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Chemical and Structural Features of Plants That Contribute to Biomass Recalcitrance

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Chemical and Structural Features of Plants That Contribute to Biomass Recalcitrance

A Dissertation submitted in partial satisfaction of the requirements for the degree of

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in

Chemical and Environmental Engineering

by

Jaclyn Diana DeMartini

March 2012

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Committee Chairperson

University of California, Riverside
ABSTRACT OF THE DISSERTATION

Chemical and Structural Features of Plants That Contribute to Biomass Recalcitrance

by

Jaclyn Diana DeMartini

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, March 2012
Dr. Charles E. Wyman, Chairperson

Currently, the primary barrier to low cost biological conversion of cellulosic biomass to renewable fuels is a plant’s recalcitrance to sugar release. The energy-intensive pretreatments and high enzyme loadings that are typically required to obtain high sugar yields result in elevated processing costs that must be reduced in order to make cellulosic fuels competitive with those derived from petroleum feedstocks. To lower processing costs, the natural recalcitrance of biomass must be overcome either through the identification and use of superior biomass candidates with reduced recalcitrance, the development of enzymes that are more effective, and/or the design and implementation of efficient pretreatment processes to reduce plants’ recalcitrance prior to enzymatic
conversion. Unfortunately, the current understanding of biomass recalcitrance is extremely limited, which makes such options difficult. Thus, this thesis will address the vital need for a better understanding of what plant structural and chemical features cause biomass recalcitrance and how they can be overcome with pretreatment.

Towards this end, three main objectives were pursued in this thesis. First, downscaled and high throughput tools were developed for screening large sets of biomass samples for composition as well as for sugar release from pretreatment and enzymatic saccharification. Next, the implementation of these tools facilitated the screening of multiple sets of biomass samples to identify superior feedstock candidates and general trends in sugar release behavior. Finally, from these trends, a more detailed analysis was undertaken for select species in order to investigate what plant structural and chemical features contribute most heavily to biomass recalcitrance and how pretreatment processes overcome these limitations to achieve high sugar yields in subsequent enzymatic saccharification.
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Chapter 1. Introduction
1.1 Overview

One of the greatest challenges of modern times is addressing the world’s ever-growing energy demands. As the world population approaches 7 billion people, the total energy consumption has grown to a staggering 505 quadrillion BTU annually (in 2008), and is estimated to increase at about 1.4% per year (EIA, 2011). In 2010, the primary energy use in the United States equaled 98 quadrillion BTU, of which over 25% was consumed by the transportation sector. This annual amount is driven almost exclusively by liquid fossil fuels, in particular by fuels derived from petroleum (EIA, 2011). In the United States, over 19.15 million barrels of petroleum (of which nearly 50% is imported) are consumed daily, with about 77% going to transportation (EIA, 2011). This large consumption translates into the release of about 2,000 million metric tonnes (4.41*10^12 lbs) of CO₂ per year by the transportation sector, equivalent to over 1/3 of the United States’ total carbon dioxide emissions (DOE/EIA-0383(2011)). Furthermore, although gasoline has historically been produced from conventional petroleum, a combination of “geologic, economic, environmental, [and] political difficulties” will likely result in increased gasoline production from hydrocarbons such as oil sands, oil shale, methane or coal, all of which result in significantly higher carbon dioxide emissions (Brandt and Farrell, 2007). The anthropogenic emission of greenhouse gases, of which carbon dioxide is reported to be the most significant, is understood to contribute directly to

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‡ Oil: Crude and Petroleum Products Explained: Available at [http://www.eia.gov/energyexplained/index.cfm?page=oil_home#tab2](http://www.eia.gov/energyexplained/index.cfm?page=oil_home#tab2)
global climate change (IPCC report, 2007). Thus, due to environmental concerns, national energy security, and domestic economic growth, there is an urgent need to develop and implement alternatives to petroleum transportation fuels.

One such source for the production of alternative liquid fuels is cellulosic biomass, which can be sustainably converted into a range of fuels including ethanol. The use of a renewable resource such as cellulosic biomass results in a closed loop cycle, where any CO$_2$ released into the atmosphere during fuel combustion is recycled back into the new crops planted for the next harvest resulting in low or net-negative carbon dioxide emissions (Farrell et al., 2006). This is opposed to the use of petroleum produced from fossil fuels, which pulls carbons previously locked beneath the ground and releases them into the atmosphere during combustion, translating into the release of 1.52*10$^{-4}$ lb CO$_2$/BTU (19 lb CO$_2$/gallon gasoline) from combustion alone (Office of Transportation and Air Quality, 2005) and a total release of 1.96*10$^{-4}$ lb CO$_2$/BTU (24 lb CO$_2$/gallon gasoline) from its production, transportation, and combustion (Brandt and Farrell, 2007).

Large quantities of ethanol, including 13.23 billion gallons in 2010, are already being produced and blended with gasoline to up to 10% in the United States (RFA, 2011). However, almost the entirety of this ethanol is produced from corn starch. Although corn ethanol is a significant improvement over gasoline produced from conventional petroleum in terms of petroleum usage, it still requires the input of fossil fuels and results in only a slight reduction in greenhouse gas emissions compared to gasoline (Fig. 1.1) (Farrell et al., 2006). Furthermore, there are also concerns over the competition with

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§ Renewable Fuels Association: Available at [http://www.ethanolrfa.org/pages/statistics#EIO](http://www.ethanolrfa.org/pages/statistics#EIO)
crops and fertile lands for the production of food versus fuel especially with the increasing demand for both, which adds to the potential drawback of ethanol produced from corn starch. Conversely, cellulosic biomass is a significantly more abundant source that can be grown on a wider range of lands than can corn, and is not limited to fertile agricultural lands. The U.S. Billion Ton Update led by the U.S. Department of Agriculture evaluated the amount of available biomass that can be sustainably harvested both now and in the future. The study found that under high-yield assumptions and at a price of $60/ton, up to 602 million dry tons of biomass could be available annually by 2022, including 100 million dry tons from forest and wood waste, 221 million dry tons from agricultural residues and wastes, and 282 million dry tons from dedicated bioenergy crops such as perennial grasses, woody crops, and annual energy crops (U.S. Department of Energy, 2011). Assuming a conversion estimate of 100 gallons of ethanol produced per dry ton of biomass, this could yield over 60 billion gallons of ethanol annually, enough to replace about 20% of the United States’ current petroleum usage (on a BTU basis) and avoid millions of pounds of CO$_2$ from being released into the atmosphere annually. Thus, there is great potential for cellulosic ethanol to make a significant dent in the U.S. petroleum usage and carbon dioxide emissions even within the next decade.
Figure 1.1. A comparison of gasoline, EtOH (ethanol) today produced almost exclusively from corn starch, and ethanol produced from cellulosic biomass. Shown is the amount of petroleum required per unit of energy produced, the amount of fossil energy required per unit of energy produced, and the amount of greenhouse gas (GHG) emissions as compared to conventional gasoline (adapted from Farrell et al., 2006).

1.2 Biological conversion of biomass to cellulosic ethanol

In the production of ethanol from cellulosic biomass, the first major step is the release of sugars that are stored as high molecular weight polymers in the plant cell wall (e.g., glucose is stored as cellulose and xylose is stored as hemicellulose). This can either be accomplished with chemicals alone via acid hydrolysis, or can be accomplished biologically using enzymes to hydrolyze wall polysaccharides. Biological conversion routes benefit from high selectivity and the potential for significant improvements due to future advances in biotechnology, and will thus be the focus of this thesis. However, the plant cell wall is recalcitrant to enzymatic degradation meaning that high enzyme
loadings and long hydrolysis times may be needed to achieve sufficiently high yields, resulting in high costs. To overcome the natural recalcitrance of biomass, a preliminary step called pretreatment is performed to disrupt the natural plant structure and remove or alter the structural and chemical components that inhibit enzyme hydrolysis (Mosier et al., 2005). There are a variety of different pretreatment methods, ranging in temperature, pressure, and pH, but the goal of all of them is to make biomass more susceptible to subsequent enzymatic hydrolysis. This step is crucial to realizing high yields of fermentable sugars from cellulose and hemicellulose.

Following pretreatment, cellulase enzymes are added to the biomass. Cellulases are a mixture comprised of (1) endoglucanases, which attack randomly along a cellulose chain to reduce its polymer size, (2) exoglucanases, which attack the cellulose chain ends in order to hydrolyze crystalline cellulose, and (3) β-glucosidase to hydrolyze cellobiose to glucose (Aden et al., 2002). Additional accessory enzymes, including xylanases and pectinases, are also often added to hydrolyze non-cellulose components of the cell wall, and in turn improve cellulase hydrolysis by removing additional barriers. Together, these enzymes produce a liquid stream containing sugar monomers such as glucose, xylose, and lesser amounts of other C5 and C6 sugars such as arabinose, mannose, and galactose. Afterwards, microorganisms convert sugars into ethanol. Many naturally-occurring microorganisms are readily able to convert glucose into ethanol; however, the efficient conversion of C5 sugars such as xylose has only been achieved in the past few decades through the use of recombinant organisms (Ingram and Doran, 1995). Ethanol from the resulting broth, which is usually on the order of 5% ethanol, is recovered
through a distillation process (Aden et al., 2002). The unutilized solid residue, which is mainly composed of lignin, is used as fuel for production of process steam and additional power that can be exported to the grid (Aden et al., 2002).

1.3 Research direction for cellulosic ethanol and thesis objectives

Despite the substantial progress that has been made in the past few decades, challenges still remain to produce economically viable cellulosic ethanol that can compete with conventionally produced gasoline. Although the biomass feedstock itself is estimated to account for the largest single expense at approximately one-third of the cost of cellulosic ethanol produced using current technologies, biomass is a rather inexpensive commodity, and at $40 per ton is equivalent to petroleum at only $13 a barrel (Wyman, 2007). Thus, effort must be directed at reducing processing costs, which account for around 67% of the estimated cost of cellulosic ethanol. Research has shown that the most expensive unit operations are pretreatment, enzymatic hydrolysis of pretreated biomass, and production of enzymes for hydrolysis, all of which are focused on overcoming the natural recalcitrance of biomass to release fermentable sugars (Wyman, 2007). To reduce the costs associated with releasing sugars in high yields, future research should be pointed in one or both of the following directions: development of improved enzymes and pretreatment strategies or identification of biomass species with reduced recalcitrance. For significant progress to occur in either of these directions, a better understanding of plant recalcitrance and how it relates to pretreatment and enzymatic digestion is sorely needed. Without a thorough understanding of recalcitrance, informed
genetic modification or selection of plants towards phenotypes with reduced defenses to deconstruction will be a challenge. Currently, it is unclear whether there are specific plant cell wall chemical or structural features that contribute heavily to biomass recalcitrance, and if so, what those structural characteristics are and why they significantly impact performance. Furthermore, it is important to learn whether certain structural features control recalcitrance for all plants, or if it is dependent on plant type, species, population, or subset of a population. It has commonly been observed that plant types and species can perform very differently when subjected to identical pretreatment and enzymatic hydrolysis conditions. Thus, what are the differences present in these various plants that cause them to react differently and result in some samples achieving high sugar yields under mild processing conditions while others are recalcitrant to sugar release? Furthermore, although it is known that pretreatment significantly improves enzymatic digestion of biomass, it is unclear what exactly happens to the plant chemistry and structure during this process and how it affects subsequent enzymatic hydrolysis for different plant samples.

This thesis seeks to address the previous observations and questions with the overall goals of elucidating the influence of plant cell wall structure on biomass recalcitrance and seeking to better understand how it is altered by pretreatment and enzymatic hydrolysis. In this direction, the first task was to screen biomass samples for sugar release from pretreatment and enzymatic hydrolysis to evaluate trends and patterns of sugar release behavior and biomass recalcitrance. However in order to effectively and confidently identify trends, large sample sets of plants, enzymes, and processing conditions had to be
tested in which very little material was often available for analysis. As a result, downscaled and high throughput analysis systems were developed, improved, and validated. High throughput (HT) systems were then utilized to facilitate a number of studies that were previously not feasible, including examining the following: the variable effects of pretreatment on the digestibility within an industrially-sized wood chip, the influence of wood maturity and grass and legume anatomy on biomass recalcitrance, and the effects of lignin content and composition on the digestibility of a large natural population of poplar trees. After employing the HT systems to screen a wide variety of samples and sample characteristics, the final phase of this thesis involved a more detailed analysis of plant cell wall structure and its influence on biomass recalcitrance for two select species. The fate of specific plant cell wall structures during pretreatment and the effects of these structures on enzymatic digestibility were investigated to identify and rank factors that influence biomass recalcitrance. Ultimately, the results in this thesis contribute significantly to the current understanding of biomass recalcitrance by identifying factors from wood maturity and grass and legume anatomy to smaller scale structural and chemical features, that influence biomass recalcitrance.

1.4 Thesis organization

Chapter 2 will begin with a literature review of information relevant to this thesis. As such, three main topics will be covered starting with the physical and chemical structure of biomass and the plant cell wall. Next, a variety of pretreatment technologies will be introduced with particular attention paid to the effects of hydrothermal and dilute acid
pretreatments on biomass structure and enzymatic hydrolysis. Finally biomass recalcitrance and factors thought to limit effective pretreatment and enzymatic degradation will be reviewed. Chapters 3 through 10 will then be split into three main foci: 1) the development and verification of downscaled and high throughput tools to screen biomass samples for composition and sugar release from pretreatment and enzymatic hydrolysis, 2) the implementation of these tools to screen plant samples against a variety of pretreatment and enzyme conditions to identify trends and patterns in sugar release behavior, and 3) a more in depth investigation into interactions between plant cell wall structure and recalcitrance to pretreatment and enzymatic digestion for select biomass species. As such, chapters 3 and 4 will cover focus area #1 including the verification and improvement of a HT pretreatment and enzymatic hydrolysis screen and the development of a novel downscaled compositional analysis, respectively. These tools were then employed in Chapters 5 through 8 in a variety of studies that evaluated a large number of diverse samples. In this case, the variability within industrially sized pretreated wood chips is evaluated in Chapter 5 to identify differences in composition and enzymatic digestion across the chips’ thickness. Limitations in sugar release performance were identified for various pretreatment conditions and causes of the wood’s differing recalcitrance were investigated. Next, the composition and sugar release of annual rings from an aspen cross section were studied in Chapter 6 to evaluate the influence of wood maturity and associated chemical and structural features on recalcitrance to sugar release. Two additional studies also utilized the HT systems to screen a variety of biomass species. In an association study presented in Chapter 7, the
sugar release of 47 natural *Populus trichocarpa* variants was tested to identify trends in sugar release behavior and evaluate the influence of lignin content and composition on recalcitrance. Finally in Chapter 8, testing of the most abundant grass and legume species from a mixed prairie plot demonstrated the importance of plant anatomy on composition and the ability to attain high sugar yields.

Chapters 9 and 10 will then cover the final main focus of this thesis. In Chapter 9, a novel tool called glycome profiling was applied to investigate the detailed effects of hydrothermal pretreatment on plant cell wall structure and chemistry. New insights into how pretreatment alters the cell wall of poplar to render it more digestible to enzymatic hydrolysis were discovered that can aid in improving future pretreatments and enzymes. However, due to the difficulty in relating specific plant cell wall changes to reduced biomass recalcitrance in Chapter 9 as a result of a multitude of changes observed to occur simultaneously during hydrothermal pretreatment, more targeted studies were undertaken in Chapter 10 to test the effect of specific cell wall components on enzymatic efficiency. Chemical and enzymatic extractions were performed on poplar and switchgrass to remove certain cell wall components. After characterizing the resulting materials and the removed components, the samples’ digestibility was tested to investigate what structures most strongly inhibit efficient enzymatic degradation and what structures do not contribute heavily to biomass recalcitrance. Furthermore, since two very different plants were evaluated in this study, including a grassy monocot and a woody dicot, the influence of plant type was evaluated to test whether structural features control the recalcitrance of both types of plants, or if not, why they react differently.
Chapter 11 moves away from detailed studies of biomass recalcitrance to examine the entire process of cellulosic ethanol production. As such, a study is presented that compares the long term carbon dioxide emissions from cellulosic ethanol and conventional gasoline. Finally, Chapter 12 summarizes the key findings from this thesis and evaluates their significance. Particular attention will be paid to Chapters 5 through 10 to identify factors that most strongly influenced biomass recalcitrance. This summary will be written from the standpoint of both enzymes and plants to identify potential strategies to address the causes of biomass recalcitrance identified in this thesis. The thesis will conclude with recommendations for future work to further pursue biomass recalcitrance and methods by which to overcome it.

1.5 References


Chapter 2. Literature Review
2.1 Abstract

Processing costs remain too high in biological conversion of lignocellulosic biomass. This is due largely to pretreatment and enzymatic hydrolysis processes, which together must overcome the natural recalcitrance of plants in order to achieve the efficient release of sugars. To date, significant research has been directed at identifying biomass characteristics that contribute to recalcitrance, as well as learning how pretreatment overcomes the recalcitrance barrier to produce digestible material. However, the literature published to date is often conflicting, and as a result, there is no clear picture about what structural features do and do not play a role.

In this chapter, the diversity of plants and their complex cell walls will first be reviewed. This will provide context for the following sections which will 1) review biomass and enzyme-related factors that have been previously proposed to influence biomass recalcitrance and 2) review the changes in biomass structure that occur as a results of hydrothermal and dilute acid pretreatments. This review will build a framework for the remainder of this thesis in order to identify gaps that still exist in our current knowledge that must be addressed.
2.2 Plant structure

2.2.1 Introduction

Plants vary greatly from one another in terms of their composition and structure at every level, from anatomical characteristics that can be easily observed all the way down to the molecular scale. Both woody and herbaceous plants are potential feedstocks for biofuels applications, and while both typically contain between 30-50% cellulose, 15-30% hemicellulose, and 10-30% lignin, they are incredibly different from each other. Grasses are classified as monocots (with Type II primary cell walls) and trees as dicots (with Type I primary cell walls) based on morphological characteristics including, but not limited to, embryo, pollen, and vascular bundle structure** (Vogel, 2008). Within grasses and trees, there are also many further divisions and considerable variety. For example, perennial grasses can be split into C3 and C4 plants based on the photosynthetic pathway that they utilize; each of these sub-groups also contain a large and diverse set of species (Galyean and Goetsch, 1993). Likewise, woody species can also be split into two main categories, hardwoods and softwoods, which vary in terms of their cell type and function, as well as composition (Fengel and Wegener, 1984).

The remainder of section 2.2 will provide an overview of general plant structure applicable to both trees and grasses. Differences that exist between monocots and dicots will also be pointed out. Topics covered will range from tissue and cell types, to chemistry for the main wall polymers, including cellulose, hemicellulose, and lignin.

** Available at http://www.ucmp.berkeley.edu/glossary/gloss8/monocotdicot.html
Finally, the integration of these cell wall components and the resulting wall architecture will be presented, with differences between monocot and dicot species also discussed.

2.2.2 Plant tissues

Plant anatomy is a key characteristic because it defines the presence and proportion of different tissues, which in turn defines cell type, composition, and structure. As such, all plants are composed of three different tissue types, including ground, dermal, and vascular tissues, each of which includes distinct cell types (Albersheim et al., 2011). The ground tissue is the main support structure and contains parenchyma, collenchyma, and sclerenchyma cells. Parenchyma cells have thin primary cell walls and include cells that are involved in food production and storage in the photosynthetic leaf and stem (called mesophyll cells). Collenchyma cells are similar, but they have much thicker cell walls and are shaped more like long fibers that are packed together to provide mechanical support to the plant. Sclerenchyma cells also have strengthening and support functions; however, these cells are usually dead and have thick lignified secondary cell walls. The second type of tissue, the dermal system, is the plant’s skin that serves to protect it from the environment; it includes epidermal and stomatal cells. The epidermis is usually composed of living cells with thick primary cell walls, and usually consists of a single layer covering the entire surface of the plant. Stomata on the other hand are openings in the epidermis that regulate gas exchange in the plant. Finally, the vascular system, which is composed of phloem and xylem, conducts water and solutes within the plant and also provides a level of mechanical support. The phloem is involved in transporting
solute within the plant, and is made up primarily of conducting cells called elements that come together to form tubes (sieve cells or tubes), whereas the xylem carries water and dissolved ions throughout the plant, and is composed primarily of vessel elements, as well as tracheids in some plants such as hardwoods. Together, the vascular system is often present in structures called vascular bundles, which include phloem and xylem cells closely packed together, in addition to sclerenchyma and parenchyma cells. Each of these individual cell types that comprise different plant tissues is distinct from others. They all vary significantly in shape, surface area to volume ratio, and cell wall thickness (Grabber et al., 1992; Grabber and Allinson, 1992; Wilson and Mertens, 1995).

In general, wood has a significantly simpler structure than grasses. For example, softwoods consist of 90-95% tracheids, which are long slender xylem cells that serve both strength and water conducting functions (Fengel and Wegener, 1984). Hardwoods have a slightly different structure in which the basic tissue for strength contains libriform fibres and fibre tracheids, among which there are conducting vessels (Fengel and Wegener, 1984). As opposed to trees, the structure of herbaceous plants is much more complex and depends heavily on anatomy. For example, a grass blade may consist of the following tissues: epidermal (22%), mesophyll (31%), parenchyma (14%), thick-walled parenchyma bundle sheath (24%), thick-walled vascular (6%), and thick-walled sclerenchyma (2%) (Wilson, 1993). However, the stem from that same grass sample may consist of the same tissues in entirely different proportions, including 2% epidermal, 2% mesophyll, 75% parenchyma, 1% phloem, 12% thick-walled parenchyma bundle sheath, and 8% thick-walled sclerenchyma (Wilson, 1993).
2.2.3 Cell wall structure and chemistry

Despite all of the variation between and within plants, the most important element of all cells from a biofuels perspective is the cell wall since a great deal of fermentable sugars is stored there. There are both primary (Type I and II cell walls) and secondary cell walls, although the secondary wall accounts for a larger mass fraction than the primary, and is laid down between the plasma membrane and primary wall. All cells have primary walls, which are typically much thinner than the secondary wall. They have a base structure of cellulose microfibrils in thin crossing layers (Fengel and Wegener, 1984), but also contain significant portions of pectin and hemicellulose (Albersheim et al., 2011). After a cell stops growing, many lay down a tougher and more insoluble secondary wall (Wilson, 1993). This wall is also comprised primarily of cellulose microfibrils and hemicellulose, but compared to the primary wall, contains significantly less pectin, and is lignified. The ultrastructure of the secondary cell wall is also different from that of the primary wall; as such, it can be comprised of up to three different layers, or lamellae. These layers include the secondary (S)1, S2, and S3 that are distinguished by their cellulose microfibril orientation (Wilson, 1993). In the S1 layer, the cellulose microfibrils are arranged in a gentle helical slope with several crossing sets of helices, whereas the S2 layer is the thickest layer in which the microfibrils are oriented in a steep vertical direction (Fengel and Wegener, 1984). Surrounding the primary and secondary cell walls is the middle lamella, which is a continuous intercellular region that serves as the “glue” holding cells together (Albersheim et al., 2011). This region is rich in pectic polysaccharides, protein, and phenolics.
In the following sections 2.2.3.1 through 2.2.3.3, the chemistry and structure of the major wall polysaccharides will be reviewed. It is important to note that much of the fundamental cell wall science is based predominantly upon the study of primary cell walls, which vary significantly in composition from secondary walls (Table 2.1). However, since secondary cell walls are an extension of the primary wall, a majority of the structures and interactions likely remain valid and applicable for both, but the proportion and relative importance of individual components will vary.

<table>
<thead>
<tr>
<th></th>
<th>Monocot grass</th>
<th>Woody dicot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary wall</td>
<td>Secondary wall</td>
</tr>
<tr>
<td>Cellulose</td>
<td>20-30</td>
<td>34-45</td>
</tr>
<tr>
<td>Hemicelluloses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylans</td>
<td>20-40</td>
<td>30-50</td>
</tr>
<tr>
<td>Mixed-linked glucans</td>
<td>10-30</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mannans and glucomannans</td>
<td>0</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Xyloglucan</td>
<td>5</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Pectins</td>
<td>5</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Structural proteins</td>
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<td>&lt;1</td>
</tr>
<tr>
<td>Phenolics</td>
<td></td>
<td></td>
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<tr>
<td>Ferulic acid and p-coumaric acid</td>
<td>1-5</td>
<td>0.5-1.5</td>
</tr>
<tr>
<td>Lignin</td>
<td>&lt;1</td>
<td>10-20</td>
</tr>
</tbody>
</table>

2.2.3.1 Cellulose

Cellulose is the most prominent component of primary and secondary cell walls. It is a particularly stable polysaccharide that provides the majority of structural and load-bearing support (Albersheim et al., 2011). Cellulose itself is a very simple, unbranched \( \beta-1,4 \)-linked glucan chain with a disaccharide repeating unit of cellobiose. The cellulose
from secondary cell walls has been reported to have a degree of polymerization (DP) of between 10,000 and 15,000, whereas it is lower in primary walls (DP less than 4,000). These long glucan chains can hydrogen-bond together to form a structure called the cellulose microfibril. As such, approximately 30 to 50 glucan chains can be held together to form a microfibril that has a diameter between 3 to 5 nm. This crystalline arrangement of cellulose microfibrils can exist in many different forms (defined as I through IV). Cellulose I is the native form found in plant cell walls and is characterized by a parallel configuration in which all of the reducing ends of the glucan chains are at the same end of the microfibril. In addition to different forms of cellulose, two crystal phases with distinct conformations and hydrogen bonding characteristics also exist, namely the $I_\alpha$ and $I_\beta$ forms (Nishyama et al., 2003). Microfibrils have also been found to have amorphous (less crystalline) regions interspersed within the crystalline structure, which are thought to affect the physical properties of the microfibril. Cellulose microfibrils are closely associated with hemicellulose, which can hydrogen bond to the microfibril surface. This interaction provides the interface between cellulose and all other cell wall components (Albersheim et al., 2011).

### 2.2.3.2 Hemicellulose

Compared to cellulose, hemicellulose is a very diverse and complex set of polysaccharides that is often highly branched and substituted (Albersheim et al., 2011). Two of the major hemicellulosic polysaccharides are xyloglucan and arabinoxylan. In the primary wall, xyloglucan is the predominant hemicellulose in dicots, while
(glucurono)arabinoxylan is the predominant one in grassy monocots. Xyloglucan has a cellulose-like backbone of β-1,4-linked glucan, but differs in that it can be substituted to various degrees with combinations of xylose, galactose, fucose, and arabinose residues. Xyloglucans can adhere strongly to the surface of cellulose microfibrils via hydrogen-bonding, which creates a network in dicots around which all other wall polymers organize. Arabinoxylan also has a cellulose-like backbone, except that it consists of β-1,4-linked xylan and can be substituted with various combinations of xylose, arabinogalacturonic acid, and 4-O-methyl glucuronic acid residues. Furthermore, unlike xyloglucan, arabinoxylans can impart acidic functionality due to the presence of acidic residues (Albersheim et al., 2011). In addition to arabinoxylan and xyloglucan, secondary cell walls can also contain fairly large quantities of hemicelluloses such as xylans and glucomannan. Glucuronoxylan for example, which consists of a backbone of β-1,4-linked xylopyranose (cyclic 6-member ring) substituted with arabinose, 4-O-methyl-D-glucuronic acid and O-acetyl residues, can account for a significant portion of secondary cell walls in dicot (Albersheim et al., 2011).

The proportions and structures of hemicellulose components change somewhat when examining the secondary cell wall as opposed to the primary (Vogel, 2008). For grasses, the main hemicellulose remains (glucurono)arabinoxylan, although it has been reported that it contains fewer side chains in the secondary wall. For the secondary cell walls of dicots, xyloglucan is no longer the predominant hemicellulose. Instead, xylans substituted primarily with glucuronic acid and methyl glucuronic acid are present at higher levels.
2.2.3.3 Pectins, arabinogalactans, and mixed-linked glucans

Pectic polysaccharides are another important component that along with hemicellulose make up the cell wall matrix (Albersheim et al., 2011). Although they account for a much lower percentage of secondary cell walls (<10%) than they do for primary walls (up to 35% in dicots), they have the ability to form gels that are highly interactive with all other matrix polysaccharides. Thus, pectins affect the physical properties and functioning of the wall including the limitation of wall porosity. There are three main classifications of pectic polysaccharides including homogalacturonan (HG), rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II). Homogalacturonan consists exclusively of D-galacturonic acid residues that can be highly methyl-esterified (up to 70%). RG-I is made up of a repeating backbone of rhamnose and galacturonic acid that can be highly substituted with arabinose and galactose, while RG-II is an extremely complex, highly branched polysaccharide in which galacturonic acid and rhamnose are the major components. Enzymatic removal of these pectic polysaccharides suggests that all three can covalently attach to one another through \( \alpha-1,4 \)-linked galacturonic acid residues (Albersheim et al., 2011).

Cell walls also contain between 1 and 10% (dry weight) of proteins, proteoglycans, and glycoproteins that are involved in enzymatic, structural, and defensive functions (Albersheim et al., 2011). For the purpose of this thesis, arabinogalactan proteins are an important class since they contain higher levels of associated carbohydrates (>90%) compared to others that contain 55% at most. As their name suggests, the carbohydrate portion of arabinogalactan proteins are rich in arabinose and galactose that are attached to
multiple sites of the core protein, and may also contain lesser amount of glucuronic acid and other sugars (Showalter, 2001).

Another set of polysaccharides that are common in grasses includes the mixed-linked glucans, which are like cellulose in that they are made entirely of $\beta$-linked glucose residues (Albersheim et al., 2011). However, about 30% of 1,3-linked, while 70% are 1,4-linked.

2.2.3.3 Lignin

In addition to carbohydrates, lignin is another extremely important component of plant cell walls that is usually present at levels of between 10-35% in secondary walls. Lignin is a complex hydrophobic phenolic polymer that provides mechanical support and a water-impermeable barrier to the secondary cell wall (Albersheim et al., 2011). It is a very large polymer: isolated lignin has been found to have a molecular weight average of 20,000 to 77,000, demonstrating that lignin in its native form within the cell wall likely has a degree of polymerization of hundreds of phenylpropane subunits. Unlike the other major polymers within the cell wall such as cellulose and hemicellulose, the structure of lignin is unclear. It forms a three-dimensional structure that differs from the other major wall polymers that tend to exhibit highly ordered linear structures. What is known about lignin is that it is primarily composed of three different subunits, including p-hydroxyphenyl (H units), guaiacyl (G units), and syringyl (S units), which are polymerized from precursor cinnamyl alcohol monolignols. The ratio of these different lignin subunits can vary greatly between different plants and tissues. For example,
conifer lignin from a softwood tree such as spruce or pine is composed primarily of G lignin with minor amounts of H, while a hardwood such as poplar contains similar amounts of S and G lignin. Grasses on the other hand contain all three lignin subunits with S/G and H/G ratios of 0.46 and 0.49, respectively, as previously reported for miscanthus grass (Albersheim et al., 2011).

2.2.4 Cell wall architecture

The secondary cell wall is an extremely complex matrix made up of many different interacting components including cellulose, lignin, and the matrix polysaccharides hemicellulose and pectin (Albersheim et al., 2011). There is no single model that fully describes the architecture of the plant cell wall and how its numerous and diverse macromolecules come together to form it. Visual evidence has shown that cellulose microfibrils are the dominant structure imbedded in a matrix of other wall polysaccharides that determine microfibril spacing. Furthermore, several studies support the concept that the wall polymers are cross-linked by numerous covalent and non-covalent bonds and interactions. One of the most common interactions is that of cellulose and hemicellulose, which interact through multiple hydrogen bonds. In dicot primary cell walls, xyloglucans are the predominant hemicellulose that can adhere strongly to the surface of cellulose microfibrils, while arabinoxylans play this role in grasses. Conformational studies suggest that both xyloglucan in dicots and arabinoxylan in monocots can assume a structure resembling that of cellulose chains. This implies that these hemicelluloses can hydrogen bond to the cellulose surface, and may also serve to
cross-link multiple microfibrils, as demonstrated in Fig 2.1. Imaging studies suggest that these cross-links are likely on the order of 20 to 40 nm long. The degree to which cellulose microfibrils are covered or coated by hemicellulose is unknown. It is possible that they form a complete monolayer around the microfibrils; however, this is not agreed upon in the literature. It has been commonly reported that the ability of xyloglucan and arabinoxylan to bind to cellulose is affected by the degree and distribution of substitutions on their backbone. There is greater binding potential for hemicelluloses with less substituted backbones. For example, non-fucosylated xyloglucan likely has greater potential to bind to cellulose than does fucosylated xyloglucan (Albersheim et al., 2011).

Figure 2.1. Possible configurations of hemicellulose-cellulose interactions. The red portions represent hemicellulose domains that could provide further cross-links to additional cellulose microfibrils. Adapted from Albersheim et al., 2011.
There are numerous other interactions between macromolecules within the cell wall besides those between cellulose and hemicellulose (Albersheim et al., 2011). As such, it is also believed that multiple wall polysaccharides can be connected to one another through diferulic acids. Hydrocinnamic acids such as ferulic acid and \( p \)-coumaric acid are thought to serve as a bridge between hemicelluloses and/or pectins. For example, ferulic acid can cross-link arabinoxylan and xyloglucan through an ester-linkage in grasses. Additionally, it has also been postulated that pectic polysaccharides are covalently interconnected with one another via glycosidic bonds. Furthermore, as stated above, certain pectic polysaccharides such as HG, have the ability to form gels through multiple calcium cross-linking bridges. Although there is no strong evidence of covalent linkages between this pectic network and cellulose or hemicellulose, their gel-like structure likely causes them to be highly interactive with all other matrix polysaccharides (Albersheim et al., 2011).

Another extremely important source of cell wall interactions occurs within lignin, as well as between lignin and polysaccharides (Albersheim et al., 2011). Its different subunits can interconnect primarily through ether linkages and carbon-carbon linkages. It is also known that lignin can covalently cross-link to polysaccharides to form what are known as lignin-carbohydrate-complexes (LCCs). Lignin has a greater affinity for hemicellulose than for cellulose, and as a result, it is likely that lignin’s only main interaction with cellulose is through hemicellulose. There are three types of potential linkages responsible for LCCs, including (1) direct ester linkages between uronic acids of the polysaccharides and hydroxyl groups of lignin monomers, (2) direct ether bonds
between polysaccharides and lignin, and (3) a glycosidic cross-link such as a ferulic acid bridge, which can serve to indirectly connect lignin and polysaccharides (Iiyama et al., 1994).

With the exception of lignin structural studies, it is important to reiterate that much of the predicted cell wall architecture is based predominantly upon the study of primary cell walls. However as stated above, a majority of the structures and interactions likely remain valid and applicable for both. Regardless, it is clear that due to the diverse structure of cell wall macromolecules and their numerous interactions with one another, the cell wall is an extremely complex and cross-linked system. It is this complexity and highly cross-linked nature that contributes heavily to biomass recalcitrance and makes the release of sugars from biomass difficult.

2.3 Biomass recalcitrance

In order to obtain high yields of sugars from biomass via biological conversion routes, severe pretreatments and high enzyme doses are often required. There has been a tremendous amount of research directed at gaining a better understanding of why the hydrolysis of native lignocellulosic biomass is slow and incomplete, but the answers remain elusive. In general, it is thought that both substrate and enzyme features, as well as their interactions, play a role in limiting breakdown (Mansfield et al., 1999).
2.3.1 Enzyme factors

Potential limiting enzyme factors include inhibition, a decrease in substrate reactivity, enzyme deactivation with time, and non-productive binding to biomass components such as lignin (Berlin et al., 2005; Yang et al., 2006; Kumar and Wyman, 2009). The number of compounds that have been demonstrated or proposed to inhibit cellulase hydrolysis is high. End product inhibition by glucose and cellobiose is well established, and can largely explain the drop off in hydrolysis rates at longer times, particularly at high solids loadings (Holtzapple et al., 1990; Kristensen et al., 2009). Research has also recently shown that additional sugars such those that are xylan-derived including xylose and xylooligomers (Kumar and Wyman, 2009; Qing et al., 2010), as well as other hemicellulose sugars such as mannose and galactose (Xiao et al., 2004) can inhibit cellulases. Furthermore, there are many more potential inhibitory products that are formed by the degradation of biomass during pretreatment. These include a range of aliphatic acids, aromatic acids, aldehydes, and ketones, all of which can vary in concentration and level of inhibition for different biomasses (Du et al., 2010; Jing et al., 2009; Nakagame et al., 2010; Palmqvist et al., 1996).

In addition to inhibition, even the nature of the hydrolysis reaction itself poses barriers since biomass is largely insoluble, and thus requires direct physical contact between multiple enzymes and the substrate (Chang and Holtzapple, 2000). A suite of enzymes must work synergistically to effectively hydrolyze lignocellulosic biomass, further complicating the reaction (Mansfield et al., 1999).
2.3.2 Biomass structural factors

As compared to enzyme-related factors, there is an even longer list of biomass substrate features proposed to contribute to recalcitrance. Unfortunately, the literature published to date is often conflicting, and as a result, there is no clear picture about what structural features do and do not play a role. That being said, it is evident that biomass recalcitrance is a multi-scale phenomenon that scales several orders of magnitudes and includes structural, molecular and chemical features (Himmel et al., 2007; Chundawat et al., 2010). At the millimeter scale, plant anatomy and tissue arrangement have often been suggested to contribute to recalcitrance (Himmel et al., 2007). As discussed in section 2.2.2, tissue and cell types differ substantially from each other. Waxy epidermal cells have been shown to restrict enzyme and microbe access, while thick-walled sclerenchyma cells (2-5 µm thick) in tightly-packed block arrangements are typically much slower and more difficult to digest than thin-walled and loosely packed mesophyll cells (0.1-0.2 µm thick) (Wilson and Mertens, 1995; Buxton and Redfearn, 1997).

At a smaller scale, the highly cross-linked nature of the cell wall also likely adds to biomass recalcitrance. Hemicellulose and lignin, and their interactions with one another have been repeatedly suggested to influence biomass recalcitrance due largely to the idea that they limit and block access of enzymes to cellulose. The removal or reduction of these components can sometimes be strongly correlated with improved digestibility. However, while some studies have shown that genetically modified plants with lowered lignin contents exhibit significantly higher susceptibility to pretreatment and enzymatic hydrolysis (Chen and Dixon, 2007; Xu et al., 2008), other studies have found no such
correlation among large natural populations (Voelker et al., 2010). The influence of lignin and xylan has also been evaluated by removing these components in pretreatment reactions and testing their subsequent digestibility. Although the removal of lignin and xylan is commonly correlated with reduced recalcitrance (Yang and Wyman, 2004; Zhu et al., 2008), their direct effect is somewhat convoluted by the observation that their removal increases porosity and surface area. These are also viewed as major factors affecting biomass recalcitrance due to the potential to decrease mass transfer limitations and enhance physical contact between enzymes and substrates (Ishizawa et al., 2007; Chang and Holtzapple, 2000; Jeoh et al., 2007; Mansfield et al., 1999). Thus, it is unclear if the specific removal of lignin and/or xylan is important, or if generally increasing the available surface area is the real key.

A number of characteristics relating to cellulose have also been commonly proposed to influence biomass recalcitrance, ranging from microfibrillar orientation to cellulose lattice structure, crystallinity and DP (Mansfield et al., 1999; Chang and Holtzapple, 2000; Kumar et al., 2009). As such, it has been reported that decreased cellulose crystallinity is associated with improved digestibility (Chang and Holtzapple, 2000; Zhu et al., 2004; Harris et al., 2009). This observation seems logical since the closely-packed hydrogen-bonded structure associated with highly crystalline cellulose may be harder to break down and less accessible than more loosely arranged cellulose (Gregg and Saddler, 1996). However, literature reports on the subject are contradictory, and the results of many pretreatment studies followed by subsequent enzymatic hydrolysis seem to suggest that crystallinity is not critical (Kumar et al., 2009; Mansfield et al., 1999). However,
unlike pretreatment studies, genetic modification can produce more targeted and specific cell wall changes. As such, one group has reported that genetically lowering the crystallinity of *Arabidopsis* by 34% increased the enzymatic efficiency by 15% (Harris et al., 2009). Studies trying to correlate cellulose DP with glucan digestibility have been likewise inconclusive (Chang and Holtzapple, 2000; Mansfield et al., 1999; Hu et al., 2011). The DP determines the relative abundance of terminal and interior $\beta$-glucosidic bonds, and thus the availability of substrates for exo- and endo-acting enzymes (Zhang and Lynd, 2004). As such, some have reported that the cellulose DP determines the hydrolysis rate, but not necessarily the overall yield (Chang and Holtzapple, 2000).

Another subset of biomass characteristics that potentially contribute to recalcitrance is the composition and structure of individual wall polymers other than cellulose. In one study, down-regulation of UDP-xylose produced genetically modified tobacco lines with reduced xylan content; contrary to expectations, mutants with reduced xylan content did not exhibit improved cellulose extractability, and furthermore, were increasingly difficult to delignify (Bindschedler et al., 2007). On the other hand, modification of the hemicellulose-cellulose network via xyloglucan transglycosylases was reported to potentially loosen cell-wall structures (Abramson et al., 2010; Fry et al., 1992) and thus reduce biomass recalcitrance. In addition to the hemicellulose backbones, their degree of acetylation is also a commonly proposed feature that contributes to biomass recalcitrance. It has been reported that acetyl groups may decrease enzyme accessibility to both cellulose and xylan and thus reduce enzyme effectiveness. Their removal via chemical reactions has been shown to result in increased enzymatic digestion in some studies (Zhu
et al., 2008; Kumar et al., 2009), similar to reports that genetically modified plants with reduced O-acetylation of xyloglucan and pectin resulted in improved enzymatic digestibility and polysaccharide extractability (Pauly and Scheller, 1999; Abramson et al., 2010). However, others studies have suggested that the impact of acetylation is minimal (Chang and Holtzapple, 2000). In general though, it is understood that extensive branching and substitutions do restrict the ability of xylanases to break down the xylan backbone (Correia et al., 2011). In addition to acetyl groups, a study by Mortimer et al. (2010) recently found that Arabidopsis mutants lacking almost all xylan substitutions (including glucuronic acid and arabinose residues) exhibited significantly improved extractability via sequential chemical extraction. Although this study did not shed light on the implications of xylan branching on enzyme accessibility, it did suggest that the absence of branching may change the cell wall structure in such a way that it is less recalcitrant to degradation. Furthermore, another set of polysaccharides that have also been proposed to influence recalcitrance is pectins. A study by Lionetti et al. (2010) found that the genetic reduction of de-methyl-esterified homogalacturonan (HGA) increased enzymatic saccharification efficiency.

