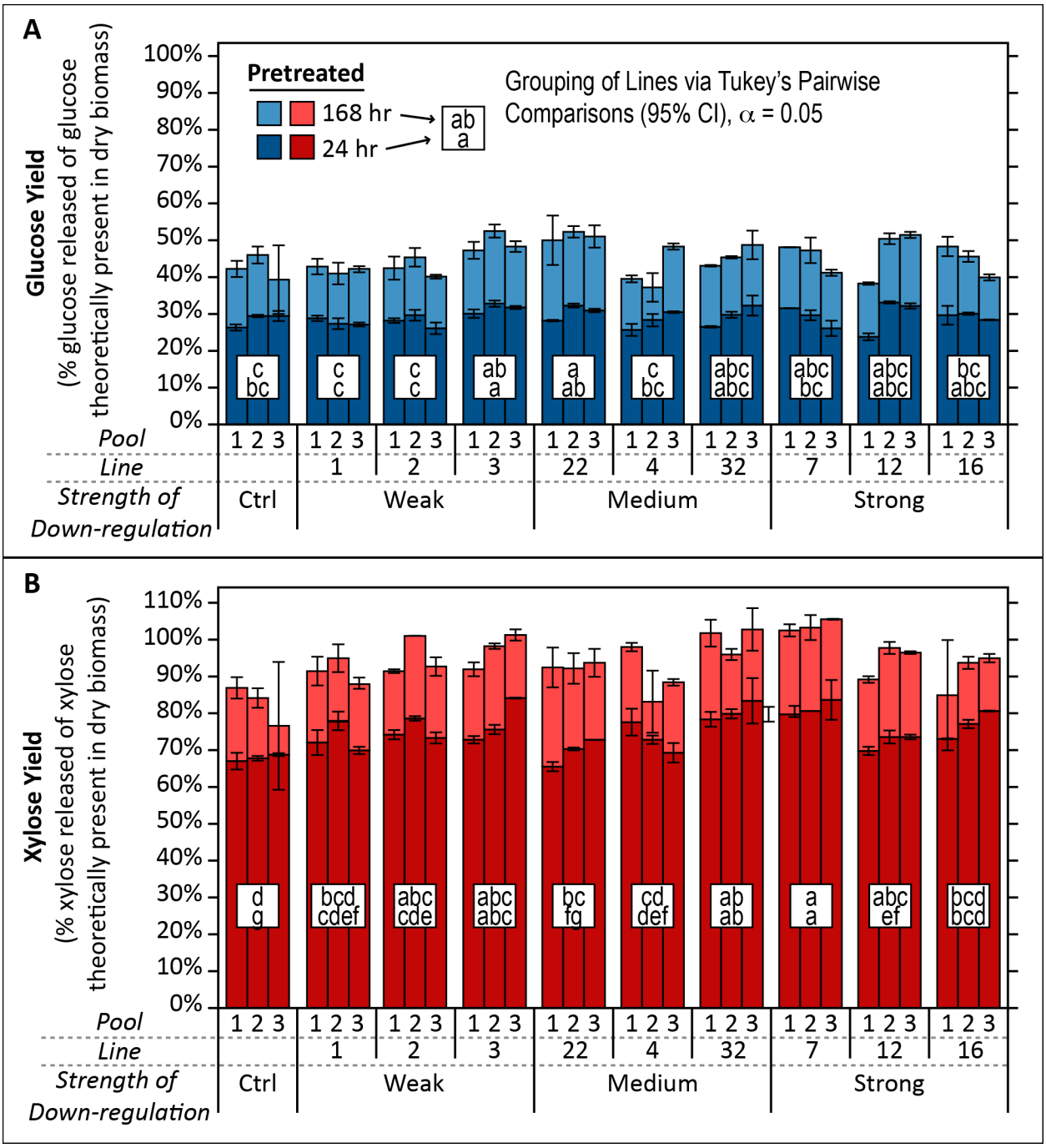

Figure D.3: **Pretreated wildtype and 4CL downregulated poplar (A) glucose and (B) xylose yields.** Sugar yields with different letters within each subplot are statistically different based on Tukey's pairwise comparisons (95% CI), ($p < 0.05$) and are not comparable between 24 h and 168 h data. All samples were pretreated using 1:1 g NH_3:g DM; 1:1 g H_2O:g DM; 180°C for 20 min. Enzymatic hydrolysis was conducted at 200 rpm and 1.25% total sugar loading with 24 mg total protein per g cell wall sugars (80% Accellerase® 1500, 10% Accellerase® XY, 10% Multifect® Pectinase).