Lignin composition and structure have also been proposed to influence biomass recalcitrance. The degree of cross-linking between lignin and hemicellulose provides strength to the cell wall, helps to reinforce it, and decrease its accessibility to enzymes. The reduction of LCCs has been proposed as a mechanism to increase digestibility (Casler, 2008; Buxton and Redfearn, 1997; Jung and Casler, 2006). Likewise, the composition of lignin is another potential feature that impacts recalcitrance. As discussed
in section 2.2.3.3, lignin is composed of three different subunits, including p-hydroxyphenyl (H units), guaiacyl (G units), and syringyl (S units). Their structure and ability to covalently crosslink with other units varies. For example, guaiacyl until can crosslink with up to three other units, while syringyl units may link with up to two (Davison et al., 2006). Intuitively then, a lower S/G lignin ratio should imply more potential for crosslinking, and thus, higher recalcitrance. A number of studies have tested this hypothesis, but they have often been limited in sample size, as well as lignin content and composition range. As a result, some studies have found no influence of S/G ratio (Reddy et al., 2005; Chen and Dixon, 2007), while others have reported positive and negative correlations with digestibility (Fontaine et al., 2003; Davison et al., 2006).

2.4 The effect of pretreatment

2.4.1 Introduction

To overcome the natural recalcitrance of biomass and achieve high sugar yields, a processing step called pretreatment is typically performed prior to enzymatic hydrolysis. Enzymatic conversion of raw, untreated biomass usually results in low yields even after very long incubation times (168 hr). However, enzymatic hydrolysis of pretreated materials can reach theoretical 100% conversion in more reasonable incubation times (<72 hrs). To date, a wide variety of pretreatments have been tested, most of which involve a high temperature and pressure reaction. Chemicals can also be added to help catalyze the reaction, including a variety of acids and bases (Wyman et al., 2005). Furthermore, a mechanical process can also accompany pretreatment reactions; as such,
an explosive condition associated with the rapid release of high pressure is found in steam explosion and Ammonia Fiber EXpansion (AFEX) pretreatments. The common denominator underlying all pretreatments is that they can significantly improve the digestibility of biomass. However, the extent to which they do this and the mechanisms by which it is accomplished are highly variable. It remains unclear how pretreatments with such different mechanisms that cause distinct changes to the biomass chemistry and structure can all result in significant digestibility improvements.

This thesis focuses mainly on hydrothermal pretreatment, which in many respects can be considered a milder version of dilute acid pretreatment. The severity of a hydrothermal pretreatment reaction can be described by the severity parameter (Overend and Chornet, 1987):

\[
R_0 = t \cdot e^{\frac{T-100}{14.75}}
\]

where \(t\) is time in minutes and \(T\) is temperature in °C. The remainder of this chapter will introduce typical hydrothermal and dilute acid pretreatment conditions and discuss their effects on biomass.

2.4.2 Hydrothermal and dilute acid pretreatment

Hydrothermal and dilute acid pretreatments typically involve reaction times ranging from seconds to minutes and temperatures between 120-220°C (Mosier et al., 2005). The most commonly used acid is dilute sulfuric acid, which is usually added at concentrations of less than 2%. The main effect of sulfuric acid pretreatment is the removal and
hydrolysis of xylan into xylose, whereas in hydrothermal pretreatment, the xylan removal is lower and a larger proportion of xylooligomers remain in solution (Mosier et al., 2005; Wyman et al., 2005; Kumar et al., 2009). In addition to the removal of xylan, it has also been shown that xylan is redistributed within the cell wall during dilute acid pretreatment. As such, xylan was found to migrate from the center of most sclerenchyma cells into the cell lumen and middle lamellae (Brunecky et al., 2009). Furthermore, it has also been reported that the acetyl side chains of xylan get progressively removed by pretreatments of increasing severity (Kumar et al., 2009).

The fate of lignin during pretreatment is likely equally important as that of hemicellulose. Although there is little removal during dilute acid and hydrothermal pretreatments, it has been reported that lignin is substantially disrupted and redistributed during pretreatment reactions (Wyman et al., 2005). As such, droplets of lignin were found on the surface of dilute acid pretreated biomass (Donohoe et al., 2008; Kristensen et al., 2008; Selig et al., 2007). Whether or not these droplets stay complexed with any carbohydrates is unclear. However, it was observed that particular regions appeared to accumulate higher concentrations of droplets, including cell corners and pits (Donohoe et al., 2008, 2011). Based on these results, it was proposed that for any thermochemical pretreatment above its melting temperature, lignin coalesces, migrates, and re-deposits on cell walls (Donohoe et al., 2008). Thus, despite the low removal of lignin observed during pretreatment, it is likely that its restructuring and redistribution exposes the internal cellulose surface for subsequent enzymatic hydrolysis.
On a molecular level, changes also occur to cellulose during pretreatment. It has been reported that both hydrothermal and dilute acid pretreatments increase the crystallinity of cellulose slightly, which has been attributed to the preferential degradation of the more labile amorphous material (Hu et al., 2011; Sannigrahi et al., 2008; Kumar et al., 2009; Foston and Ragauskas, 2011). The level of increase as measured by the crystallinity index (CrI) is somewhat minor, ranging between about 1-8% depending on biomass and pretreatment severity (Kumar et al., 2009; Foston and Ragauskas; Sannigrahi et al., 2008). Studies have also shown that there is an increase in the relative amount of the cellulose \( \text{I}_\beta \) form that occurs concurrently with changes to the crystallinity (Sannigrahi et al., 2008; Foston and Ragauskas, 2011). Furthermore, multiple studies demonstrate that the cellulose DP also changes during dilute acid and hydrothermal pretreatments. A significant drop has been reported, such that the DP of pretreated materials is usually about one-third of the starting biomass (Kumar et al., 2009; Hu et al., 2011).

Application of microscopic techniques has also provided further insight into the effects of hydrothermal and dilute acid pretreatments on biomass. Despite the long-held notion that these pretreatments significantly disrupt the natural cell and cell wall structure, recent findings have shown that the tissue and cell structures remain largely intact (Kristensen et al., 2008; Donohoe et al., 2011). At a magnification of 600X, pretreated cells appear expanded or swollen, but are largely very similar to the untreated material (Donohoe et al., 2011). At a greater magnification of up to 20,000X, differences in the ultrastructure of the untreated and pretreated cell walls can be seen. Partial defibrillation of cellulose microfibrils and delamination of cell wall lamella has been
observed (Kristensen et al., 2008; Donohoe et al., 2011). Together with the removal of hemicellulose and the relocalization of lignin, these observed changes likely further increase the porosity of the pretreated material and help to improve enzyme accessibility. As such, multiple studies have demonstrated that there is an increase in the available surface area following pretreatment, as well as a shift in the pore size distribution from smaller to larger volume pores (>3 nm) (Thompson et al., 1992; Ishizawa et al., 2007). This is a significant increase from untreated biomass in which 80% of pores have been reported to have diameters smaller than 0.8 nm (Adani et al., 2007). To put these values in perspective, a water molecule has an approximate diameter of 0.28 nm, while the catalytic core of a cellobyohydrolase (CBHI) enzyme from *Trichoderma reseii* has the approximate dimensions of 5 x 6 x 4 nm (Mansfield et al., 1999).

**2.5 Closing thoughts**

The plant cell wall contains an enormous amount of fermentable sugar that has the potential to produce significant amounts of biofuels. However, although there has been a tremendous amount of research directed at understanding why the hydrolysis of native lignocellulosic biomass is slow and incomplete, the answers remain elusive. This review highlights the tremendous complexity of the cell wall, including its individual macromolecules and how they are integrated together within the wall. The complexity and highly cross-linked nature likely contribute heavily to biomass recalcitrance and make the efficient release of sugars from biomass difficult. Unfortunately, the lack of a model to fully describe the architecture of plant cell walls makes it difficult to confidently
identify structural features that contribute to biomass recalcitrance. Regardless, it is evident that recalcitrance is a multi-scale phenomenon that spans several orders of magnitudes and includes structural, molecular and chemical features.

Pretreatment processes can effectively overcome this natural recalcitrance. There is a long list of changes that occur in biomass as a result of pretreatment, almost all of which have been proposed at one time or another to increase biomass digestibility. However, unraveling all of these ideas to determine which factors do and do not impact recalcitrance has yet to be accomplished. This objective is complicated by the fact that the effectiveness of pretreatment can vary by species, plant, plant fraction, as well as by pretreatment condition. It is still unclear why a certain plant, or fraction of a plant, can respond better to a given pretreatment than another. The plant features that contribute to biomass recalcitrance and the mechanism(s) by which pretreatment overcomes this recalcitrance must be clarified.

2.6 References


Chapter 3. High throughput pretreatment and enzymatic hydrolysis (HTPH) systems*

* The first paragraph of the abstract and section 3.2 was reprinted from High Throughput Pretreatment and Hydrolysis Systems for Screening, in Aqueous Pretreatment of Plant Biomass for Biological and Chemical Conversion to Fuels and Chemicals, edited by Professor Charles E. Wyman, Copyright (IN PRESS) John Wiley and Sons Ltd. Please contact publishers for details.
3.1 Abstract

*Due to the limited understanding of biomass recalcitrance, there was a need to generate and screen large numbers of plant samples against a variety of pretreatment conditions, including various times, temperatures, and chemical addition, as well as a wide range of enzyme sources and formulations. Because of the time-consuming and laborious nature of biomass analytical techniques, conventional testing of sugar release from pretreatment and enzymatic hydrolysis was not feasible, and as a result, high throughput pretreatment and hydrolysis (HTPH) systems that were capable of providing basic sugar release data for large sample sets were developed.

In section 3.2, HTPH systems published in the literature will be reviewed with a detailed examination of how the basic required steps were accomplished, including material preparation, material distribution, the pretreatment reaction, and sample preparation and analysis. Additionally, HTPH philosophy, as well as difficulties and limitations associated with these systems will be presented, followed by a review of successful applications to date and possible future applications. Section 3.3 will then focus on the system development for the University of California Riverside (UCR) HTPH system, as well as select tests that were performed to verify its performance. Finally, section 3.4 will discuss improvements that were made in order to integrate the UCR system with a robotics platform to increase its throughput and improve accuracy; afterwards, a more detailed analysis of the HTPH performance will be presented.
3.2 High throughput pretreatment and hydrolysis systems for screening biomass species: an introduction and review of HTPH systems*

3.2.1 Introduction: a need for high throughput technologies

The primary barrier to low cost biological conversion of lignocellulosic biomass to renewable fuels and chemicals is a plant’s recalcitrance, or the resistance of cell walls to deconstruction by enzymes or microbes (Lynd et al., 2008; Wyman, 2007). However, the discovery and use of biomass species with reduced recalcitrance, when combined with optimized pretreatment processes and enzyme mixtures, have the potential to improve the commercial viability of fuel and chemical production from lignocellulosic biomass (Lynd et al., 1991; Lynd et al., 1999). Unfortunately, the current understanding of biomass recalcitrance is limited, making it difficult to rationally select superior plant species without prior sugar release testing. As a result, there is a need to generate and screen a large variety of plants to identify those that exhibit both superior and sub-par sugar release. To this end, there are two central methodologies in generating and screening plants: the generation of mutants to see what effect targeted modifications have; and the evaluation of natural variants to identify outliers for further characterization in order to relate observed behavior to structural features and biomass characteristics. Both methodologies require thousands of samples to be tested. When considering that each sample should be screened against a variety of pretreatment conditions, including various times, temperatures, and chemical addition, as well as a wide range of enzyme sources and formulations, the number of experiments easily reaches the tens of thousands.
Due to the time-consuming and laborious nature of biomass analytical techniques, conventional testing of sugar release from pretreatment and enzymatic hydrolysis is not feasible. As a result, in the past five to ten years, there has been a push to develop high throughput pretreatment and hydrolysis (HTPH) systems that are capable of providing basic sugar release data in a fast and automatable manner, while using significantly less biomass than conventional techniques. The benefits of implementing a fast and automatable procedure are clear: the throughput of analysis can be dramatically increased, and the screening of multiple biomass-pretreatment-enzyme formulation combinations can be possible in a greatly-reduced time frame and with lower costs. Additionally, equally important benefits can be realized due to the low material requirements of HTPH systems. For one, results can be obtained sooner. For genetically modified mutants, it can often take years for plants to adequately mature to produce sufficient amounts of material for analysis; this however is not a concern with downscaled HTPH systems for which only mg-amounts of biomass would be required. Additionally, when screening trees with HTPH systems, entire trees do not have to be destroyed for analysis; instead, a small core sample can be obtained and analyzed without harming the tree. Finally, the reduced material requirement also allows analysis of individual biomass fractions that was previously not possible, in addition to making more biomass material available for other analyses.
3.2.2. Review of past high throughput systems and application to pretreatment and enzymatic hydrolysis

The development and use of high throughput (HT) technologies is relatively new. Since the 1990’s, there has been a push to automate and increase the throughput in a variety of fields, ranging from pharmaceuticals and drug discovery to genetic sequencing. In fact, there have also been efforts directed at downscaling enzymatic hydrolysis of lignocellulosic biomass. Conventionally, enzymatic hydrolysis is typically performed in either 20 mL scintillation vials or 125 mL Erlenmeyer flasks, into which on the order of 0.2 to 3 g, respectively, of wet pretreated solids are weighed (Brown and Torget, 1996; Selig et al., 2008). However in 2006, “A Rapid Microassay to Evaluate Enzymatic Hydrolysis of Lignocellulosic Substrates” was reported that was based on a 96-well microplate for which each ~300 μL well served as a reactor for enzymatic hydrolysis (Berlin et al., 2005). In 2007, the use of a standard 96-well plate was similarly reported for cellulase accessibility experiments (Jeoh et al., 2007), while more recently, additional reports have been made of microplate-based approaches to evaluate sugar release from the enzymatic hydrolysis of both untreated and pretreated biomass (Chundawat et al., 2008; Navarro et al., 2010). Other downscaled systems, such as 1.5 mL plastic Eppendorf tubes and 2 mL glass high performance liquid chromatography (HPLC) vials, have also been used (Selig et al., 2009; Decker et al., 2009). Although these systems provided a significant advancement in scaling down and automating biomass analyses, the development of downscaled and high throughput pretreatment still remained a hurdle. Without pretreatment, sugar yields are extremely low, and in order to adequately screen plant samples for their applicability in conversion to fuels and chemicals, a pretreatment
step is required prior to enzymatic hydrolysis (Wyman, 2007). However, extending high throughput applications to pretreatment processes presents a number of difficulties. Whereas enzymatic hydrolysis is performed at $\leq 50^\circ$C and near-neutral pH, pretreatments are often associated with high temperatures, pressures, and corrosive chemicals, virtually ruling out use of glass HPLC vials and plastic microplates and Eppendorf tubes.

To date, a variety of conventional pretreatment reactors have been utilized at the laboratory bench scale. For three of the most commonly used pretreatments, hydrothermal (hot water-only), dilute acid, and dilute alkali, metal tubes or stirred tank reactors are often employed. Such reactors can safely handle the acid or base concentrations (usually $<2\%$) at the elevated pressures and temperatures that are used (90-220$^\circ$C). Table 3.1 summarizes features of a variety of batch pretreatment reactors that have been reported in the literature in order to demonstrate reactor types, reaction volumes, and biomass material requirements that are often encountered in laboratory experiments. The table does not provide a comprehensive review of all reactor types, but instead demonstrates the range of instruments and conditions that have been employed in the past. For such reactor systems, steam and fluidized sand or oil baths are commonly used as heat sources in order to achieve fairly uniform heating, as well as fast heat-up. Due to the large vessel size conventionally required for a single pretreatment reaction and the space available in employed heating devices, only a limited number of pretreatments can be performed simultaneously.
Table 3.1. Types of batch pretreatment reactors commonly employed in conventional laboratory processes, with pretreatment chemical environment and required reaction mass listed for each.

<table>
<thead>
<tr>
<th>Reactor type</th>
<th>Reaction mass (g)</th>
<th>Pretreatment Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>11mm Pyrex glass tubes</td>
<td>0.4</td>
<td>Dilute acid</td>
<td>Chen and Lee, 1996</td>
</tr>
<tr>
<td>300-mL metal Parr stirred tank reactor</td>
<td>2</td>
<td>Dilute acid</td>
<td>Foston and Ragauskas, 2010</td>
</tr>
<tr>
<td>Batch metal tube reactors – 12.5mm OD, 0.8255-mm wall thickness, 10 cm length</td>
<td>6</td>
<td>Dilute acid</td>
<td>Lloyd and Wyman, 2003</td>
</tr>
<tr>
<td>Batch metal tube reactors – 1.5in schedule 40 pipe nipples</td>
<td>7.5</td>
<td>Calcium hydroxide</td>
<td>Kaar and Holtzapple, 2000</td>
</tr>
<tr>
<td>500-mL metal stirred autoclave</td>
<td>40</td>
<td>Liquid hot water</td>
<td>Negro et al., 2003</td>
</tr>
<tr>
<td>1-L Parr metal stirred tank reactor</td>
<td>50</td>
<td>Dilute acid</td>
<td>Torget et al., 1992</td>
</tr>
<tr>
<td>2-gal metal Parr stirred tank reactor</td>
<td>500</td>
<td>Dilute acid</td>
<td>Spindler et al., 1990</td>
</tr>
</tbody>
</table>

Although there was some success in scaling down pretreatments to use less than 1 g (Lloyd and Wyman, 2003), there still remained a number of time-consuming steps required after pretreatment in order to prepare the materials for subsequent enzymatic hydrolysis: 1) pretreated solids and liquids are separated; 2) solids are washed; and 3) wet chemistry compositional analysis is performed on pretreated washed solids. Enzymatic hydrolysis is then typically performed on the washed pretreated solids, with enzyme addition based on the composition of pretreated biomass (Brown and Torget, 1996; Selig et al., 2008). The amount of sugars released by both pretreatment and enzymatic hydrolysis is measured, with a post hydrolysis procedure often applied to determine oligomeric sugar concentrations (Sluiter et al., 2006). The procedures are tedious and time-consuming, require significant amounts of material, and do not easily lend themselves to automation or increased throughput. As a result, unlike enzymatic hydrolysis that is more readily scaled down as a result of the mild reaction conditions, the development of HT pretreatments lagged behind.
3.2.3 Current HTPH systems

To date, descriptions of four high throughput pretreatment and enzymatic hydrolysis (HTPH) systems have been published, all of which were based on the diagram shown in Figure 3.1b (Decker et al., 2009; Gomez et al., 2010; Santoro et al., 2010; Selig et al., 2010; Studer et al., 2010). As opposed to conventional pretreatment and enzymatic hydrolysis (Figure 3.1a) that involve large scale pretreatments from which pretreated solids are then distributed to multiple hydrolysis experiments, HTPH systems involved downscaled pretreatments that were then used directly for subsequent enzymatic hydrolysis.

![Diagram of HTPH systems](image)

Figure 3.1. Comparison of (a) conventional pretreatment and subsequent enzymatic hydrolysis laboratory experiments with (b) HTPH approach in which the same reactor is employed for both pretreatment and enzymatic hydrolysis to avoid processing between the two operations. Adapted from Studer et al (2011b).
To accomplish this, three of the HTPH systems employed a process termed ‘co-hydrolysis’ (Decker et al., 2009; Selig et al., 2010; Studer et al., 2010; Studer et al., 2011b) or ‘one-tube process’ (Santoro et al., 2010) in which both pretreatment and enzymatic hydrolysis were performed in the same reactor without the need for conventional procedures such as solid/liquid separation and solid washing in between the pretreatment and hydrolysis steps. Figure 3.2 outlines the differences between conventional pretreatment and enzymatic hydrolysis with new co-hydrolysis or one-tube processes. Work by Santoro et al. (2010) and Studer et al. (2010) demonstrated that these processes achieved the same sugar yields as conventional washed solids hydrolysis. Further detailed analyses showed that due to the release and/or generation of inhibitors in the pretreatment hydrolyzate that was not separated from pretreated solids prior to enzymatic hydrolysis, certain conditions existed, including solid and enzyme loadings, for which co-hydrolysis was most comparable to conventional processes (Studer et al., 2011b). As a result, particular attention must be paid in HTPH systems that employ co-hydrolysis or one-tube processes to select conditions that best mimic the sugar yields obtained from conventional pretreatment and enzymatic hydrolysis. To avoid this concern, one of the HTPH systems (Gomez et al., 2010) varied somewhat from the other three: after pretreatment, several rinses with a buffered solution were applied prior to enzymatic hydrolysis. This process closely mimicked the solid/liquid separation and solid washing that is typically performed in conventional processes, such that the pH of the pretreated material was brought to the same value as that of enzymatic hydrolysis and the majority of inhibitors generated during pretreatment were removed.
Figure 3.2. Flow diagrams of conventional pretreatment and enzymatic hydrolysis versus an HTPH approach that employs a co-hydrolysis or one-tube process. Adapted from Studer et al. (2011b).

Although all four HTPH systems were based on the same principle (Fig 3.1b), each system varied in terms of its reactor configuration and processing conditions. Developed through support of the BioEnergy Science Center (BESC), the HTPH system at the University of California Riverside (UCR) is pictured in Figure 3.3 and was based on a 96-well plate design in which the base plate was constructed of either aluminum or brass, and the reactor wells were made of Hastelloy. The original design (Studer et al., 2010) included an aluminum base plate into which Hastelloy wells that employed a reaction mass of 250 mg were press fit (Fig 3.3a). More recently, an updated well plate design was developed in which the Hastelloy wells were larger, employing a reaction mass 450 mg, and in which the wells themselves were free-standing via a small pin at the well bottom to allow wells to sit upright on the brass base plate, as opposed to press fit. This modification enabled a robotic platform’s gripper to pick up and move individual vials to
more accurately tare and add ingredients. For sealing in both cases, the well plate was clamped between a top and bottom stainless steel plate with a flat silicone gasket. For pretreatment, the well plate assembly was placed into a custom-built steam chamber in which steam could penetrate the space between individual wells and provide 360° heat transfer. At the completion of the pretreatment reaction, steam was vented and cooling water was flooded into the chamber. Therefore, UCR’s HTPH system was capable of pretreating 96 biomass samples in one plate, with multiple plates potentially heated at once in the steam chamber.

As shown in Figure 3.4, the National Renewable Energy Laboratory (NREL) also developed an HTPH system through support of the BioEnergy Science Center (BESC) based on gold-plated aluminum or Hastelloy stackable 96-well plates for hydrothermal or dilute acid pretreatments for which the total reaction mass employed by each well was 255 mg (Decker et al., 2009; Selig et al., 2010). After loading, each plate was sealed by placing an adhesive-backed aluminum foil Teflon® gasket between plates, and then up to 20 custom-made 96-well plates could be stacked together and clamped within a 2-gal Parr reactor for pretreatment with indirect steam. Holes were machined into each well plate to serve as channels through which steam could travel and provide more rapid and uniform heating during pretreatment. After the pretreatment reaction was complete, cooling water was forced through the channels in the plate. NREL’s HTPH system was capable of pretreating up to 1,920 biomass samples simultaneously.
Figure 3.3. UCR’s HTPH reactor system including the original reactor design in which Hastelloy wells with a 250 mg reaction mass were (a) press fit into an aluminum plate clamped between two stainless steel plates during pretreatment, and (b) the updated reactor with larger free-standing Hastelloy wells (450 mg reaction mass) being loaded by a Symyx Core Module.

Figure 3.4. The NREL HTPH reactor system uses the Symyx Powdernium to dispense biomass into (a) the wells of the 96-well reactor plate with a reaction mass of 255 mg per well with (b) 20 reactor plates stacked together in a modified 2-gal Parr reactor for pretreatment. Reproduced from Decker et al. (2009).
Figure 3.5. GLBRC’s HTPH system, including (a) iWall robotics platform for milling and dispensing. More detailed views of the weighing substation (b), balance and vibro-feeder dispensing from input (upper) to output (lower) tube (c), diagram of weighing substation (d), and bar code scanner sub-station (e) are also shown. Reproduced from Santoro et al. (2010) with permission from Springer.

The Great Lakes Bioenergy Research Center’s (GLBRC) HTPH system shown in Figure 3.5 was based on a 96 tube Stabo-rack that held 1.4 mL polypropylene microtubes, each of which employed a reaction mass of ~750 mg (Santoro et al., 2010). After loading, the tube racks were sealed with an elastopolymer seal and placed into a water bath for pretreatment. At the completion of the pretreatment reaction, the tube racks were cooled on ice. GLBRC’s HTPH system could pretreat 3 96-tube racks simultaneously, or 243 biomass samples at a time.

The fourth HTPH system shown in Figure 3.6 was developed by researchers at the University of York and the University of Dundee, and was based on a standard plastic 96-well plate (Gomez et al., 2010). In this system, each well employed a pretreatment
reaction volume of 350 μL. After loading, the plates were sealed with a silicone cover and placed onto a heating block for pretreatment. This system could pretreat 360 biomass samples at a time.

Figure 3.6. HTPH system described by Gomez et al., (2010) including general view of robotics platform for milling and dispensing (A), and schematic of robot’s different sub-stations. Figure reproduced from Gomez et al. (2010).

Although it did not measure the sugar release from combined pretreatment and enzymatic hydrolysis, a fifth high throughput system accomplished downscaled and high throughput pretreatment coupled with an alternative measure of biomass-pretreatment performance. In this system, a standard polystyrene 96-well plate was employed (~200 mg reaction mass per well) in which various ionic liquids could be tested for their ability to dissolve the cellulose portion of biomass samples (Zavrel et al., 2009). For
pretreatment, a block containing heating rods through its interspaces, was heated by temperature-controlled water. The block itself also acted as a seal to prevent water uptake by the ionic liquids. This HTPH system was capable of pretreating and measuring \textit{in-situ} 96 biomass samples at a time.

3.2.4 Key steps in HTPH systems

For all HTPH systems, there were four basic steps that had to be accomplished: 1) material preparation, 2) material distribution, 3) the pretreatment reaction, and 4) sample preparation and analysis. In this section, we will outline each step and summarize techniques that have been applied to accomplish them to date.

3.2.4.1 Material preparation

Preparing biomass materials for high throughput pretreatment and enzymatic hydrolysis experiments was a crucial step which impacted all downstream processes, as well as the validity of any results. The material had to be homogeneous so that each small sample was representative of the larger mass, with three primary steps involved, including sampling, particle size reduction, and any subsequent conditioning to obtain the prepared sample. In NREL’s HTPH system, biomass material was knife milled until it passed through a 1 mm screen (Selig et al., 2010). No further conditioning was reported. Similarly, UCR’s HTPH system also employed knife-milled material; however, milling was typically followed by sieving in order to obtain a 20-80 mesh fraction (0.18<x<0.85 mm) (Studer et al., 2010). Zavrel et al (2009) reported the use of wood chips produced
by sawing in their HT pretreatment system; the resulting particle sizes were in the range of 1-2 mm in length.

Unlike the above three systems that required fairly time-consuming manual material preparation steps, the HTPH systems reported by Santoro et al (2010) and Gomez et al (2010) were unique in that they performed size reduction automatically via grinding robotics platforms, namely iWALL (TECAN, Mannedorf, Switzerland) and Labman Automation (Stokesley, North Yorkshire, UK), respectively. With the former, dried plant material (20-40 mg) was manually loaded into 2-mL screw cap microtubes along with three 7/32 inch stainless steel balls. These tubes were then loaded into racks and placed in the robotics system. Pulverization of the biomass was accomplished by ball milling to produce a fine powder for which the particle geometric mean diameter was between 0.034 and 0.055 mm (depending on biomass type), and more than 90% of the particles were smaller than 0.35 mm for all plant types tested. The system described by Gomez et al (2010) similarly employed automated shaking at approximately 5000 rpm using three ball bearings within biomass-filled vials. The particle size range of the resulting material was not reported.

3.2.4.2 Material distribution

Following preparation, materials had to be distributed to the pretreatment reactors. For the small amounts of biomass that were used in HT technologies, accurate solids distribution could have been a tedious and time-consuming step. Prior to the development of HTPH systems, some of the downscaled and HT enzymatic hydrolysis setups that were discussed in section 2 applied creative methods to accomplish small
scale solids distribution. For example, Berlin and coworkers (2005) took advantage of the uniform nature of biomass paper to produce “handsheets” of ethanol organosolv pulped poplar, from which 6 mm disks that weighed on the order of 1-2 mg were obtained using a paper punch. Single disks were then manually distributed into each individual well for subsequent enzymatic hydrolysis experiments. This method was similar to one developed by Decker et al. (2003) in which 2.65 mg filter paper disks were produced and distributed into the wells of a standard microtiter plate for an automated filter paper assay to determine cellulase activity. Others (Chundawat et al., 2008; Navarro et al., 2010) accomplished small scale biomass solids distribution into 96-well plates by suspending a batch of solids in a slurry with water or buffer (1-5% w/w). While maintaining a well-mixed environment, homogeneous aliquots of slurry were transferred to individual wells of a 96-deepwell microplate for testing.

For HT pretreatment and enzymatic hydrolysis systems, some papers reported that solids were manually weighed into the individual wells of microtiter plates (Studer et al., 2010; Zavrel et al., 2009). In the case of the initial UCR HTPH design described by Studer et al. (Studer et al., 2010) a small brass weighing cup that held a volume corresponding to the target mass to be dispensed (2.5 mg) was used to facilitate the distribution of biomass to the individual wells. However, the weighing of mg-quantities of biomass was time-consuming and reduced the throughput of these systems compared to what was sought. As a result, there was a shift by all four HTPH systems to use of solids dispensing robotics platforms. At NREL, a Symyx MTM Powdernium powder dispensing system (Symyx, Sunnyvale, CA) was used for distributing 5 mg of biomass to
each well of their 96-well plate reactor (Decker et al., 1009; Selig et al., 2010). The robotic platform’s deck could accommodate up to 80 plastic 10 mL biomass-dispensing hoppers at a time, each of which could handle small biomass amounts (50-100 mg). In this system, the entire reactor plate was moved to a modified Sartorius LP330 balance, which recorded (to 0.1 mg) the final weight of biomass that was dispensed into each well. After solids distribution, an automated pipetting system (Biomek FX) was used for all subsequent liquid handling steps. The total reaction mass employed by each well was 255 mg, of which 250 µL of water or dilute acid was added to 5 mg of milled biomass.

At UCR, a Symyx Core Module with Standard Configuration 2 was used for dispensing 4.5 mg of biomass per well for the updated well plate design (DeMartini et al., 2011). The robotics platform could accommodate 10 metal 25 mL biomass-dispensing hoppers, each of which could be loaded with a range of biomass amounts (5-5,000 mg). UCR’s system differed from NREL’s in that individual wells of the microtiter reactor were moved into a Sartorius WZA65-CW balance one at a time, allowing determination of the precise amount of each ingredient added to a single well (to 0.01 mg). Subsequent liquid handling steps were either accomplished by the liquid handling setup on the same Core Module robotics platform or with multichannel pipettes (8 channel pipetter, Eppendorf, Hamburg, Germany). In this system, 445.5 µL of water was typically added to 4.5 mg of milled biomass for hydrothermal pretreatment. For dilute acid and dilute alkali pretreatments, the reaction was performed with 85.5 µL of acid or 40.5 µL of base added to 4.5 mg of biomass.
In GLBRC’s HTPH system, the same iWALL robotics platform used for biomass milling was also used for solids dispensing (Santoro et al., 2010). After automated milling within a 1.4 mL tube, vials were sent to a de-clogging station to break up clumped material, followed by transfer to a piercing station at which a 1 mm hole was bored into the base of each vial. Another empty vial was placed below onto a Mettler Toledo SAG 205 balance into which the original vial filled with milled biomass dispensed 1.5 mg of material through a funnel by the action of a vibro-feeder. The amount of biomass dispensed into each tube was then recorded by the balance (to 0.01 mg) and stored. Subsequent liquid transfers were accomplished using a PerkinElmer (Waltham, MA) Janus workstation, which dispensed 750 µL of pretreatment liquid, typically dilute NaOH, into each tube.

The system reported by Gomez et al. (2010) was similar to the last one described above. The major differences included use of an alternate robotics platform, namely a Labman Automation platform (Stokesley, North Yorkshire, UK), that dispensed 4.0 mg of material from the original vials filled with milled biomass into the individual wells of a 96-well microplate. The amount of biomass in each plate was monitored by placing the entire microplate on a balance (to 0.1 mg) during dispensing. Subsequent addition of 350 µL of pretreatment liquid, either dilute NaOH or H₂SO₄, were performed with the same robotics platform.
3.2.4.3 Reactions

After material distribution, the next key steps in any HTPH system were the pretreatment and enzymatic hydrolysis reactions themselves. NREL performed either water-only or dilute acid pretreatment in their system at around 2% solids loading. As an example, they reported a hydrothermal pretreatment condition of 40 min at 180°C (Selig et al., 2010), and more recently have also employed dilute acid pretreatment typically with 0.3% $\text{H}_2\text{SO}_4$ for 30 min at 180°C. UCR’s HTPH system was compatible with hydrothermal (Studer et al., 201), dilute acid (Gao et al., in preparation), and dilute alkali pretreatments (Li et al., in preparation). For hydrothermal pretreatments, a solids loading of 1% was employed, while for dilute acid and alkali, 5% and 10% solid loadings were used, respectively. Variable temperatures (between 120 to 180°C) and times (between 10 to 300 min) were selected based on the biomass to be tested. For dilute acid and alkali pretreatments, concentrations of 0.5-1% $\text{H}_2\text{SO}_4$ and 1% NaOH, respectively, were applied. Both dilute acid and dilute alkali pretreatments could also be performed in GLBRC’s HTPH system. For example, they reported pretreatment at 90°C in 0.025% NaOH for 3 hours with a solids loading of 0.2% (Santoro et al., 2010). Similarly, Gomez et al (2010) employed both dilute acid and alkali pretreatments but at different conditions: 1% $\text{H}_2\text{SO}_4$ or 0.5N NaOH at 90°C for 30 min at a solids loading of about 1.1% were reported. Finally, Zavrel et al (2009) employed an ionic liquid pretreatment using a variety of imidazolium-based solvents. A total of 200 µL of various imidazolium-based ionic liquids was added to between 4 to 12 mg of biomass per well, for a corresponding
solids loading of 2-6%. In this setup, pretreatments were performed at 50°C for between 8 and 24 hours.

Following pretreatment, enzymatic hydrolysis was performed in the same reactor in a co-hydrolysis or one-tube processing format for three of the HTPH systems (Santoro et al., 2010; Selig et al., 2010; Studer et al., 2010). In general, a higher enzyme loading was employed in these formats as opposed to conventional washed solids hydrolysis to offset the effects of inhibitors possibly present in the pretreated biomass slurry as a result of not separating the pretreated solids and liquids. For hydrothermal pretreatment by the NREL system, an enzyme loading of 70 mg cellulase per g initial biomass supplemented with 2.5 mg/g initial biomass β-glucosidase was applied for a 72 hr static incubation at 40°C (Selig et al., 2010). The enzyme addition was made to the entire pretreated biomass slurry in combination with 1M sodium citrate buffer to bring the pH of the pretreatment slurry to ~5.0. UCR used a similarly high enzyme loading, typically 75 mg cellulase per g glucan + xylan in the initial biomass, supplemented with 25 mg/g of xylanase for a 72 hr incubation at 50°C and with shaking at 150 rpm (Studer et al., 2010). Diluted enzyme was added to the pretreated biomass slurry in combination with 1M citrate buffer and 1 g/L of the biocide sodium azide. For dilute acid (Gao et al., in preparation) and dilute alkali (Li et al., in preparation) pretreatments, the pretreated slurry was diluted with water 5 or 10 times, respectively, prior to enzyme addition to achieve a total reaction mass of 450 mg. GLBRC’s HTPH system employed an enzyme loading of 30 mg cellulase per g glucan in the initial biomass, which was added to the pretreated biomass slurry in each vial as a solution that also contained 30mM citrate buffer and 0.01% sodium azide.
Hydrolysis was performed at 50°C with end-over-end rotation for a 20 hr incubation time (Santoro et al., 2010).

As mentioned previously, the HTPH system developed at the University of York and University of Dundee (Gomez et al., 2010) more closely mimicked conventional solid/liquid separation and solid washing by applying several rinses with a buffered solution prior to enzymatic hydrolysis. Due to this, a lower enzyme loading of 6.3 FPU/g of material (~14 mg cellulase/g biomass (Garcia-Aparicio et al., 2011)) was typically employed using a mixture of cellulase (Celluclast, Novozymes, Bagsvaerd, Denmark) supplemented with β-glucosidase (Novozyme 188, Novozymes, Bagsvaerd, Denmark) at a 4:1 ratio, respectively. Enzymatic hydrolysis was reported to be carried out in 25 mM sodium acetate buffer at 50°C for 8 hours with constant shaking at 120 rpm.

3.2.4.4 Sample measurement

The four HTPH systems reported here all measured the amount of sugar released from combined pretreatment and enzymatic hydrolysis. To accomplish this, UCR employed HPLC equipped with a refractive index detector for measurement (Studer et al., 2010). As such, hydrolysates were transferred to HPLC-compatible vials or microplates following the completion of enzymatic hydrolysis. The benefits of this approach included that it was well-established and enabled the measurement of a variety of sugars besides just glucose and xylose. However, HPLC was time-consuming and could require between 15-30 minutes per sample, equating to 24-48 hours for analysis of all 96 samples from a single well plate. Alternatively, both NREL and GLBRC’s systems
employed enzyme-based assays (Santoro et al., 2010; Selig et al., 2010). As such, after enzymatic hydrolysis, NREL’s system required dilution and transfer of hydrolyzates to 96-well flat-bottomed polystyrene plates in which glucose was detected via a modified glucose oxidase/peroxidase (GOPOD) assay and xylose was detected with a xylose dehydrogenase assay (Megazyme International Ireland, Wicklow, Ireland) by measuring their absorbance at 510 and 340 nm, respectively (Selig et al., 2010). GLBRC’s HTPH system similarly employed GOPOD and xylose dehydrogenase assays following transfer to 384-well microtiter plates (Santoro et al., 2010). The benefit of sugar analysis by enzyme-based assays was the speed at which they could be completed: it was estimated that a plate of 96 samples could be analyzed in 20 minutes (Decker et al., 2009).

However it has also been reported that due to the specificity of the assays, certain sugars such as xylooligomers, could interfere with xylose measurements (Selig et al., 2010).

Colorimetric assays, which could easily be applied in high-throughput setups, have also been employed for sugar release measurements. In HT enzymatic hydrolysis systems (Navarro et al., 2010; Jager et al., 2011), a standard dinitrosalicylic acid (DNS) assay was used to measure the reducing sugars released during enzymatic hydrolysis. Alternatively, the HTPH system reported by Gomez et al (2010) measured glucose equivalents released by enzyme action using a modified 3-methyl-2-benzothiazolinonehydrozone (MTBH) assay. In this approach, a mixture of hydrolyzate, NaOH, MBTH, and dithiothreitol (DTT) with a final volume of 250 μL was incubated at 60°C for 20 min, after which an oxidizing reagent was added and optical measurements were taken at 620 nm in an optical well plate. As with enzyme-based assays,
colorimetric measurements such as the modified MTBH assay employed by Gomez et al (2010) had the benefit of fast detection; however, possible interference among sugars and differing response for various sugars could be concerns (Decker et al., 2009; Gomez et al., 2010).

In addition to testing sugar release from combined pretreatment and enzymatic hydrolysis, Zavrel et al (2009) presented a different approach to evaluating pretreatment-biomass combinations by monitoring the *in-situ* dissolution of cellulose by ionic liquids. In their HT pretreatment system, scattered and transmitted light was measured continuously during pretreatment to follow the size and number of cellulose particles as a function of pretreatment time. Although this system was unable to quantitatively measure sugar release, greater solubilization of crystalline cellulose was associated with enhanced sugar release in subsequent enzymatic hydrolysis, and thus provided an alternative measure of cellulose digestibility.

Table 3.2 summarizes key characteristics for each of the four HTPH systems.
Table 3.2. Summary of key characteristics for each of the four HTPH systems including the reaction volumes utilized in pretreatment and enzymatic hydrolysis, solids loading, chemical type and concentration, temperature, and heat source.

<table>
<thead>
<tr>
<th>Institution</th>
<th>Reaction volume (μL) (pretreatment/EH*)</th>
<th>Solids loading (%**/mg)</th>
<th>Pretreatment type</th>
<th>Temp/acid concentration</th>
<th>Heating medium</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>NREL</td>
<td>A</td>
<td>300/300</td>
<td>1.7/5.0</td>
<td>Hydrothermal</td>
<td>180°C/0%</td>
<td>Steam</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>250/250</td>
<td>2.0/5.0</td>
<td>DILUTE acid/H₂SO₄</td>
<td>180°C/0.3%</td>
<td>Steam</td>
</tr>
<tr>
<td>UCR</td>
<td>A</td>
<td>450/450</td>
<td>1.0/4.5</td>
<td>Hydrothermal</td>
<td>120-180°C/0%</td>
<td>Steam</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>85.5/450</td>
<td>5.0/4.5</td>
<td>DILUTE acid/H₂SO₄</td>
<td>120-180°C/0.5-1%</td>
<td>Steam</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>40.5/450</td>
<td>10.0/4.5</td>
<td>DILUTE alkali/NaOH</td>
<td>120-180°C/1%</td>
<td>Steam</td>
</tr>
<tr>
<td>GLBRC</td>
<td>A</td>
<td>750/750</td>
<td>0.2/1.5</td>
<td>DILUTE alkali/NaOH</td>
<td>90°C/0.025%</td>
<td>Water bath</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>350/750</td>
<td>1.1/4.0</td>
<td>DILUTE acid/H₂SO₄</td>
<td>90°C/1.0%</td>
<td>Heating block</td>
</tr>
<tr>
<td></td>
<td>***</td>
<td></td>
<td></td>
<td>DILUTE alkali/NaOH</td>
<td>90°C/0.5N</td>
<td></td>
</tr>
</tbody>
</table>

*The reaction volume for enzymatic hydrolysis is the total slurry volume (water, dilute acid, or dilute alkali) prior to enzyme, buffer, biocide addition.

**Solids loading is described as % biomass weight per reaction volume.

***University of York (UY); University of Dundee at SCRI (UD).

3.2.5 HTPH philosophy, including difficulties and limitations

HTPH technologies provided a significant step in screening large numbers of plant samples for their recalcitrance to sugar release. However, it was important to recognize the differences between conventional and HT pretreatment and enzymatic hydrolysis testing, particularly that the latter was primarily a screening tool that provided a platform from which sugar release trends, as well as superior and sub-par outliers, could be identified for further analysis. Along these lines, HTPH processes typically only measured total monomeric sugar release from combined pretreatment and enzymatic hydrolysis. In conventional methods, sugar release is tracked from the individual steps by separately analyzing both the solid and liquid phase, which reveals more information about the fate and mass balance of sugars, including those in both monomeric and oligomeric form (Wyman et al., 2005). Although it was not a requirement for HTPH systems to measure only total monomeric sugar release from combined pretreatment and
enzymatic hydrolysis, and HTPH systems could be applied to measure sugar release from individual stages, the limitation of measurements from co-hydrolysis or one-tube processes applied in HTPH systems needed to be recognized. However, it should also be mentioned that solid and liquid separation processes do not represent the most attractive commercial operations due to high capital costs for this operation, as well as the additional opportunity for contamination. Thus, although HTPH systems that employed co-hydrolysis or one tube processes may have varied somewhat from conventional ones, they may have better simulated commercial practices.

The lack of solid liquid separation following pretreatment in HTPH systems also had strong implications on enzyme loadings used in hydrolysis of the pretreated biomass slurry. Since the purpose of HTPH systems was primarily to screen multiple plants for reduced recalcitrance, this required the selection of conditions that best highlighted these differences. Thus, application of high enzyme loadings allowed determination of differences in substrate digestibility, as opposed to enzyme inhibition or activity. Another key point in ensuring that differences in substrate digestibility were uncovered was the selection of proper pretreatment conditions. Typically, low pretreatment severities that were below the optimal pretreatment condition were selected to reduce sugar degradation that may have resulted in certain samples exhibiting lower sugar yields. Employing a lower pretreatment severity ensured that digestibility differences would be enhanced, and that plants which performed well at reduced temperatures and times could be easily identified from others that might have performed well at higher severity pretreatments.
Despite the significant progress in HTPH systems, difficulties still remained. One such difficulty, as with any downscaled system, was that errors were easily amplified at this small scale. This included pipetting errors, such as the inclusion of a single air bubble that may have resulted in inaccurate sugar concentrations and erroneous sugar release results. Furthermore, the possible presence of starch and extractives in plant materials introduced another potential source of difficulty with HTPH technologies. For example, with the HTPH analysis methods discussed previously, glucose released from starch could not be differentiated from glucose released from cellulose, and as a result, some differences in the amount of glucose release that were observed between samples could have been due to varying levels of endogenous sugars in the native biomasses. Likewise for extractives, particularly free sugars, their fate during pretreatment had not yet been established, and as a result, the mass of sugar released per mass of biomass could have been influenced. Unfortunately, the removal of starch and extractives is conventionally performed using 0.1 to 5-20 g of material, respectively, with equipment and techniques that are not currently designed for large numbers of samples and high throughput applications (Sluiter and Sluiter, 2005; Sluiter et al., 2005). A simple way to address possible starch interference in HTPH systems was to test the enzymes used for their ability to hydrolyze starch into glucose. As such, both Santoro et al (2010) and Studer et al (2011a) reported non-detectable, or minimal levels of starch hydrolysis, respectively. Alternatively, researchers at NREL have developed downscaled and higher-throughput methods to remove starch and extractives prior to HTPH testing to ensure that
endogenous sugars did not interfere with sugar release results (Decker; personal communication).

However, perhaps the biggest concern with scaling down pretreatment and enzymatic hydrolysis processes was obtaining homogeneous biomass samples, which could be influenced by a range of steps including sampling, milling, and sieving. For example, an HTPH system was applied to measure the ring-by-ring composition and sugar release from combined pretreatment and enzymatic hydrolysis across an aspen wood cross section to show that both varied significantly across the radial direction (DeMartini et al., 2011). Additionally, Garlock and coworkers (2009) reported that corn stover composition and performance in Ammonia Fiber EXpansion (AFEX) pretreatment varied considerably by anatomical fraction. These findings demonstrated that when only a small portion of a plant was being sampled for analysis, such as was the case in HTPH testing, the location from which that sample was taken could impact sugar release results. Additionally, once the sample had been obtained, subsequent milling and sieving processes used to prepare materials for analysis could also affect results. It had been reported that in evaluating milled corn stover, larger particle size fractions were richer in corn cob and stalk portions that were more recalcitrant to hydrolysis than smaller size fractions, which contained higher amounts of leaves and husk (Chundawat et al., 2007). Along these lines, it had also been noted that although sieving was often necessary to achieve homogeneous samples for biomass analyses, this material preparation process itself could interfere with obtaining representative samples (Sluiter et al., 2010). The issue had not been found to be as significant for woody samples that are generally very
homogeneous; however, herbaceous materials can contain larger fraction of fines (<80 mesh or <0.180 mm) that are composed of considerable amounts of inorganic materials. The removal of these components could affect composition and sugar release performance and may not have provided results that were representative of the entire plant since certain anatomical fractions segregate into this size fraction (Sluiter et al., 2010). Together, these studies all stressed the importance of taking great care in sampling and material preparation steps in HTPH analyses to ensure that the mg-level of materials employed were representative of the entire plant sample being tested. Conversely, due to the downscaled and high throughput nature of HTPH systems, this limitation could be easily be tested for by running many replicates to check for biomass inhomogeneity, which was a task that was previously not feasible with larger-scale conventional pretreatment and enzymatic hydrolysis methods.

3.2.6 Applications to date

To date, HTPH systems have enabled a number of research projects that were previously not possible, including studies that fall under both plant generation and screening methodologies presented in the Introduction. One of the first reports of a large scale project to screen sugar release from combined pretreatment and enzymatic hydrolysis of hundreds of plants was by Santoro et al (2010) in which 1,200 Arabidopsis samples were tested. These samples contained T-DNA insertions in genes that were believed, or known, to play a role in cell wall metabolism and possibly digestibility. As such, several Arabidopsis lines were successfully identified to exhibit significantly higher
glucose and xylose release than most others. Voelker et al (2010) also applied an HTPH system (Decker et al., 2009; Selig et al., 2010) to screen four to seven field-grown poplar trees per transgenic event (14 events, 100 samples in total) that had undergone transgenic down-regulation of the Pt4CL1 gene, which was previously reported to reduce lignin content in cell walls. In contrast to previous studies, they found that trees with reduced lignin contents did not yield substantially higher saccharification potential; instead, very little difference was found in the sugar release from combined hydrothermal pretreatment and enzymatic hydrolysis among all tested trees. Again, due to the sheer number of samples, Selig et al (2010) similarly employed an HTPH system to screen 755 natural poplar variants from the Pacific Northwest of North America. In this study, sugar release from combined hydrothermal pretreatment and enzymatic hydrolysis was independent of total lignin content but strongly related to the lignin syringyl to guaiacyl (S/G) ratio. In an extension of this study, a much smaller sub-sample of natural poplar variants from the same population was tested under a variety of hydrothermal pretreatment conditions (47 samples x 3 pretreatment conditions) using HTPH technology (Studer et al., 2011a). In this case, glucose release had a strong negative correlation with lignin content only for trees with low S/G (<2) ratio, while xylose release was dependent on the S/G ratio alone and not lignin content. Furthermore, certain trees featuring average lignin content and S/G ratios were identified to exhibit exceptionally high sugar release, demonstrating that factors beyond lignin content and S/G ratio influenced recalcitrance.

HTPH systems are not only useful for screening large numbers of samples but also for their ability to provide sugar release results from very minimal amounts of biomass.
material. Along these lines, DeMartini and Wyman (2011) applied HTPH technology to test sugar release from combined pretreatment and enzymatic hydrolysis of the individual annual rings of 26 and 8 year old aspen trees. Although there were only about 35 samples in total tested at a single pretreatment condition, the small sample amounts (<100 mg) necessitated downscaled technology. In this case, sugar release (g sugar released per g biomass) varied significantly across the radial direction of the tree, but sugar yields (g sugar released per g available sugar) did not, suggesting that wood maturity impacted composition much more than recalcitrance.

3.2.7 Future applications

The development of HTPH technology has opened the door for downsizing sample size and increasing the throughput for a variety of biomass applications. HTPH systems produced large quantities of sugar release data to gain a better understanding of factors influencing biomass recalcitrance. However, results from these screening studies typically reported the amount of sugar released per amount of total biomass because at the time that most of the studies were performed, there was no method to accurately determine the carbohydrate content of the large numbers and small amounts of samples available. However, to gain a better sense of a plant’s recalcitrance, sugar yields should be determined as the amount of sugar released per amount of sugar available. To meet this need, downscaled and high throughput compositional analysis approaches have recently been developed (DeMartini et al., 2011; Selig et al., 2011). Both are based directly on conventional two-stage acid hydrolysis compositional analysis (Sluiter et al.,
2008) but are scaled down to use significantly less material (between 60-100 times less material, equivalent to 3 or 5 mg biomass per test). For this duty, a 96-well plate format similar to the HTPH procedure was employed at NREL (Selig et al., 2011), and an array of 48 1.5 mL glass HPLC vials with the same plate clamping mechanism was used to support UCR’s HTPH system (DeMartini et al., 2011). The method reported by Selig et al (2011) was capable of measuring glucan and xylan contents of much larger sample sets than reported by DeMartini et al (2011); however, the latter benefitted in that it could estimate whole ash and Klason lignin contents (through the measurement of acid insoluble residue (AcIR)).

Furthermore, the development of HTPH technologies also enabled large scale pretreatment kinetic studies that would have previously required hundreds or thousands of individual experiments at large scale but can now be accomplished with minimal time and labor requirements. Along these lines, recent work by Zhang et al (in preparation) employed UCR’s HTPH system to study the kinetics of hemicellulose and cellulose conversion to sugar degradation products such as furfural, 5-hydroxymethyl-2-furaldehyde (5-HMF), and levulinic acid, by evaluating a number of different pretreatment times, temperatures, acids, and acid concentrations. In about a month’s time, approximately 4,000 samples were tested.

In addition to employing HTPH technology to screen sugar release from combined pretreatment and hydrolysis or developing pretreatment degradation kinetics, there are opportunities to expand it to microbial screening. For example, Cianchetta et al (2010) reported the development of a miniaturized cultivation system based on flat bottom 24-
well plates to assess large numbers of fungal strains. They tested over 300 *Trichoderma* strains on cellulose powder to quickly and easily identify superior and sub-par cellulase producers. The extension of this technology to real biomass substrates, including pretreated materials, presents an important opportunity.

3.2.8 Conclusions and recommendations

Testing the sugar release of thousands of samples in combination with various pretreatment and enzymatic hydrolysis conditions is no longer an impossible feat. The development of HTPH systems has been a major advancement in biofuels research by providing a platform for screening large sets of plant samples in order to identify biomass outliers and trends in recalcitrance. The downscaling of pretreatment reactions to microplate- or small tube-based formats, in addition to the development of co-hydrolysis or one-tube processes, has enabled automation and increased throughput, as well as greatly reduced material requirements for recalcitrance assays. Although some difficulties arise with the scaling down of biomass analyses, HTPH systems can be powerful tools if care is particularly paid to biomass sampling and distribution. With the continued application of these systems, new insights can be gained into biomass recalcitrance that will aid in identification of superior feedstock candidates.

3.3 Development and certification of UCR HTPH system

Section 3.3 focuses on the HTPH system development at UCR. Results from a variety of tests that were performed to validate the system will be presented. Complete
information on the UCR HTPH system can be found in two publications by Studer et al. (2010, 2011b).

3.3.1 Development and overview of system

To develop an HTPH system capable of identifying differences in sugar yields of at least 10% from among hundreds of biomass samples with reduced time and material requirements, a number of options were considered ranging from fully automated robotics platforms to custom built devices. After gathering and analyzing detailed information for all options, it was determined that the selection with the highest degree of confidence was a custom built 96-well plate format to serve as the pretreatment and enzymatic hydrolysis reactor, and a custom-built steam chamber to provide indirect heating from saturated steam during pretreatment. The well plate reactor had the same footprint as a standard 96-well microplate; however, the HTPH system was constructed of metal to withstand the high temperatures, pressures, as well as acid concentrations associated with pretreatment. In addition to the development of the well plate reactor, other modifications had to be made to the conventional pretreatment and enzymatic hydrolysis processes in order to facilitate high throughput screening, resulting in the development of an approach termed co-hydrolysis (Fig 3.2) (Studer et al., 2011b). The time consuming step of solid and liquid separation that is difficult to scale down was eliminated, and enzymes were instead added directly to the pretreated biomass slurry. Using this approach, the compositional analysis step typically required after pretreatment was eliminated, and enzymes were added based on the composition of the unpretreated
biomass. In order for co-hydrolysis to be a valid approach that serves to mimic conventional methods and its corresponding sugar release, it was determined that low solids loading (0.5-1% w/w) and high enzyme loading (about 100 mg enzyme protein / g glucan + xylan in raw biomass) had to be employed. Low solids loading was used to minimize the release of possible enzyme inhibitors, while the high enzyme loading was employed to ensure that any potential inhibition by compounds that were released during pretreatment and enzymatic hydrolysis did not interfere with enzyme action.

3.3.2 Verification of HTPH system

Before the HTPH system could be used to screen biomass variants, a number of questions had to be answered to ensure that future sugar release results were a true reflection of substrate differences and reaction properties, as opposed to an artifact of the HTPH system itself. For example, did the well plate hold its seal during pretreatment or was there leakage from some or all of the wells? During pretreatment, were the temperature histories of all 96 wells identical? Were heat-up and cool-down periods rapid? Furthermore, were sugar yields across all 96 wells identical, such that performance was independent of plate position?

3.3.2.1 Confirmation of no leaking

The first issue to consider was the sealing ability of the well plate since differential pressures between the wells and surrounding environment can reach 1 MPa during heat-up and cool-down periods of pretreatment. For this reason, a fluorometric leak test
commonly employed to calibrate multichannel pipettes was employed. 250 µL of phosphate buffer (pH 9, for optimal fluorescein signal) was accurately measured into each well using a multichannel pipette (8 channel pipetter, 30-300 µL, Eppendorf, Hamburg, Germany). The well plate was sealed and held at a temperature of 180°C for 55 minutes in the steam chamber. After rapid cooling and opening of the well plate assembly, 20 µL (8 channel pipetter, 10-100 µL, Eppendorf) of a 1.35 µM fluorescein solution was added to a final concentration of 100 nM. After mixing by repetitive pipetting, 200 µL of the solutions were transferred to a solid black, flat-bottom microplate appropriate for fluorescent measurements using the same multichannel 30-300 µL pipette. The solutions were excited at 485 nm, and the fluorescence intensity was measured at 525 nm (SpectraMax M5e, Molecular Devices, Sunnyvale, CA). The fluorescence reading of each well provided a direct measure of the liquid volume in the corresponding HTPH well following pretreatment. A control was also performed in which a plate underwent identical steps as described above except that the control plate was never sealed or cooked. The mean of the ratios of the fluorescent readings of the cooked versus the control plate was 1.00 ± 0.04. Furthermore, the standard deviation of the liquid volumes in the 96 wells was 4.2% after pretreatment, compared to 2.3 % measured in the control experiment. These results proved that no significant leaking from the wells occurred during pretreatment, and that the well plates were sufficiently sealed.
3.3.2.2 Determination of temperature histories

In order to ensure that 1) the temperature histories of all 96 wells were the same, regardless of whether the well is positioned on the edge of the plate or the center, and 2) the heat-up and cool-down times were rapid, temperature profiles during pretreatment were recorded for a number of locations throughout the plate. Type K thermocouples (Wilcon Industries, Lake Elsinore, CA) were inserted into the center of the liquid phase of select wells. Thermocouple cables were passed through, and cast in a pipe connecting to the steam chamber such that the temperatures could be logged outside by a data logger (CR10X Measurement & Control Datalogger, Campbell Scientific, Inc., Logan, UT). The well plate containing thermocouples was positioned horizontally in the steam chamber. After sealing the plate, saturated steam was allowed to enter the chamber to achieve the desired temperature of 180°C. After holding at 180°C for a few minutes, the plate was then rapidly cooled by shutting the steam inlet valve to the chamber, opening the chamber’s vent, and immediately flooding it with cold water. The temperature profiles of Figure 3.7 show that it took approximately 30 seconds for the individual wells to heat up from 120°C to the target temperature of 180°C. Furthermore, it took only 10 seconds after the steam was vented and the chamber flooded with water for the temperature to fall below 120°C. Such rapid heat-up and cool-down times were a marked improvement from conventional laboratory pretreatment technology that utilized heating mechanisms such as electric heaters and sand baths, the latter of which required a heat-up time between 1-4 minutes for various reactor configurations. Figure 3.7 also proves that the temperature profiles of different wells, regardless of position on the plate were almost
identical, except for a small difference near the end of the cool-down. Thus, it was demonstrated that the HTPH system provided rapid, accurate, and consistent heating and cooling.

![Heat-up and cool-down temperature profiles](image)

**Figure 3.7.** Heat-up and cool-down temperature profiles for water in the wells of HTPH plate heated in a chamber with condensing steam and cooled by flashing the steam and flooding with cold water. The color of the lines encode for the position of the thermocouples in the well-plate, as shown in the 96-well plate schematic. The 2 dashed horizontal lines represent the error of thermocouples Type K. Reproduced from Studer et al. (2010) with permission from John Wiley and Sons.

### 3.3.2.3 Sugar yields across the plate

To ensure that sugar yields across all 96 wells were uniform, known masses of milled poplar were loaded into each well at a total reaction mass of 250 mg and 1% solid loading. Thus, 2.63 mg of biomass (assuming about 5% moisture content) and 247.4 µL of deionized (DI) water were added to each well. The plate was then subjected to pretreatment at 180°C for 55 minutes. After pretreatment, 20 µL of a mixture of 1M citric acid buffer (pH 4.95), sodium azide solution (1g/L), and enzyme were pipetted into
each well (8 channel pipetter, 10-100 µL, Eppendorf) corresponding to a total protein
loading of 75 mg of cellulase (Spezyme CP) plus 25 mg of xylanase (Multifec Xylanase)
/ g of total glucan and xylan in the raw biomass. Following enzyme addition, the plate
was re-sealed and placed on its side in an incubation shaker (Multitron Infors-HT, ATR
Biotech, MD) at 50°C for 72 hours, shaking at 150 rpm. Following the completion of
enzymatic hydrolysis, total glucose and xylose release from all 96 wells was measured
using HPLC (Alliance 2695, Waters, Milford, MA) with an Aminex HPX-87H column
(BioRad, Hercules, CA) heated to 65°C using 0.005M sulfuric acid as the eluent. As
shown in Figure 3.8, variations among sugar yields from the individual wells were not
correlated to position on the well plate, demonstrating that performance was independent
of plate position. In addition, the standard deviation of the total sugar yields across all 96
wells was only 4.1 %, which proved that the plate was capable of detecting sugar release
differences greater than 10% as originally targeted. This low standard deviation proved
that the HTPH system provided consistent data, and that the small sample size being
utilized is indeed a representative sample.
3.4 In-depth analysis and improvement of well plate

This thesis relied heavily on the UCR HTPH system to screen multiple biomass species. Although the system was demonstrated as a high throughput method to successfully screen biomass samples for enhanced sugar release by employing water-only pretreatment at a specific operating condition (Studer et al., 2010), the system needed to be further studied and improved in order to extend its application and increase its throughput and level of automation.

3.4.1 Development and automation of an improved HTPH system

The original well plate format developed by Studer et al. (2010) in which the wells were press fit into a bottom aluminum plate did not allow for automation with a solids
and liquid dispensing robotics platform. As a result, the wells were re-designed to feature a pin at their bottom corresponding to a hole in the base plate in which they could freely sit. This allowed the wells to be picked up by grippers on a robotics platform and moved onto a balance to be individually weighed and loaded with biomass (Fig 3.3b).

Furthermore, the size of the wells was also increased in the new plate design to allow for larger liquid volumes for analysis. As with the original well plate, a leak test (performed as described in section 3.3.2.1) confirmed that there was no leaking in the new reactor.

After the development of an improved well plate, the next goal was to integrate it with an automated solid and liquid dispensing robotics platform (Core Module, Freeslate (formerly Symyx), Sunnyvale, CA) in order to increase the throughput and reduce manual labor and weighing errors. The robotics platform is described in section 3.2.4.2. To integrate the updated HTPH system with the robot, a number of protocols were written using the robotics platform’s user interface program Automation Studio (Version 1.1.2.14, Symyx, Sunnyvale, CA). After optimizing the protocols and robot hardware settings, the sugar yields across the well plate were measured following the methods reported in section 3.3.2.3 to test whether improved results could be obtained. The only differences in the methods included the use of the robotics platform for dispensing biomass into individual wells instead of by hand, the use of a higher biomass weight – 4.5 mg versus 2.5 mg – to account for the larger well reaction volume, and the application of a less severe pretreatment time – 27.8 min versus 55 min. Whereas the standard deviation of the total sugar yields across all 96 wells was previously 4.1% when biomass was weighed in by hand, the same value dropped to below 2% with the updated well
plate reactor loaded by the robotics platform. The lower standard deviation achieved by the new automated HTPH system was likely due to more accurate and reproducible weighing by the robotics platform.

3.4.2 In-depth analysis of well plate performance

One particular area that still needed to be addressed was the lack of research to compare the performance of pretreatment and enzymatic hydrolysis as separate steps in the HTPH system as compared with conventional washed solids hydrolysis. It was previously shown by Studer et al. (2010) that total glucose and xylose yields from combined pretreatment and enzymatic hydrolysis were nearly the same in the well plate as conventional washed hydrolysis with performed in tube reactors. However, determining the sugar release from each separate step could provide more in-depth data about the HTPH system’s performance as opposed to total sugar release from the combined processes. Furthermore, since co-hydrolysis was employed in the well plate, the presence of inhibitors such as lignin and sugar degradation products (Palmqvist et al., 1996) and oligomers (Kumar and Wyman, 2009) was a concern. If the purpose of the HTPH system was to screen feedstocks for reduced recalcitrance, the identification of considerations for selecting appropriate pretreatment and enzymatic hydrolysis conditions and the effect of these selected conditions was crucial. For example, a sub-optimal pretreatment condition is often selected for biomass screening studies in the HTPH system in order to reduce sugar degradation and enhance differences between plant samples. However, lower severity pretreatments (particularly water-only pretreatment)
often leave xylan-derived sugars in oligomeric form, which have previously been demonstrated to be inhibitory to cellulases (Qing et al., 2010). Thus, what pretreatment condition should be selected in order to balance the effects of sugar degradation products from more severe pretreatments and xylooligomers from milder treatments?

To accomplish this, replicate wells were loaded with *Populus trichocarpa* at a solids loading of 1.0%, equivalent to 4.50 mg dry biomass in a total reaction mass of 450 mg. Following water-only pretreatment for 27.8 min at 180°C (a previously identified slightly sub-optimal condition for poplar), the hydrolyzate from some wells was removed and analyzed. The monomeric sugar content and presence of degradation products such as furfural and 5-hydroxymethylfurfural (5-HMF) in the hydrolyzate was examined by HPLC. Furthermore, a scaled-down post hydrolysis method was developed based on a larger scale procedure in which the liquid contents were hydrolyzed with strong acid (Sluiter et al., 2006). This method was performed on the hydrolyzate samples to deduce total oligomer concentrations, while the xylooligomer presence and distribution was also investigated using high performance anion exchange chromatography with pulsed amperometric detection HPAEC-PAD on a Dionex DX-600 Ion Chromatograph system (Li et al., submitted). The remaining wells continued through enzymatic hydrolysis to determine the resulting sugar release from the final stage. In this way, the performance of individual steps conducted in the well plate was evaluated.

Injection of the pretreated hydrolyzates on the HPLC showed that very little xylan (6.2%) was converted into its monomeric form during pretreatment, and no glucan was converted into glucose. Post-hydrolysis of the pretreated liquid demonstrated that only
1% of glucan was solubilized during pretreatment into glucooligomers whereas a significant portion of xylan was converted into xylooligomers during pretreatment, with the total xylan yield (including both xylose and xylooligomers) totaling 69.5%. In total, the concentration of xylan-derived oligomers was just below 1 g/L. Furthermore, Dionex results indicated that the majority of xylooligomers in solution were of low degree of polymerization (DP). As such, approximately 80% of oligomers were between X2 and X10 (xylooligomers with chain lengths of between 2 and 10, respectively). In addition to sugar monomers and oligomers, injection of the raw hydrolyzates on HPLC also showed low levels of other potential inhibitors such as acetic acid and furfural at concentrations of 0.1 and 0.05 g/L, respectively, and an even lower concentration of 5-HMF that was below the detection limit of the refractive index detector employed.

Figure 3.9 displays the sugar yields from the separate steps of pretreatment and enzymatic hydrolysis, as well as the total yields from the combined processes. Results are compared for those obtained from co-hydrolysis in the HTPH system to those obtained from pretreatment in 10 mL tube reactors followed by conventional washed solids hydrolysis with a total reaction mass of 25 g††. As shown, glucan and xylan yields from the individual and combined steps were very similar for the two approaches. The xylan yield during pretreatment was slightly higher in the conventional tube reactor (75.4%) than in the HTPH system (69.5%), while the glucan yield during enzymatic hydrolysis was slightly higher in the HTPH system (72.9%) than it was with the conventional washed solids hydrolysis method (66.3%). Overall, this study demonstrated

†† Sugar yield data from conventional washed solids hydrolysis were obtained, and generously provided by Ms. Heather McKenzie.
two important findings. First, very similar patterns of sugar yields were observed between the HTPH and conventional methods, with the level of variability in the results being consistent with that commonly experienced in biomass pretreatment and enzymatic hydrolysis experiments. Thus, the applicability of the HTPH system in mimicking conventional methods was further proved. Additionally, it was also shown that the potential of enzyme inhibition due to degradation products is fairly low due to their low concentrations. Conversely, the concentration of xylooligomers was higher and in the range that has been previously reported to be inhibitory to cellulases (Qing et al., 2010). Thus, when choosing a pretreatment condition for screening, the pros and cons of increasing xylooligomer concentration at lower severity pretreatment but increasing degradation products at higher severity must be weighed. However, the high enzyme loading utilized in this study appeared to overcome any potential inhibition, as demonstrated by the very similar performance of the conventional washed solids hydrolysis method.
3.5 Acknowledgments

I would like to sincerely thank Dr. Michael Studer (now at the Institute of Process Engineering, ETH Zurich, Switzerland) for his help and guidance on this work. The development and verification of co-hydrolysis and UCR’s HTPH system, including the design of the steam chamber and first generation well plate were led by Dr. Studer. Additionally, I would also like to thank Ms. Heather McKenzie for helpful discussions and the sugar yield data from conventional washed solids hydrolysis of poplar shown in Figure 3.9.
3.6 References


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Chapter 4. Small scale and automatable high-throughput compositional analysis of biomass*
4.1 Abstract

Conventional wet chemistry methods to determine biomass composition are labor- and time-intensive and require larger amounts of biomass (300 mg) than is often available. To overcome these limitations and to support a high throughput pretreatment and hydrolysis (HTPH) screening system, this paper reports on the development of a downscaled biomass compositional analysis that is based on conventional wet chemistry techniques but is scaled down by a factor of 100 to use significantly less material. The procedure is performed in readily available high performance liquid chromatography vials and can be automated to reduce operator input and increase throughput. Comparison of the compositional analyses of 3 biomasses determined by the downscaled approach to those obtained by conventional methods showed that the downscaled method measured statistically identical carbohydrate compositions as standard procedures and also can provide reasonable estimates of lignin and ash contents. These results demonstrate the validity of the downscaled procedure for measuring biomass composition to enable the calculation of sugar yields and determination of trends in sugar release behavior in HTPH screening studies.
4.2 Introduction

As conventional petroleum reserves dwindle and concerns over associated anthropogenic greenhouse gas emissions grow, alternative renewable energies should be at the forefront of research (Brandt and Farrell, 2007; Lynd, 1991). Currently, the only promising resource for large-scale sustainable production of liquid fuels for transportation is lignocellulosic biomass (Lynd, 1991; Farrell et al., 2006; Perlack et al., 2005; Ragauskas et al., 2006). However, the primary obstacle to low cost biological production of renewable fuels from lignocellulosic biomass is a plant’s recalcitrance or resistance to deconstruction by enzymes or microbes (Lynd et al., 2008; Wyman, 2007). Thus, optimizing pretreatment and enzymatic hydrolysis processes, improvement of enzymes applied, and identification and use of biomass species with reduced recalcitrance are attractive routes to making fuels from lignocellulosic biomass more commercially viable (Lynd et al., 1991; 1999). In pursuing these objectives, accurate and rapid determination of composition, in particular sugar contents, is essential to determining yields accurately and identifying plant-pretreatment-biocatalyst combinations that provide performance advantages.

Conventional wet chemistry techniques employed to determine biomass composition generate accurate and quantitative compositional data through a two-step acid hydrolysis sequence to breakdown structural carbohydrates into components that can be more easily quantified by chromatography and gravimetric methods (Sluiter et al., 2008). Unfortunately, the process is both exacting and labor intensive and also requires a relatively large amount of material (300 mg). These disadvantages are particularly
problematic in feedstock studies that aim to identify promising biomass variants for enhanced sugar release in which thousands of samples can be generated for screening and limited amounts of material may be available. As a result, alternative, higher-throughput, and smaller scale methods are sorely needed for compositional analyses. Currently, near infrared (NIR) spectroscopy (Hames et al., 2003; Kelley et al., 2004) and pyrolysis molecular beam mass spectrometry (py-MBMS) (Evans and Milne, 1997; Sykes et al., 2008; Sykes et al., 2009) coupled with multivariate analytical techniques have been shown to rapidly and accurately determine biomass composition. However, these methods require sophisticated and fairly costly equipment and extensive calibration data from a distinct set of biomasses that must closely resemble the samples to be tested. Besides the challenges of acquiring biomasses with a sufficient compositional range to support calibration, a large number of calibration samples must be analyzed by the above-mentioned traditional wet chemistry methods to provide a sufficient range of compositions.

This paper reports on the development of a simple, downscaled biomass compositional analysis that was developed to support a high throughput pretreatment and enzymatic hydrolysis system used to screen thousands of biomass samples and identify those with reduced recalcitrance (Decker et al., 2009; Studer et al., 2010). Previously, results from these screening studies could only be expressed as the amount of sugar released per amount of total biomass because there was no available method to accurately determine carbohydrate content of the small amounts of material collected (<50 mg in some cases). Thus, to allow the calculation of glucan and xylan yields, and the
estimation of lignin and ash contents, a method was developed that is based on the well-established two-stage acid hydrolysis but is scaled down by a factor of 100 to use significantly less material. Furthermore, downsizing the procedure also lend itself to automation, resulting in far less operator input and higher-throughput for large sample sets compared to standard wet chemistry procedures. Results from the use of the high throughput method were compared to those from application of the conventional method for three different biomasses to verify that the scaled-down method produced identical sugar compositions to the standard procedure, and also to evaluate the method’s accuracy in measuring lignin and ash contents. Testing was performed with a robotics platform to facilitate all weighing and solid dispensing steps in order to demonstrate that the downscaled method can be automated to reduce operator input and increase throughput. However, this approach can also be applied with simple equipment available in most laboratories that already perform biomass compositional analysis, as shown by performing the downscaled analysis manually for one biomass material. Furthermore, the present study employed readily available high performance liquid chromatography (HPLC) vials as reaction vessels for downscaled analysis, but other, more elaborate systems, e.g., based on well-plate formats, could also be used instead (Studer et al., 2010).
4.3 Materials and Methods

4.3.1 Materials

Three biomass materials were tested: *Populus deltoides* (8492 National Institute of Standards and Technology), sugarcane bagasse (8491 National Institute of Standards and Technology), and Alamo switchgrass (BioEnergy Science Center internal standard material). The NIST materials comprised of a mesh fraction of -20/+74 (0.85 mm > x > 0.19 mm), while the switchgrass had a mesh fraction of -20/+80 (0.85 mm > x > 0.18 mm). All materials were well-mixed, had a moisture content of 5%, and were analyzed as received.

4.3.2 Downscaled compositional analysis

The downscaled method reported here is an adaptation of the well-established laboratory analytical procedures ‘Determination of Structural Carbohydrates and Lignin in Biomass’ and ‘Determination of Ash in Biomass,’ refined by NREL researchers (Sluiter et al., 2008 and Sluiter et al., 2005, respectively). The first method is based on a two-stage acid hydrolysis, originally developed by NREL in 1996, adopted as the standard method by ASTM, E1758-01, and most recently updated by NREL in 2008. The major differences in the conventional and downscaled methods include 1) the solid liquid separation step, which is accomplished by centrifugation and decanting in the downscaled version, versus filtration in the conventional method, 2) the lack of mixing in the first hydrolysis step of the downscaled procedure due to the impractical nature of mixing the contents of such small reactors, and 3) the measurement of the entire acid
insoluble residue (AcIR) in the downscaled analysis to estimate the lignin content, which unlike the conventional Klason lignin procedure, includes acid insoluble ash.

4.3.2.1. Carbohydrates and lignin

For the downscaled system, 1.5 mL high recovery glass HPLC vials that weigh approximately 2550 mg each (Agilent, Santa Clara, CA) were placed in a muffle furnace (Isotemp Programmable Muffle Furnace, Fisher Scientific, Pittsburg, PA) at 575°C for a minimum of 8 h. After cooling, the vials were weighed and loaded with 3.0 mg (± 0.15 mg) of dry *P. deltoides*, bagasse, or switchgrass by a liquid and powder dispensing robot (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance and 10 25 mL biomass-dispensing hoppers with either 8 or 12 mm openings, Symyx Technologies, Sunnyvale, CA). In regards to the robotics platform, it should be mentioned that the biomass-dispensing hoppers did not require significantly more material than the target amount to be dispensed, and that the Sartorius balance featured a readability of 0.01 mg and a repeatability of 0.03 mg. After vial weighing and biomass addition steps, 30 µL of 72% H₂SO₄ was pipetted manually into all vials (10-100 µL pipetter, Eppendorf, Hamburg, Germany). Then, the vials were held at 30°C (± 0.5°C) on the Core Module’s platform for 60 min, followed by removing them from the heat source and manually pipetting 840 µL of deionized (DI) water (100-1000 µL pipetter, Eppendorf, Hamburg, Germany). Nine independent replicates were prepared for all three biomasses.
A set of glucose and xylose sugar recovery standards (SRS) was also prepared to support correction for losses due to sugar degradation during the second hydrolysis stage (Sluiter et al., 2008). The glucose and xylose concentrations of the 3 standards ranged from 1.8-7.6 g/L and 1.0-4.5 g/L, respectively. For each, 300 µL of standard, 540 µL of DI water, and 30 µL of 72% H₂SO₄ was manually pipetted into 1.5 mL high recovery vials.

The vials containing biomass and those containing the sugar recovery standards were placed in an aluminum reactor block (Symyx Technologies, Sunnyvale, CA) that could house 48 1.5-2 mL glass vials. The block was then clamped between two aluminum plates with a flat Silicone gasket (thickness 1.5875 mm, durometer hardness A40), and the resulting sandwich was clamped together using four ¼ inch-20 threaded bolts (6.35 mm-20) placed in each corner of the plate, thereby sealing all of the vials. The technique was used to save time as compared to closing each vial individually and has been successfully applied in similar cases that involve much higher absolute and differential pressures between the inside of the reaction vessel and the surrounding environment (Studer et al., 2010). The reactor block was then placed in an autoclave (HA-MII Hirayama, Westbury, NY) and held at 121°C for 60 min.

After allowing the vials to cool to room temperature, the reactor block was opened. A sealing tape (Nunc, Rochester, NY) was secured to the top of all vials, and the entire reactor block was centrifuged (CS-6R Centrifuge, Beckman, Fullerton, CA) for 10 min at a force of 4,200 g using a 96 well plate carrier adaptor (Microplate carriers SX4750, VWR International, West Chester, PA). In contrast to the conventional technique, the
downscaled method uses centrifugation and decanting because the filtration and weighing of such small amounts of residue are not practical. After spinning down, the sealing tape was removed, and up to 840 µL of the sugar recovery standards as well as of the hydrolyzate supernatant was transferred to 2 mL polypropylene (PP) centrifuge tubes (Safe-Lock 2.0 mL test tubes, Eppendorf, Hamburg, Germany), while the acid insoluble residues were left in the glass HPLC vials. The liquid hydrolyzates were neutralized in the centrifuge tubes by adding CaCO₃ in steps until the pH was between 5 and 6 (using approximately 50 mg of CaCO₃ in total), as monitored by pH indicator strips (EMD Chemicals, Gibbstown, NJ) and as described in more detail by Sluiter et al. (2008). Tubes were then centrifuged (5415 D, Eppendorf, Hamburg, Germany) for 5 min at 18,200 g, and 300 µL supernatant of the neutralized hydrolyzate was transferred to a 0.5 mL PP 96 well plate (Agilent, Santa Clara, CA) for HPLC analysis.

The acid insoluble residues remaining in the glass vials were washed two times by centrifugation and re-suspension with 1 mL of DI water for each wash, with centrifugation at 4,200 g for 10 min. Afterwards, the reactor block containing vials with washed residues was placed in an oven set to 105°C for at least 36 h. Then, the vials were removed from the oven, allowed to cool in a desiccator, and weighed by the Core Module to determine the final acid insoluble residue (AcIR) content.

Additionally, the entire procedure described above was also performed manually for *P. deltoides* without the use of a robotics platform but instead employed a laboratory microbalance for weighing (MX5, Mettler Toledo, Columbus, OH). The MX5 balance featured a readability of 0.001 mg, and at a load of 0 to 2 g, a repeatability of 0.0008 mg.
To facilitate manual weighing of 3 mg of biomass, a specially made aluminum weighing beaker that held a volume corresponding to the target mass was used to scoop biomass into vials (Studer et al., 2010).

4.3.2.2. Determination of ash content

To determine the ash content, 15 ± 0.75 mg of biomass was weighed out by the Symyx Core Module into previously dried (at 575°C) 1.5 mL glass HPLC vials. Next the vials were placed in a muffle furnace and heated to 575°C following the Furnace Temperature Ramp Program described elsewhere (Sluiter et al., 2008). Then, the vials were removed, allowed to cool in a desiccator, and weighed by the Core Module. The ash content was calculated as the difference in weight between the vial plus ash, minus the weight of the empty vial, all divided by the original dry weight of the sample and multiplied by 100. The downscaled ash procedure was also performed manually for *P. deltoides* using a laboratory microbalance for weighing (MX5, Mettler Toledo, Columbus, OH).

4.3.3 Conventional procedures

The conventional procedure to determine sugar, AcIR, and Klason lignin content was performed following the NREL Laboratory Analytical Procedure “Determination of Structural Carbohydrates and Lignin in Biomass” (Sluiter et al., 2008) using 300 mg material per test, while the NREL Laboratory Analytical Procedure “Determination of
Ash in Biomass” (Sluiter et al., 2005) was used to determine the ash content with 500-2000 mg material per test.

4.3.4 Sugar analysis

Sugar concentrations were measured by HPLC (Agilent 1200 Series RI detector, Santa Clara, CA) using an Aminex HPX-87H column (BioRad, Hercules, CA) heated to 65°C and using 0.005M sulfuric acid as the eluent. However, since sample hydrolyzates were neutralized, an Aminex HPX-87P column could also have been used for measurement of additional sugars.

4.3.5 Statistical analysis

The carbohydrate and lignin/AcIR analysis of each biomass was performed using 9 independent replicates for both the conventional and downscaled methods, while the ash analysis was based on 6 independent replicates. To test whether the two methods produced carbohydrate compositional data that were statistically the same, an equivalence test was performed to compare glucan and xylan results from the conventional and downscaled analyses. Irrelevant difference criteria were defined to be ±10% of the mean value for glucan and xylan, which was defined based on the composition uncertainties for \textit{P. deltoides} and bagasse reported by NIST at a 95% confidence level. The reported errors of these materials ranged from ±10% for glucan to ±15% for xylan, and were determined by gathering compositional data performed on the NIST materials in a 2001 inter-laboratory study (National Institute of Standards &
Technology, 2001). For the current study, a non-paired t-test was performed on the measured glucan and xylan data for all 3 biomasses to determine the corresponding 95% confidence interval (CI), which had to fall within the defined irrelevance range to confirm that the compositional data from the downscaled method was statistically identical to that of the conventional analysis. The glucan and xylan composition data from the downscaled and conventional methods were also compared to reference values for *P. deltoides* and bagasse by testing whether the measured values fell within the NIST-reported 95% confidence interval calculated from the standard deviation of the mean (SDOM) for these standard materials. No error analysis was available for the BESC switchgrass used.

To evaluate AcIR measurements, values determined by the downscaled and conventional methods were compared to one another. To further gage whether AcIR measurements were within the correct range, Klason lignin measurements from the conventional method were evaluated against the reference lignin values. For ash contents, measurements taken by the downscaled and conventional methods were compared to one another, and also evaluated against the reference values to assess their ability in estimating the ash content.

### 4.4 Results and Discussion

As demonstrated in Figure 4.1, we achieved our primary goal of developing a downscaled method that measures virtually identical carbohydrate compositions as standard wet chemistry procedures. The carbohydrate measurements from the
downscaled and conventional methods were in good agreement with each other and produced comparable standard deviations. Furthermore, there was no discernible trend to suggest that the downscaled method consistently resulted in over- or under-estimation of the composition as compared to the conventional method. To compare the results produced by both methods, relative differences were reported in percent with the conventional measurements serving as the reference. Accordingly, the average glucan content determined by the downscaled method was slightly lower than the value determined by the conventional method for bagasse and *P. deltoides*, producing a 0.8 and 3.6% lower glucan content, respectively. However for switchgrass, the glucan content measured by the downscaled method was 3.8% higher than the conventional result. For xylan, the values resulting from the downscaled method were slightly higher than what was determined by the conventional method for all three biomasses. For *P. deltoides*, the xylan content measured by the downscaled method was 1.3% higher than that measured by the conventional method, while it was 1.4 and 0.6% higher for bagasse and switchgrass, respectively.
Figure 4.1. Glucan and xylan contents of bagasse, *P. deltoides* and switchgrass, determined by the downscaled and the conventional methods, along with the reference values. The compositions are displayed as mass percent, and the error bars represent the uncertainty at the 95% confidence level as reported by NIST for the reference materials and calculated from the SDOM of 9 independent measurements for the downscaled and conventional analyses.

The carbohydrate data produced by the two methods was further tested for statistical equivalence by comparing a 95% confidence interval of a heteroscedastic t-test with the above-defined levels of scientific indifference. For data to be statistically equivalent, the calculated 95% confidence interval had to be smaller, and fall within the defined indifference interval. Based on this criterion, the glucan and xylan measurements of all biomass materials tested were statistically identical for the downscaled and conventional analysis methods, as shown in Table 4.1. These results demonstrate that we were able to quantify the carbohydrate contents of lignocellulosic biomasses using the newly introduced downscaled method to obtain identical results as conventional methods.
Table 4.1. To compare whether glucan and xylan contents measured by the downscaled and conventional methods are statistically identical, the level of scientific indifference and the 95% confidence intervals for a heteroscedastic t-test are shown for compositions of NIST bagasse, NIST P. deltoides, and BESC switchgrass analyzed by the two methods. Results are considered identical if the 95% confidence interval falls within the defined indifference interval of ±10% of the mean determined by the conventional analysis.