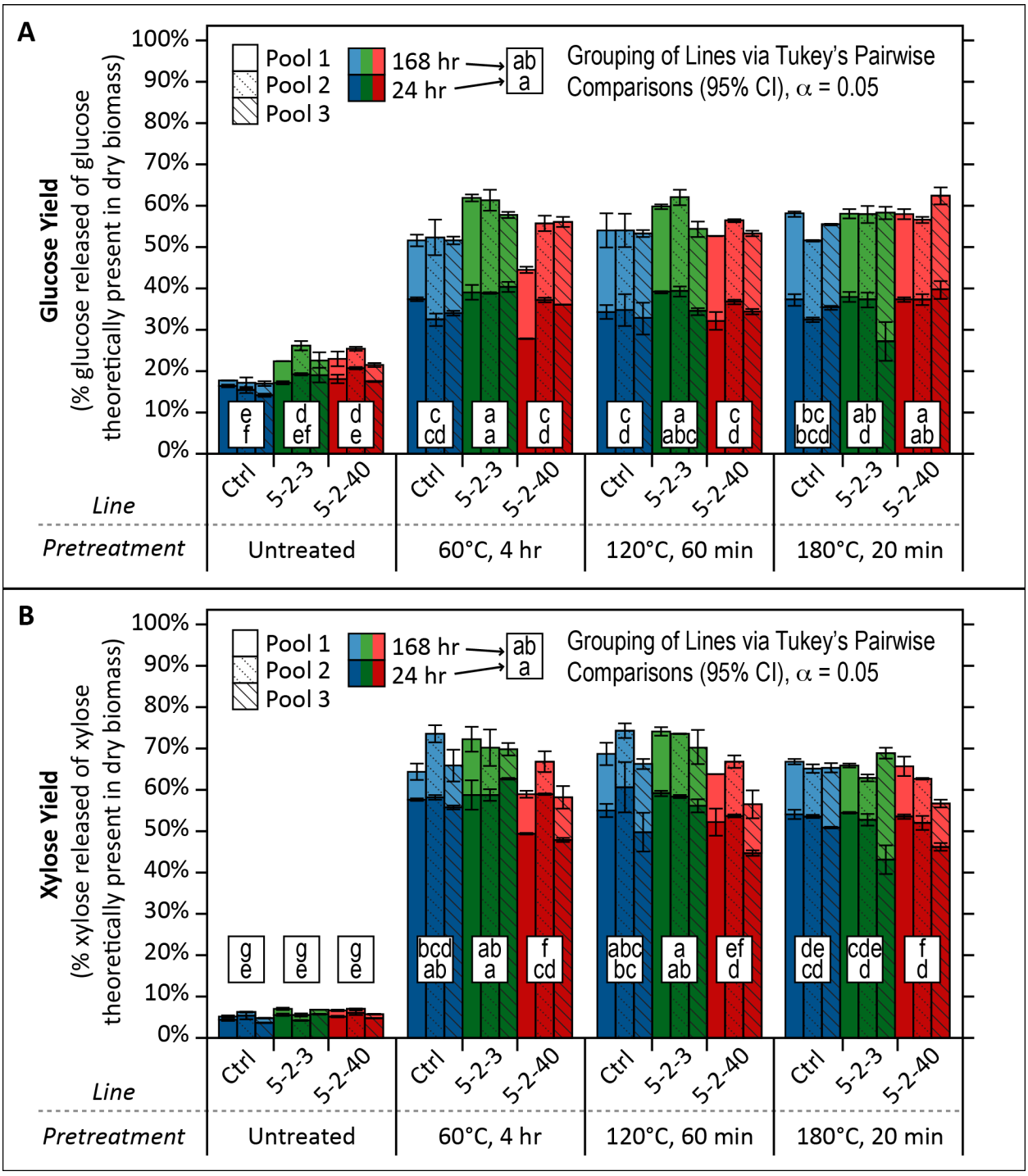

Figure D.4: Control and CCR downregulated poplar (A) glucose and (B) xylose yields. Sugar yields with different letters within each subplot are statistically different based on Tukey's pairwise comparisons (95% CI), ($p < 0.05$) and are not comparable between 24 h and 168 h data. Samples were pretreated using 1:1 g NH_3:DM and 1:1 g H_2O:g DM. Enzymatic hydrolysis was conducted at 200 rpm and 1.25% total sugar loading with 24 mg total protein per g cell wall sugars (80% Accellerase® 1500, 10% Accellerase® XY, 10% Multifect® Pectinase).

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