<table>
<thead>
<tr>
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<th>NIST Bagasse</th>
<th>NIST P. deltoides</th>
<th>BESC Switchgrass</th>
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<tr>
<td>Glucan</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Automated</td>
<td>[-3.81, -0.68, 1.41] 3.81</td>
<td>[-4.19, -2.39, 0.21] 4.19</td>
<td>[-2.89, -2.38, 0.20] 2.89</td>
</tr>
<tr>
<td>Manual</td>
<td>[-4.19, -2.81, -0.82] 4.19</td>
<td></td>
<td></td>
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<tr>
<td>Xylan</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Automated</td>
<td>[-2.01, -1.44, 0.90] 2.01</td>
<td>[-1.51, -0.70, 0.26] 1.51</td>
<td>[-1.74, -1.27, 1.10] 1.74</td>
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<tr>
<td>Manual</td>
<td>[-1.51, 0.17, 0.65] 1.51</td>
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* Results are expressed as follows:
[- Indifference interval [Upper 95% CI, Lower 95% CI] + Indifference interval]

We also sought to demonstrate that the downscaled procedure can be performed manually. Thus, the composition of P. deltoides was also analyzed by performing all steps, including weighing and biomass dispensing, manually. The glucan content measured by the manual downscaled version was 4.3% lower than that measured by the conventional method, and was 0.9% lower than the automated downscaled measurement. The xylan content that was measured by the manual downscaled process was 2.7% higher than the conventional measurement, and was 1.2% higher than that measured by the automated downscaled method. Thus, the downscaled method can be performed manually to measure statistically identical glucan and xylan contents as the conventional wet chemistry method, which is important for labs that do not have robotics systems available.

To ensure that our measurements were reasonable, we also compared the values measured by conventional and downscaled methods to reference values reported by NIST. Direct comparison between the measured and reference values should not be used
as a strict guideline because the latter were obtained using extractive-free material, while values measured in this study were obtained from raw biomass. We did not use extractive-free materials because the small amounts of material that will be available in future applications of the downscaled analysis will not suffice to accomplish an extraction step. Figure 4.1 shows that the measured carbohydrate values were in agreement with the reference values, as demonstrated by all measurements falling within the 95% confidence intervals of the NIST standards *P. deltoides* and bagasse. For switchgrass, although there was no error analysis available for comparison, both the conventional and downscaled measured values differed from the reference values by less than 10%, which is in line with the 10 to 15% uncertainties reported for the NIST *P. deltoides* and bagasse.

After confirming that the downscaled method produced statistically identical carbohydrate measurements to the conventional method, we evaluated the feasibility of applying it to lignin and ash measurements. The lignin content determined by the Klason lignin procedure is a lumped measure that includes all acid insoluble non-sugar and non-ash components. The downscaled analysis however, does not allow determination of the ash content of the Klason lignin acid insoluble residue because 3 mg of starting material is not sufficient to accurately quantify ash content (see discussion below). Therefore the acid insoluble residue (AcIR) contents were quantified by both the downscaled and conventional methods, and the resulting values were compared in Figure 4.2 and Table 4.2. While AcIR contents measured by the downscaled method were 1.1 and 3.5% lower than that measured by the conventional method for switchgrass and *P. deltoides*,

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respectively, the downscaled measurement for bagasse was 19.8% higher than that from the conventional method. When performed manually, the AcIR content of *P. deltoides* measured by the downscaled procedure was 11.1% higher than that measured by the conventional method. All downscaled AcIR measurements were within a 20% relative error range as compared to conventional AcIR measurements, which is only slightly higher than the errors reported for the lignin values of NIST bagasse (14.9%) and *P.deltoides* (19.5%).

**Figure 4.2.** For bagasse, *P. deltoides* and switchgrass, Klason lignin, acid insoluble residue (AcIR), and ash contents determined by the conventional analysis, AcIR and ash contents determined by the downscaled analysis, and reference Klason lignin and ash contents are reported. The compositions are displayed as mass percent, and the error bars represent the uncertainty at the 95% confidence level as reported by NIST for the reference materials and calculated from the SDOM of 9 independent measurements for the downscaled and conventional AcIR and Klason lignin analyses and 6 independent measurements for the ash analyses.
Table 4.2. For bagasse, *P. deltoides*, and switchgrass, mean acid insoluble residue (AcIR) contents determined by the conventional and downscaled methods, as well as Klason-lignin (K-lig) contents determined by the conventional method. Values are compared to the reference K-lig values. All data includes corresponding 95% confidence intervals as reported by NIST for the reference materials and calculated from the SDOM of 9 independent measurements for the downscaled and conventional analyses. Also shown is the mean acid insoluble residue content and corresponding 95% confidence interval for *P. deltoides* as measured by the downscaled method performed manually.

<table>
<thead>
<tr>
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<th>NIST Bagasse</th>
<th>NIST <em>P. deltoides</em></th>
<th>BESC Switchgrass</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Downscaled</td>
<td>Conv</td>
<td>Conv</td>
</tr>
<tr>
<td></td>
<td>AcIR</td>
<td>AcIR</td>
<td>K-lig</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Automated</td>
</tr>
<tr>
<td></td>
<td>AcIR</td>
<td>K-lig</td>
<td></td>
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<tr>
<td></td>
<td>AcIR</td>
<td>K-lig</td>
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<td></td>
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<tr>
<td>27.3 ± 1.8</td>
<td>22.8 ± 0.3</td>
<td>21.6 ± 0.3</td>
<td>22.3 ± 2.5</td>
</tr>
</tbody>
</table>

To help gauge whether AcIR contents measured by the downscaled and conventional approaches were within the correct range, we next compared conventionally-measured Klason lignin values with reference lignin values reported by NIST, and found that all Klason lignin measurements were statistically identical to reference values (Figure 4.2 and Table 4.2). Because of this good agreement between reference and conventionally-measured lignin values, we can assume that the conventional measurements of AcIR were an accurate reflection of the biomass’ acid insoluble residue content. Furthermore, since we previously determined that conventional and downscaled AcIR measurements were comparable to one another, we can then deduce that the AcIR contents measured by the downscaled analysis were also reasonable. Thus, even though the Klason lignin content cannot be directly measured by the downscaled method, we have shown that this approach can determine the AcIR content of biomass, which can provide meaningful information about lignin content since Klason lignin is just the AcIR minus the insoluble ash content, which is a relatively low percentage of biomasses (<10%).
that are particularly low in ash, such as woody materials, this AcIR measurement can provide an estimate of the Klason lignin content, while for materials that are high in ash content, such as herbaceous plants, the AcIR measurement will significantly overestimate the Klason lignin content. However, it is also important to keep in mind that the primary purpose of this method is to support a high throughput screening system from which sample outliers will be selected for further study to undergo more thorough testing, including a detailed analysis of lignin content and composition.

In the process of developing the downscaled Klason lignin method, it was determined that while 3 mg of raw biomass was sufficient to determine carbohydrate and acid insoluble residue contents, this amount was insufficient to determine an accurate ash content for all biomass materials tested (Table 4.3). Reasonable ash values were determined for bagasse and switchgrass using 3 mg of material, but due to the low ash content of *P. deltoides*, this same amount produced erratic ash contents. If only 3 mg of material were used to measure the ash content of a biomass containing 1% ash (such as *P. deltoides*), the balance would have to be capable of accurately weighing 0.03 mg. Although this weight is above the detection limit of the Sartorius balance used in the automated downscaled experiments, it is already at the limit of the balance’s repeatability. As a result, to develop a single method that could be used to more accurately estimate the ash content of all biomasses tested, it was established that a minimum mass of 15 mg had to be used, which for a biomass containing 1% ash, now results in an ash weight (0.15 mg) that is 15 times greater than the balance’s detection limit and 5 times greater than its repeatability. All values measured by the conventional
and downscaled procedures were in the same range as one another, as well as with reference values (Figure 4.2 and Table 4.3). The results suggest that 15 mg of material used in the downscaled method is sufficient to estimate the ash content of a diverse set of materials.

![Table 4.3. Mean ash values with corresponding 95% confidence intervals calculated from the SDOM for bagasse, P. deltoides, and switchgrass as determined by the downscaled (automated) and conventional methods compared to the reference values. Shown are the ash contents determined with both 3 and 15 mg in the downscaled analysis, as well as with 300 mg in the conventional analysis. Data from performing the downscaled analysis manually with 15 mg of P. deltoides is also listed.](image)

In addition to the methods and materials presented here, which are capable of analyzing up to 64 different biomasses in triplicate per run, there are also important opportunities to further simplify and improve it. For example, while our current robotics platform houses 10 25 mL biomass-dispensing hoppers, other Symyx robotics platforms can house up to 144 10 mL hoppers on deck at a time, with an option for additional off-deck hoppers. In this paper, we have demonstrated the ability to scale down and automate the compositional analysis procedure, but the degree of automation will depend on the specific robotics platform used. Additionally, the solid and liquid dispensing robot can also be applied to dispense water and acid, as well as decant and transfer liquids. Neutralization with CaCO$_3$ or other bases can also be accomplished with a pH probe that is installed on many laboratory automation systems. Thus, only the autoclaving,
centrifugation, and ashing steps need to be performed manually. Furthermore, although we neutralize samples prior to HPLC analysis to provide flexibility in column usage, samples can be directly injected on an Aminex HPX-87H column to eliminate the neutralization step and significantly speed analysis. Conversely, we also demonstrated that the downscaled method works equally well in determining accurate glucan and xylan contents when it is performed manually without the use of a robotics platform. Other possible equipment simplifications include the use of crimp caps instead of the top and bottom plates with a flat gasket, which were also found to seal glass vials during high temperature hydrolysis (data not shown). Furthermore, to spin down solids in the HPLC vials, these vials could alternatively be inserted into larger centrifuge tubes for centrifugation instead of employing the reactor block and well plate carrier adaptor as reported here.

Besides these variations, the method can also be extended to measure additional biomass components. Once plant material has been broken down in the two-stage hydrolysis into components that can be more easily quantified, the measurement of a wide range of sugars is only limited by the selectivity and resolution of the chromatography column. Finally, the ability to process very small amounts of biomass is not only applicable in determining the composition of raw materials, it can also be very beneficial in following the progress of laboratory-scale hydrolyses or fermentations with solid substrates, for which the small samples available are inadequate for conventional methods.
4.5 Conclusions

In support of a high throughput pretreatment and enzymatic hydrolysis system, a rapid and simple method was developed that can determine statistically identical carbohydrate contents of very small amounts of biomass as conventional wet chemistry procedures and enables the subsequent calculation of sugar yields in screening studies. Furthermore, the method can also measure acid insoluble residue and ash contents to help identify compositional outliers that warrant further study and also identify correlated trends in sugar release data. The result of the modified procedure presented here is a new method that can process a much higher number of samples per time than is possible with the conventional method, and also can also dramatically reduce the amount of material that must be sacrificed for compositional analysis.

4.6 Acknowledgments

We gratefully acknowledge support for this research by the Office of Biological and Environmental Research in the DOE Office of Science through the BioEnergy Science Center (BESC). The authors also thank Dr. Markus Kalisch of the Department of Statistics at ETH Zurich for help with the statistical analysis. We also want to recognize the Ford Motor Company for their support of the Chair in Environmental Engineering at the University of California Riverside (UCR).
4.7 References


Chapter 5. Is Effective Hydrothermal Steam Pretreatment Limited in Industrially Sized Wood Chips?

* This whole chapter will be submitted under the following citation:
DeMartini JD, Foston M, X Meng, Ragauskas AJ, Wyman CE. “Is Effective Hydrothermal Steam Pretreatment Limited in Industrially Sized Wood Chips?”
5.1 Abstract

Although mechanical size reduction of woody biomass consumes a significant amount of energy, it is a crucial step in reducing recalcitrance obtaining high sugar yields in biological conversion of cellulosic biomass into fuels and chemicals. However, most laboratory research is performed on materials that are significantly smaller than what is applicable in a commercial setting (2 x 2 x 0.5”), and as a result, there is limited understanding of the effects of steam explosion pretreatment on larger wood chips, and what these effects may have on subsequent enzymatic hydrolysis. To address these concerns, this study employed novel downscaled and high throughput analysis systems to examine whether differences exist in the composition and digestibility within a single pretreated wood chip due to heterogeneous pretreatment across its thickness. As such, it was demonstrated that effective pretreatment can be limited at short pretreatment times, resulting in digestibility spatial effects and overall lower sugar yields in subsequent enzymatic hydrolysis. Various techniques including heat transfer modeling, Simons’ Stain testing, and magnetic resonance imaging (MRI) were also applied to probe the effects of water-only steam explosion pretreatment within and between pretreated wood samples to shed light on potential causes of variation.
5.2 Background

Woody biomass represents an important source of lignocellulosic biomass for sustainable production of organic chemicals and liquid fuels. Up to 142 million dry tons of sustainably-sourced forest biomass and wood waste will be available in 2012 (U.S. Department of Energy, 2011). This amount has the potential to dramatically increase through the future use of dedicated woody bioenergy crops, for which it has been estimated that between 100-300 million dry tons could be produced annually by 2030 (U.S. Department of Energy, 2011). However, woody biomass is recalcitrant to enzymatic sugar release and thus often requires significant size reduction and severe pretreatments to achieve economically viable product yields. Due to its large size and high density, the mechanical size reduction of wood consumes between 5-10 times more energy than that of agricultural residues (Zhu et al., 2010). The use of wood chips that are larger in size can result in substantially reduced energy requirements for milling, translating into lower production costs. As such, chips on the order of 50.8 x 50.8 x 12.7 mm (2 x 2 x 0.5”) are thought to be representative of what is currently used in the pulp and paper industry, and what may be applicable in a commercial cellulosic ethanol plant (Todd Lloyd, personal communication).

Unfortunately, much of the laboratory research focused on sugar release from woody biomass by pretreatment and enzymatic hydrolysis has utilized small particle sizes (typically <2mm) that are not economically feasible in an industrial setting. As such, there is limited understanding of the effects of steam explosion pretreatment on subsequent enzymatic hydrolysis for larger chips. To better understand whether effective
steam explosion pretreatment is inhibited by size in an industrially-relevant wood chip, we sought to address the following questions:

i) Do composition and/or digestibility vary across the thickness of an industrially-sized pretreated wood chip?

ii) Does pretreatment render the exterior of a wood chip more digestible than the interior? If so, why?

iii) Does pretreatment efficacy for an industrially-sized wood chip vary for different pretreatment times? If so, why?

As summarized by Vidal Jr. et al. (2011), a number of previous studies have provided information relevant to these questions by examining the effect of particle size on various pretreatment regimes. Although these studies generally tested particle sizes <12 mm, the majority reported that for woody biomass following steam explosion, the larger sized materials either exhibited similar (Negro et al., 2003), or higher glucose yields in subsequent enzymatic hydrolysis (Cullis et al., 2004, Ballesteros et al., 2000) as compared to smaller sized particles. These studies suggest that chip size, at least up to 12 mm, does not limit effective pretreatment in terms of preparing biomass for enzymatic digestion. However, little work has been done to look at larger wood chips, and furthermore, to examine whether differences exist in the composition and digestibility within a single pretreated wood chip due to heterogeneous pretreatment across its thickness. This is due in large part to the significant amounts of material that are typically required for biomass composition and enzymatic sugar release analysis. To overcome this limitation, we applied downscaled processes (Studer et al., 2010;
DeMartini et al., 2011) to study potential variation in both composition and sugar release of sub-sections (or slices) taken across the thickness of a pretreated wood chip measuring 50.8 x 38.1 x 12.7 mm (2 x 1.5 x 0.5”) in length, width, and thickness, respectively. Various other techniques including heat transfer modeling, Simons’ Stain testing, and Magnetic Resonance Imaging (MRI) were also applied to probe the effects of water-only steam explosion pretreatment within and between pretreated wood samples to shed light on potential causes of variation.

5.3 Results and discussion

5.3.1 Composition

The composition of the raw, unpretreated aspen wood was found to be 51.0% glucan, 22.3% xylan, and 21.0% acid insoluble residue (AcIR). The AcIR content is an approximation of the Klason lignin content since it is a measure of the total acid insoluble residue including acid insoluble ash. However, since the whole ash content of Populus tremuloides wood is typically fairly low, <4% (DeMartini et al., 2011b), the AcIR is a good estimate of the Klason lignin content.

Figure 5.1 displays the glucan, xylan, and AcIR contents as a function of sub-section location across the thickness (which measures 12.7 mm or 0.5” in total in that direction) of each of the four pretreated wood chips. Additionally, the composition is also shown at the far right end of the x-axis for wood that was milled prior to pretreatment. In comparing the compositions across the thickness of a single pretreated chip, preliminary assessment revealed no drastic differences in the glucan, xylan, or AcIR content.
Compositions of the exterior samples (1 and 8) were not considerably different than the interior samples (2 to 7) of the same chip. In fact, for the 4-min pretreated chip (Fig. 5.1A), the tested parameters of glucan, xylan, and AcIR content were not found to be significantly different between the interior and exterior of the chip. However, for the 8, 12, and 18-min pretreated wood chips (Fig. 5.1B-1D), the difference in glucan and xylan content between the interior and exterior sub-samples were extremely statistically significant (p<0.001). In these pretreated wood chips, the glucan contents of the exterior sub-samples 1 and 8 were slightly higher than that of interior sub-samples 2 through 7, while the xylan content followed the opposite trend in which xylan content was lower on the exterior than it was for interior sub-samples. These results demonstrate that there was compositional variability in an industrially-sized wood chip pretreated for longer times (>4 min), with the exterior surfaces of the wood chip showing signs of more severe pretreatment, in particular increasing hemicellulose solubilization and a corresponding increase in the relative glucan content. However, it is interesting that the wood chip pretreated for the shortest time, in which one may expect to see greater variation across the section due to less equilibration time, showed no such trends.

In comparing Figures 5.1A through 5.1D, another key observation is that in general, the glucan and AcIR contents increase, while the xylan contents decrease with increasing pretreatment time. The average glucan, xylan, and AcIR contents of the 4-min pretreated chip were 50.6, 23.8, and 20.6%, respectively, whereas the same values were 52.0, 20.5, and 22.0% in the 18-min pretreated chip. These changes were more pronounced in the pretreated milled wood in which the 4-min pretreated material had a composition of
58.2% glucan, 19.4% xylan, and 19.8% AcIR, while the 18-min pretreated milled material had a composition of 63.9% glucan, 9.8% xylan, and 27.4% AcIR. These trends reflect the increasing solubilization of hemicellulose during water-only steam explosion with longer pretreatment times, and the corresponding increase in the relative glucan and lignin contents. The difference in the composition of the wood chip and the milled wood pretreated under the same condition demonstrates that the latter was more sensitive to these effects. Xylose removal was always higher for the pretreated milled material than it was for the chip pretreated at the same condition.

Figure 5.1. Compositional analysis of aspen wood chips and milled aspen wood pretreated at 180°C for 4 (A), 8 (B), 12 (C), and 18 (D) minutes. Glucan, xylan, and AcIR contents of the steam pretreated milled aspen are shown on the far right of the x-axis, while chip sub-samples are shown to the left, and are labeled 1-8 to represent the 8 layers into which the pretreated wood chips were fractionated, with 1 and 8 being exterior layers, and 2-7 being interior. Downscaled compositional analysis was performed in triplicate, with error bars representing the corresponding standard deviation.
5.3.2 Sugar yields

We next sought to determine whether differences were present in the digestibility both between chips, and within a single chip across its thickness. Figure 5.2 plots the 168-hr glucose, xylose, and glucose + xylose (total sugar) yields from enzymatic hydrolysis as a function of sub-section location across the thickness of each of the four pretreated wood chips. Additionally, the sugar yields are also shown at the far right end of the x-axis for wood that was milled prior to pretreatment. Yields reflect the amount of sugar released in enzymatic hydrolysis out of the available sugar in the pretreated biomass, as measured by downscaled compositional analysis (Fig. 5.1).

Figure 5.2 demonstrates that there was no clear trend in the glucose or xylose yields across the thicknesses for the 8, 12, or 18-min pretreated chips. In particular, glucose and xylose yields at the chips’ exteriors were not higher than those of the interiors, suggesting that a 180°C pretreatment for greater than 8 min did not result in significant spatial effects. However for the 4-min pretreated chip, there was a clear trend of decreasing glucose and xylose yields from the exterior to interior of the chip, suggesting that the shortest pretreatment tested resulted in overall lower sugar yields, as well as digestibility spatial effects across the chip’s thickness. The glucose yields decreased from an average of 58.4% for the exterior samples to 45.3% for the innermost two samples. Similarly, the average difference in xylose yield was 60.1% versus 47.0% for the exteriors and interiors, respectively.

It is also important to note that chips pretreated for longer times generally exhibited higher yields for all sub-samples, as expected. There was a steep drop off in sugar yields
for the wood chip pretreated for the shortest amount of time (Fig. 5.2A), for which the average glucose yield was only about 50%, as compared to average glucose yields of between 83-89% achieved by the 8 to 18 min pretreated chips (Fig. 5.2B-2D). Results were similar, but less drastic, for the 4-min pretreated milled material as compared to the 8-18 min pretreated milled materials. As such, the 168 hr glucose yields increased from 84.5% for the shortest pretreatment time to 94.0% for the 18-min pretreated milled wood. Thus, final glucose yields in enzymatic hydrolysis were significantly higher for the 4-min pretreated milled wood than they were for the 4-min pretreated wood chip, but were very similar between the chips and milled wood that were subjected to longer pretreatment times.

Figure 5.2. 168-hour sugar yields from enzymatic hydrolysis of each sub-sample of the pretreated aspen wood chip, as well as milled pretreated aspen. Shown are results from chips and milled wood pretreated at 180°C for 4 (A), 8 (B), 12 (C), 18 (D), and 28 (E) minutes. Yields reflect the amount of sugar released in enzymatic hydrolysis out of the available sugar in the pretreated biomass, as measured by compositional analysis. Error bars represent the standard deviation of triplicate runs.
Figure 5.3 plots the time profiles of glucose yields during enzymatic hydrolysis for all pretreated chip and milled samples. The profiles of the chip sections were very similar for wood chips pretreated for 8, 12, and 18 minutes. On the other hand, Figure 5.3A demonstrates that significant differences in yields from the 4-min pretreated chip sections became apparent particularly after 48 hours of hydrolysis, and continued to further differentiate until 168 hours. Thus, digestibility differences increased with hydrolysis time for this chip. It is also interesting to compare the profiles of the chip sections to those of the corresponding pretreated milled materials. As such, the 4-minute milled material exhibited higher yields than the chip sections at all hydrolysis points, whereas the milled material pretreated for longer times exhibited higher yields than the corresponding chips only at the earlier hydrolysis time points. These milled materials had higher final digestibility after 168 hours of hydrolysis.
5.3.3 Investigating performance and variability

Based on the results, three questions were pursued. First, why was the 4-min pretreated chip the only one tested that exhibited digestibility differences across its thickness? Furthermore, what structural and/or chemical features caused the difference in digestibility in this pretreated chip? Finally, why did the milled material exhibit significantly higher digestibility than the corresponding pretreated chip only for the
shortest pretreatment time, whereas the final glucose yields were similar in the 8, 12, and 18-min pretreated chips and milled materials?

5.3.3.1 Heat conduction modeling

To address the first question, heat transfer throughout a wood chip during pretreatment was modeled in order to estimate whether this could be a factor in limiting effective pretreatment across an entire chip thickness. To this end, a solution to two-dimensional heat conduction through a rectangular cross section was applied (MacLean, 1932). It was reported that the solution converges quickly, so only the first seven terms were necessary (Simpson, 2001). Equation 1 approximates the temperature at a given point within a wood chip,

\[ T = T_s + \left( T_0 - T_s \right) \left( \frac{16}{\pi^2} \right) \times \]

\[ \left\{ \sin \left( \frac{\pi x}{a} \right) \sin \left( \frac{\pi y}{b} \right) \exp \left[ -\pi^2 t \left( \frac{a_x}{a^2} + \frac{a_y}{b^2} \right) \right] + \left( \frac{1}{3} \right) \sin \left( \frac{3\pi x}{a} \right) \sin \left( \frac{\pi y}{b} \right) \exp \left[ -\pi^2 t \left( \frac{a_x}{a^2} \right) \right] + \left( \frac{1}{5} \right) \sin \left( \frac{5\pi x}{a} \right) \sin \left( \frac{\pi y}{b} \right) \exp \left[ -\pi^2 t \left( \frac{25a_x}{a^2} + \frac{a_y}{b^2} \right) \right] + \left( \frac{1}{7} \right) \sin \left( \frac{7\pi x}{a} \right) \sin \left( \frac{\pi y}{b} \right) \exp \left[ -\pi^2 t \left( \frac{49a_x}{a^2} + \frac{a_y}{b^2} \right) \right] + \left( \frac{1}{9} \right) \sin \left( \frac{9\pi x}{a} \right) \sin \left( \frac{\pi y}{b} \right) \exp \left[ -\pi^2 t \left( \frac{a_x}{a^2} + \frac{49a_y}{b^2} \right) \right] \right\} , \]

(1)

where \( T_s \) is the surface temperature (attained immediately), \( T_0 \) is the initial temperature, \( a \) and \( b \) are the cross-sectional dimensions, \( \alpha_x \) and \( \alpha_y \) are the thermal diffusivities in the x
and y directions, respectively, and t is time. To calculate the temperature at the center of the chip, the following conditions were set: x=a/2 and y=b/2. Furthermore, diffusivity in the radial and tangential directions can be assumed to be very similar since they are both against the wood grain, so $\alpha_x$ is equal to $\alpha_y$ (Simpson, 2001). The assumptions applied for the wood chip used in this study are listed in Table 5.1. The diffusivity values were obtained from Abasaeed and Lee (1991), and represent a range of values for conduction in both the radial and longitudinal directions, as well as conduction in the radial direction in hemicellulose-free wood; the values were determined experimentally for the hardwood species southern red oak.

<table>
<thead>
<tr>
<th>$T_s/T_{in}$</th>
<th>180° C</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_0/T_{init}$</td>
<td>22° C</td>
</tr>
<tr>
<td>$a/TH$</td>
<td>0.5” (0.0127 m)</td>
</tr>
<tr>
<td>$b/W$</td>
<td>1.25” (0.03175 m)</td>
</tr>
<tr>
<td>$\alpha_x=\alpha_y$</td>
<td>$1.27\times10^{-7}$ m²/s¹</td>
</tr>
<tr>
<td>$1.74\times10^{-7}$ m²/s²</td>
<td></td>
</tr>
<tr>
<td>$2.63\times10^{-7}$ m²/s³</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>22.26%</td>
</tr>
<tr>
<td>G</td>
<td>0.425†</td>
</tr>
<tr>
<td>Constants: a/b/c/d</td>
<td>60.44/-3.032/3.080/-0.2662</td>
</tr>
<tr>
<td>Constants: e/f/g/h</td>
<td>1.720/-0.2560/-0.0945/0.2156</td>
</tr>
</tbody>
</table>

† For conduction in radial direction (against grain)
‡ For conduction in radial direction (against grain)
in hemicellulose-free wood
§ For conduction in longitudinal direction (with grain)
¶ The specific gravity was taken to be the average of 0.35 and 0.5‡‡

Based on this, the temperature at the center of the wood chip was plotted versus pretreatment time in Figure 5.4 for the three different diffusivity values. The results

‡‡ Specific gravity values were obtained from [http://www.engineeringtoolbox.com/wood-density-d_40.html](http://www.engineeringtoolbox.com/wood-density-d_40.html), and agreed with other reports in literature.
show that the temperature at the center of the wood chip increased rapidly during the first couple of minutes of the pretreatment reaction, and then asymptotically approached the target temperature of 180°C. The inset table in Figure 5.4 summarizes the time it takes to reach a specific center temperature for the different diffusivity values. As displayed, it is predicted to take between 3.7 and 7.6 minutes for the center of the chip to reach within 5°C of the target temperature, depending on the assumed wood thermal diffusivity value.

Figure 5.4. Predicted temperature at the center of a wood chip (of the chip dimensions used in this study) versus pretreatment time (at 180°C) based on a solution to 2-dimensional heat conduction through a rectangular cross section (MacLean, 1932; Simpson, 2001). Temperature profiles are given for 3 thermal diffusivity values to represent a range of possible values, dependent on grain direction, wood density, and hemicellulose content (Abasaeed and Lee, 1991). The inset table displays the numerical value of the estimated time it takes for the center of the wood chip to reach a specific temperature for the assumed diffusivity values.

An additional analysis was also performed to provide a second estimate of the heating time. The analysis by Simpson (2006) is based on a multiple regression analysis in which heating times for a large combination of variables (including chip size, temperature, wood specific gravity, etc.) was calculated using heat conduction equations, and then fitted to the regression model shown in equation 2.
\[ T = a(T_{ht})^b(T_{ctr})^c(T_{init})^d(TH)^eW^fM^gG^h \]  

where \( T_{ht} \) is the heating temperature, \( T_{ctr} \) is the target center temperature, \( T_{init} \) is the initial temperature, \( TH \) is the chip thickness, \( W \) is the chip width, \( M \) is the chip moisture content (\%), \( G \) is the specific gravity of the wood chip, and \( a-h \) are pre-determined regression coefficients. The inputs applied in this model are also listed in Table 5.1. As such, this model estimated that it should take approximately 4.4 min for the center of the wood chip to reach its target temperature. This analysis agreed fairly well with the one presented in Figure 5.4. Both suggested that it should take in the range of about 5 minutes for the center of the wood chip to reach the target temperature, or very close to it. Thus, it seems probable that the 4-min pretreatment applied in this study was not sufficient to attain the target temperature uniformly throughout the thickness of the wood chip. However, the entire thickness of wood chips pretreated for 8-18 minutes was modeled to reach the target temperature, or very close to it, which seems to support the sugar yield results presented in Figure 5.2.

5.3.3.2 Composition and enzyme inhibition

After observing that effective pretreatment was likely limited for shorter reaction times due to insufficient heating throughout the chip, the next question to address was whether any specific structural and/or chemical features could be identified in the pretreated wood to cause the differing digestibility. If the interior sections of the 4-min pretreated chip exhibited lower enzymatic sugar yields than the exterior chips, what changes in the wood due to pretreatment caused this?
The first possibility examined was whether or not the composition caused differential yields across the 4-min pretreated chip. Thus, the 168-hr glucose yields were plotted versus xylan and AcIR content. These factors were selected because xylan removal is thought to correlate positively with glucose yield due to a reduction in enzyme restriction (Knappert et al., 1980; Yang and Wyman, 2004), while lignin content (which is by far the largest component of AcIR) has often been reported to be inversely related to glucose yield due to restriction of enzyme access and non-productive binding (Chang and Holtzapple, 2000; Berlin et al., 2005; Chen and Dixon, 2007). However, there was no correlation or trend to suggest that either of these factors caused the differential sugar yields in the 4-min pretreated chip.

The second possibility that was investigated focused on the potential presence of soluble sugars that may have influenced compositional analysis results and/or caused enzyme inhibition during hydrolysis. Because compositional analysis and enzymatic hydrolysis were performed on the pretreated unwashed solids, the presence of soluble sugars such as xylose or xylooligomers may have resulted in an overestimated xylan content for some samples, perhaps influencing the sugar yield results discussed above. Additionally, if the interior samples had a higher xylooligomer to xylose ratio than the exterior samples, this may have caused a higher level of enzyme inhibition for the interior samples (Qing et al., 2011), resulting in the lower yields observed in these samples (Figure 2A). To test this, the dried pretreated samples from the 4-min pretreated chip were washed in hot water to remove soluble sugars. The liquid washates were tested for monomeric and oligomeric sugars, while the washed solids underwent compositional
analysis and enzymatic hydrolysis. The liquid analysis demonstrated that there were fairly low levels of soluble sugars in the pretreated samples: less than 6.5% of xylan was left as soluble sugar in the chip after 4 minutes of pretreatment. Furthermore, based on post-hydrolysis of the liquid washates, the xylooligomer to xylose ratio was slightly higher for the exterior samples than the interior ones, which is the opposite of what one might expect based on the sugar yield data. The average ratio of xylooligomers to xylose was 2.0 for the two exterior samples, while the same average was 1.1 for all six interior samples. However as stated above, the level of all soluble xylan-derived sugars was very low; furthermore, no glucan-derived sugars were detected.

Compositional analysis of the washed solids further supported the liquid analysis. In line with Figure 5.1A, there was still no variation in glucan or xylan content across the thickness of the washed pretreated chip samples. The xylan content did decrease uniformly by about 3.5% on average for all sub-samples, but no compositional variation within the washed chip was revealed. Finally, the washed solids also underwent enzymatic hydrolysis testing as was performed on the unwashed samples. As with Figure 5.2A, there was substantial variation in glucose and xylose yields across the chip thickness in an identical trend as previously found. The only difference between the 168-hr sugar yields of the washed and unwashed chip samples was a uniform slight increase in the glucose yields by about 5.8% on average. Thus, although soluble sugars such as glucose, glucooligomers, xylose, and xylooligomers were not the cause of variable yields across the 4-min chip’s thickness, the washing of solids and removal of soluble components did slightly increase the digestibility of all samples to the same degree.
Additional potential inhibitors from steam pretreatment include sugar-degradation products such as 5-hydroxymethylfurfural and furfural, acetic acid (Palmqvist et al., 1996), or lignin-degradation products (Panagiotou and Olson, 2007; Sanderso, 1965). However, high levels of these inhibitors are quite unlikely due to the short pretreatment time.

To further probe the cause(s) of the variability observed in the 4-min pretreated chip, nuclear magnetic resonance will be utilized to directly monitor the amount of adsorbed water (moisture content) and the proportion of bound to free water protons (pore surface area to volume ratio). The amount of adsorbed water and strength of association with the lignocellulosic substrate has been shown to be directly correlate with a combination of recalcitrance relevant characteristics, primarily pore surface area to volume ratio along with the ultrastructural and chemical state of the biomass (Foston et al., 2010; Blumich et al., 2003; Elder et al., 2006). While several studies have been published which use NMR without any spatial information to determine moisture content (Nanassy, 1976; Sharp et al. 1978; Hartley et al. 1994, 1996), this study will apply spatially resolved NMR to provide information regarding potential variability between and within wood chips.

5.3.3.3 Moisture content and local water mobility: results from MRI

To further probe cause(s) of the variability observed within the 4-min pretreated chip, as well as between chips pretreated for different times, MRI was utilized. Water can be found spatially localized in biomass on cellulose fibril surfaces, capillaries of lumens or in-between fibers and within the voids of the lignocellulosic matrix (Felby et al., 2008;
Menon et al., 1987; Friox et al., 1975; Araujo et al., 1993). The amount of adsorbed water and strength of association with the lignocellulosic substrate has been shown to be directly correlated with a combination of recalcitrance relevant characteristics, primarily pore surface area to volume ratio along with the ultrastructural and chemical states of the biomass (Foston et al., 2010; Blumich et al., 2003; Elder et al., 2006). Nuclear magnetic resonance (NMR) can be utilized to directly monitor the amount of adsorbed water (moisture content) and the proportion of bound to free water protons (pore surface area to volume ratio). Biomass with reduced hydrophilic character, e.g. biomass with altered composition, would absorb less water and have a lower ratio of bound to free water as compared to biomass with lower lignin contents. However, compositional differences between samples in this study were very minimal so this is not expected to influence results. On the other hand, biomass with increased pore size distributions would absorb more water while still displaying a lower ratio of bound to free water. Several studies have been published which use NMR without any spatial information to determine moisture content (Nanassy et al., 1976; Sharp et al. 1978; Hartley et al. 1994, 1996) and pore size distributions (Foston et al., 2010; Felby et al., 2008; Haggkvist et al, 1998; Li et al., 1993). Magnetic Resonance Imaging (MRI) is a spatially resolved technique which has also been used for moisture measurements on biomass (Olson et al.,1989; McCarthy et al., 1991; Nilsson et al., 1996). MRI is not frequently used to analyze biomass in part due to the expense of equipment and expertise needed. Moreover, it is also particularly difficult to measure the moisture contents in biomass below the fiber saturation point with conventional imaging techniques because of the relatively short spin-spin relaxation
(T₂) times associated with water molecules bound to the macromolecules in the cell wall. Because of molecular motional averaging, protons on mobile moieties such as free water give rise to narrow line-widths and long T₂ values; conversely, protons on rigid or bound moieties are associated with broad line-widths and short T₂ values. Recent advances in MRI methodology such as zero echo time (ZTE) imaging can deliver images of structures that were previously invisible in MRI due to very short relaxation times (Herlihy et al., 2005). Utilizing such techniques, we were able to spatially monitor the relaxation rates (T₂) and proton density of adsorbed water in the untreated and pretreated wood chips.

Figure 5.5. Cross-sectional slices of ZTE images resulting on Populus wood chips, both untreated and pretreated in a steam gun at 180 °C for 4 and 18 min conditioned at 100% RH for 14 days. Red represents high proton density and water content. The z-direction corresponds to the chip length and the x-direction corresponds to the thickness dimension.
ZTE images were obtained for untreated *Populus* wood chips, as well as chips pretreated in a steam gun at 180°C for 4 and 18 minutes (Figure 5.5). All chips were conditioned at 100% relative humidity (RH) for 14 days prior to analysis. The image color map indicates the amplitude of the NMR signal in which red represents the highest density of mobile protons and consequently the highest water content. Higher water content also indicates a larger total pore volume. The series of images displayed in Figure 5.5 show that it was possible to acquire images of wood, depicting moisture profiles with high spatial resolution. As such, variation in the moisture content was observed even for the untreated chip, demonstrating that there was natural variation in the moisture content within a single sample of wood. Furthermore, Figure 5 demonstrates that the relative moisture content and related pore volume generally increased with pretreatment, while also displaying that higher proton densities existed near the bottom corners of almost all of the images. The higher proton densities at the bottom of the chips may be due in part to the effects of gravity during the conditioning period, in which the chip was laying on its x-y surface. The images of the two pretreated chips further demonstrate that the proton density matched well with the grain of the wood, suggesting that the wood chip was hydrated primarily through the natural pore structure during pretreatment. However, it is interesting to observe that the images of the pretreated samples (4 and 18 minutes) were fairly similar, suggesting that moisture content and total pore volume cannot fully explain the digestibility differences observed between the 4 and 18-minute pretreated chips. Furthermore, the variation in the x-y face of the pretreated chips was relatively small; the densities were similar, ranging primarily from blue to
green. Thus, it does not appear as if variation in moisture content and related total pore volume within pretreated wood chips explains the variability in enzymatic sugar yields across the thickness (x-direction) of the 4-minute pretreated chip.

In addition to moisture content, MRI was also utilized to examine the local mobility of absorbed water within and between wood chips. Spin echo images were recorded for 8 echo times between 7.9 and 63.0 ms, recording 20 slices (1 mm thick) in the x-y plane approximately every 2 mm along the length of the wood chip. Figure 6 shows 5 cross-sectional $T_2$ images resulting from spin echo MRI experiments on conditioned untreated and pretreated *Populus* wood chips. The $T_2$ values represent the local mobility of absorbed water and thus, the amount of bound versus unbound water within the pores. This also indicates differences between the ratio of surface area to volume. The images clearly showed an increase in $T_2$ values of absorbed water with pretreatment, displaying an average increase of about 20 ms from the untreated to 18-minute pretreated chip. This suggests that there was a relative increase in the proportion of free water, which consequently indicates a decrease in the pore surface area to volume ratio in pretreated chips. The 4-minute pretreated chip images display a distinct ring-like pattern in the x-y plane with higher $T_2$ values near the perimeter of the chip cross section. The 18-minute sample also showed a heterogeneous spatial distribution of $T_2$ values across the chip’s thickness; however, unlike the 4-minute sample, the spatial effects were less localized and more evenly distributed throughout all images.
Figure 5.6. T$_2$ images resulting from spin echo MRI experiments on Populus wood chips, both untreated and pretreated in a steam gun at 180 °C for 4 and 18 min conditioned at 100% RH for 14 days. Red represents high water local mobility. The z-direction corresponds to the chip length and the x-direction corresponds to the thickness dimension.

When combined with the observations from Figure 5.5, these results match well with the digestibility data. As discussed in section 2.2, the following two observations were made: 1) the overall glucose yields of wood chips increased with increasing pretreatment time, and 2) the 4-minute sample was the only pretreated chip to exhibit digestibility spatial effects across its thickness. From Figure 5.6, it can be observed that the local water mobility 1) increased with pretreatment, and 2) displayed a ring-like pattern in which the mobility was higher at the edges of the 4-minute pretreated chip than in the interiors. These results suggest that higher water mobility, meaning lower surface area to volume ratio, was positively correlated with digestibility of the wood chip samples. At first glance, it may appear counter intuitive that lower surface area to volume ratio is characteristic of samples exhibiting higher digestibility. However, Figure 5.5
demonstrated that pretreated chips had higher moisture contents. Furthermore, a decreasing ratio of pore surface area to volume (which for a sphere, simplifies to 6 over the diameter) results in an increase to the pore diameter. Thus, wood chips and regions of pretreated chips that exhibited higher digestibility were characterized by pores with relatively larger diameters. These findings suggest that differences in pore size, which can be related to enzyme accessibility, were at least partly responsible for the variable glucose yields observed within and between wood chips. Previous research has clearly demonstrated that enzymatic digestibility of pretreated biomass is directly related to cellulase accessibility to cellulose (Jeoh et al., 2007).

5.3.3.4 Total pore volume and relative accessibility: Results from Simons’ Stain

From Figure 5.3 it was observed that the milled material exhibited significantly higher 168-hr glucose yields than the corresponding pretreated chip only for the 4-min pretreatment time; conversely, the final glucose yields were very similar between the chip and milled material for the 8, 12, and 18-minute pretreatments. Furthermore, although the 4-minute milled material exhibited higher yields than the chip sections at all hydrolysis points (24, 48, and 168 hr), the milled material pretreated for longer times showed higher glucose yields only at the earlier hydrolysis time points (24 and 48 hr) (Fig 5.3).

It seems intuitive that smaller particle sizes could result in more effective pretreatment due to a higher surface area to volume ratio. However, more effective pretreatment of the milled material (e.g., higher glucose yields in subsequent enzymatic
hydrolysis) was only observed for the 4-minute pretreatment condition. Under all other tested pretreatment conditions, the significantly smaller-sized milled material performed worse initially in hydrolysis, and exhibited only slightly higher glucose yields at the end of hydrolysis. This observation is especially curious when you consider that the xylan removal was higher in the milled material than in the chips. Thus, why is the initial hydrolysis rate lower for materials with higher xylan removal?

To further investigate these observations, a Simons’ Stain test was performed that is based on competitive adsorption on two different-sized molecular dyes (Chandra et al., 2008). The results provide insights into total pore surface area and the relative accessibility (ratio of large to small pores) of the biomass sample. As displayed in Figure 5.7, Simons’ Stain testing was employed to compare 1) the raw aspen, 2) the 4-min pretreated chip, 3) the 4-min pretreated milled material, 4) the 18-min pretreated chip, and 5) the 18-min pretreated milled material. The results indicated that pretreatment increased the relative accessibility for all pretreated samples as compared to the raw wood. Additionally, although the total pore surface area was similar between the 4-min pretreated chip and the untreated material, the total pore surface area was higher in all other pretreated samples. In comparing the 4-min pretreated chip and 4-min pretreated milled material, the results indicated that both the total pore surface area and relative accessibility were higher in the milled material than they were in the chip. For the 18-min pretreated samples, although the total pore surface area was somewhat higher in the milled material versus the chip, the opposite was true for the relative accessibility: the 18-min pretreated chip exhibited higher relative accessibility than the milled material.
Figure 5.7. Simons’ Stain result for untreated wood, 4-min pretreated chip and milled materials, and 18-min pretreated chip and milled materials. Shown is the total pore surface area of both dyes (mg dye/g of substrate) and the dye accessibility ([mg large dye/g substrate] / [mg small dye/g substrate]).

As in the previous section, these results corresponded well with the enzymatic hydrolysis data and point to the idea that accessibility is a key characteristic in determining digestibility. The relative accessibility measured by Simons’ Stain can be strongly correlated with enzyme accessibility to cellulose (Esteghlalian et al., 2008), particularly at earlier hydrolysis times when accessibility is likely a more significant limitation. As such, the accessibility of the 4-min milled material was higher than the accessibility of the 4-min chip, just as the glucose yields were higher for the 4-min milled material than for the 4-min pretreated chip. Conversely, the accessibility of the 18-min pretreated milled material was lower than that of the 18-min pretreated chip, corresponding to the lower glucose yields at the early hydrolysis time points (24 and 48 hr) for the milled material.

Although MRI and Simons’ Stain results both support the concept that enzyme accessibility was a key factor in the digestibility of the pretreated samples in this study,
the cause of the differing accessibility is not entirely clear. The higher accessibility observed in the pretreated materials as compared to the untreated wood is logical since pretreatment removes and re-locates barriers such as lignin and hemicellulose (Donohoe et al., 2008; Kristensen et al., 2008; Brunecky et al., 2009; Liu and Wyman, 2005). The same reasoning should also be able to be applied to the accessibility of the milled material versus the chip. For the same pretreatment condition, the milled material exhibited higher xylan removal than the chip, and thus should have higher accessibility. This was indeed the case for the 4-minute pretreatment condition, but not for the 18-minute pretreatment, in which the chip exhibited higher accessibility than the milled material. The cause of this observation is unknown. However, we believe that two factors could possibly be playing a role. For one, the explosive nature of the reaction has been reported to significantly change to ultrastructure of wood (Grouss et al., 1986; Donaldson et al., 1988; Mosier et al., 2005). It is possible that milled materials following longer pretreatment times, in which more cell wall material is removed or relocated, may not be able to stand up to the explosive release of pressure. As a result, there may be some degree of unwanted pore collapse in the pretreated milled material that results in reduced accessibility as compared to the pretreated chips, which may better withstand steam explosion pretreatment. Another potential contributing factor relates to the fact that all pretreated materials were allowed to air-dry before subsequent testing. It has been previously reported that drying pretreated materials may cause their pore structure to collapse (Jeoh et al., 2007). It is possible that the air-drying of pretreated materials may have affected the milled and chip materials differently, particularly at longer
pretreatment times in which the difference between xylan removal between the chip and milled materials was greater. However, it has also been reported that this collapse should be associated with reduced enzymatic hydrolysis and lower sugar yields. In this study, the final glucose yields in the 18-min pretreated samples were high regardless of whether a chip or milled material was tested. Thus, the cause of the differing accessibility in the pretreated milled and chip materials remains unclear, yet we believe that accessibility likely played a key role in defining enzymatic digestibility.

5.4 Conclusions

This study discovered that under certain reaction conditions, efficient and uniform pretreatment is limited in industrially-sized wood chips. While pretreatments longer than 8 minutes at 180°C achieved uniform yields across the entire thickness of the resulting wood chips, digestibility varied substantially within chips pretreated for short times (4 minutes). Based on analysis of the pretreated chips and milled materials, enzyme inhibition was not found to be a factor in the variable glucose yields observed in the 4-min pretreated chip. Instead, heat transfer modeling suggested that attaining the target pretreatment temperature throughout the entire thickness of the wood chip is important in obtaining uniformly high sugar yields within the chip. Furthermore, MRI and Simons’ Stain testing both demonstrated that structure of the biomass, in particular the size distribution of pores and related enzyme accessibility, were key factors dictating the digestibility of wood samples.
5.5 Methods

5.5.1 Biomass samples and material preparation

Trembling Aspen (Populus tremuloides) samples were obtained by Benchmark International in High Level, Alberta, Canada. Trees were destructively sampled to collect approximately 20-80 mm thick disks, or cross sections, from individual trees at 0.3 meters from the point of germination. The sections were sent to the University of California Riverside, USA, where they were frozen at 0°C until use. The cross section used in this study was from an 80-90 year old tree.

After allowing the cross section to thaw in a refrigerator, chips were obtained using a chisel. All chips were taken from the outer portion of the section, or the mature wood, to minimize variability within a single chip. Chips had the following dimensions: 50.8 x 38.1 x 12.7 mm (2 x 1.5 x 0.5”) in length, width, and thickness, respectively. The moisture content of the chips was approximately 22%. Additional material was taken from the cross section and milled through a 20-mesh screen (< 0.85 mm) and allowed to air dry until the moisture content reached about 6%.

5.5.2 Pretreatment

For steam explosion pretreatment without the addition of any external catalysts, a single chip was placed into a woven metal mesh (Particle-sifting woven wire cloth type 316, 8 x 8 mesh, 0.025” wire diameter, McMaster Carr, CA, USA) basket, which was then suspended in a 4L Hastelloy steam reactor in the fashion of a tea bag. Steam for pretreatment was provided by a Fulton steam boiler (FB-075-L, Fulton Companies,
Pulaski, NY), which was controlled by setting the boiler pressure to the saturated steam pressure corresponding to the target temperature of 180°C. Pretreatments were performed at 180°C for 4, 8, 12, or 18 minutes. At the end of the reaction time, the temperature and pressure was suddenly dropped by opening a valve at the bottom of the vessel, during which all pretreatment liquid was discharged and not collected for analysis. Afterwards the chip, which remained intact, was removed from the steam reactor for further analysis.

For pretreatment of the milled material, about 4.5 g of material was loaded into a cylindrical catalyst basket whose surface (except the top and bottom faces) was made of metal that was similar in mesh size to that of the basket used for the chip pretreatment. The catalyst basket was suspended and pretreated in the steam chamber as described above.

5.5.3 Material preparation

For the pretreated milled wood, the material was allowed to air dry after pretreatment until the moisture content reached approximately 6%. Immediately following pretreatment, the pretreated chips were fractioned into eight sub-sections across the chips’ 12.7 mm (0.5”) thickness. Thus, a single pretreated chip produced eight sub-sections that were each 50.8 x 38.1 x 1.59 mm (2 x 1.5 x 0.063”) in length, width, and thickness, respectively. 32 samples were produced in total from the four pretreated chips. After fractionation, all chip sub-sections were air-dried for two days until the moisture
content reached approximately 6%. These samples were then milled through a 20-mesh screen (< 0.85 mm) and collected for further analysis.

Additionally, some of the milled material from the 4-min pretreated chip sub-samples was also washed to remove any soluble sugars or other components that may have remained with the wood after pretreatment. As such, 15 mg of dried biomass from all 8 chip sub-samples of the 4-min pretreated chip were weighed in duplicate into 1.5 mL glass HPLC vials by a solid and liquid dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance and 10 25-mL biomass-dispensing hoppers, Symyx Technologies, Sunnyvale, CA). 800 μL of deionized (DI) water was then added to all vials. Next, vials were placed into an ultrasonic cleaner for 90 min sonicating at 50°C. Afterwards the vials were centrifuged (Allegra X-15R, Beckman Coulter, Fullerton, CA), and the liquid washates were removed for further analysis. The solids were washed with DI water two times by centrifugation and re-suspension and then were allowed to dry at room temperature until the moisture content was less than 6%. The dried washed solids were saved for compositional analysis and enzymatic hydrolysis.

5.5.4 Compositional analysis

Glucan, xylan, and acid insoluble residue (AcIR) contents were determined for the pretreated milled wood and each of the chip sub-sections (as well as the washed solids) with a downscaled wet chemistry compositional analysis coupled with high performance liquid chromatography (HPLC) and gravimetric methods to allow analysis of the small
amounts of materials (DeMartini et al., 2011a). The procedure is nearly identical to conventional procedures (Sluiter et al., 2008) but uses 100 times less biomass (3 mg versus 300 mg) and can be automated using a solid and liquid dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance and 10 25-mL biomass-dispensing hoppers, Symyx Technologies, Sunnyvale, CA) due to the use of 1.5 mL glass HPLC vials as reactors.

Sugar concentrations were measured by HPLC (Alliance 2695 equipped with 2414 RI detector, Waters, Milford, MA) on an Aminex HPX-87H column (BioRad, Hercules, CA, USA) heated to 65°C and with 0.005 M sulfuric acid as the eluent (0.6 mL/min flow rate). AcIR contents were determined by gravimetric methods to provide an estimate of Klason lignin content. Unlike the conventional method, this downscaled procedure measures the total acid insoluble residue including the acid insoluble ash. However, due to the low whole ash contents of Populus tremuloides (DeMartini et al., 2011b), the acid insoluble residue should provide a good estimate of the Klason lignin content.

5.5.5 Enzymatic hydrolysis

All chip samples were subjected to enzymatic hydrolysis in a downscaled well plate reactor described in detail elsewhere (Studer et al., 2010, DeMartini et al., 2011c), in which individual wells employed a reaction mass of 450 mg. In this study, 4.4 mg of dry biomass was loaded into individual wells of a custom-built metal well plate using a robotics platform (Core Module, Symyx Technologies, Sunnyvale, CA). After biomass was loaded into the well plate, it was removed from the robot’s deck, and 435.2 µL of
deionized (DI) H₂O was pipetted into all wells (8 channel pipetter, 30-300 µL, Eppendorf, Hamburg, Germany) to achieve a solids loading of approximately 1% w/w. After allowing the biomass to soak overnight, 23.8 µL of a mixture of 1 M citric acid buffer (pH 4.95), sodium azide solution (1 g/L), and enzyme mixture was pipetted into each well (8 channel pipetter, 10-100 µL, Eppendorf, Hamburg, Germany). The mixture contained 5.227 mL of buffer, 1.045 mL of sodium azide solution, and 0.858 mL of a dilute cellulase (Spezyme CP, lot no: 3016295230, 116 mg protein/mL) and xylanase (Multifect, lot no: 301-04021-015, 56.6 mg protein/mL) (Genencor, PaloAlto, CA, USA) solution prepared at a protein mass ratio of 3:1, respectively, to which DI water was added at a volume ratio of 3:1. The resulting enzyme loading corresponded to 30 mg cellulase + 10 mg xylanase per gram glucan in the raw material was applied, respectively, which had a composition of 49.8% glucan, 17.6% xylan, and 19.8% AcIR. After the addition of the enzyme/buffer/biocide solution, the plate assembly was sealed as described previously (Studer et al., 2010), and placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD, USA) at 50°C and 150 rpm.

Replicate plates were prepared to allow sampling at different time points, including 24, 48, and 168 hours after incubation. At each time point, the respective well plate was removed from the shaker, and the slurry from each individual well was transferred to 1.5 mL polypropylene (PP) centrifuge tubes (Safe-Lock 1.5 mL test tubes, Eppendorf, Hamburg, Germany) for centrifugation (5415 D, Eppendorf, Hamburg, Germany) for 5 min at 18,200 g, and transfer of 300 µL of hydrolyzate to HPLC vials for analysis.
5.5.6. MRI

Chips were never frozen, and were stored at 4°C prior to conditioning. Untreated and pretreated chips were conditioned in a sealed desiccator at 25°C at ~ 100% relative humidity over a 0.01 (w/v) NaN₃ solution for 14 days. Magnetic resonance images were taken on a 7 Tesla Bruker Pharmascan (300 MHz \(^1\)H frequency) with a Doty CP (circular polarized) rf-coil with an interior diameter of 60 mm. During image acquisition, the samples were kept in sealed plastic bags. Zero time echo (ZTE) images were collected with a pulse time of 1 ms and a pulse power giving a nominal \(\beta = \pi/72\) (5°) at the resonant frequency, 10 ms repetition delay, collecting 16 averages and recording 128 data points sampling for a 0.64 ms acquisition time. The ZTE method is based on a non-selective and signal acquisition under constant gradient (Hanfer, 1994; Kuethe, 1999). The image voxel size was ~ 0.5 mm and the slice thickness was ~ 1.0 mm. The T₂ images were collected by a multi-slice multi-echo (MSME) acquisition similar in form to that of Carr and Purcell written in the form \(\beta_x - [\tau - 2\beta_y - \tau]N\)-echo, where \([\tau - 2\beta_y - \tau]N=\text{TE}\) and characterized by a pulse time of 1 ms with a pulse power giving a nominal \(\beta = \pi/2\) (90°) at the resonant frequency, giving an effective TE of 7.9 ms for the first echo, 8 echoes were recorded and in each echo signal from the wood sample 64 data points were acquired at a repetition delay of 5000 ms. The image voxel size and slice thickness is ~ 1.0 mm.
5.5.7 Simons’ Stain

DB (Pontamine Fast Sky Blue 6BX) and DO (Pontamine Fast Orange 6RN) dyes were obtained from Pylam Products (Garden City, NY). A modified version of the Simons’ staining (SS) procedure developed previously was used (Chandra et al., 2008). The fractionation of the orange dye was performed by filtering a 1% (w/v) solution of orange dye through a 100 K ultrafiltration membrane using an Amicon ultrafiltration apparatus under 28 psi nitrogen gas pressure (Esteghlalian et al, 2001). The orange dye solution was poured into the apparatus and filtered until 20% of the original solution was left. 1.0 mL of the retained dye on the filter was dried in a 50°C vacuum oven for 5 days, and the weight of the solid residue was measured to determine the concentration of the filtered solution. This result was then used to dilute the filtered orange solution to the concentration required (10 mg/mL) for the Simon staining.

100 mg of biomass samples were weighed into five 15 mL centrifuge tubes. 1.0 mL of phosphate buffered saline solution (pH 6, 0.3M PO₄, 1.4 mM NaCl) was also added to each centrifuge tube. Both the DB solution (10 mg/mL) and DO solution (10 mg/mL) were added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5 mL) to a series of five tubes, each containing the biomass sample and the buffer solution, thus creating a 1:1 mixture of DO and DB dyes at increasing concentrations. DI water was then added to each tube to make the final volume 10.0 mL. All of these tubes were then incubated at 70°C with shaking at 200 rpm for 6 hours. Afterwards, the tubes were centrifuged at 10,000 rpm for 8 min to remove all of the solids. The supernatant was then placed in a cuvette and the absorbance was read on a Lambda 35 UV-vis spectrophotometer at 455
nm and 624 nm. The concentration of the DO and DB dyes (C\textsubscript{O} and C\textsubscript{B}, respectively) in the supernatant was determined using following two equations (Lambert-Beer law for a binary mixture) (Esteghlalian et al, 2001) that were solved simultaneously:

\[ A_{455\text{nm}} = \varepsilon_{O/455} L C_O + \varepsilon_{B/455} L C_B \]  
(1)

\[ A_{624\text{nm}} = \varepsilon_{O/624} L C_O + \varepsilon_{B/624} L C_B \]  
(2)

where A is the absorption of the mixture, \( \varepsilon \) is the extinction coefficient, and L is the path length (in this case 1cm). The extinction coefficients were calculated previously by preparing standard calibration curves of each dye at 455 and 624 nm. The coefficients used in this study were \( \varepsilon_{O/455} = 35.86 \), \( \varepsilon_{B/455} = 2.58 \), \( \varepsilon_{O/624} = 0.22 \), and \( \varepsilon_{B/624} = 15.26 \text{ L g}^{-1} \text{ cm}^{-1} \). The amount of dye adsorbed by the biomass was then determined by the difference in the concentration of the initial added dye and the concentration of the dye in the supernatant. Total adsorption is reported as mg of dye per gram of biomass substrate. Additionally, the accessibility is calculated by dividing the amount of adsorption of the large orange dye, by the amount of adsorption of the small blue dye, all multiplied by 100.

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5.7 References


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Chapter 6. Changes in Composition and Sugar Release Across the Annual Rings of Populus Wood and Implications on Recalcitrance

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6.1 Abstract

Understanding structural characteristics that are responsible for biomass recalcitrance by identifying why it is more difficult for some plants, or portions of plants, to release their sugars would be extremely valuable in overcoming this barrier. With this in mind, this study investigated the recalcitrance of wood by considering the effects of aging in two *Populus tremuloides* cross sections. By applying our novel small scale systems, including a multi-well pretreatment and enzymatic hydrolysis system and a downscaled compositional analysis procedure, we were able to follow ring-by-ring compositions and sugar release patterns. Observed variations were then related to structural changes that occur across the radial direction of trees, providing an important step toward understanding the influence of these changes on recalcitrance.
6.2. Introduction

There is an urgent need for utilization of renewable resources to reduce our dependence on petroleum and associated greenhouse gas emissions. However, options are limited, and currently the only promising resource for large-scale sustainable production of organic chemicals and liquid fuels is lignocellulosic biomass (Lynd et al., 1991; Perlack et al., 2005; Farrell et al., 2006). The cellulose and hemicellulose fractions that comprise about two thirds to three quarters of such materials can be broken down to release sugars that in turn can be converted biologically or chemically into a wide range of fuels, chemicals, and materials (Wyman, 2007). Woody biomass represents one potentially important source of lignocellulosic materials, but lignocellulosic biomass, and particularly woody material, is recalcitrant to sugar release. As a result, severe pretreatments are needed to achieve reasonable sugar yields, which result in some losses through degradation while still requiring high enzyme loadings in the subsequent enzymatic hydrolysis operation. The resulting high energy inputs and high enzyme protein doses translate into high costs for conversion that have stymied commercialization. Unfortunately, the understanding of biomass recalcitrance is limited, making it difficult to optimize harvest age and conversion technologies, and also limiting rational genetic engineering efforts to produce less recalcitrant species.

Although there is little consensus on what specific structural characteristics cause biomass, or wood in particular, to be recalcitrant, some of the most commonly proposed features include the degree of lignification (Mansfield et al., 1999; Chang and Holtzapple, 2000; Himmel, 2007; Chen and Dixon, 2007), the distribution of microfibrils
and matrix polymers (Himmel, 2007), the level of cellulose crystallinity and degree of polymerization (Mansfield et al., 1999; Chang and Holtzapple, 2007), the acetyl content (Chang and Holtzapple, 2007), and the available surface area (Mansfield et al., 1999; Chang and Holtzapple, 2007). To expand on current knowledge, we sought to examine the effect of age on composition and sugar release of *Populus* wood and thereby develop a better understanding of recalcitrance in trees. In particular, we sought to evaluate the influence of structural changes resulting from juvenile-to-mature aging processes on recalcitrance and sugar yields by addressing the following questions:

1) Are significant differences present in the composition of, and sugar release from juvenile versus mature wood?

2) Do these differences translate into digestibility and recalcitrance being defined by the maturity of wood?

3) Can the recalcitrance of wood be correlated to structural changes that are caused by juvenile-to-mature aging? If not, can it be related to other structural, compositional, or environmental factors?

In pursuit of answers, we investigated the radial variation in composition and performance in pretreatment and enzymatic hydrolysis for two different-aged *Populus tremuloides* trees to determine whether significant radial variation existed, and if so, whether it followed any observable trends. Results were then related to structural changes that are known to occur across the radial direction of trees so that the influence of these changes could be evaluated for their influence on sugar yields.
Previous studies have examined the variation in composition across individual annual rings of a tree’s cross section (Shupe et al., 1997; Bertaud and Holmbom, 2004; Sykes et al., 2008), however, the evaluation of ring-by-ring sugar release from pretreatment and enzymatic hydrolysis has been limited by the significant amounts of material that are typically required. As a result, conversion data is usually developed from large amounts of materials that include the entire cross section of the tree. To overcome this limitation, downscaled processes can be employed to study the variation in both composition and sugar release of individual annual rings, of which there is often very small amounts of material (less than 1 g). Such a study is valuable in understanding how wood age affects sugar release because the use of samples from a single tree eliminates the influence of genetic factors associated with comparison of multiple trees and also reduces the impact of environmental factors. The result is that observed differences can be more confidently attributed to wood age and juvenile-to-mature transitions within a tree.

To facilitate the analysis of radial variation within trees, cross sections from 8 and 26 year old *Populus tremuloides* trees were fractionated into their individual annual rings and analyzed for composition using a novel downscaled wet chemistry method (DeMartini et al., 2011). Each ring was then subjected to a hot water pretreatment followed by enzymatic hydrolysis in a similar high throughput, scaled-down system as used for the compositional analysis (Studer et al., 2010). Composition, sugar release, and resulting sugar yields were then determined for each ring and analyzed for variation.
6.3. Materials and methods

6.3.1 Biomass samples

Trembling Aspen (Populus tremuloides) samples were obtained by Benchmark International within a 2 km radius in High Level, Alberta, Canada. Variable aged stands were located, and trees were destructively sampled to collect approximately 20-80 mm thick disks, or cross sections, from individual trees at 0.3 meters from the point of germination. The sections were grouped into age class in 10 year increments, labeled, and sent to the University of California Riverside, USA.

6.3.2 Cross section fractionation

Cross sections classified as 1-10 and 20-30 years in age were chosen for this study. They were sanded with decreasingly course sand paper (grit #50 through #150) to produce clean and smooth surfaces. Cross sections were then cut into different pieces, including two bulk sections that were prepared to serve as control materials representative of the entire cross section. For both cross sections, Bulk 1 included the bark from its section, while for Bulk 2, the bark was removed.

The remaining strip of wood was fractionated into its individual annual rings. Annual rings were usually identified by general observation. However, to aid in distinguishing between rings in the 20-30 year section, in which ring width was generally more compressed, a thin strip of the ring section was cut and examined under a microscope (Nikon Optiphot-66 equipped with Imaging Source IS-2CU USB CCD Camera, Tokyo, Japan). In this way, individual rings were identified and then fractionated using a wood
chisel and hammer. The 20-30 year section was fractionated into 26 rings plus the bark, and the 1-10 year section was fractionated into 8 rings plus the bark. Following fractionation, all samples were milled (Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philidelphia, PA, USA) until they passed through a 20-mesh screen (< 0.85 mm).

6.3.3 Compositional analysis

Glucan, xylan, and lignin contents were determined by performing a novel downscaled compositional analysis that is nearly identical to conventional wet chemistry procedures (Sluiter et al., 2008) but uses 100 times less biomass. The entire process, which is described in detail elsewhere (DeMartini et al., 2011), was performed in 1.5 mL high recovery glass high performance liquid chromatography (HPLC) vials (Agilent, Santa Clara, CA, USA) using only 3 mg dry biomass per test. It should be noted that the lignin contents determined by this method are approximate values because the analysis method measures the total acid insoluble residue, which also includes acid insoluble ash. However, since the whole ash contents of the Bulk 1 and 2 materials are quite small, 2.8 ± 0.5 and 1.2 ± 0.3%, respectively, the acid insoluble residue should provide a good estimate of the Klason lignin content for the ring samples.

6.3.4 Pretreatment and enzymatic hydrolysis

All samples were subjected to combined pretreatment and enzymatic hydrolysis to determine resulting sugar release using a novel high throughput pretreatment and
enzymatic hydrolysis (HTPH) system described elsewhere (Studer et al., 2010). In this process, 2.5 mg dry, milled biomass was loaded into individual wells of a custom-built metal well plate in which both pretreatment and enzymatic hydrolysis were performed. Next, 247.5 µL deionized (DI) H₂O was pipetted into all wells (8 channel pipetter, 30-300 µL, Eppendorf, Hamburg, Germany) to achieve a solids loading of 1% w/w. The well plate was then clamped between two stainless steel plates with a flat silicone gasket (thickness 1.5875 mm, durometer hardness A40) in between. The sealed plate assembly was placed in a custom-built steam chamber for pretreatment with condensing steam (Studer et al., 2010).

After pretreatment, the reaction was quenched with cold water and the plate assembly was removed from the chamber and opened. 20 µL of a mixture of 1 M citric acid buffer (pH 4.95), sodium azide solution (1 g/L), and enzyme mixture was pipetted into each well (8 channel pipetter, 10-100 µL, Eppendorf, Hamburg, Germany). The mixture contained 5 mL of buffer, 1 mL of sodium azide solution, and 2.0 mL of a dilute cellulase and xylanase solution prepared at a protein mass ratio of 3:1, respectively, to which DI water was added at a volume ratio of 3:1. The resulting enzyme loading corresponded to 75 + 25 mg of cellulase and xylanase protein, respectively, per g of glucan + xylan in raw biomass for the 8 year old Bulk 1 material, which had a composition of 40.7% glucan, 17.0% xylan, 27.8% lignin + ash. After enzyme addition, the plate was re-sealed and placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD, USA) at 50°C for 72 hours at 150 rpm.
Following enzymatic hydrolysis, the slurry was transferred to, and centrifuged in 1.5 mL polypropylene (PP) centrifuge tubes (Safe-Lock 2.0 mL test tubes, Eppendorf, Hamburg, Germany). 300 µL of the contents were transferred to a PP 96-well plate (Agilent, Santa Clara, CA, USA) for HPLC analysis.

Prior to testing all individual ring samples in the HTPH system, the bulk materials were used to establish a pretreatment optimization curve. Based on the resulting sugar release data, a slightly sub-optimal pretreatment condition was selected for testing all ring samples to reduce sugar degradation. The selected pretreatment condition was a 70 min reaction at 160°C.

6.3.5 Sugar analysis and results

Sugar concentrations were measured on an Aminex HPX-87H column (BioRad, Hercules, CA, USA) heated to 65°C used in a separation module (Agilent 1200, Agilent, Santa Clara, CA, USA) equipped with a refractive index detector (1200 Agilent) using 0.005 M sulfuric acid as the eluent.

Sugar release results (g/g) were defined as the amount of glucose (or xylose) monomer released into solution per the amount of dry biomass used, while sugar yields (%) were defined as the amount of glucose (or xylose) monomer released into solution divided by the maximum amount of glucose (or xylose) that could be released, based on the given glucan (or xylan) content, times 100.
6.3.6 Statistical analysis

An unpaired two-tailed student’s t test was used to evaluate whether differences observed between juvenile and mature wood were significant. Unless otherwise stated, parameters were considered to be significantly different for p<0.05. Analysis was performed using Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA).

6.4. Results and discussion

6.4.1 Composition results

Figure 6.1 demonstrates that the composition of individual annual rings from the 8 and 26 year old cross sections were highly variable within the sections. In particular, rings from the young tree (excluding the bark) ranged from 27.5 to 42.1% in glucan content, from 16.2 to 21.2% in xylan content, and 26.9 to 38.2% in lignin content. For rings from the 26 year old section (again excluding bark), the glucan content ranged from 29.0 to 48.9%, while the xylan and lignin contents ranged from 13.1 to 18.8% and 21.5 to 33.5%, respectively. Compositional variation across both sections were also found to follow the same trend, namely that glucan content increased in the direction of pith to bark, lignin content decreased in the same direction, while the variation in xylan content across the sections showed no clear trend.
6.4.2 Sugar release results

Significant variation was also discovered in sugar release from individual annual rings resulting from pretreatment and enzymatic hydrolysis, as displayed in Figure 6.1. For the younger tree, the xylose release varied slightly from 0.15 to 0.18 g/g, while the glucose and glucose + xylose releases varied from 0.26 to 0.41 g/g and 0.41 to 0.57 g/g, respectively. Similar results were found for rings from the 26 year section, in which the xylose release ranged from 0.15 to 0.20 g/g, and the glucose and glucose + xylose releases varied from 0.27 to 0.47 g/g and 0.42 to 0.66 g/g, respectively.

Figure 6.1. Ring-by-ring composition including glucan, xylan, and lignin contents (in mass percent), as well as ring-by-ring sugar release (in g sugar released / g biomass) for glucose, xylose, and glucose + xylose. Results are shown for the 8 year old (a) and 26 year old (b) section, with the horizontal axis plotting the age of each ring from pith (left) to bark (right), where the final data point represents the bark material. Error bars represent the standard deviation of triplicate experiments.
6.4.3 Sugar yield results

Figure 6.2 shows that sugar yields calculated from the composition and sugar release data varied much less than the composition and sugar release (g/g). The xylose and glucose yields of the 8 year’s individual rings ranged between 72.7 to 88.6%, and 85.4 to 91.8%, respectively, corresponding to a range of glucose + xylose yields of 81.5 to 88.7%. For the 26 year section, both the yields themselves and the extent of variation were somewhat higher than that of the 8 year old tree. In particular, the xylose and glucose yields varied between 90.9 to 104.7% and 70.2 to 96.0%, respectively, while the glucose + xylose yields ranged between 76.3 to 98.4%. However, closer examination shows that although the overall variation was larger for the older tree, this was due primarily to rings aged 7 and 8, which performed significantly worse than the remaining rings within that same cross section. If these two rings were not included in the analysis, the glucose and glucose + xylose yields only varied by between 82.3 to 96.0% and 87.8 and 98.4%, respectively, which is similar to the variation observed for the younger tree. The performance of rings 7 and 8 will be discussed in more detail in section 3.5.
Figure 6.2. Xylose, glucose, and glucose + xylose yields (%) for the 8 year old (a) and 26 year old (b) cross section. The horizontal axis plots the age of each ring from pith (left) to bark (right), where the final data point represents the bark material. Error bars represent the standard deviation of triplicates.

6.4.4 Evaluating variability

Analysis of the composition and sugar release patterns of individual annual rings from two *Populus tremuloides* cross sections revealed substantial within tree radial variation. Figure 1 demonstrates that compositional variation was more pronounced for the older tree, in which the glucan, xylan, and lignin contents varied among the 26 annual rings by up to 41, 30, and 36% of their respective values. However, the 8 year old tree also covered a large range, with glucan, xylan, and lignin contents varying by up to 35, 24, and 36% of their respective values. While the variability and observed trends of increasing glucan and decreasing lignin contents from pith to bark agreed with results
from past work (Shupe et al., 1997; Bertaud and Holmbom, 2004; Sykes et al., 2008; Fengel and Wegener, 1984), the extent of variation was unexpected.

Figure 6.1 further demonstrates that these trends were not gradual changes within the 26 year tree, but instead two major regions were discernible in the cross section. The first region encompassed mostly, if not all, of the juvenile wood (rings approximately aged 1 - 6 years) and was characterized by low glucan and slightly higher lignin contents. Rings aged 7 and 8 years appeared to transition to the second region that included more mature wood (approximately rings aged 9-26 years) and was characterized by higher glucan and somewhat lower lignin contents. The tested parameters of glucan and lignin content were found to be significantly different between the two groups. This trend was not observed in the 8 year cross section, likely due to the younger age and the resulting reduced, or non-existent presence of mature wood.

By subjecting rings to a high throughput and downscaled pretreatment and enzymatic hydrolysis screen, the sugar release across each cross section was found to vary significantly, and in a remarkably similar manner to the observed composition trends. Overall, glucose release (g sugar/g biomass) increased from pith to bark, and xylose release fluctuated slightly. Glucose release was also found to be significantly different between the juvenile and mature regions described above for compositional characteristics. Comparison of these two distinct regions suggested that they could be distinguished by darker colored wood, which may reflect the presence of heartwood caused by the deposition of extractives into the dead xylem long after cells have become metabolically inactive (Sjostrom, 1993; Taylor, 2002). Once the cell is dead, wood
chemistry and structure do not change, suggesting that the observed sugar release patterns were not a direct reflection of the presence of heartwood or sapwood but instead may be due to juvenile-to-mature transitions over the life of a tree. Although these transitions vary from genotype to genotype and with environment (Bendtsen and Senft, 1986; Peszlen, 1994), heartwood formation is related to age, which may explain the correlation seen between sugar release and darker wood color.

6.4.5 Investigating glucan digestibility

After observing significant variations in sugar release, it was surprising to discover that there was very little variation in glucose yield within each cross section, and that the ring-by-ring composition explained almost all of the observed trends and differences in sugar release performance. In fact, with the exception of bark, which has been previously shown to be inhibitory to enzymatic and microbial actions (Walch et al., 1992; Robinson et al., 2002), only rings 7 and 8 from the 26 year section deviated significantly from the other rings in terms of glucose yield. The glucose yields of rings 7 and 8 were 70.2 and 73.2%, respectively, while the average glucose yield of all other rings in that set was 90.9%. Because Figure 6.3 shows a general trend of decreasing glucose yield with increasing lignin content, we first tried to explain the performance of these two rings by checking whether they exhibited higher lignin contents. Rings 7 and 8 had lignin contents of 29.3 and 30.3%, which were slightly higher than the average of all rings (26.8%). However, this alone could not explain the low yields because other rings with virtually the same lignin content performed substantially better and exhibited glucose
yields of between 89.4 and 92.3%, a 20% improvement. Thus, factors other than lignin and carbohydrate contents apparently affected sugar yields, and as a result, we sought to investigate other possible causes for the low yields observed in these two rings.

Figure 6.3. Glucose and xylose yields (%) plotted versus lignin content for the 26 year old section. Dotted lines represent the linear fit of yield data in each subplot to emphasize the observed trends in sugar yields.

Monthly and annual weather histories were examined for High Level, Alberta, with particular attention paid to the growing season, but no correlation could be found between the low glucose yields of rings 7 and 8 and average or extreme temperatures or precipitation (http://www.climate.weatheroffice.gc.ca/climateData/canada_e.html). Forest fire history was also investigated, but no correlation was found between sugar yields and fires in the growth area and the time of interest (http://fire.cfs.nrcan.gc.ca/home-accueil-eng.php). It is possible that the low yields observed for rings 7 and 8 may correspond to the transition between heartwood and sapwood, which is characterized by a high accumulation of extractives (Bertaud and Holmbom, 2004; Sykes et al., 2006). However, this zone is also reported to have high
cellulose and low lignin contents, in contrast to what we found for these two rings. Thus, other unmeasured biochemical or anatomical changes that occurred initially during transition from juvenile to mature wood and that stabilized after the transition was complete appeared to be responsible for the low glucose yields observed in these rings.

6.4.6 Mechanistic implications for recalcitrance

With the exception of rings 7 and 8, results from this study demonstrated that the digestibility of the *Populus* trees examined did not change with age, and in particular, that the recalcitrance of wood was not defined by juvenility, maturity, or changes caused by this transition. Although the exact age of demarcation between juvenile and mature wood varies, it is almost certain that the transition will occur within the 26 year life span of the older tree, making results from this cross section particularly applicable in the context of studying wood maturity and recalcitrance (Bendsten and Senft, 1986). As did Bendtsen and Senft (1986), we assumed that rings aged 6 years or less were representative of juvenile wood and that rings aged 20 years and older were representative of mature wood. In this way, we could be confident that our defined juvenile and mature woods were safely distant from the age of demarcation. Analysis of these two regions demonstrated that there was no statistically significant variation between them in terms of glucose yields, confirming that wood maturity did not define conversion potential.

Evaluating structural characteristics of juvenile and mature wood could provide clues for understanding factors that control the recalcitrance of *Populus tremuloides* wood and help explain these findings. A review of the literature on wood quality as related to
mechanical and anatomical properties demonstrated that the radius, length, and cell wall thickness of fiber increases from juvenile to mature wood, resulting in enhanced strength and stiffness of mature wood (Yanchuk et al., 1984; Bendsten and Senft, 1986; Peszlen, 1994; Lei et al., 1996; Bao et al., 2001), while microfibril angle (MFA) has consistently been demonstrated to be higher in juvenile wood than in mature wood (Bendsten and Senft, 1986; Lichtenegger et al., 1999; Evans et al., 2000; Barnett and Bonham, 2004). While little work has been reported that systematically studied the effect of these or other structural features in wood on recalcitrance, a multitude of studies on the digestibility of grasses as ruminant feedstocks have demonstrated similar structural characteristics to be important. For example, the distribution and relative amounts of various tissue types have been observed to affect digestibility in grasses because they define cell shape, size, wall thickness, and corresponding surface area to cell wall volume, all of which have been suggested to impact digestibility (Grabber et al., 1992; Grabber and Allinson, 1992; Wilson and Mertens, 1995; Buxton and Redfearn, 1997). It has also been proposed that the structural heterogeneity and complexity of cell wall components such as microfibrils may play a role in biomass recalcitrance (Himmel et al., 2007). Investigating whether these structural characteristics affect conversion of \textit{Populus tremuloides} to sugars may help advance our understanding of recalcitrance in wood. However, the lack of significant variation in glucose yields between the defined juvenile and mature wood suggests that structural differences in cell radius, length, and wall thickness, as well as MFA, could be ruled out of impacting the digestibility of \textit{Populus tremuloides} wood.
6.4.7 Influence of lignin content

The plot in Figure 6.3 of xylose and glucose yields versus lignin content was prepared to determine the influence of lignin content on sugar release for the individual rings of the 26 year old cross section. A dotted line showing the linear fit of each subplot is included to represent the observed trends in sugar yields. While the glucose yield decreased somewhat with increasing lignin content, the xylose yield appears to have increased slightly for rings with higher lignin content.

In addition to evaluating the impact of structural features on recalcitrance, it has been reported that digestibility can be affected by cell wall composition, particularly lignin composition and content. Increased lignin content has been shown to adversely effect glucan digestibility, possibly due to restriction of enzyme access to cellulose and increased non-productive binding of enzyme (Chang and Holtzapple, 2000; Berlin et al., 2005; Chen and Dixon, 2007). The negative influence of lignin was observed in the older cross section of this study, in which the glucose yield dropped with increasing lignin content. However, much less clear is the effect of lignin on hemicellulose convertibility, which was shown to generally increase with higher lignin contents. It is also important to note that the lignin and xylan contents were inversely related, and that samples with higher lignin contents exhibited lower xylan contents. These observations were surprising because lignin is known to impart a high level of mechanical strength to cell walls by forming numerous crosslinks with polysaccharides (Carpita and Gibeaut, 1993; Iiyama et al., 1994). As a result, increased lignin content should produce hemicellulose that is more tightly bound within the cell wall matrix which in turn would be expected to
have negative consequences on xylose release and conversion. Further investigation will be required to determine the cause of this trend.

6.4.8 Implications for harvest age

Results from this study have implications on the optimal harvest age of trees to be converted to sugars for fermentation to ethanol and other products. Because it was observed that sugar yields were fairly consistent across the rings and that juvenile to mature changes occurring in the wood did not affect digestibility, sugar yields should be similar for trees harvested at a later age as those harvested earlier in their life. In fact, comparison of sugar yields of the 8 and 26 year sections showed that on average, the glucose and xylose yields were higher for the older tree. However, despite the proximity of the two trees used in this study, it is unknown if they were suckers off of the same parent and thus directly related to one another. As a result, the difference in yields cannot be attributed exclusively to age, but may in fact be an effect of natural variation.

This study confirmed that the older tree had a larger proportion of mature wood which contained high levels of glucan, which was further demonstrated to be converted to glucose with the same efficiency as the glucan in juvenile wood. Thus, there will be an optimum harvest age when glucan content and tree productivity maximize the mass of sugar produced per mass of feedstock per unit time. Although the growth of almost all trees follows an S-shaped sigmoidal curve, the rates of growth and final tree height will vary by genotype and by site location (Chen et al., 2002), and thus broad generalizations about optimum harvest times are difficult to make. However, the results of this study
strongly suggest that this optimum is not likely to occur during the juvenile growth phase, which could have implications on the 6-10 year rotations that are typically proposed for short rotation woody crops. Additional studies are needed to examine cross sections of varying ages for trees of interest to determine whether this observation can be generalized to all *Populus tremuloides* trees as well as to other hardwoods.

### 6.5. Conclusions

Our novel small-scale high throughput multi-well pretreatment and hydrolysis system and downscaled compositional analysis procedure were able to determine ring-by-ring compositions and sugar release patterns of two *Populus tremuloides* trees of different ages. Although significant within tree radial variation in composition and sugar release was found, digestibility remained almost constant. These results suggest that wood maturity does not influence the recalcitrance of *Populus tremuloides* wood and further allowed us to speculate that a number of structural features that have previously been proposed to impact digestibility, did not effect the recalcitrance for the wood used in this study.

### 6.6 Acknowledgments

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6.7 References


**Web References**


Chapter 7. Lignin content in natural Populus variants affects sugar release*

* This whole chapter has been published under the following citation:
7.1 Abstract

The primary obstacle to producing renewable fuels from lignocellulosic biomass is a plant’s recalcitrance to releasing sugars bound in the cell wall. From a sample set of wood cores representing 1100 individual undomesticated *Populus trichocarpa* trees, 47 extreme phenotypes were selected across measured lignin content and ratio of syringyl and guaiacyl units (S/G ratio). This subset was tested for total sugar release through enzymatic hydrolysis alone, as well as combined hot water pretreatment and enzymatic hydrolysis, using a high-throughput screening method. The total amount of glucan and xylan released varied widely among samples, with total sugar yields up to 92% of the theoretical maximum. A strong negative correlation between sugar release and lignin content was only found for pretreated samples with an S/G ratio <2.0. For higher S/G ratios, sugar release was generally higher and the negative influence of lignin was less pronounced. When examined separately, only glucose release was correlated with lignin content and S/G ratio in this manner, while xylose release depended on the S/G ratio alone. For enzymatic hydrolysis without pretreatment, sugar release increased significantly with decreasing lignin content below 20%, irrespective of the S/G ratio. Furthermore, certain samples featuring average lignin content and S/G ratios exhibited exceptional sugar release. These facts suggest that factors beyond lignin and S/G ratio influence recalcitrance to sugar release and point to a critical need for deeper understanding of cell wall structure before plants can be rationally engineered for reduced recalcitrance and efficient biofuels production.
7.2 Introduction

Lignocellulosic biomass is the only sustainable resource in terms of cost, availability and scale, that can be converted into liquid fuels to reduce the prevailing role of petroleum in providing energy for the world’s transportation needs (Dinus et al., 2001; Ragauskas et al., 2006) and to decrease the emissions of fossil CO$_2$ damaging the world’s climate (Tuskan and Walsh, 2001). The primary obstacle to producing liquid transportation fuels by bioconversion methods is the release of sugars in high quantities at low costs from recalcitrant lignocellulosic biomass feedstocks (Lynd et al., 1991; 1999). Genetic modifications of plants to make them less recalcitrant is a promising path to address this challenge on the feedstock side, but the effort would be greatly aided by improving understanding of the fundamental relationship between cell wall composition and sugar release via pretreatment and enzymatic hydrolysis.

In this paper we focus on the influence of lignin content and ratio of its syringyl and guaiacyl units (S/G ratio) on recalcitrance to sugar release because these two traits were previously identified as dominant factors (Davison et al., 2006). Although it is generally perceived that low lignin contents increase the ability of cellulosolytic enzymes to hydrolyze plant fibers (Chang and Holtzapple, 2000; Chen and Dixon, 2007; Dien et al., 2009; Vermerris et al., 2007; Wyman et al., 2009), only a limited number of studies investigated the effect of lignin S/G ratio on sugar release through combined pretreatment and enzymatic hydrolysis. While some found no clear trend (Chen and Dixon, 2007; Jackson et al., 2008), Li et al. (2010) demonstrated that an Arabidopsis mutant containing mainly S-lignin showed a much higher sugar yield after hot water pretreatment and
enzymatic hydrolysis compared to the wild type and the S-deficient plant. Furthermore, a high S/G ratio is known to enhance the efficiency of kraft pulping (Huntley et al., 2003; Lapierre et al., 2009; Li et al., 2008; Samuel et al., 2010) but adversely affects xylose release through dilute acid hydrolysis (Davison et al., 2006). However, the mentioned studies are characterized by small population sizes or by coverage of narrow ranges in lignin content and S/G ratio. Thus we initiated an unrivaled large scale screening program by collecting 1100 samples of a natural population of undomesticated *Populus trichocarpa* trees, quantifying the lignin content and S/G ratio, and selecting 47 extreme phenotypes across the entire range of measured traits. This subset was analyzed for sugar release by using our high-throughput pretreatment and enzymatic hydrolysis pipeline (Studer et al., 2010) to address the following questions:

1. How does lignin content and lignin S/G ratio correlate with recalcitrance to monosaccharide sugar release?

2. Do changes in pretreatment process parameters influence the sugar release from each individual in the investigated sample set in the same manner, or do subsets within the population exist that are particularly susceptible to specific processing conditions? Furthermore, are there certain samples that achieve high yields if pretreatment is eliminated entirely?
3. And finally, can biomass materials with exceptionally high or low sugar release be identified for further investigations that permit drawing conclusions on factors impacting recalcitrance?

7.3 Results

We established a large collection of biological samples from 1100 geographically distributed, undomesticated *Populus trichocarpa* genotypes and analyzed them for lignin content and the S/G ratio. The sampled trees covered a wide span in lignin content (15.7 to 27.9%) and S/G ratio (1.0 to 3.0) (Fig. 7.1). A total of 47 samples were selected for in-depth analysis of recalcitrance to sugar release: 30 were selected based on their extreme values in lignin content and composition, while the additional 17 were selected in an orthogonal manner along average S/G (2.0) and lignin (~22.5%) values (Fig. 7.1). To measure sugar release, these samples were subjected to coupled pretreatment and enzymatic hydrolysis by a mixture of cellulase and xylanase using our high-throughput pretreatment and hydrolysis technique (HTPH) (Studer et al., 2010). In addition, samples were enzymatically hydrolyzed without pretreatment.
Figure 7.1. Characterization of the complete Populus association sample set, including the selected and analyzed 47 Populus samples. Relationships are shown between S/G ratios and lignin contents. 30 samples selected based on their extreme values in lignin content and composition, as well as 17 additional ones selected in an orthogonal manner along an average S/G ratio (2.0) and lignin content (~22.5%) were tested for their recalcitrance to sugar release. The dots (·) mark the complete 1100 sample set, while the larger symbols mark the 47 analyzed samples. Samples exhibiting the highest (black markers) or lowest (grey markers) total sugar release from pretreatment and enzymatic hydrolysis (solid circles ○, ●) or just enzymatic hydrolysis (solid squares ■, □) are highlighted. The labels appoint interesting samples discussed in more detail in the text.

The total amount of glucan and xylan released was widely variable among the 47 tested genotypes. Sugar release ranged from 0.25 to 0.67 g of glucose and xylose per g of dry raw biomass (35 to 91% of the theoretical sugar yield) for pretreatment at 180°C, from 0.20 to 0.68 (28 to 92%) for pretreatment at 160°C, and from 0.17 to 0.58 (23 to 83%) at 140°C, demonstrating that total sugar release generally dropped at lower pretreatment temperatures (Fig. 7.2, Fig S1\textsuperscript{88}). Without pretreatment, sugar yields also varied widely but were considerably lower, ranging from 0.05 to 0.40 g/g dry biomass (4 to 56%).

\textsuperscript{88} All supporting figures and tables can be accessed online at the following web address: http://www.pnas.org/content/suppl/2011/03/24/1009252108.DCSupplemental/pnas.201009252SI.pdf
Figure 7.2: Total glucose plus xylose release for pretreatment of poplar at different temperatures followed by enzymatic hydrolysis using cellulase and xylanase and their relationship to lignin content. Samples were pretreated in just water at 180°C for 18 min (a, e, i), 160°C for 28 min (b, f, j), and 140°C for 464 min (c, g, k) or directly subjected to enzymatic hydrolysis without pretreatment (d, h, l). Each marker represents the mean value of three replicates for all pretreatment conditions and two replicates for just enzymatic hydrolysis. The sugar releases are displayed in g of sugars per g of raw biomass. The maximum theoretical sugar release based on the composition of the *Populus* standard is represented by the declining dotted line (a-d). The markers distinguish samples featuring S/G ratios <2.0 (□) and ≥2.0 (○). The numbers denote the slope of the trend lines of the two sub-groups for low and high lignin contents with the respective standard deviations. The black arrow points to sample no.014, the red arrow to no. 152.

Total sugar release from pretreatment and enzymatic hydrolysis was negatively correlated with lignin content for all pretreatment temperatures. Furthermore, if the S/G
ratio of the samples was also taken into account, the negative correlation became much stronger for samples with low S/G ratios (<2.0). This observation held for all pretreatment temperatures (Fig. 7.2a-c), as the corresponding trend lines showed statistically identical slopes of -0.03 (Table S1). In contrast, samples with larger S/G ratios (≥2.0) generally showed higher sugar release and the negative influence of lignin was less pronounced, with the slopes of regression lines approximating the slope of the theoretical maximum yield curve (-0.01), reflecting the expected tradeoff between carbohydrate and lignin contents (Fig. 7.2a-c). Separate analysis of glucose and xylose release revealed that glucose followed the trends with lignin content and composition (Fig. 7.2e-g), while xylose release was independent of lignin content but generally higher for the high S/G subset (Fig. 7.2i-k, Table S1). Furthermore, we identified unusual outliers that clearly did not follow the described dependency on lignin content for the respective S/G group. For example, biomass no. 014 showed a low sugar yield relative to its peers after pretreatment and hydrolysis at all temperatures and biomass no. 152 showed a comparably high sugar release for the low S/G group.

As indicated above, higher S/G ratios were beneficial to high monosaccharide release. Total sugar release tended to increase with increasing S/G ratios (Fig. 7.3a-c), which was also the case for glucose and xylose release if analyzed separately (Fig. S2a-c, e-g and Table S1). For samples enzymatically hydrolyzed without pretreatment, sugar yields were generally low except for individuals with lignin contents below 20%. In this subgroup, digestibility increased considerably with decreasing lignin content, yielding up to 56% of
the theoretical sugar yield, higher than sugar release from most of the pretreated samples featuring an S/G value <2.0 (Fig. 7.2d, 7.3d). Interestingly, in these samples, mainly glucose was released while xylose remained virtually untouched (Fig. 2h, l and S2d, h).

**Figure 7.3.** Total sugar release from pretreatment and enzymatic hydrolysis of poplar correlated to lignin S/G ratio and lignin content. The latter is indicated over the marker size. Samples were pretreated at 180°C (a), 160°C (b), and 140°C (c) or directly subjected to enzymatic hydrolysis without pretreatment (d). Each marker represents the mean value of three replicates for all pretreatment conditions and two replicates for just enzymatic hydrolysis. The sugar releases are displayed in g of sugars per g of raw biomass.

From the 47 samples, we identified individual samples that exhibited unusually high total sugar release (i.e., the three samples with the highest sugar release for each pretreatment condition (Table 7.1, Fig. 1)). Depending on pretreatment temperature, different individual biomasses ranked in the top three, with the exception of one biomass (no. 909), which gave the second highest mass yields for all three pretreatment temperatures (Table 7.1). However, when considering a larger subset consisting of the top 20% of all tested variants, the same seven individuals ranked in the top nine for all hot water pretreatments, and interestingly, all samples except two individuals (no 819 and 462) were members of the orthogonally selected control group featuring average lignin and S/G values (Fig. 7.1). Only two of these seven well-performing pretreated samples
(no. 819 and 909) were also represented in the top nine for the no pretreatment case (Table S2). The worst nine performing biomasses were the same for all pretreatments and featured either very low S/G ratios (S/G ≤ 1.2) or very high lignin contents (≥ 27.8%) (Table S2, S3). In contrast, the S/G ratios of samples representing the lowest tier for direct enzymatic hydrolysis of unpretreated biomass covered the entire spectrum analyzed, and also featured average lignin contents. Only three of these nine samples (no. 163, 290 and 829) were present on both negative lists (Table S2, S3).

<table>
<thead>
<tr>
<th>Rank</th>
<th>Sample ID</th>
<th>Sugar release (g/g) and rank (number) for the other pretreatments</th>
<th>Lignin content (%)</th>
<th>S/G ratio (-)</th>
<th>dbh (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>273</td>
<td>0.672 ± 0.014*</td>
<td>22.42</td>
<td>2.0</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>909</td>
<td>0.667 ± 0.030*</td>
<td>22.57</td>
<td>2.0</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>349</td>
<td>0.654 ± 0.015*</td>
<td>22.48</td>
<td>2.0</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>383</td>
<td>0.600 ± 0.015*</td>
<td>21.81</td>
<td>2.0</td>
<td>26</td>
</tr>
<tr>
<td>5</td>
<td>909</td>
<td>0.667 ± 0.030*</td>
<td>22.57</td>
<td>2.0</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>349</td>
<td>0.654 ± 0.015*</td>
<td>22.48</td>
<td>2.0</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>383</td>
<td>0.600 ± 0.015*</td>
<td>21.81</td>
<td>2.0</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>909</td>
<td>0.667 ± 0.030*</td>
<td>22.57</td>
<td>2.0</td>
<td>24</td>
</tr>
<tr>
<td>9</td>
<td>349</td>
<td>0.654 ± 0.015*</td>
<td>22.48</td>
<td>2.0</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 7.1. Total sugar release for the top three performing *Populus* samples at each pretreatment condition tested with their performance and relative rankings listed for the other conditions applied. Shown are the mean values from three replicates for all pretreatment conditions and two replicates for just enzymatic hydrolysis together with standard errors of the mean. The sugar releases are reported in grams sugars per grams raw biomass. dbh, diameter on breast height (i.e., the diameter of the sampled tree).

7.4 Discussion

We studied a selection of wood increment core samples from a large natural population of *Populus trichocarpa* trees with considerable natural variation in cell wall...
composition to determine fermentable sugar release from pretreatment in hot water followed by enzymatic hydrolysis. We selected pretreatment with just water at moderate temperatures to mimic pragmatic, environmental friendly, future large-scale conditions. In order to enhance differences in sugar release between individual samples, pretreatment times were adjusted to correspond to an overall severity of 3.6 (see equation 7.1), which is below the optimum severity of 4.1 established for our internal Populus biomass standard (Fig. S3). A relatively high enzyme loading was used to overcome potential inhibition by substances released or formed during pretreatment (Studer et al., 2010), which would be washed away in the base case cellulosic ethanol process (Wyman et al., 1996), and also to be sure that changes in recalcitrance could be distinguished from limitations in enzyme performance. Despite the high enzyme loading, profound differences in sugar yield were found for different pretreatment severities (Fig. S3), allowing us to conclude that the chosen conditions were suitable to investigate the influence of S/G ratio and lignin content on recalcitrance for the selected biomasses. This judgment was indeed confirmed by the broad range of sugar release measured (Fig. 7.2 and 7.3).

We found that sugar release depended on both lignin content and lignin composition, i.e., yields tended to increase with increasing S/G ratios and to decrease with lignin content. At the current state of investigations, we can only speculate on reasons for the higher reactivity of S-rich lignin. Generally, S-rich lignin features predominantly linear chains with less crosslinking than G-rich lignin due to the methoxylated, and thereby blocked, C-5 position in the syringyl unit (Stewart et al., 2009), resulting in fewer highly
stable 5-5 and β-5 linkages (Stewart et al., 2009; Kishimoto et al., 2010; Sannigrahi et al., 2010). The higher occurrence of β-β-units (resinols) in S-rich lignin leads to shorter chain lengths and thus lower molecular weights (Kishimoto et al., 2010), which potentially alter thermoplastic properties including lowered melting points (Li et al., 2010). The relative amount of chemically labile β-O-4 ether linkages has been shown to remain constant independent of the S/G ratio if at least some syringyl units are present (Stewart et al., 2009; Kishimoto et al., 2010). Mainly these linkages are cleaved not only during Kraft pulping (Lapierre et al., 1999), but more importantly also during hydrothermal (Li et al., 2007) and dilute acid pretreatment (Samuel et al., 2010). Steam explosion of *Populus tremuloides* reduced the relative amount of the remaining β-O-4 linkages from 78% to 19% when the severity (log\(R_0\)) was increased from 3.2 to 4.5 (Li et al., 2007).

Furthermore, the S/G ratio has been shown to drop during dilute acid pretreatment (Samuel et al., 2010), pointing to a higher reactivity of S-lignin, a trend that has also been confirmed by the faster cleavage of β-O-4 linkages in S-lignin under alkaline conditions (Tsutsumi et al., 1995). Higher pretreatment severities result in a presumably higher degree of β-O-4 cleavage that leads to higher sugar yields after enzymatic hydrolysis (Fig. S3). Thus we deduce that the increase in digestibility from combined pretreatment and enzymatic hydrolysis with increased S/G ratios is mainly related to the more labile β-O-4 bonds in S-lignin during pretreatment.

The S/G ratio also determines the dependency of sugar release on lignin content. Although the common assumption is, that high lignin content adversely affects enzymatic hydrolysis (Chang and Holtzapple, 2000; Dien et al., 2006; Vinzant et al., 1997), we
observed no influence of lignin content on sugar release for high S/G ratios (≥2.0) (Fig. 7.2a-c). The chosen S/G ratio of 2.0 should not be considered a sharp threshold, as further analysis suggested a gradually increasing dependency of sugar release on lignin content with decreasing S/G ratio (Fig. S4) but the sample size must be increased to statistically support this conclusion.

These results can only partly be validated by literature as lignin composition is often not recorded (Dien et al., 2009; Vermerris et al., 2007). However, Chen and Dixon (2007), who investigated samples featuring very low S/G ratios (0.3–1.0), found a similar strong dependency of release on lignin content. Overall, the finding that the sugar yield does not depend on lignin content for high S/G ratios was unexpected and points to the possibility that pretreatment modifies lignin to such an extent that it does not impact enzymatic hydrolysis. Nevertheless, high lignin contents are not desired due to the displacement of fermentable carbohydrates in the biomass and its nonproductive binding to enzyme (Palonen et al., 2004).

For enzymatic hydrolysis of Populus samples without prior pretreatment, the highest sugar release was found for lignin contents less than 20% (Fig. 7.2d). Furthermore, because hydrolysis was independent of the S/G ratio (Fig. 7.3d), as also confirmed by Li et al. (2010), enzyme penetration appears to be unaltered by the above described higher amount of crosslinks in G-rich lignins. Interestingly, virtually only glucose was released while xylan was not (Fig. 7.2h, l), a remarkable result in light of the conventional perception that either hemicellulose and/or lignin needs to be removed or relocated to enable high glucose yields by enzymatic hydrolysis (Chang and Holtzapple, 2000;
Chapple et al., 2007; Converse, 1993; Donohoe et al., 2008; Sanchez and Cardona, 2008). This raises the question as to whether these samples contained unusually high amounts of endogenous sugars, starch or tension wood. Although samples were not routinely tested for free sugars and starch, analysis of nine random samples yielded 0.2 to 0.6% (w/w) endogenous glucose or starch based on the raw biomass (Table S4), which is in good agreement with expected values (Essiamah and Eschrich, 1985; Sauter and Wellenkamp, 1998), while young trees may contain higher amounts of sugars of up to 10% (Novaes et al., 2010). Furthermore, the amylase activity expressed by the employed enzyme mixture is low, with expected sugar yields from starch below 10% (Table S5). Taken together, it is unlikely that starch or endogenous sugar content can account for the high sugar release of up to 0.4 g/g for low lignin samples. Tension wood in Populus formed by external stimuli, such as wind or gravitropic sway (Dinus et al., 2001) contain an additional inner gelatinous layer of almost pure, highly oriented and crystalline cellulose microfibrils (Boyd, 1997; Timell, 1969) that could account for the relatively large amounts of glucose released without pretreatment. However, tension wood is also known to have an increased S/G ratio (Pilate et al., 2002; Koehler and Telewski, 2006) compared to normal wood, while the select samples were all below 2.0. Therefore it is not likely that tension wood accounted for the observed high sugar yields in these samples. Attributing the high sugar release to tree age can also be ruled out, as the best performing trees covered a wide range in diameters (i.e., age) between 8 and 92 cm.

In spite of the correlations between sugar release and lignin content and/or S/G ratio described above, several samples performed considerably better even though they
showed average values in the analyzed cell wall traits (Table 7.1). As a result, other strictures or factors must be influencing recalcitrance to sugar release, possibly including 1) the presence of incorporated \( p \)-hydroxybenzoates acylated (Lapierre et al., 1999; Boerjan et al., 2003) or acetylated (Weng et al., 2008) monolignols, 2) the amount of free phenolic groups in lignin (Lapierre et al., 1999), 3) the amount and structural features of xylan (e.g., chain length, side-chain substitution pattern) (York and O’Neill, 2008), 4) differences in cell and tissue anatomy (Dinus et al., 2001), 5) insect herbivory or pathogen attack (Pilate et al., 2002), and/or 6) the proximity of the sampling point to abscised lateral branches (Sykes et al., 2008). None of the above co-variants were quantified in the current study, though all areas containing visual defects were avoided during sampling, but rather extreme cell wall phenotypes were selected and analyzed for sugar release independent of their genetic or environmentally induced origins. Thus, a deeper understanding of cell wall structure, anatomy and biochemistry is critically needed as plants are being systematically engineered for reduced recalcitrance and efficient biofuels production.

7.5 Materials and Methods

7.5.1 Populus association samples

One-thousand-one-hundred Populus trichocarpa (Torr. & Gray) trees were systematically sampled from within a 1.7° latitudinal gradient from northwest Washington to central Oregon. The sampling took place in December 2008. Each sampled tree was selected based on a range of diameters (Table 1, S3), upright form, and
lack of obvious physical or biological damage. We were not able to account for historical differences in microclimate or influence by external stimuli, such as gravitropic or biologic factors. The geographical location of each tree was recorded and wood cores were extracted using a 3-thread 0.17” (4.3 mm) core and 12” (304.8 mm) Haglöf increment borer to depth or to the center of the tree. The core samples were air-dried and knife milled along their entire length (Thomas-Wiley Mini-Mill, Thomas Scientific, Swedesboro, NJ) to a particle size <20 mesh (0.85 mm) by Oak Ridge National Laboratory (ORNL). A homogenized subsample was obtained and then sent to the National Renewable Energy Lab (NREL) for analytical pyrolysis.

7.5.2 Biomass analysis

All biomass samples were analyzed for lignin content and S/G ratio by analytical pyrolysis, as described elsewhere (Sykes et al., 2008; Evans and Milne, 1987; Sykes et al., 2010; Tuskan et al., 1999). Briefly, approximately 4 mg of ground *Populus* material was pyrolyzed for 2 min at 500°C, and the pyrolysis vapors were entrained in helium flowing at 2 L/min to a mass spectrometer. Spectra were collect over a m/z range from 30 to 450 using 22.5 eV electron impact ionization. Lignin content was determined by summing the relative intensity of the major lignin peaks (m/z ratios of 120, 124, 137, 138, 150, 152, 154, 164, 167, 178, 180, 181, 182, 194 and 210) and multiplying the sum by a correction factor calculated from the mass spectrum of a standard *Populus deltoides* (NIST 8492, National Institute of Standards and Technology, Gaithersburg, MD, USA) and its known absolute lignin content. S/G ratios were determined by summing the
intensity of the syringyl peaks at 154, 167, 168, 182, 194, 208 and 210 and dividing by
the sum of intensity of guaiacyl peaks at 124, 137, 138, 150, 164 and 178. All pyrolysis
mass spectra are known to be genetically controlled and heritable (Yin et al., 2010).

7.5.3 Pretreatment and enzymatic hydrolysis

All biomass samples were subjected to a combined high-throughput pretreatment and
enzymatic hydrolysis process based on a 96 well-plate format to test for sugar release as
described elsewhere (Studer et al., 2010). Briefly, approximately 2.6 mg of Populus
material was weighed into an individual Hastelloy well on a 96 well-plate. Then,
247.4 µL of deionized water was added to each well to produce a range of solids
concentrations from 0.70 to 1.17% (w/w) and the biomass was incubated at room
temperature for 4 h. All pretreatments were conducted at a log\(R_0\) severity of 3.6 (equation
1) by heating the well-plate with condensing steam to temperatures of 180, 160 and
140°C for 17.6, 68.1 and 464.4 min, respectively. Severity was defined as:

\[
R_0 = t \cdot e^{\frac{T-100}{14.75}}
\]

(1)

where \(t\) is in minutes and \(T\) in °C (Chum et al., 1990).

After pretreatment 20 µL of an enzyme/buffer/sodium azide mixture, as specified
below, was pipetted into each well without any preceding separation or washing steps.
Spezyme CP (lot no. 3016295230, 116 mg protein/mL, 62 FPU/mL) and Multifect
Xylanase (lot no. 301-04021-015, 56.6 mg protein/mL) (Genecore, PaloAlto, USA) were
employed as cellulolytic enzymes with a loading of 75 + 25 mg of cellulase and xylanase
protein, respectively, per g of glucan + xylan in raw biomass for the *Populus* standard, which had a composition of 46.2% glucan, 14.8% xylan and 27.0% lignin. The final concentrations of citric acid buffer (pH 4.95) and sodium azide were 0.05 M and 0.01 g/L, respectively. The samples were incubated in a shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD) at 50°C for 72 h at 150 rpm and then analyzed for sugars in the supernatant. If pretreatment was omitted samples were only soaked for 4 h in water and then directly subjected to enzymatic hydrolysis as described.

For each independent biomass sample, three analytical replicates were performed for all pretreatment conditions and two for enzymatic hydrolysis without pretreatment.

### 7.5.4 Sugar analysis

Cellobiose, glucose and xylose concentrations were measured using high performance liquid chromatography. An Aminex HPX-87H column (BioRad, Hercules, CA) heated to 65°C was used in a separation module (Alliance 2695, Waters, Milford, MA) equipped with a refractive index detector (2414, Waters) using 0.005 M sulfuric acid as the eluent in an isocratic mode.

### 7.5.5 Statistical analysis

Linear regression of equation 2 using a dummy variable $z$ with values of 0 or 1 to distinguish the respective sample sets was applied to test the null hypothesis of no statistical difference in slope and intercept:

$$y = a_1 + b_1 \cdot x + z(a_2 + b_2 \cdot x)$$  \hspace{1cm} (2)
The null hypothesis was rejected at the 0.05 level. Regression analysis was performed using Igor Pro (Wavemetrics Inc., Lake Oswego, OR, USA).

7.6 Acknowledgements

Support by the Office of Biological and Environmental Research in the DOE Office of Science for the BioEnergy Science Center (BESC) made this research possible. The authors thank Karen Huaying Xu (UC Riverside) for help on the statistical analysis; Kristen Reichel (NREL), Steve Thomas (NREL), Justin Anderson (NREL), Geoffrey Turner (NREL) and Angela Ziebell (NREL) for HTP screening of plant cell wall chemistry traits; Lee Gunter (ORNL), Sara Jawdy (ORNL), Nancy English (ORNL), Xiaohan Yang (ORNL), Gancho Slavov (WVU) and Steve DiFazio (WVU) for sample collection, design, preparation and shipping; and Susan Holladay (ORNL) for LIMS data organization. The authors would also like to extend their appreciation to Eugene Nothnagel (UC Riverside) and Simone Brethauer (ETH) for their valuable discussions and insights.

7.7 References


Chapter 8. Composition and hydrothermal pretreatment and enzymatic saccharification performance of grasses and legumes from a mixed-species prairie

* This whole chapter has been published under the following citation:
8.1 Abstract

Mixtures of prairie species (mixed prairie species; MPS) have been proposed to offer important advantages as a feedstock for sustainable production of fuels and chemicals. Therefore, understanding the performance in hydrothermal pretreatment and enzymatic hydrolysis of select species harvested from a mixed prairie is valuable in selecting these components for such applications. This study examined composition and sugar release from the most abundant components of a plot of MPS: a C3 grass (*Poa pratensis*), a C4 grass (*Schizachyrium scoparium*), and a legume (*Lupinus perennis*). Results from this study provide a platform to evaluate differences between grass and leguminous species, and the factors controlling their recalcitrance to pretreatment and enzymatic hydrolysis.

Significant differences were found between the grass and leguminous species, and between the individual anatomical components that influence the recalcitrance of MPS. We found that both grasses contained higher levels of sugars than did the legume, and also exhibited higher sugar yields as a percentage of the maximum possible from combined pretreatment and enzymatic hydrolysis. Furthermore, particle size, acid-insoluble residue (AcIR), and xylose removal were not found to have a direct significant effect on glucan digestibility for any of the species tested, whereas anatomical composition was a key factor in both grass and legume recalcitrance, with the stems consistently exhibiting higher recalcitrance than the other anatomical fractions.

The prairie species tested in this study responded well to hydrothermal pretreatment and enzymatic saccharification. Information from this study supports recommendations as to which plant types and species are more desirable for biological conversion in a
mixture of prairie species, in addition to identifying fractions of the plants that would most benefit from genetic modification or targeted growth.
8.2 Background

The only known resource that can promise to support large-scale, sustainable production of organic chemicals and liquid fuels and reduce dependence on petroleum is lignocellulosic biomass (Lynd et al., 1991; Perlack et al., 2005; Farrell et al., 2006). However, owing to the large amounts of biomass and land that would be required to satisfy the world’s growing energy demands, there are concerns that biofuels would compete with food for fertile land, and may also threaten biodiversity if natural lands are dedicated to monoculture bioenergy crops (Tilman et al., 2006). For lignocellulosic biofuels to be produced as sustainably as possible, the ideal feedstock would achieve high biomass yields with minimal or no irrigation and fertilization, be grown on degraded and abandoned agricultural lands, and be converted at high yields to sugars and subsequent fuels and/or chemicals. One such potential feedstock is mixed prairie species (MPS), which has been reported to grow well on agriculturally degraded lands with minimal fertilization, irrigation only during establishment, and low inputs otherwise (Tilman et al., 2006). Tilman et al. (2006) found that mixtures exhibiting high levels of biodiversity, in particular those including legumes, also benefit from a self-supply of nitrogen, potentially reducing or eliminating the need for nitrogen fertilizer.

Several studies have looked at cell wall digestibility and sugar release of individual legume or grass species (Spindler et al., 1990; Torget et al., 1990; Torget et al., 1992; Gaylean and Goetsch, 1993; Jung and Vogel, 1992; Dien et al., 2006), but to our knowledge, none has investigated the performance of both grass and legume components that are grown together within a mixed plot. Thus, this study aimed to gain a better
understanding of how composition and performance in pretreatment and enzymatic hydrolysis vary for three of the most abundant components within a mixture of prairie species: a C3 grass, a C4 grass, and a legume.

Owing to the strongly heterogeneous nature of the mixture and the individual species themselves, composition and sugar release within each species were examined by fractionating plants and analyzing the resulting anatomical components. To facilitate the analysis, members of each class of plant (a C3 grass (*Poa pratensis*; PP), a C4 grass (*Schizachyrium scoparium*; SS) and a legume (*Lupinus perennis*; LP)) were fractionated into their anatomical components, and the resulting mass fractions analyzed (Table 8.1). Each anatomical component was then fractionated by particle size, creating a total of 36 samples that were analyzed for chemical composition using a downscaled wet-chemistry method (DeMartini et al., 2011). Each of the 36 samples was then subjected to hydrothermal pretreatment followed by enzymatic hydrolysis in a similar high-throughput, scaled-down system as used for the compositional analysis (Studer et al., 2010). With this approach, we evaluated differences in composition and sugar-release performance between both grass and leguminous species, and those between anatomical fractions of each plant. This information allowed us to investigate whether there are factors that control the recalcitrance of both grasses and legumes, which could help in identifying fractions of the plants that would most benefit from genetic modification or targeted growth. Finally, this work might also support recommendations as to which plant types are more desirable for biological conversion in a mixture of prairie species.
Table 8.1. Anatomical composition of tested mixed prairie species

<table>
<thead>
<tr>
<th>Plant part</th>
<th>Anatomical component mass fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lupinus <em>perennis</em></td>
</tr>
<tr>
<td>Stem</td>
<td>37.5</td>
</tr>
<tr>
<td>Leaf</td>
<td>49.3</td>
</tr>
<tr>
<td>Petiole</td>
<td>13.3</td>
</tr>
<tr>
<td>Sheath</td>
<td>–</td>
</tr>
<tr>
<td>Flowers</td>
<td>–</td>
</tr>
</tbody>
</table>

aMass fraction of major anatomical components for the three species examined in this study, *L. perennis* (LP), *S. scoparium* (SS), and *P. pratensis* (PP). LP included the stem, petiole and leaf, while SS and PP were divided into the stem, sheath, leaf, and flower. Each of these components was weighed to determine its anatomical mass fraction.

8.3 Results and discussion

8.3.1 Analysis of grass and legume anatomical fractions

8.3.1.1 Composition of anatomical fractions

The composition of the anatomical fractions for the three species was compared (Figure 8.1). The galactan and arabinan contents of the samples are not included in Figure 8.1 because all of their values were below 3.5%. As shown, the flower fraction from PP had the highest glucan content of any fraction (44.5%), and the stem portion of all species exhibited the next highest: 29.0% for LP, 34.9% for PP, and 35.0% for SS. The stem portion also had the highest xylan content: 10.8% for LP, 21.1% for PP, and 25.2% for SS. With the exception of the leaf fraction from LP, which exhibited the largest AcIR
content (42.6%), the AcIR contents of the remaining species and anatomical fractions ranged from 17.5% (PP sheath) to 26.7% (LP stem).

Figure 8.1. Composition by anatomy. Glucan, xylan, and acid-insoluble residue (AcIR) contents as measured by downscaled wet chemistry for each anatomical fraction of the three species tested. Error bars represent the overall standard error from triplicate analyses of each particle-size fraction.

Differing compositions between anatomical fractions have been previously reported for both grasses and legumes. Jung and Vogel (1992) showed that the leaves of grasses, including big bluestem (*Andropogon gerardii*) and switchgrass (*Panicum virgatum*), have lower cellulose content than grass stems. Similarly, Aman (1993) found that in a legume such as red clover (*Trifolium pratense*), the leaves contained less than half of the sugar content of stems, whereas the lignin content of both legume and grass leaves tended to be lower than that for stems (Jung and Vogel, 1992; Aman, 1993). In the present study, although the patterns of sugar concentrations within anatomical fractions agreed with previous studies, the AcIR content, which can serve as an estimate of lignin content, did not entirely coincide. For the two grass species tested, no significant differences were
seen between the AcIR content of the stem and leaf fractions. By contrast, the AcIR content of LP leaf was much larger than that of all the other fractions, a particularly unexpected finding, as this fraction contains mostly non-lignified mesophyll cells. The high average AcIR content for LP leaf was mainly associated with two particle-size fractions of this component, the -20/+40 and -40/+60 mesh fractions, which exhibited AcIR contents of 72% and 48%, respectively, compared with values of 17% to 35% for all the other particle-size fractions tested in this study. Because of these unexpectedly high values, these samples were reanalyzed and confirmed to have particularly high AcIR contents. The cause of this behavior is unknown, but it may be attributed in part to the preferential sieving of the anatomical components. Generally, the lignin in legumes is more localized than in grasses (Wilson, 1993). It is possible that the more highly lignified leaf midrib will be more prevalent in the larger particle-size fractions. Alternatively, the high AcIR content in these particular samples may not mean that they have a significantly higher lignin content, but instead may be due in part to an elevated content of acid-insoluble ash and extractives, including proteins and inorganic materials. These components may also explain why the AcIR contents of the grasses were almost identical for both the leaf and stem sections even though the stem portion was expected to have a higher lignin content.

To test whether elevated protein content might have been the cause of the high AcIR values in the LP leaf samples, the nitrogen content of all particle-size fractions of this species was analyzed. The resulting protein values were estimated to be in the range of 7% to 11% for LP leaf depending on particle size, about twice the protein content of 4%
to 6% for LP stem. Nonetheless, although protein content might have resulted in a slightly higher AcIR content for some MPS samples, it cannot fully account for the particularly high AcIR content in the LP leaf samples. Thus, it is possible that some other form of unidentified water or ethanol extractives may be responsible for the high AcIR content in legume leaves.

8.3.1.2 Sugar yields of anatomical fractions

The glucose, xylose, and total sugar (glucose + xylose) yields from combined pretreatment and enzymatic hydrolysis of the anatomical fractions of each species were analyzed (Figure 8.2). The leaf fraction exhibited the highest glucose yield within each species, ranging from 76.7% in LP to 88.7 and 90.6% in PP and SS, respectively. Conversely, the lowest glucose yields were generally seen in the stem fraction, an observation that was most pronounced in LP and SS, with yields of 59.6 and 61.6%, respectively. The only anatomical fraction that exhibited lower glucose yields than the stem was the flower portion of PP, with a glucose yield of 76.3%, which was slightly lower than that of the same plant’s stem (77.7%). Conversely, unlike glucose yields, no clear correlation was found between xylose yield and anatomical fraction. Although xylose yields varied between fractions, ranging from 57.2% in LP petiole to 91.8% in PP stem, no single anatomical fraction exhibited consistently high or low xylose yields for all three species tested.
Figure 8.2. Sugar yields by anatomy. Glucose, xylose, and total sugar (glucose + xylose) yields from combined pretreatment and enzymatic hydrolysis for the anatomical fractions of *Lupinus perennis*, *Schizachyrium scoparium*, and *Poa pratensis*. Yields represent the amount of sugar released per amount of sugar available in the biomass (for example, glucose released/glucose in biomass). Error bars represent the standard error of experiments performed with six replicates for each particle-size fraction.

Other researchers have also reported the influence of anatomical fraction on sugar yields. For example, past work has evaluated sugar yields from husks, leaves, cobs, and stalks of corn (*Zea mays*) in pretreatment and enzymatic hydrolysis (Chundawat et al., 2007; Montross and Crocheck, 2004; Garlock et al., 2009). Both Montrass and Crocheck (2004) and Garlock *et al.* (2009) found the leaves to be less recalcitrant than stalks. Similar work by Anderson *et al.* (2008) also found that grass leaves generally exhibited higher *in vitro* dry-matter digestibility compared with stems.
8.3.2 Analysis of overall species

8.3.2.1 Composition of grass and legume species

Based on the compositions and mass fractions of each of the anatomical parts, the AcIR, glucan, and xylan compositions of the three species were compared. The C4 grass SS contained the highest levels of both glucan (33.4%) and xylan (22.4%), followed by the C3 grass PP with 32.7% glucan and 16.8% xylan (Figure 8.3). The legume LP contained the lowest levels of sugars, with only 21.3% glucan and 9.2% xylan. By contrast, LP contained 33.5% AcIR, whereas the two grasses had 19.9% (PP) and 22.1% (SS). The lower sugar contents seen for the legume compared with the grasses is not indicative of all species of their kinds. While Torget et al. (1990) found the legume Sericea lespedeza to contain less glucan and xylan than both switchgrass (Panicum virgatum) and weeping lovegrass (Eragrostis curvula), they later observed that the legume flatpea hay (Lathyrus sylvestris L.) had a slightly higher glucan content than reed canary grass (Phalaris arundinacea) (Torget et al., 1992). Others have shown that whereas glucan content shows no clear trend between grasses and legumes, the xylan content is typically much lower in legumes than in grasses (Aman, 1993). However, it should also be noted that although some legumes may have a lower content of neutral sugars than do monocot grasses, they probably contain higher amounts of acidic sugars, as do other dicots. As for lignin content, it tends to be significantly higher in legumes than in grasses (Torget et al., 1990; Torget et al., 1992; Aman, 1993), consistent with the AcIR results in this study.
8.3.2.2 Sugar yields of grass and legume species

The overall sugar yields for the three species were calculated as the percentage of the maximum possible from yields of the individual anatomical fractions and their corresponding mass fractions (Figure 8.4). The highest overall glucose, xylose, and total sugar (glucose + xylose) yields were for the C3 grass PP, with yields of 86.6%, 80.5%, and 84.6%, respectively. The next highest yields were for the other grass tested, SS, with values of 73.6% glucose, 79.0% xylose, and 76.1% glucose + xylose. Finally, the legume LP responded the most poorly to pretreatment and enzymatic hydrolysis, with glucose, xylose, and glucose + xylose yields of 70.0%, 76.2%, and 72.3%, respectively.
Figure 8.4. Sugar yields by species. Glucose, xylose, and total sugar (glucose + xylose) yields calculated from combined pretreatment and enzymatic hydrolysis of each anatomical fraction and the corresponding mass fractions of each fraction for *Lupinus perennis*, *Schizachyrium scoparium*, and *Poa pratensis*. Yields represent the amount of sugar released per amount of sugar available in the biomass (for example, glucose released/glucose in biomass). Error bars represent the overall standard error based on the variance of results for each anatomical fraction.

The lower yields from the legume LP are in agreement with past studies that found legume cellulose to be more recalcitrant than that of grasses (Spindler et al., 1990; Torget et al., 1990; Torget et al., 1992; Dien et al., 2006). Dien *et al.* (2006) attributed this to the differences in plant cell wall structure, and noted that lignin is more uniformly distributed among tissues of grass compared with those of legumes, so that perhaps the lower sugar yields of legumes are associated with those tissues containing higher levels of lignin. Furthermore, in evaluating the differing performance of the two grasses, Galyean and Goetsch (1993) reported that cool-season grasses, such as *Poa pratensis*, are more digestible than warm-season grasses, including *Schizachyrium scoparium*, consistent with the results from this study. Those authors attributed this differing behavior to the unique
proportions and arrangements of tissues in warm- and cool-season grasses (Galyean and Goetsch, 1993). For example, owing to differences in their photosynthetic pathways and optimal growing temperatures, warm-season grasses have a larger proportion of less digestible stem material (1993). Additionally, the digestibility of the stem and leaf materials themselves differs between warm- and cool-season grasses: digestion of warm-season tissues was reported to be lower, possibly due to a higher concentration of phenolic compounds and a more tightly packed, radial arrangement of tissues (Galyean and Goetsch, 1993).

8.3.3 Evaluating factors that influence glucose yields and glucose release

8.3.3.1 Interpretation of statistical analysis

Although we found that on average, certain anatomical fractions and plant species exhibited greater glucan digestibility, we also evaluated the influence of a larger array of sample characteristics, still including anatomical fraction and species. In particular, analyses of covariance (ANCOVA) and of variance (ANOVA) were performed to investigate, respectively, 1) whether there are individual factors or a combination of factors that influence glucan digestibility and glucose mass release of MPS, and 2) how these factors may be related to each other. To address the first question, the ANCOVA test was performed with three factors (those defined by the experimental setup: plant species, anatomical fraction, and particle size) and four continuous covariates (AcIR content, glucan content, xylan content, and xylose yield). ANCOVA was selected because it takes into account both the effects of individual variables and the interactive
effects of multiple variables. When we tested the influence of all of these factors and
covariates in a full ANCOVA, we found that only anatomical fraction and AcIR content
had a significant effect on glucan digestibility (glucose released/glucose available). To
further examine this finding, a reduced model was used, which evaluated the two
significant variables (anatomical fraction and AcIR content) identified in the full model.
Using this refined model, we found that the anatomical fraction had a highly significant
influence on glucan digestibility ($P<0.001$), whereas the influence of AcIR content was
less important ($P = 0.11$). The same analysis was also performed to evaluate the
influence of the same set of variables on the glucose mass release (glucose released/total
dry biomass). In this case, the full model suggested that anatomical fraction and glucan
content had a significant influence. A reduced model confirmed this finding, with both
variables having a highly significant influence ($P<0.001$) on glucose release.

The ANOVA test was performed to evaluate how each of the individual four
covariates defined in the previous analyses were related to the three factors (plant species,
anatomical fraction, and particle size). Although ANOVA does not consider interactive
effects, it is useful in determining the influence of controllable experimental factors on
results and in future model development to predict glucan digestibility and glucose mass
release with these factors. Thus, glucose yield, glucose mass release, AcIR content,
glucan content, xylan content, and xylose yield were defined as the response variables.
The significance of the factors’ influence on the response variables is summarized in
Table 8.2. As shown by the low $P$ values, all of the factors considered influenced most of
the response variables. For example, anatomical fraction had an effect on AcIR content
and glucose mass release \((P = 0.02)\) and an even stronger influence on glucan content \((P < 0.001)\), xylan content \((P = 0.001)\), xylose yield \((P < 0.001)\), and glucan digestibility \((P < 0.001)\). Unlike anatomical fraction, particle size significantly influenced only two variables, the glucan and xylan contents, whereas plant species influenced all variables except glucan digestibility. The results from this test further suggest that the only experimental factor that significantly influenced the glucan digestibility of all samples was the anatomical fraction, whereas both plant species and anatomical fraction influenced glucose mass release. Particle size did not influence either glucan digestibility or glucose mass release.

Table 8.2. Interactive effects between response variables and experimental factors

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Plant species</th>
<th>Anatomical fraction</th>
<th>Particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcIR content</td>
<td>&lt;0.001</td>
<td>0.03</td>
<td>0.11</td>
</tr>
<tr>
<td>Glucan content</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>Xylan content</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.01</td>
</tr>
<tr>
<td>Xylose yield</td>
<td>0.05</td>
<td>&lt;0.001</td>
<td>0.80</td>
</tr>
<tr>
<td>Glucan digestibility</td>
<td>0.34</td>
<td>&lt;0.001</td>
<td>0.12</td>
</tr>
<tr>
<td>Glucose mass release</td>
<td>&lt;0.001</td>
<td>0.02</td>
<td>0.45</td>
</tr>
</tbody>
</table>

aThe significance of interactive effects is represented by the \(P\)-value. The significance \((P\)-value\) of the influence of the experimental factors (plant species, anatomical fraction, and particle size) on response variables (AcIR content, glucan content, xylan content, xylose yield, glucan digestibility, and glucose mass release) is displayed. The \(P\) values are calculated from individual ANOVA tests and are displayed for each interaction.
8.3.3.2 The importance of anatomical composition

Both statistical analyses stressed the importance of anatomical fraction on sample composition, glucan digestibility, and glucose mass release. ANOVA showed that anatomical fraction had a strong effect on glucan, xylan, and AcIR contents, whereas ANCOVA analysis clearly showed that when both individual and interactive effects were considered, anatomical fraction was the variable that most significantly influenced glucan digestibility of the MPS considered here. The strong influence of anatomical fraction is logical because it will define the distribution and relative amounts of various tissue types, which in turn affect cell shape, size, wall thickness, and corresponding cell wall surface area to volume ratio. All of these factors have previously been suggested to affect digestibility (Grabber et al., 1992; Grabber and Allinson, 1992; Wilson and Mertens, 1995; Buxton and Redfearn, 1997). In general, the leaf fraction exhibited higher glucose yields; the stem fraction tended to be significantly more recalcitrant to glucose release; and the flower, sheath, and petiole fractions exhibited intermediate performance compared with leaf and stem. These observations can be attributed to differences in tissue type and distribution for the anatomical fractions. Leaves are primarily composed of thin-walled (approximately 0.15 µm thick) and loosely arranged mesophyll cells with a high proportion of intercellular airspace and few wall contacts between cells, rendering them easily disrupted and digested (Wilson, 1993; Wilson and Mertens, 1995). Conversely, stems contain high levels of vascular tissue that are rich in recalcitrant and highly lignified xylem elements (Wilson, 1993; Chundawat et al., 2007). Furthermore, stems also contain a higher proportion of thick-walled parenchyma (approximately 1.0
µm) and sclerenchyma (approximately 2.4 µm) cells (Wilson and Mertens, 1995) that are probably more difficult to break down. The general anatomy of sheaths and petioles has been reported to be between that of the leaf blade and stem (Wilson, 1993), possibly explaining their intermediate performance.

In contrast to the anatomical fraction, the particle size, AcIR content, xylose yield, or xylan content did not strongly influence glucan digestibility of the MPS samples. Zeng et al. (2007) also reported that smaller particle sizes did not consistently result in higher glucose yields from hydrothermal pretreatment and enzymatic hydrolysis. Although it might seem intuitive that smaller particle sizes improve enzyme effectiveness because of their higher surface area to volume ratio, it may be that pretreatment disrupts biomass particles and increases accessible surface area and pore volume sufficiently that differences caused by particle size are masked (Zeng et al., 2007; Mansfield et al., 1999).

The lack of significant influence of AcIR content on glucose yield was somewhat unexpected, as glucose yield has been commonly reported to be inversely related to lignin content (which is by far the largest component of AcIR). However, as explained above (section on ‘Composition of anatomical fractions’), AcIR content only provides an estimate of the Klason lignin amounts because the downscaled analysis procedure measures the total acid-insoluble residue including the acid-insoluble ash. The lack of influence of AcIR content on glucose yield disagrees with many past studies showing that increased lignin content adversely affected glucan digestibility, caused in part by restriction of enzyme access and non-productive binding (Chang and Holtzapple, 2000; Berlin et al., 2005; Chen and Dixon, 2007). It is possible that AcIR measurements of
grasses and legumes are not a sufficient estimate of their lignin content, and that if glucose yields were evaluated versus the true lignin content, a significant effect might be seen. However, it is still interesting to note that AcIR as a whole, which is comprised mostly of lignin, does not seem to have a significant direct negative effect on yields.

The absence of a significant influence of xylose yield on glucan digestibility was also unexpected, because it has often been reported that glucan digestibility correlates positively with hemicellulose removal (Knappert et al., 1980; Grohmann et al., 1989; Yang and Wyman, 2004). Yet, we found no strong correlation to support this hypothesis in the grasses and legumes tested in this study. As an example, for two different samples that both exhibited a xylose yield of 75%, the corresponding glucose yields were 57% and 93%, suggesting that other factors must also contribute significantly to glucan digestibility.

8.3.4 Implications for biomass in mixed-species prairies

Good glucose and xylose yields were obtained for the most common grass and legume species that comprised the plot of MPS, especially considering that pretreatments were performed with water alone, at a maximum temperature of 180°C, owing to pressure limitations in the steam chamber. Use of acid or higher temperatures would probably improve yields and might be particularly beneficial for legumes (Torget et al., 1990; Torget et al., 1992). This study also provides insight into possible strategies to improve the conversion characteristics of MPS. The results indicate that plant anatomy is a key factor in grass and legume recalcitrance, and furthermore, that leafy material
responds better to hydrothermal pretreatment and enzymatic hydrolysis than do stems, suggesting that genetic modification of stems could be most productive. Also along these lines, methods to increase the leaf:stem ratio would improve overall glucose yields from combined pretreatment and enzymatic hydrolysis. However, despite the differences in glucan digestibility between the various anatomical fractions, the mass of sugar produced per total mass of biomass must also be considered. As such, in this study, the stem released more sugar in LP and PP (280 and 500 g glucose + xylose/kg dry biomass, respectively) than did the leaves (200 and 430 g glucose + xylose/kg dry biomass, respectively), whereas in SS, the amounts were identical for both (440 g glucose/kg dry biomass). This consideration diminishes the consequences of lower sugar yields from stems, and suggests that breeding targets for improved conversion should be directed at stems in order to capitalize on their higher sugar contents.

A similar analysis was used for the three species tested. Although PP exhibited higher glucose yields than SS, the mass of sugar produced per total feedstock mass was almost identical for the two grasses. By contrast, the legume LP released almost half as much glucose + xylose as the grasses. Nevertheless, despite the low sugar yields and mass release exhibited by LP, legumes constitute an integral part of MPS because of their ability to fix nitrogen and thereby reduce or eliminate the need for nitrogen fertilizer. Hence, one potential solution could be to plant other legumes that offer similar nitrogen-fixing benefits but can produce higher structural carbohydrate contents and sugar yields than LP. Alternatively, it may be advantageous to select a harvest time that maximizes the grass:legume ratio so that the agricultural benefits of legumes can still be obtained.
while reducing their negative effect on the biological conversion yields. For cool-season
legumes such as LP, this is a very real possibility. In the upper Midwest of the USA, in
which the plants for this study were grown, most of the legumes have dried and fallen to
the soil surface, releasing nitrogen, by mid-July, whereas the warm-season grasses are
near peak growth at this time. Thus, if harvest time occurs during the period at which the
grasses begin to senesce, such as late September in the upper Midwest USA, the
glass:legume ratio will strongly favor grasses. Additionally, nitrogen loss will be
reduced if a harvest time that occurs after the senescence of warm-season grasses is
chosen.

8.4 Conclusions

Analysis of the most abundant legume and C3/C4 grass species within a mixture of
prairie species showed that the grasses contained higher levels of sugars compared with
the legume. The grasses also exhibited higher sugar yields from combined hydrothermal
pretreatment and enzymatic hydrolysis, demonstrating that they are the more desirable
components in the MPS for conversion to sugars and subsequent fuels and chemicals. In
analyzing the influence of a variety of sample characteristics on the recalcitrance of grass
and legume MPS, we found no evidence to suggest a direct significant effect of particle
size, plant composition, or xylose yield. Instead, plant anatomy was found to be the most
influential factor for both glucan digestibility and glucose mass release, suggesting that
breeding and harvest methods to control anatomical composition might be an important
route to improving sugar yields from MPS.
8.5 Methods

8.5.1 Biomass samples

A plot of MPS containing 16 different species was planted in the spring of 1994 in Cedar Creek Ecosystem Science Reserve, Minnesota, MN, USA. The species in this plot were grown on nutrient-poor sandy soils, burned annually in the spring, and grown without irrigation or fertilization before the samples were obtained in 2008 by the University of Minnesota. The plot produced 3.38 tons/hectare of fully dried above-ground harvested biomass, and samples for this study were collected from a section 6 × 0.1 m wide. A portion of the plot was collected and sent unsorted to the University of California Riverside (UCR) to represent the entire mixture, while another portion was sorted and sent to UCR as individual species, from which their weight and corresponding percentage of the plot that they comprised was determined. Table 8.3 shows the 12 species that were identified in the plot at harvest in 2008, with the percentage weight of the plot that each represents. It is important to note that the biomass fractions shown are from a sampling performed in early July, whereas the biomass tested in this study is from late autumn when the C4 grasses, including *Andropogon gerardi*, *Sorghastrum nutans*, and *Schizachyrium scoparium*, would probably have outgrown the others, and therefore would make up a larger fraction of the biomass.

8.5.2 Material preparation

From the 12 sorted species, the most abundant C3 grass (PP), C4 grass (SS), and legume (LP) were selected for further analysis. Each of these air-dried materials
(moisture content approximately 7%) was first divided into its anatomical components. For the legume, this comprised the stem, petiole, and leaf, whereas for the grasses, the materials were divided into stem, sheath, leaf, and flower. Each of these fractions was weighed to determine the anatomical mass fraction for each species (Table 8.1).

**Table 8.3. Plant species comprising the plot\(^a\) of mixed prairie species**

<table>
<thead>
<tr>
<th>Species name</th>
<th>Classification</th>
<th>Mass fraction, %</th>
<th>Cumulative mass fraction(^b), %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Schizachyrium scoparium</em></td>
<td>C4 grass</td>
<td>31.5</td>
<td>31.5</td>
</tr>
<tr>
<td><em>Lupinus perennis</em></td>
<td>Legume</td>
<td>27.3</td>
<td>58.8</td>
</tr>
<tr>
<td><em>Andropogon gerardii</em></td>
<td>C4 grass</td>
<td>14.7</td>
<td>73.5</td>
</tr>
<tr>
<td><em>Poa pratensis</em></td>
<td>C3 grass</td>
<td>12.1</td>
<td>85.6</td>
</tr>
<tr>
<td><em>Lespedeza capitata</em></td>
<td>Legume</td>
<td>8.4</td>
<td>94.0</td>
</tr>
<tr>
<td><em>Monarda fistulosa</em></td>
<td>Non-leguminous forb</td>
<td>3.7</td>
<td>97.7</td>
</tr>
<tr>
<td><em>Sorghastrum nutans</em></td>
<td>C4 grass</td>
<td>1.3</td>
<td>99.0</td>
</tr>
<tr>
<td><em>Asclepias tuberosa</em></td>
<td>Non-leguminous forb</td>
<td>0.2</td>
<td>99.2</td>
</tr>
<tr>
<td><em>Achillea millefolium</em></td>
<td>Non-leguminous forb</td>
<td>0.1</td>
<td>99.3</td>
</tr>
<tr>
<td><em>Agropyron repens</em></td>
<td>C3 grass</td>
<td>0.1</td>
<td>99.4</td>
</tr>
<tr>
<td>Miscellaneous litter</td>
<td>–</td>
<td>0.8</td>
<td>100.2</td>
</tr>
</tbody>
</table>

\(^a\)The plot from which these species were obtained produced 3.38 tons/hectare of fully dried aboveground harvested biomass.

\(^b\)Cumulative mass fraction does not add up to 100.0% because of rounding.

After fractionation into anatomical components, each of the subsamples was then milled (Wiley Laboratory Mill Model 4; Arthur H. Thomas Company, Philadelphia, PA,
USA) until it passed through a 20-mesh screen (<0.85 mm). Material was collected and then sieved using USA Standard Testing Sieves (Fisher Scientific Company, Pittsburg, PA, USA), from which different particle-size fractions were collected: -20/+40 mesh (425<\(x<850\) µm), -40/+60 mesh (250<\(x<425\) µm), -60/+80 mesh (180<\(x<250\) µm), and <80 mesh (\(x<180\) µm). All subsequent experiments were performed on the individual particle-size fractions obtained from the anatomical components of each species. It should be noted that for PP, there was not enough flower, sheath, or stem material to produce all four particle-size fractions. As a result, the PP flower sample included all material that fell through the 20-mesh screen, whereas the PP sheath and stem samples were collected in two fractions: -20/+60 and <60 mesh.

8.5.3 Compositional analysis

Glucan, xylan, arabinan, galactan, mannan and AcIR contents were determined by performing a downscaled wet-chemistry compositional analysis coupled with high-performance liquid chromatography (HPLC) and gravimetric methods to allow analysis of the small amounts of materials available (DeMartini et al., 2011). This procedure is nearly identical to conventional procedures (Sluiter et al., 2008), and produces virtually identical results; however, it uses 100 times less biomass (3 mg versus 300 mg) and can be automated using a solid- and liquid-dispensing robotics platform (Core Module Standard Configuration 2 equipped with Sartorius WZA65-CW balance and 10 biomass-dispensing hoppers of 25 mL capacity; Freeslate, Inc., Sunnyvale, CA, USA). After two-stage acid hydrolysis in the downscaled system, the neutralized sugar hydrolyzates were
measured by HPLC, while the AcIR contents were determined by gravimetric methods to provide an estimate of Klason lignin content. Unlike the conventional method, this downscaled procedure measures the total AcIR including the acid-insoluble ash, and as a result, cannot measure lignin content directly. Additionally, the composition of the unsorted material was determined by the conventional scaled-up procedure described by Sluiter et al. (2008).

To test for the protein content in the LP leaf samples, the nitrogen content was measured (EATM 112 N/Protein plus CHNS/O Analyzer; CE Elantech, Lakewood, NJ, USA) with L-aspartic acid (10.52% N, 36.09% C) as a standard. A nitrogen factor of 6.25 was used to estimate the resulting protein content of the samples (Hames et al., 2005).

8.5.4 Pretreatment and enzymatic hydrolysis

All samples were subjected to hydrothermal pretreatment followed by enzymatic hydrolysis to determine total glucose and xylose released from the combined operations using a high-throughput pretreatment and enzymatic hydrolysis (HTPH) system described in detail previously (Studer et al., 2010). In this study, 4.5 mg of dry biomass were loaded into individual wells of a custom-built metal well plate using a robotics platform (Core Module; Freeslate, Inc.). The well plate used in this study differs from that described by Studer et al. (2010) in that the wells are larger, employing a reaction mass of 450 mg as opposed to 250 mg. In addition, the individual wells themselves are free-standing on a brass plate, as opposed to being press-fit into an aluminum plate as per
the previous report, enabling the robot’s four-pronged gripper to pick up and move individual wells to the balance for accurate weighing and biomass dispensing. After the well plate was loaded with biomass samples, it was taken off the robot’s deck, and 445.2 µl of deionized water was transferred into all wells using an eight-channel pipettor (30-300 µl; Eppendorf, Hamburg, Germany) to achieve a solids loading of 1% w/w. A flat silicone gasket (thickness 1.5875 mm, durometer hardness A40) was laid on top of the open ends of the wells with a thin stainless steel sheet (0.1016 mm) then placed on top of the gasket. This entire assembly was then clamped between two stainless steel plates using spring washers (flat load 1,500 N) and wing nuts. Next, the sealed plate assembly was placed in a custom-built steam chamber for pretreatment with condensing steam (Studer et al., 2010) provided by a Fulton steam boiler (FB-075-L, Fulton Companies, Pulaski, NY, USA).

After a pretreatment of 44 minutes, the reaction was quenched by opening the valve to the chamber to release the steam, followed by flooding the chamber with cold water. Afterwards, the plate assembly was removed and opened, and 33.7 µl of a mixture of 1 mol/l citric acid buffer (pH 4.95), sodium azide solution (1 g/L), and enzyme was added to the pretreated biomass slurry in each well using an eight-channel pipettor (10-100 µl, Eppendorf). The resulting mixture contained 6.750 mL of buffer, 1.350 mL of sodium azide solution, and 2.002 mL of a dilute cellulase (Spezyme CP, lot no: 3016295230, 116 mg protein/mL) and xylanase (Multifect, lot no: 301-04021-015, 56.6 mg protein/mL) (both Genencor, Palo Alto, CA, USA) solution prepared at a protein mass ratio of 3:1, respectively, to which deionized water was added at a volume ratio of 3:1. The resulting
enzyme loading corresponded to 75 mg of cellulase + 25 mg xylanase per gram of glucan + xylan in the raw biomass for the unsorted material, which had a composition of 26.7% glucan, 12.5% xylan, and 20.3% Klason lignin, as determined by the conventional analytical procedure. After addition of the enzyme/buffer/biocide solution, the entire plate assembly with silicone gasket was re-assembled and placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, Laurel, MD, USA) set at 50°C and 150 rpm.

After 72 hours, the well plate was removed from the shaker, and the slurry from each individual well was transferred to 2.0 mL polypropylene (PP) centrifuge tubes (Safe-Lock 2.0 mL test tubes, Eppendorf). After centrifugation (5415 D; Eppendorf) at 18,200 g for 5 minutes, 300 µL of hydrolyzate were transferred to HPLC vials for analysis.

Before running all subsamples in the HTPH system, the unsorted MPS was used to establish a pretreatment yield curve from which a condition could be chosen for subsequent testing. The -20/+40 and -40/+60 mesh fractions of the unsorted MPS were used in the optimization, which was performed at five different hydrothermal pretreatment times, all at a temperature of 180°C. Additionally, the two size fractions of the unsorted MPS were subjected directly to enzymatic hydrolysis without any prior pretreatment. Based on these results, a suboptimal pretreatment severity of 4.0, corresponding to 44 minutes at 180°C, was selected to reduce xylose degradation but still achieve reasonably high sugar yields.
8.5.5 Sugar analysis

Sugar concentrations for compositional analysis were measured by HPLC (Alliance 2695 equipped with 2414 RI detector; Waters, Milford, MA, USA) with an Aminex HPX-87P column (BioRad, Hercules, CA, USA) heated to 85°C using distilled and deionized water as the eluent, while sugar concentrations from HTPH testing were measured on an Aminex HPX-87H column (BioRad, Hercules, CA, USA) heated to 65°C using the same HPLC configuration but with 0.005 mol/L sulfuric acid as the eluent. Both measurements used an eluent flow rate of 0.6 mL/min.

8.5.6 Statistical analysis

As mentioned above, each anatomical fraction was further divided into subsamples based on particle size (see ‘Material preparation’), for which compositional analyses were performed in triplicate, and HTPH sugar release was measured in six replicates. To determine the composition and sugar release of an entire anatomical fraction, the following equation was used to combine results for the subsamples sorted by particle size:

\[ X = \sum m_i \bar{x}_i \]  

(Equation 1),

where \( X \) is the desired composite result for the entire anatomical fraction (such as glucan content or glucose yield), \( m_i \) is the mass fraction of sub-sample \( i \) with a particular particle-size range, and \( \bar{x}_i \) is the corresponding average result (such as glucan content or glucose release) for sub-sample \( i \) for the set of replicates analyzed. To enable calculation
of the overall standard error for an entire anatomical fraction, the variance was computed and summed over each subset, as shown below:

\[
Y = \sqrt{\sum m_i^2 \text{variance}(\bar{x}_i)} = \sqrt{\sum m_i^2 \left(\frac{s_i^2}{n}\right)}
\]

(Equation 2),

where \(Y\) is the standard error of a result (such as glucan content or glucose yield) for an entire anatomical fraction, \(m_i\) and \(\bar{x}_i\) are as defined above, and the variance of \(\bar{x}_i\) is calculated by dividing the square of its standard deviation \(s_i\) by the number of replicates \(n\). The same statistical approach was applied to analyze compositions, sugar release, and corresponding standard errors, for an entire species based on the results for that species’ anatomical fractions. Error bars on plots represent the overall standard error as described above.

All statistical analysis was performed using the SAS software package (version 9.2; SAS Institute, Cary, NJ, USA). To evaluate the influence of individual or combinations of sample characteristics on glucan digestibility and glucose mass release, ANCOVA was performed. The three factors were those defined by the experimental setup, including plant species, anatomical fraction, and particle-size fraction. The four continuous covariates were AcIR content, glucan content, xylan content, and xylose yield. To further evaluate how the individual four covariates, as well as the glucan digestibility and glucose mass release, were related to the three factors (plant species, anatomical fraction, and particle size), we used ANOVA. For both analyses, all samples were evaluated including the individual particle sizes of the separate anatomical fractions for all 3 species (36 samples in total).
8.6 Acknowledgments

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8.7 References


Chapter 9. Application of monoclonal antibodies to investigate plant cell wall deconstruction for biofuels production

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DeMartini JD, Pattathil S, Avci U, Szekalski K, Mazumder K, Hahn MG, Wyman CE.
9.1 Abstract

To better understand how hydrothermal pretreatment reduces plant cell wall recalcitrance, we applied a high throughput approach ("glycome profiling") using a comprehensive suite of plant glycan-directed monoclonal antibodies to monitor structural/extractability changes in *Populus* biomass. The results of glycome profiling studies were verified by immunolabeling using selected antibodies from the same toolkit. The array of monoclonal antibodies employed in these studies is large enough to monitor changes occurring in most plant cell wall polysaccharides. Results from these techniques demonstrate the sequence of structural changes that occur in plant cell walls during pretreatment-induced deconstruction, namely, the initial disruption of lignin-polysaccharide interactions in concert with a loss of pectins and arabinogalactans; this is followed by significant removal of xylans and xyloglucans. Additionally, this study also suggests that lignin content per se does not affect recalcitrance; instead, the integration of lignin and polysaccharides within cell walls, and their associations with one another, play a larger role.
9.2 Introduction

Lignocellulosic biomass is the only sustainable resource for large-scale production of liquid transportation fuel that has the potential to significantly reduce the world’s dependence on petroleum (Farrell et al., 2006; Lynd et al., 1991; Ragauskas et al., 2006). One of the primary barriers to low cost biological conversion of lignocellulosic biomass to renewable fuels is the plants’ recalcitrance, which refers to the resistance of cell walls to deconstruction by enzymes or microbes (Lynd et al., 2008; Wyman, 2007). To overcome this obstacle, biomass is subjected to pretreatment prior to enzymatic hydrolysis in order to disrupt the plant cell wall’s structure and thereby allow hydrolyzing enzymes better access to the cellulose core (Mosier et al., 2005). However, few details are known as to the exact effects of pretreatment on cell wall structure and composition, thus making it difficult to intelligently design and optimize biomass deconstruction.

To gain insight into the effects of pretreatment, laboratories conventionally perform a general wet chemistry compositional analysis to determine basic carbohydrate and lignin content of the untreated and pretreated biomass (Sluiter et al., 2008). More recently, though, investigations into the effects of pretreatment on biomass and cell wall structure have been improved through the application of microscopic techniques. Such studies can provide visual evidence of disruptions to microfibrils (Kumar et al., 2009), the creation of pits or holes in the cell wall (Kristensen et al., 2008), the fate of lignin (Kristensen et al., 2008; Donohoe et al., 2008), and the distribution of xylan throughout the cell walls of pretreated materials (Brunecky et al., 2009). However, detailed information regarding the deconstruction of plant biomass and its polysaccharide components remains far from
complete. Such information is essential to rationally design processes that more effectively prepare biomass for the subsequent step of enzymatic hydrolysis while keeping costs low. Optimized pretreatment processes, when combined with improved enzyme mixtures and the use of biomass species with reduced recalcitrance, have the potential to make the production of fuels from lignocellulosic biomass more commercially viable (Lynd et al., 1991).

Beyond the chemical and microscopic techniques mentioned above, tools that can analyze plant cell wall composition and structure remain limited (Somerville et al., 2004). To address this concern, this study employed a novel glycome profiling technique in which cell wall glycan-directed monoclonal antibodies (mAbs) were used to monitor structural/extractability changes in untreated and hydrothermally-pretreated *Populus* biomass. The worldwide collection of plant cell wall glycan-directed mAbs is now sufficiently large that they can be used to monitor cell wall structural changes involving most major classes of plant polysaccharides (Pattathil et al., 2010). The combination of glycome profiling, immunolocalization, along with sugar release data from the enzymatic hydrolysis of untreated and hydrothermally-pretreated *Populus* biomass made it possible to draw correlations between plant cell wall structure and digestibility.
9.3 Results and Discussion

9.3.1 Cell wall changes in hydrothermally-pretreated biomass revealed by glycome profiling

We used three approaches to investigate how poplar cell walls are deconstructed during hydrothermal pretreatments of different lengths (11, 28, and 70 minute): 1) wet chemistry compositional analysis of the untreated and pretreated poplar solids for glucan, xylan, and acid insoluble residue contents; 2) glycome profiling of sequential chemical extracts of the untreated and pretreated biomass samples; and 3) immunolabeling to study the in situ spatial distribution of carbohydrate epitopes in intact untreated and pretreated biomass materials. All three approaches showed dramatic changes in the composition and structure of the biomass during deconstruction by hydrothermal pretreatment, with increasingly long pretreatments yielding greater changes. The wet chemistry compositional analyses provided the most limited information regarding changes, demonstrating that the xylan content decreased with increasing pretreatment time, ranging from 17% in the untreated material to 5% in the 70 minute pretreated material (Figure 9.1). Furthermore, the analyses also demonstrated that there was a corresponding increase in glucan content from 42 to 62%, as well as a slight increase in acid-insoluble residue content (which closely approximates Klason lignin in poplar) from 25 to 33% for the untreated and 70 minute pretreated materials, respectively, reflecting the preferential solubilization of non-glucan biomass components by hydrothermal pretreatment. These results are consistent with typical results under similar conditions (Kristensen et al., 2008).
Figure 9.1. Composition of untreated and hydrothermally-pretreated *Populus trichocarpa* biomass. Glucan, xylan, and acid insoluble residue (Klason lignin) contents were determined as described in Materials and Methods. Tests were performed in triplicate, with the error bars representing the corresponding standard deviations.

Significantly more information on the deconstruction of poplar cell walls by hydrothermal pretreatment was revealed by the glycome profiling and immunolocalization studies. Glycome profiling employs a set of increasingly harsh sequential extractions to solubilize different carbohydrate components from the cell walls, depending on how tightly these components are bound into the walls. For example, extracts released by mild reagents such as oxalate and carbonate tend to be enriched in arabinogalactans and pectic components, while the extracts resulting from the harsher alkaline extractions tend to be enriched in hemicellulosic polysaccharides (xylans and xyloglucans). More tightly bound lignin components of the cell wall materials are removed using a chlorite treatment, and a post chlorite 4M KOH treatment solubilizes additional carbohydrates after removal of lignin. These wall extracts can then be screened with a comprehensive mAb toolkit to define what glycan components are solubilized in each extraction step.
The results (Figure 9.2) demonstrate that the glycome profiles of the hydrothermally-pretreated biomass samples were entirely different from that of the unpretreated biomass, even for the mildest pretreatment condition of 11 minutes at 180°C. Specifically, there were three distinct differences in the glycome profiles between the untreated and mildly pretreated material: 1) a loss of almost all lignin-bound arabinogalactan and xylan epitopes in the chlorite extract, suggesting that these lignin-carbohydrate associations in the wall were particularly labile; 2) a significant reduction in pectic and arabinogalactan epitopes (those recognized by the AG, RG-I/AG and pectic backbone antibody groups); and 3) a slight shift of xylan components from harsher extracts (1 M KOH through 4 M KOH PC) to milder extracts (oxalate and carbonate). However, the amounts of xylan epitopes recognized by the xylan-3 and -4 groups of mAbs remained largely unchanged after the 11 minute pretreatment. The levels of xyloglucan (both fucosylated and non-fucosylated) epitopes also appeared largely unaffected by the mild pretreatment.

Poplar biomass subjected to longer hydrothermal pretreatment times (28 and 70 minutes) showed a further reduction in wall polysaccharide epitopes recognized by the entire mAbs toolkit. In particular, pectic and arabinogalactan epitopes disappeared more completely under the longer pretreatments, suggesting that these epitopes were significantly more sensitive to deconstruction than were hemicellulose epitopes. Furthermore, an increasing loss of xylan epitopes, as well as fucosylated and non-fucosylated xyloglucan epitopes, was also observed in poplar biomass pretreated for 28 and 70 minutes. Nonetheless, significant amounts of xylans and non-fucosylated xyloglucan epitopes remained strongly bound in the cell wall after intermediate
hydrothermal pretreatment, as seen in the 4 M KOH and 4 M KOH PC extracts of this pretreated biomass. For the 70 min pretreated material, while most of the wall polysaccharide epitopes recognized by the antibodies in the toolkit were gone, some xylan and xyloglucan epitopes remained, particularly in the 4M KOH extract.

Figure 9.2(A-D). Glycome profiling of untreated and hydrothermally-pretreated Populus trichocarpa biomass. Sequential extracts were prepared from untreated (A), 11 min pretreated (B), 28 min pretreated (C), and 70 min pretreated (D) biomass. The extracted materials released from each biomass sample by various reagents (as labeled at the bottom of each map) were loaded onto the ELISA plates and were screened against an array of plant glycan-directed monoclonal antibodies. The legend panel on the right of the figure displays the nature of the polysaccharides predominantly recognized by these mAbs. Antibody binding is represented as colored heat maps, with dark blue signifying no binding, white representing intermediate binding, and bright red representing the strongest binding. The bar graphs at the top indicate the amount of material recovered at each extraction step per gram of alcohol insoluble residue (AIR).
9.3.2 Molecular sieve chromatography of wall extracts and immunolabeling of biomass

The ELISA assays carried out for glycome profiling were performed with loading of equal amounts of carbohydrate of each extract onto the plates. However, for many of the extracts from hydrothermally pretreated biomass, there was far lower total signal for many antibodies than was observed for the equivalent extracts from untreated poplar biomass. For example, the chlorite extract of the mildly pretreated poplar contained considerable carbohydrate material, but almost no antibody binding (Figure 9.2). This is in contrast to the untreated material in which the chlorite extract contained significant amounts of both pectic and hemicellulosic epitopes. This observed loss of signal in the ELISAs for pretreated samples could arise for two reasons: 1) a mass removal of epitope structures (resulting from the breaking of bonds during the hydrothermal pretreatment process), or 2) the inability of smaller-sized components that may be produced by hydrothermal pretreatments to adsorb onto the ELISA plate since it is known that small polysaccharides, such as rhamnogalacturonan II (which has a molecular mass of 5-10 kDa (O’Neill et al., 2004)), do not adhere to ELISA plates (Pattathil et al., 2010). The size of the extracted materials (except for the oxalate fractions, where the amounts of material were too low to permit analysis) was examined by molecular sieve chromatography, and the results demonstrated that extracts from the hydrothermally pretreated materials contained shorter polysaccharide chains compared to the equivalent extracts from unpretreated samples. These results suggest that the absence of antibody binding to the extracts from pretreated biomass could largely be explained by the cleavage of the polysaccharides to small fragments. However, they do not exclude the
possibility that the epitopes themselves have been removed or destroyed by the pretreatments.

To test for epitope destruction in situ that might be correlated with the glycome profiling results described in the previous section, immunolabeling of the untreated and pretreated poplar was performed (Figure 9.3) to reveal information on the in-situ spatial distribution of carbohydrate epitopes in intact biomass before and after deconstruction. Accordingly, antibodies selected from diverse antibody groups based on the glycome profiling results, in addition to the carbohydrate-binding module, CBM2a, which binds to crystalline cellulose (Blake et al., 2006), were used for the glycan epitope localizations. With the exception of crystalline cellulose (CBM2a binding) and the xylan epitope recognized by CCRC-M149 (of the xylan-3 group), which showed no appreciable decline as a result of hydrothermal pretreatment, a general decline in wall polysaccharide epitopes recognized by the selected mAbs was observed with increasing pretreatment time (Figure 3). Thus, immunolabeling of many pectic (HG and RG-I) and arabinogalactan (AG) related epitopes declined or disappeared even after the shortest hydrothermal pretreatment, consistent with the results of glycome profiling. Immunolabeling of the fucosylated xyloglucan epitope by CCRC-M1 also declined appreciably after mild pretreatment, while labeling of a non-fucosylated xyloglucan epitope by CCRC-M88 was still evident even after the 70 minute pretreatment. Each of the xylan epitopes examined showed different immunolabeling patterns in the biomass samples. CCRC-M150 (xylan-2 group) exhibited no binding to any of the samples, whereas CCRC-M108 (xylan-1 group) showed a low level of labeling in untreated
biomass that disappeared after mild pretreatment. CCRC-M138 (xylan-4 group) labeled untreated and mildly pretreated biomass strongly, but labeling declined dramatically after 28 and 70 minute pretreatments. Thus, immunolabeling clearly showed that epitopes were lost in the pretreatment process, further supporting the conclusion that the significant changes observed in glycome profiling largely reflected the removal of epitopes during deconstruction by pretreatment.
Figure 9.3. Immunofluorescent labeling of untreated (A), 11 min pretreated (B), 28 min pretreated (C), and 70 min pretreated (D) Populus trichocarpa biomass with selected mAbs representative of different groups of antibodies that recognize distinct epitopes present on various plant cell wall glycans, as indicated at the tops of the images. Sections (250 nm) were taken from biomass samples, and (immuno)labeling was carried out as described in Materials and Methods. Scale bars = 50 µm.
9.3.3 Past approaches and new insights

Detailed studies of cell wall deconstruction caused by pretreatment have heretofore been limited by the availability of tools capable of analyzing plant cell walls. Chemical analyses provided basic compositional data (Figure 9.1), but in general did not supply more detailed compositional information or structural characteristics. However, a substantially increased collection of plant cell wall glycan-directed monoclonal antibodies is now available that is sufficiently large and diverse to monitor changes occurring in most major plant cell wall polysaccharides (Pattathil et al., 2010). A previous study employed four cell wall glycan-directed probes in a Comprehensive Microarray Polymer Profiling (CoMPP) technique (Moller et al., 2007) to examine the effects of hydrothermal pretreatment on wheat straw (Alonso-Simon et al., 2010). The results of the previous study and the current study reported here are largely in concurrence, despite the fact that our study employed different pretreatment conditions with a woody dicot, which has a significantly different structural makeup than wheat straw. To wit, significant reductions in hemicellulose contents were observed in both studies only under the harshest pretreatment conditions. Although the work by Alonso-Simón and coworkers (2010) was an important step toward providing more detailed analysis of cell walls following deconstruction by pretreatment, the study only monitored four glycan epitopes covering three wall polymers. The current study utilized 155 antibodies against a broad diversity of wall glycan structures, with each major wall polymer class being monitored by antibodies against multiple epitopes on each of those polymers (Pattathil et al., 2010). This larger antibody toolkit yielded a more complete
picture of changes that occurred during hydrothermal pretreatment and provided greater insight into the polymers that were attacked most quickly by hydrothermal pretreatment. In the case of the poplar biomass examined here, these were the arabinogalactans and lignin-associated glycans. In addition, we were able to document that not all polysaccharides of a given class were equally affected by the pretreatment. Thus, the changes in the poplar biomass resulting from pretreatment were complex, in keeping with the known complexity of cell walls.

Furthermore, although hydrothermal pretreatment has been previously reported to remove a significant portion of hemicellulose (Kumar and Wyman, 2009; Liu and Wyman 2005), the resolution and scope of biomass monitoring was improved in the current study. Here we found that arabinogalactans of various types were the first wall components that were lost upon pretreatment of poplar, a result that has not been reported previously. On the other hand, hemicelluloses such as xylans and xyloglucans required harsher pretreatments to be removed. Both glycome profiling and immunolabeling further suggested that the fucosyl-containing epitope present on xyloglucans is removed more easily than were non-fucosylated xyloglucan epitopes. Furthermore, some xylan epitopes showed remarkable resistance to the pretreatment.

This study also sheds new light on lignin-carbohydrate associations in poplar, and their fate during deconstruction by hydrothermal pretreatment. Although the lignin-carbohydrate associations observed in the chlorite extractions of this study likely do not represent all such associations that exist in the cell walls of poplar, the results suggest that there are at least two major classes of polymers that are associated with lignin, the xylans
and pectins/arabinogalactans. In fact, arabinogalactans have been previously found to be
removed concurrently with lignin during delignification of lupin by chemical treatments
(Monro et al., 1972; Selvendran et al., 1974), although the nature of these lignin-
carbohydrate complexes still remains largely a mystery. The fate of lignin and its
associations with carbohydrates during pretreatment is equally unclear. Although it has
been previously reported that the morphology of lignin changes as a result of
pretreatment, including an increase in the degree of condensation (Sannigrahi et al.,
2008) and a re-localization (Donohoe et al., 2008), there is no clear-cut consensus on its
fate. Some data (Donohoe et al., 2008) suggest that in dilute acid pretreatment above the
melting temperature of lignin, lignin coalesces within the cell wall, migrates out, and then
re-deposits as droplets on the biomass surface. It remains less clear whether the lignin
droplets stay complexed with carbohydrates during removal and re-deposition. Glycome
profiling results reported here suggest that the lignin-carbohydrate associations that we
were able to monitor, including lignin-pectic/arabinogalactan and some lignin-xylan
associations, were disrupted by even the mildest hydrothermal pretreatment.

Additionally, labeling of the biomass with CBM2a showed that the distribution of
crystalline cellulose did not appear to change between the untreated and pretreated
materials. Some past studies measured slight increases in cellulose crystallinity
following dilute acid pretreatment of biomass (Kumar et al., 2009; Sannigrahi et al.,
2008), while others (Kristensen et al., 2008) reported that hydrothermal pretreatment did
not result in an increase in the degree of cellulose crystallinity. Our immunolabeling
results, although not quantitative, support the latter claim. It is interesting that despite the
significant changes observed in the hemicellulose, pectin, and arabinogalactan structures during deconstruction by hydrothermal pretreatment, there was no apparent disruption to the overall crystalline cellulose structure of the cell wall at any of the pretreatment times tested in this study.

9.3.4 Relating cell wall changes to digestibility

Considerable differences were observed in the glucan digestibility of *Populus trichocarpa* biomass pretreated for various times (Figure 9.4). Overall, glucose yields were lower than expected, which we suspect may be due in part to the drying of pretreated materials prior to enzymatic hydrolysis, since this has been previously reported to negatively affect enzymatic digestibility (Jeoh et al., 2007). Despite this, enzymatic hydrolysis of all of the pretreated materials resulted in increased glucose release for all enzyme loadings when compared to the untreated biomass. The largest increase in glucose yield between temporally adjacent materials (e.g., untreated vs. 11 min pretreated, 11 min vs. 28 min pretreated, or 28 min vs. 70 min pretreated) occurred between the untreated and 11 minute pretreated materials. These results can then be related to the hierarchy of changes that were observed to take place in the cell wall, namely the initial disruption of lignin-arabinogalactan/pectin and some lignin-xylan interactions, which occurred in concert with the loss of arabinogalactans in the 11 minute pretreatment. These changes were associated with an increase in digestibility of up to 24% as compared to the untreated material, depending on enzyme loading. These changes were then followed by the increasing loss of some, but not all, xylans and most
xyloglucans in the 28 and 70 min pretreatments, resulting in further increases in glucose yields upon subsequent enzymatic digestion.

Figure 9.4. Sugar release data from enzymatic hydrolysis of untreated and hydrothermally-pretreated Populus trichocarpa biomass. Poplar biomass samples were digested with cellulase supplemented with xylanase at three enzyme loadings as described in Materials and Methods. Results are expressed as glucose yields for tests performed in triplicate, with error bars representing the corresponding standard deviations.

Besides carbohydrate composition and structure, lignin is also known to play an important role in enzymatic hydrolysis of biomass because it appears to increase nonproductive binding of hydrolyzing enzymes and restrict access of enzymes to cellulose (Kumar and Wyman, 2009; Berlin et al., 2005; Chen and Dixon, 2007; Lloyd and Wyman, 2005; Mansfield et al., 1999). Our results show that glucose yields improved even though lignin removal during hydrothermal pretreatment was minimal (Figure 1), in agreement with previous findings (Kristensen et al., 2008). However, glycome profiling demonstrated that pretreatment altered lignin’s role in the cell wall in terms of its association with pectins, arabinogalactans, and some xylans. Thus, these data
support the concept that it is not lignin content per se that affects recalcitrance. Rather, the integration of lignin and polysaccharides within the cell wall, and their associations with one-another and with other wall components, appear to play a larger role.

9.4 Conclusions

A diverse collection of cell wall glycan-directed monoclonal antibodies can monitor structural/extractability changes in pretreated biomass at greater resolution and scope than was previously possible. Results using these antibodies demonstrated that significant changes occur to the lignin and polysaccharide composition, structure, and integration within the *Populus* cell wall even under mild hydrothermal pretreatment conditions. Interestingly though, not all polysaccharides of a given class respond in the same manner to hydrothermal pretreatment, with some carbohydrate structures being more recalcitrant than others. For the first time, more detailed information is available on cell wall changes that occur during hydrothermal pretreatment which result in improved enzymatic digestibility. Although it is difficult to relate a specific cell wall characteristic to reduced biomass recalcitrance due to the multitude of changes that were observed to occur simultaneously during hydrothermal pretreatment, this information hints as to what structures may or may not contribute to recalcitrance. This not only provides a platform from which more targeted studies can be undertaken to further test the effect of specific cell wall components, it can also aid in the optimization of future pretreatment strategies for improved biofuels production.
9.5 Material and Methods

9.5.1 Plant material

A single genotype of *Populus trichocarpa* grown at Oak Ridge National Laboratory (ORNL) was used in this study. The logs were debarked, split, and then chipped (Yard Machines 10HP, MTD Products Inc., Cleveland, OH). Two forms of the material were tested: 1) knife milled (Model 4 Wiley Mill, Thomas Scientific, Swedesboro, NJ) biomass containing a 20 mesh (<0.85 mm) to 80 mesh (>0.18 mm) particle size fraction and 2) chips taken from a cross section of the same tree’s lateral branch. The chips were approximately 200 x 200 x 40 mm in width, length, and thickness, respectively. Both materials were air dried to a moisture content of around 5%.

9.5.2 Pretreatment

Both forms of *Populus trichocarpa* biomass were subjected to hydrothermal pretreatment as follows. Half-inch (12.7 mm) outer diameter stainless steel tube reactors that were 12.5 inches (317.5 mm) in length were loaded at 5% (w/v) solids concentration with a total reaction mass of 25 g. Both ends of the tubes were closed by stainless steel tube fittings and caps (Swagelok, San Diego Fluids System Technologies, CA) (Lloyd and Wyman, 2005) and heated with condensing steam by placing them horizontally in a custom-built steam chamber (Studer et al., 2010). Pretreatment was performed on both materials at a temperature of 180°C for reaction times of 11, 28, and 70 min, based on previous work performed in the UC Riverside laboratory which showed that xylan
removal during pretreatment peaked between 28 and 70 min for the same *Populus trichocarpa* biomass.

Following pretreatment, the tube reactors were opened, the contents filtered, and the filtrate collected. The resulting solids were washed with 50 mL of DI water and the liquids were frozen.

### 9.5.3 Compositional Analysis

Glucan, xylan, and acid-insoluble residue (which closely approximates Klason lignin) contents were determined for both the untreated and pretreated solids using a downscaled compositional analysis described elsewhere (DeMartini et al., 2011). This analysis is based on conventional wet chemistry techniques to determine biomass composition but is scaled down by a factor of 100.

### 9.5.4 Enzymatic Hydrolysis

Enzymatic hydrolysis was performed in 1.5 mL high performance liquid chromatography (HPLC) vials at 2% (w/v) solids concentration. Air-dried biomass (20 mg), DI water, and a mixture of 1M citric acid buffer (pH 4.95), sodium azide solution (1 g L⁻¹) was dispensed into vials in triplicate to bring the total reaction mass to 1000 mg. Total protein loadings of 20, 60, and 100 mg of enzyme protein per gram of total glucan plus xylan in the untreated poplar (raw BESC poplar had composition of 42% glucan, 17% xylan, and 25% Klason lignin) were applied at a 3:1 ratio of cellulase (Spezyme CP, Lot-Nr. 3016295230) to xylanase (Multifec, Genencore, Palo Alto, CA, Lot-Nr. 261
The cellulase loadings corresponded to 7.5, 22.5, and 37.5 Filter Paper Units (FPU) per gram of total glucan plus xylan in the raw biomass. The vials within the reactor block were then sealed in the same manner as described elsewhere (DeMartini et al., 2011), and the assembly was placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, MD) at 50°C for 72 hours with shaking at 150 rpm.

9.5.5 ELISA Sample Preparation and Analysis

Approximately 250 mg (dry weight) each of untreated and pretreated milled Populus solids (20-80 mesh size) were sequentially washed with absolute ethanol and 100% acetone. The washed residues were then vacuum-dried overnight. The dried biomass samples were subjected to sequential extraction with increasingly harsh reagents in order to isolate fractions enriched in various cell wall components. All extractions were done in 10 mg mL$^{-1}$ suspensions based on the starting dry biomass weight used. First, the biomass was suspended in 50 mM ammonium oxalate (pH 5.0) and incubated overnight with constant mixing at room temperature. After incubation, the mixture was centrifuged at 3400 g for 15 min at room temperature. The resulting supernatant was decanted and saved as the ammonium oxalate fraction, and the pellet was subsequently washed by re-suspension in the same volume of deionized water and centrifuged again as previously described except that the subsequent supernatant was decanted and discarded. Following the same protocol, the pellet was then subjected to additional sequential extractions using in turn 50 mM sodium carbonate (pH 10) containing 0.5% (w/v) sodium borohydride, and 1 M KOH and 4 M KOH, each containing 1% (w/v) sodium borohydride. The pellet
remaining after the final KOH extraction was then treated with sodium chlorite (100 mM) (Ahlgren and Goring, 1971) in order to breakdown lignin polymers into smaller components. Lastly, the pellet left following the sodium chlorite treatment was subjected to a final extraction with 4 M KOH containing 1% (w/v) sodium borohydride to isolate material that had previously been secured within the walls by lignin (4 M KOH PC). The resulting residual pellet was not analyzed any further. The 1M KOH, 4M KOH, and 4M KOH PC extracts were neutralized with glacial acetic acid. All extracts were dialyzed against four changes of DI water (with an approximate sample to water ratio of 1:60) for 48 hours at room temperature and subsequently lyophilized.

After estimating the total sugar contents of the cell wall extracts using the phenol-sulfuric acid method (Dubois et al., 1956; Masuko et al., 2005), the extracts were dissolved in DI water to a concentration of 0.2 mg mL\(^{-1}\). Next, all extracts were diluted to the same sugar concentration of 60 µg sugar mL\(^{-1}\) for loading onto ELISA plates (Costar 3598). Diluted extract (50 µL) was added to each well and allowed to evaporate overnight at 37°C until dry. The ELISAs were conducted as described (Pattathil et al., 2010) using an array of 150 monoclonal antibodies specific to epitopes from most major groups of plant cell wall polysaccharides. Negative controls consisting of water blanks without antigen were included in all assays and their absorbance subtracted from all samples. None of the monoclonal antibodies that were used show backgrounds in the ELISA assays. ELISA data are presented as heat maps in which antibodies are grouped based on a hierarchical clustering analysis of their binding specificities against a diverse set of plant glycans (Pattathil et al., 2010).
9.5.6 Monoclonal antibodies and CBM

CCRC, JIM, and MAC series of monoclonal antibodies used in this study were obtained as hybridoma cell culture supernatants from the Complex Carbohydrate Research Center collection (available through CarboSource Services; http://www.carbosource.net). The xylan-3 and xylan-4 antibody groupings recognize distinct xylan epitopes and will in general be referred to as xylan-directed antibodies throughout the manuscript. Please note that links to detailed descriptions of all antibodies can be found in Table 9.1. The carbohydrate-binding module, CBM2a, which binds to crystalline cellulose (Blake et al., 2006), was obtained from Dr. Harry Gilbert (University of Newcastle, Newcastle upon Tyne, United Kingdom).
Table 9.1. List of all individual glycan-directed monoclonal antibodies (mAbs) applied in glycome profiling. The mAbs are grouped into the polysaccharides that are predominately recognized by the mAbs. Each listing links to a detailed description of each mAb.

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<td>CCRC-M108</td>
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Xylan 2

CCRC-M109
CCRC-M119
CCRC-M115
CCRC-M110
CCRC-M105
CCRC-M117
CCRC-M113
CCRC-M120
CCRC-M118
CCRC-M116
CCRC-M114
CCRC-M154
CCRC-M150

Xylan 3

CCRC-M160
CCRC-M137
CCRC-M152
CCRC-M149
CCRC-M144
CCRC-M146
CCRC-M145
CCRC-M155

Xylan 4

CCRC-M153
CCRC-M151
CCRC-M148
CCRC-M140
CCRC-M139
CCRC-M138

Seed

CCRC-M75
9.5.7 Microscopy

Treated and untreated samples were fixed in 1.6\% (v/v) paraformaldehyde plus 0.2\% (v/v) glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.1) for 2 hours. Samples were washed with the same buffer (3 times, 15 minutes each) and water (2 times, 10 minutes each). Samples were dehydrated through a 35\%, 50\%, 70\%, 95\% (v/v), and 100\% ethanol series for 25 minutes each and gradually infiltrated with LR White resin (1:3 resin:100\% ethanol; 1:1 resin:100\% ethanol; 3:1 resin:100\% ethanol; 3 times resin; each step 24 hours). Samples were placed into gelatin capsules with fresh LR White and polymerized under ultraviolet light at 4°C for 48 hours. Sectioning and immunolocalization with plant glycan-directed monoclonal antibodies were carried out as previously described (Pattathil et al., 2010). The immunolocalization of bound CBM2a required an additional anti-polyhistidine antibody (H-1029; Sigma) and wash step before
applying Alexa Fluor 488-conjugated secondary antibody (goat anti-mouse, A11001, Invitrogen).

9.5.8 Molecular sieve chromatography

The extracted polysaccharides were dissolved in 50 mM ammonium formate (pH 5.0) at a concentration of 5 mg mL\(^{-1}\). A 200 µL aliquot of this solution was run on a Superdex-75 column (GE Healthcare USA) at a flow rate of 0.5 mL min\(^{-1}\) using 50 mM ammonium formate (pH 5.0) buffer as eluent on a Dionex HPLC system (Dionex Ultimate 3000). Various glycan peaks were monitored using a refractive index detector (Shodex RI-101).

9.6 Acknowledgments

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9.7 References


Chapter 10. Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass

* This whole chapter will be submitted under the following citation:
DeMartini JD, Pattathil S, Miller JS, Hahn MG, Wyman CE. “Investigating plant cell wall components that affect biomass recalcitrance in poplar and switchgrass.”
10.1 Abstract

One of the key barriers that must be overcome in order to achieve low cost biological conversion of cellulosic biomass into renewable fuels and chemicals is the recalcitrance of plants to deconstruction by chemical, enzymatic, and/or microbial routes. A deeper understanding of biomass recalcitrance is sorely needed so that specific cell wall chemical and structural features that limit the release of sugars can be identified in different plants. In this study, two phylogenetically different plants, the monocot switchgrass (*Panicum virgatum*) and the woody dicot poplar (*Populus trichocarpa*) were studied. Sets of samples were generated from each via chemical and enzymatic extractions that varied in composition and structure. Differences between poplar and switchgrass samples were identified, and their enzymatic digestibility was tested at two enzyme loadings, including an industrially-relevant low enzyme loading and a significantly higher loading to shed light on purely substrate-related features that limit sugar release. As a result, correlations were drawn between cell wall chemical and structural features that contribute to the recalcitrance of poplar and switchgrass, as well as those that do not appear to play a large role.
10.2 Introduction

Cellulosic biomass is currently the only available resource for large-scale production of renewable fuels, chemicals, and biomaterials (Lynd et al., 1991; Ragauskas et al., 2006). In order to convert cellulosic biomass into any of these products, sugars that are stored as high molecular weight polymers in the plants’ cell walls must first be deconstructed and released into solution as smaller sugar units. The efficient release of sugars at high yields is vital to the production of low cost products. To achieve this goal, one of the key barriers that must be addressed is the recalcitrance of biomass, which refers to the resistance of plant cell walls to deconstruction by chemical, enzymatic, and/or microbial routes. Biomass recalcitrance is likely a multi-scale phenomenon that includes plant structural, molecular, and chemical features (Himmel et al., 2007; Chundawat et al., 2010), and can largely be overcome by pretreatment processes that often include the use of chemicals, and high temperatures and pressures to disrupt the plant cell wall prior to enzymatic hydrolysis (Mosier et al., 2005). Unfortunately, the literature published to date is often conflicting, and as a result, there is no clear picture about what features most strongly limit efficient sugar release. A deeper understanding of biomass recalcitrance is sorely needed so that specific cell wall chemical and structural features that limit the release of sugars can be identified. Only in this way can improved enzymes and pretreatment processes, as well as superior biomass feedstocks be intelligently designed and implemented.

One of the major difficulties in identifying features that contribute to biomass recalcitrance is the complexity of plant cell walls and the lack of high throughput and
reliable tools to analyze them. To address this concern, cell wall glycan-directed monoclonal antibodies (mAbs) are now widely used as efficient probes for studying plant cell wall structure and biosynthesis (Blake et al., 2006; Pattathil et al., 2010). A comprehensive collection of plant cell wall glycan-directed monoclonal antibodies is now available to analyze structural changes occurring in most major carbohydrate components of plant cell walls (Pattathil et al., 2010). Since cell walls account for the majority of plant biomass, these mAbs are instrumental in analyzing cellulosic feedstocks both in situ and in vitro (Avci et al., In press). In a previous study (DeMartini et al., 2011a), we employed a mAbs based Glycome Profiling technique to track the cell wall composition and structure of untreated and pretreated poplar (Populus trichocarpa) biomass. Observed chemical and structural changes were then related to improvements in subsequent enzymatic digestibility to identify features that potentially influence biomass recalcitrance. As stated in the paper, it was difficult to relate a specific cell wall characteristic to reduced biomass recalcitrance due to the multitude of changes that were observed to occur simultaneously during hydrothermal pretreatment (DeMartini et al., 2011a). However, the study provided hints regarding structures that may or may not contribute to biomass recalcitrance, and provided a platform for this current study in which more targeted research was carried out to further probe the effect of specific cell wall components on recalcitrance.

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

10.3 Results and Discussion

10.3.1 Characterization of poplar and switchgrass

Poplar and switchgrass represent two potentially important bioenergy crops in North America that differ incredibly in terms of their anatomy, composition, and structure. To characterize the differences between poplar and switchgrass, the glucan, xylan, and acid insoluble residue (AcIR, which provides an estimate of Klason lignin) contents were determined by wet chemistry compositional analysis. As displayed in Figure 10.1, switchgrass contained less glucan (36.1%) and AcIR (21.0%) than poplar (48.2% glucan, 24.7% AcIR), but contained a higher proportion of xylan (19.4%) than poplar did (15.5%).
Figure 10.1. Composition of poplar (A) and switchgrass (B) samples, including both wild type and extracted materials. Glucan, xylan, and acid insoluble residue (which approximates Klason lignin) contents were determined as described in Materials and Methods. Tests were performed in triplicate, with the error bars representing the corresponding standard deviations.

To provide more detailed information on compositional and structural differences between poplar and switchgrass, Glycome Profiling was applied to both biomass materials. Glycome Profiling employs a set of sequential chemical extractions to solubilize different cell wall components, depending on how tightly these components are bound into the walls. The resulting wall extracts are then screened with a comprehensive mAb toolkit to provide insight as to what glycan components exist in the cell wall, and how tightly they are integrated within it. The Glycome Profiles of poplar and switchgrass shown in Figure 10.2 differ substantially, further demonstrating the differences that exist between the cell wall chemistry and structure of the two species. To highlight this, specific differences in the binding patterns of hemicelluloses such as xylan and xyloglucan antibodies, as well as pectic, arabinogalactan, and β-glucan antibodies will be reviewed. Furthermore, additional differences involving the interactions and integration of lignin within the cell wall will also be evaluated.
Figure 10.2. Glycome Profiling of poplar and switchgrass biomass. Sequential extracts prepared by applying various reagents (as labeled at the bottom of each map) to both biomass materials. Extracts were loaded onto the ELISA plates and screened against an array of plant glycan-directed monoclonal antibodies. The legend panel on the right of the figure displays the nature of the polysaccharides predominantly recognized by these mAbs. Antibody binding is represented as colored heat maps, with black signifying no binding, pink/red representing intermediate binding, and bright yellow representing the strongest binding. The bar graphs at the top indicate the amount of material recovered at each extraction step per gram of alcohol insoluble residue (AIR).

10.3.1.1 Hemicellulose and related lignin associations

Small amounts of xylan-5 and xylan-7 epitopes were released from poplar by the mild oxalate and carbonate treatments, as demonstrated by the high binding intensity but small
amount of cell wall material removed in these particular extractions (red bars above profiles). This suggests that there were subsets of xylan in poplar that were bound very loosely within the cell wall. Conversely, only trace amounts of xylan were removed from switchgrass in the mildest treatments, and significant amounts were not observed until the 1M KOH extraction. As such, in the first alkali extraction (1M KOH), very high levels of xylan epitopes (groups 3-7) were removed from switchgrass, as demonstrated by both the high binding intensity and the large amount of cell wall material removed. In poplar, xylan epitopes (groups 4-7) were also observed in the 1M KOH extract; however, the reduced binding intensity and lower amount of cell wall material removed by this extraction demonstrates that there was not as much semi-loosely bound xylan as there was in switchgrass.

In the 4M KOH treatment, significant amounts of additional xylan were removed from both poplar (groups 4-7) and switchgrass (groups 3-7), with the binding intensity of xylan epitopes again stronger in switchgrass than in poplar. A range of xyloglucans, both fucosylated and non-fucosylated, was also released by the 4M KOH treatment in both poplar and switchgrass. Based on the binding intensity in poplar, xyloglucan comprised a larger fraction of the 4M KOH extract than did xylan. The opposite was true for switchgrass, in which xylan epitopes were present at higher levels than xyloglucan.

The chlorite extraction fragments lignin, and removes it and associated carbohydrates from the cell wall. As such, xylans were the predominant polysaccharide associated with lignin in poplar (xylan 5-7) and switchgrass (xylan 3-7). Based on the mAbs binding intensity, the extent of lignin-polysaccharide associations appears more extensive in
switchgrass than it was in poplar. No xyloglucans were present in the chlorite extract of either poplar or switchgrass, suggesting that this hemicellulose was not associated with lignin. Finally, the 4M KOH post chlorite (PC) extraction was applied to isolate very tightly bound material that had previously been secured within the walls by lignin and associated carbohydrates. As such, a wide range of polysaccharides was released in both poplar and switchgrass, including xyloglucan, xylan, and pectins/arabinogalactans.

10.3.1.2 Pectins, arabinogalactans, β-glucans, and related lignin associations

In addition to hemicellulose, the presence and structure of pectins and arabinogalactans within the cell wall was also evaluated with Glycome Profiling. The majority of pectic and arabinogalactan epitopes were removed by the mild oxalate and carbonate extractions in both poplar and switchgrass, demonstrating that they were fairly loosely held within the cell walls of both plants. Additionally, there were also more tightly bound epitopes that were removed in subsequent KOH and chlorite extractions. The presence of pectic and arabinogalactan epitopes in the chlorite extract suggests that in addition to xylan, they too were associated with lignin in both plant types. Closer comparison between poplar and switchgrass reveals subtle differences between the presence and structure of pectins and arabinogalactans. A wider variety of these epitopes were recognized in poplar than in switchgrass, including those recognizing the homogalacturonan and rhamnogalacturonan backbones of pectin, as well as arabinogalactan. Furthermore, the extractability of different classes of pectins and arabinogalactans varied between poplar and switchgrass.
Another subset of binding that varied between poplar and switchgrass occurred for antibodies recognizing β-glucan. Switchgrass exhibited binding to β-glucan epitopes in all chlorite and KOH extracts, while poplar did not contain any β-glucan.

10.3.1.3 Summary of Glycome Profiles

The profiles revealed that poplar and switchgrass differed significantly in terms of cell wall composition, as well as structure and extractability, which agrees well with past reports on the composition of monocot and dictot cell walls (Vogel, 2008). Glycome Profiling is a semi-quantitative method, so although we cannot determine the exact content of each of these classes of polysaccharides, we can make observations about their relative amounts. As such, xylans were present at much higher levels in switchgrass than they were in poplar. Interestingly though, there were subsets of very loosely-bound xylan in poplar that were not present in switchgrass. Xyloglucan, was also observed in both poplar and switchgrass, but unlike xylan, it comprised a larger fraction of poplar than it did in switchgrass. Glycome Profiling also demonstrated that two subsets of polysaccharides were directly associated with lignin in both poplar and switchgrass: xylan and pectins/arabinogalactans. However, in switchgrass, the degree of lignin-carbohydrate associations was more extensive, particularly with xylan. Finally, the presence of mixed-linked glucans was also observed in switchgrass but not in poplar, which is also in agreement with past reports (Vogel, 2008).
10.3.2 Development and characterization of poplar and switchgrass samples produced by chemical and enzymatic extraction

A total of 12 poplar and switchgrass samples were produced via enzymatic and chemical extractions to create a set of samples that varied in composition and structure. The composition of the resulting samples was analyzed by wet chemistry compositional analysis. Additionally, Glycome Profiling was applied to the soluble extracts to determine which polysaccharides were removed from the cell walls of poplar and switchgrass by each treatment/extraction. As such, endopolygalacturonase and pectin-methylesterase (EPG/PME) were applied to remove pectins and arabinogalactans, whereas a sodium chlorite treatment was applied to remove lignin. Finally, a set of alkali extractions was also conducted with various concentrations of KOH to remove primarily xylans. The reaction mechanism of each of the extractions is summarized in Table 10.1, as well as the primary effects as observed through Figures 10.2 and 10.3.

As displayed in Figure 10.3, the EPG/PME treatment was successful in removing a portion of pectins and arabinogalactans. In poplar, subsets of xylan (groups 4-7) were also removed in concert with pectins and arabinogalactans, whereas in switchgrass, no other epitopes were recognized by the mAbs applied. In the chlorite extract, wet chemistry compositional analyses demonstrated that a large portion of AcIR was removed in poplar (57% lower AcIR content) and switchgrass (53% lower AcIR content) (Figure 10.1). Glycome Profiling further showed that various classes of polysaccharides were removed with lignin in the chlorite treatment. In particular, significant portions of pectins and arabinogalactans were removed from both poplar and switchgrass in concert with lignin. Additionally, xylan (predominantly xylan-5) epitopes were also removed
from the cell wall of poplar by the chlorite treatment, pointing to a high degree of interlinking between lignin, xylan-5, and pectins/arabinogalactans. Conversely, there were very low amounts of xylan-7 released in the chlorite extraction from switchgrass.

The last set of extractions involved KOH treatments to remove large portions of xylans in both poplar and switchgrass, as well as lesser amounts of pectins and arabinogalactans (Figure 10.3).

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<tr>
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<tr>
<td>0.5M KOH/24 hr</td>
</tr>
<tr>
<td>1M KOH/1 hr</td>
</tr>
</tbody>
</table>

1 The KOH solutions contain 0.5-1% (w/v) sodium borohydride to prevent peeling reactions from occurring at the reducing ends of the polysaccharides.
2 All base extracts are immediately adjusted to pH 5 with glacial acetic acid. Some precipitation may occur when the neutralized extracts are dialyzed to remove salts.
Although the extractions performed in this study resulted in the removal of a variety of cell wall components, the use of Glycome Profiling and wet chemistry compositional analysis allowed us to track each of these changes. Furthermore, as discussed in the methods and materials, the extractions used in this study were conducted at room temperature (except the chlorite extraction performed at 70°C) and atmospheric pressure. As compared to the hydrothermal pretreatment study discussed in the introduction that employed high temperature and pressure reactions (DeMartini et al., 2011a), the use of milder reaction conditions likely reduced changes to the composition and ultrastructure of the biomass. This was demonstrated by the wet chemistry compositional analysis of the extracted materials, which was much more similar to that of the corresponding untreated biomass (Figure 10.1) than the untreated versus pretreated materials in the previous study (DeMartini et al., 2011a). Furthermore, this was also reflected in the physical appearance of the resulting materials, which was very similar to that of the raw materials in this current study. The only exception was the chlorite residue that turned white in color. In hydrothermal pretreatments, the resulting materials were substantially darker in color and slightly smaller in particle size.
**Figure 10.3.** Glycome Profiling of poplar and switchgrass samples extracts. Chemical (0.1M KOH/24 hr, 0.25M KOH/24 hr, 0.5M KOH/24 hr, and 1M KOH/1 hr) and enzymatic (EPG/PME) extractions were applied to both biomass materials. In order to characterize which components were removed by these extractions, the resulting extracts were loaded onto the ELISA plates and screened against an array of plant glycan-directed monoclonal antibodies. Antibody binding is represented as colored heat maps, with black signifying no binding, pink/red representing intermediate binding, and bright yellow representing the strongest binding.
10.3.3 Digestibility of poplar and switchgrass samples

Figure 10.4 plots the glucose yield in enzymatic hydrolysis as a function of incubation time for the switchgrass (Figure 10.4A and 10.4B) and poplar (Figure 10.4C and 10.4D) samples. Yields are shown for hydrolysis conducted at both an industrially-relevant low enzyme loading (Figure 10.4A and 10.4C) and a significantly higher loading to shed light on purely substrate-related features that limit sugar release (Figure 10.4B and 10.4D). The glucose yields for the untreated materials were low for both poplar (<11%) and switchgrass (<17%) at both enzyme loadings. Equally low were the glucose yields exhibited by the EPG/PME-treated residues for both poplar and switchgrass. They performed almost identically to the untreated materials. However, besides these residues, the digestibility of all other materials varied greatly, particularly for switchgrass. Significantly higher glucose yields were obtained for the chlorite and KOH-treated residues than for the corresponding untreated material. As such, at low enzyme loading, all of the KOH-treated switchgrass residues achieved incredibly high yields, particularly the 0.25M and 0.5M samples that exhibited final glucose yields of 87 and 89%, respectively. In contrast, the KOH-treated poplar samples only achieved a maximum glucose yield of 40% at low enzyme loading. At high enzyme loading, these glucose yields did increase in poplar: final glucose yields of between 55 and 67% were achieved. However, these yields were still lower than that of the KOH-treated switchgrass samples at both low and high enzyme loadings. In switchgrass, the KOH-treated materials reached glucose yields of theoretical 100% yields with high enzyme loading.
As compared to the KOH treatments, the chlorite residues did not perform as well in switchgrass. At low and high enzyme loadings, the 168-hr glucose yield of the chlorite-extracted switchgrass was 39 and 66%, respectively. In the chlorite-treated poplar, the final glucose yield was 40% at low enzyme loading and 75% at high enzyme loading.

**Figure 10.4.** Enzymatic hydrolysis time profiles of switchgrass (A, B) and poplar residues (C, D), at low enzyme loading (A, C) and high enzyme loading (B, D). The low and high enzyme loadings corresponded to 15+3.2 mg cellulase+β-glucanase per g glucan in the raw biomass, respectively.

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

To date, there have been numerous substrate features proposed to contribute to biomass recalcitrance. Unfortunately, the literature published to date is often conflicting, and as a result, there is no clear picture about what plant features do and do not play a role in limiting sugar release. Additionally, results from studies performed on one plant
type may not translate to others, as supported by this current study. Regardless of plant type however, it seems apparent that recalcitrance is a multi-scale phenomenon that scales several orders of magnitudes (Himmel et al., 2007; Chundawat et al., 2010), and may include plant features ranging from tissue distribution to molecular composition. The complexity and highly cross-linked nature of the cell wall itself likely contributes to biomass recalcitrance (Himmel et al., 2007), particularly the presence and integration of hemicellulose and lignin, both of which have been proposed to limit access of enzymes to cellulose. The removal or reduction of these components has been strongly correlated with improved enzymatic digestibility (Yang and Wyman, 2004; Zhu et al., 2008). For example, studies have demonstrated that plants genetically modified for lower lignin content exhibited higher susceptibility to sugar release by pretreatment and enzymatic hydrolysis (Chen and Dixon, 2007; Xu et al., 2008). To complicate matters though, it has also been reported that there was no correlation between lignin content and sugar release from pretreatment and enzymatic hydrolysis in a large natural population of poplar (Voelker et al., 2010). As opposed to lignin content, there is significantly less literature available that has studied the effects of reduced hemicellulose content via genetic modification and natural variation. In one study, tobacco lines with genetically reduced xylan content did not result in improved cellulose extractability (Bindschedler et al., 2007); however the range of xylan content in the modified lines was somewhat limited (16.8-23.5%), and the extractability was determined by chemical extraction, not by pretreatment and/or enzymatic hydrolysis.
Lignin and hemicellulose content likely do not tell the whole story; the composition of lignin and hemicellulose may also be equally important. For example, extensive hemicellulose branching and substitutions are understood to restrict the ability of enzymes to degrade wall polysaccharides (Correia et al., 2011). Along these lines, the degree of acetylation on the xylan backbone is one such commonly proposed feature. Chemical removal of acetyl groups (Zhu et al., 2008; Kumar et al., 2009), as well as genetic modification of plants for reduced O-acetylation (Pauly and Scheller, 1999; Abramson et al., 2010) have both been demonstrated to increase enzymatic digestibility. However, there have also been other studies suggesting that the impact of acetylation is minimal (Chang and Holtzapple, 2000). In addition to acetylation alone, a study by Mortimer et al. (2010) demonstrated that modified Arabidopsis lines lacking almost all xylan substitutions exhibited improved cell wall extractability. Although these results may not be directly applicable to sugar release by pretreatment and enzymatic hydrolysis, it did suggest that reduced xylan branching may result in cell wall material that is less recalcitrant to deconstruction.

As with hemicellulose, the composition and structure of lignin within the cell wall can also vary substantially, and may influence biomass recalcitrance. Its integration within the wall and associations with other wall components provides significant strength (Albersheim et al., 2011). As a result, the reduction of lignin-carbohydrate associations has been proposed as a mechanism to increase digestibility (Casler, 2008; Buxton and Redfearn, 1997; Jung and Casler, 2006). Likewise, altered lignin composition is also thought to impact a plant’s recalcitrance since its structure and ability to crosslink with
other subunits can vary. However, while some studies have reported correlations between lignin composition (syringyl to guaiacyl, S/G, ratio) and digestibility (Fontaine et al., 2003; Davison et al., 2006; Studer et al., 2011), others found no influence between the two (Reddy et al., 2005; Chen and Dixon, 2007).

Another set of polysaccharides applicable to this study that has been previously proposed to influence biomass recalcitrance is pectins. As such, Lionetti et al. (2010) reported that the genetic reduction of de-methyl-esterified homogalcturonan (HGA) increased enzymatic saccharification efficiency. Unfortunately, there is very little other work that has evaluated the influence of pectin on biomass digestibility, so the effect is unclear.

Based on the results from this current study, there were multiple cell wall components that influenced biomass recalcitrance, as well as some that did not appear to play a role. Furthermore, significant differences were observed between poplar and switchgrass, presumably due in large part to cell wall structural differences between dicots and monocots, respectively. Despite this theme, one finding common to both of the plants tested in this study was that the removal of pectins and arabinogalactans in the EPG/PME residue had no effect on enzymatic digestibility at either enzyme loading. The EPG/PME residue of poplar also had a small portion of xylan-5 removed; however, this did not result in reduced recalcitrance. The amount of cell wall material removed by this extraction was fairly minimal (less than 20 mg/g biomass), which matches with the low pectin content in secondary cell walls, particularly in monocots such as switchgrass (Vogel, 2008). However, pectin has also been reported to have properties that contribute
to limiting the porosity of cell walls, which may be important with respect to enzyme and solvent accessibility (Albersheim et al., 2010).

Besides the EPG/PME residue, all other samples exhibited significantly improved enzymatic digestibility as compared to the untreated material. In switchgrass, the largest jump in glucose yield was associated with the removal of methyl glucuronoxylan, arabinoxylan, and unsubstituted straight chain xylan, as well as some pectins/arabinogalactans that were removed at the same time (Fig. 10.3). Depending on the extraction condition (KOH concentration), and the resulting amount of cell wall material removed, the final glucose yields increased by between 54 and 75% at low enzyme loading and 66 to 84% at high enzyme loading, as compared to the untreated switchgrass. While the removal of methyl glucuronoxylan, arabinoxylan, unsubstituted straight chain xylan, and pectins/arabinogalactans also improved the digestibility of poplar, the effects were not as substantial. As such, the final glucose yields increased by a maximum of 31% at low enzyme loading and 57% at high enzyme loading.

One of the possible causes for the differing effects in poplar and switchgrass is that in general, the same KOH extraction removed more cell wall material from switchgrass than it did from poplar. As a result, the available surface area and porosity may be higher, improving enzyme accessibility. However, when comparing KOH extractions that had similar amounts of mass removal (e.g. 0.1M KOH/24 hr extraction in switchgrass and 1M KOH/1 hr extraction in poplar), the switchgrass residue still exhibited higher glucose yields than did the poplar residue. This suggests that the structure of xylan and its integration within the cell wall was likely different between the two plants in such a way
that its removal from poplar and switchgrass had differing effects on subsequent enzymatic digestibility. It is difficult to relate this observation to the structural makeup of the cell walls of monocots versus dicots because there is no agreed upon model of plant cell walls, particularly secondary walls. Based on primary cell wall models however, it is well known that there are distinct classes of hemicellulose that are integrated differently within different types of plants. For example, in dicot primary cell walls, xyloglucan is the main hemicellulose that interacts and “coats” cellulose, whereas in monocot grasses, xylans (primarily glucurono-arabinoxylan) play this role (Albersheim et al., 2011). Although hemicellulose proportions vary between primary and secondary cell walls (Vogel, 2008), it may be that xylans are the primary hemicellulose that interact with, and coat cellulose in the secondary cell walls of monocot switchgrass, whereas other classes, such as xyloglucan, serve this purpose in poplar. In this case, the removal of xylans from switchgrass would do more to improve enzyme accessibility and resulting glucose yields than it would from poplar. This would also explain why the removal of xyloglucan from switchgrass was not necessary to achieve 90% and 100% yields at low and high enzyme loading, respectively. In poplar on the other hand, the removal of xyloglucan could be necessary to achieve equally high glucose yields if it coats the cellulose surface and blocks enzyme access. The removal of xyloglucan was not tested in this current study.

Another possible reason for the larger improvement observed in the digestibility of the KOH-treated switchgrass over the KOH-treated poplar is the fate of lignin-carbohydrate associations in each. Based on Figure 10.2, lignin-carbohydrate
associations differed between the two plants: switchgrass exhibited a higher degree of lignin-xylan associations than poplar. Additionally, ferulic acid is present at high levels in grasses, and is often esterified or linked through ether bonds to carbohydrates. Furthermore, dehydroferulic acid that is esterified to carbohydrates can be esterified to lignin (Albersheim et al., 2011). Thus, KOH treatments may also reduce the degree of lignin-xylan associations through de-esterification reactions (Table 10.1), which would provide another route towards reducing the strength and recalcitrance of the switchgrass cell wall.

In addition to hemicellulose removal, this study also demonstrated that lignin removal can provide another route toward improving the digestibility of poplar and switchgrass. In both chlorite residues, the AcIR content was about 50% lower than that of the corresponding untreated material (Figure 10.1). In switchgrass, the removal of this fraction of lignin improved glucose yields by about 24 and 50% as compared to the untreated biomass for low and high enzyme loading, respectively. In poplar, glucose yields increased more substantially, by 31 and 65% over the untreated biomass for low and high enzyme loadings, respectively. The amounts of mass removed in the chlorite extractions were very similar between both poplar and switchgrass; however, the removal improved the digestibility of poplar more so than it did in switchgrass. The exact reasoning for this is unknown, but as with the differing effects of hemicellulose removal in the KOH treatments, we believe that it is due to differing cell wall structure between poplar and switchgrass, particularly differing lignin-carbohydrate associations.
10.4 Conclusions

A set of samples varying in composition and structure was generated via chemical and enzymatic extractions applied to two phylogenetically different plants, the monocot switchgrass and the woody dicot poplar. Wet chemistry compositional analysis and cell wall glycan-directed monoclonal antibodies applied in Glycome Profiling allowed us to characterize the resulting samples in order to relate cell wall chemical and structural changes to limitations in sugar release. The results from this study demonstrated that there was significant variability in the cell wall structure and composition of poplar and switchgrass, which presumably caused the differences in recalcitrance to sugar release between the two plants. Although both lignin and hemicellulose influenced the enzymatic digestibility of poplar and switchgrass, the degree of influence varied between them. KOH treatments that removed primarily xylans produced switchgrass that obtained 100% glucose yields in subsequent enzymatic hydrolysis, whereas chlorite treatments to remove lignin had a more beneficial effect in poplar. These varying causes of biomass recalcitrance make it exceedingly difficult to apply knowledge obtained from one plant to another, and also stress the need for improved pretreatments and enzymes individually optimized for different plants.

10.5 Materials and methods

10.5.1 Plant material

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

10.5.2 Generation of poplar and switchgrass samples

Poplar and switchgrass were sequentially washed with absolute ethanol and 100% acetone. The washed residues were then vacuum-dried overnight to produce the Alcohol Insoluble Residue (AIR). All extractions were performed in 10 mg mL⁻¹ suspensions based on the starting dry biomass weight used. For the EPG/PME sample, poplar and switchgrass AIR were incubated with purified type II Aspergillus niger EPG-1 and 2 (~1 units/100 mg AIR from Complex Carbohydrate Research Center, University of Georgia) (Benen et al. 1999) and purified A. niger PME (~1 units/100 mg AIR) in 50 mM Sodium acetate, pH 5.0, for 48 h at 25°C with mixing. The pellet was recovered by centrifugation at 3660g for 20 min and washed three times with sterile water. The supernatants were stored as EPG/PME extract and the washed pellet was recovered as EPG/PME treated residue. For the chlorite extract, 0.5 g of poplar and 1.0 g of switchgrass AIR were each treated with three additions of 0.25 g of sodium chlorite and 100 µL of glacial acetic acid at 70°C. 0.5-1%(w/v) sodium borohydride was included during the extraction to protect...
reducing ends and prevent additional reactions. The resulting extract and residues were recovered as described for the EPG/PME residue. For the KOH extractions, 1.25 g of poplar and 1.5 g of switchgrass were each incubated with 0.1M, 0.25M, and 0.5M KOH for 24 hours at room temperature. At the end of the reaction, all extracts were immediately neutralized with Glacial Acetic Acid to prevent excess modifications. The final KOH extraction involved the incubation of 6.0 g poplar and 8.0 g switchgrass AIR in 1M KOH for 1 hour at room temperature. The resulting extracts and residues from the KOH treatments were recovered as described above.

10.5.2 Glycome Profiling

Glycome Profiling is an ELISA-based method involving the application of plant glycan-directed monoclonal antibodies (mAbs) to cell wall extracts. It is described in detail elsewhere (Pattathil et al., 2010; DeMartini et al., 2011a; Avci et al., In press). Monoclonal antibodies were obtained from the Complex Carbohydrate Research Center collection (available through CarboSource Services; http://www.carbosource.net). A description of the mAbs used in this study can be found in Table 10.2.

10.5.3 Compositional analysis

The composition of all poplar and switchgrass samples was determined using a scaled-down wet chemistry method described in detail elsewhere (DeMartini et al., 2011b). Glucan, xylan, and acid insoluble residue (AcIR) contents were measured. In poplar, AcIR closely approximates the Klason lignin content due to its low ash content
(0.6%), whereas in switchgrass, the AcIR content is slightly higher than the Klason lignin content due to its higher ash content (5.2%). All compositional analyses were performed in triplicate.

10.5.4 Enzymatic hydrolysis

Enzymatic hydrolysis of all poplar and switchgrass samples was performed in a downscaled and high throughput 96-well plate reactor system (Studer et al., 2010; DeMartini et al., 2011c) at 2%(w/v) solids concentration with a total reaction mass of approximately 440 mg prior to the addition of enzyme. As such, 8.80 mg of dry biomass was added to each well by an automated solid and liquid dispensing robotics platform (Core Module, Freeslate Inc., Sunnyvale, CA, USA). Afterwards, 430.6 µl of deionized (DI) water was transferred into all wells using an eight-channel pipettor (30-300 µl; Eppendorf, Hamburg, Germany). After allowing the biomass to soak overnight, a mixture of 1 mol/L citric acid buffer (pH 4.95), sodium azide solution (1 g/L), and diluted enzyme was added to each well (10-100 µl, Eppendorf). Two enzyme loadings were applied, both using a combination of cellulase (Spezyme CP, lot no: 3016295230, 116 mg protein/mL) and β-glucosidase (Novozymes 188, lot no: 037K0968, Novozymes, Franklinton, NC, USA) diluted with DI water to a volume ratio of 2:1. The high enzyme loading corresponded to approximately 120 mg cellulase protein + 25 mg β-glucosidase protein per gram glucan in the biomass, while the low loading corresponded to approximately 15 mg cellulase protein + 3.2 mg β-glucosidase protein per gram glucan in the biomass. Depending on the enzyme loading and biomass, the volume of
enzyme/buffer/sodium azide solution added to each well varied from 28.2 µl to 43.9 µl.

After enzyme addition, the wells were sealed as described elsewhere (Studer et al., 2010), and the reactor was placed on its side in an incubation shaker (Multitron Infors-HT, ATR Biotech, MD) set to 50°C and 150 rpm. Replicate plates were prepared for sampling at different hydrolysis time points, including at 2, 4, 24, 48, 96, and 168 hrs. At the desired sampling time, the corresponding well plate was removed from the shaker, and the slurry from each individual well was transferred to 2.0 mL polypropylene (PP) centrifuge tubes (Safe-Lock 2.0 mL test tubes, Eppendorf). Tubes were centrifuged at 18,200g for 5 minutes (5415 D; Eppendorf), after which 300 µL of hydrolyzate was transferred to HPLC vials for analysis. All enzymatic hydrolysis experiments were performed in triplicate.

10.5.5 Sugar analysis

Sugar concentrations from compositional analysis and enzymatic hydrolysis testing were measured by high performance liquid chromatography (HPLC) (Alliance 2695 equipped with 2414 RI detector; Waters, Milford, MA, USA). An Aminex HPX-87H column (BioRad, Hercules, CA, USA) heated to 65°C was used with 0.005 mol/L sulfuric acid as the eluent at a flow rate of 0.6 mL/min.
**Table 10.2.** List of all individual glycan-directed monoclonal antibodies (mAbs) applied in glycome profiling. The mAbs are grouped into the polysaccharides that are predominately recognized by the mAbs. Each listing links to a detailed description of each mAb.

<p>| Non-Fucosylated Xyloglucan-1 | CCRC-M95       | CCRC-M101      |
| Non-Fucosylated Xyloglucan-2 | CCRC-M104      | CCRC-M89       |
| Non-Fucosylated Xyloglucan-3 | CCRC-M93       | CCRC-M87       |
| Non-Fucosylated Xyloglucan-4 | CCRC-M58       | CCRC-M55       |
| Non-Fucosylated Xyloglucan-5 | CCRC-M54       | CCRC-M51       |
| Non-Fucosylated Xyloglucan-6 | CCRC-M57       |</p>
<table>
<thead>
<tr>
<th>Category</th>
<th>Sample IDs</th>
</tr>
</thead>
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<tr>
<td>Fucosylated Xyloglucan</td>
<td>CCRC-M102, CCRC-M39, CCRC-M106, CCRC-M84, CCRC-M1</td>
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<tr>
<td>Xylan-2</td>
<td>CCRC-M119, CCRC-M115, CCRC-M110, CCRC-M105</td>
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<td>CCRC-M117, CCRC-M113, CCRC-M120, CCRC-M118, CCRC-M116, CCRC-M114</td>
</tr>
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<td>Xylan-4</td>
<td>CCRC-M154, CCRC-M150</td>
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<tr>
<td>Xylan-5</td>
<td>CCRC-M144, CCRC-M146, CCRC-M145, CCRC-M155</td>
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</tbody>
</table>
RG-I/Arabinogalactan
10.6 References


Chapter 11. Cellulosic ethanol and gasoline for 100 years: A comparison of carbon dioxide fluxes associated with production and use of ethanol from cellulosic energy crops grown in different regions of North America to the production and use of gasoline from various fossil fuel feedstocks over 100 years

* The coauthors for this chapter are Heather McKenzie and Qing Qing. All three authors contributed equally to this work.
11.1 Introduction

There is a great deal of concern about the effects of global warming, caused by anthropogenic emissions of greenhouse gases such as carbon dioxide. Consequently, efforts are being made to reduce greenhouse gas emissions. In 2006, direct CO$_2$ emissions from the transportation sector accounted for 34% (Conti and Sweetnam, 2007), the largest single fraction, of the United States’ total emissions. If the emissions associated with petroleum fuel production were included, transportation emissions would account for 38.2% of the 2006 emissions (Conti and Sweetnam, 2007). Historically, gasoline has been produced from conventional petroleum but a combination of “geologic, economic, environmental, [and] political difficulties” (Brandt and Farrell, 2007) will likely result in increased gasoline production from hydrocarbons such as oil sands, oil shale, methane or coal. The use of these feedstocks results in much higher greenhouse gas emissions. Therefore, alternatives to petroleum transportation fuels are vital to significantly reduce overall emissions.

Ethanol produced from cellulosic biomass is one frequently proposed solution. However, questions have been raised about the balance of carbon dioxide sequestered in the biomass and soil and emitted from farming, conversion of biomass to ethanol, distribution and combustion of ethanol. The objective of this paper is to develop detailed carbon fluxes for production and use of ethanol derived from an energy crop grown on a 50 mile radius for 100 years. Mixed prairie grass and poplar were selected as example energy crops. Marginal land and cropland in the United States and Canadian temperate forests were selected as possible locations for an energy crop field and biorefinery,
whereas the creation of an energy crop field in a tropical rainforest is an example of poor land management. In addition, the carbon dioxide flux for the production and consumption of gasoline produced from various hydrocarbon feedstocks was developed for comparison to ethanol. A sensitivity analysis was conducted to verify the models’ robustness.

A possible way to cut soaring carbon dioxide emissions and reduce global warming is sequestration of carbon dioxide. In fact, commercial scale sequestration of carbon dioxide from power stations and in geological formations has already been implemented (Lake, 1989). Other large point sources include oil refineries, petrochemical fertilizer and gas processing plants, steel works and pulp and paper mills (Davison et al., 2001). The primary options for underground CO$_2$ storage are depleted oil and gas reservoirs, deep saline reservoirs and unminable coal seams (Davison et al., 2001). However, the cost of separating CO$_2$ from dilute gas streams such as flue gas from a fossil fuel power plant is considerable using current technology (Kheshgi and Prince, 2005). In the case of ethanol production, major carbon dioxide sources are from fermentation of plant-derived sugars, lignin combustion, and the end-use combustion of ethanol. If the CO$_2$ from fermentation and lignin combustion could be collected and sequestered, the net release of carbon dioxide would become significantly negative. Thus, the impact of sequestering CO$_2$ from fermentation and lignin combustion on carbon dioxide flux from cellulosic ethanol production and use will also be examined in this paper.
11.2 Development of CO₂ Emissions from Ethanol Production

In this paper, ethanol production from poplar or mixed prairie grass, grown in a 50 mile radius of three different types of land over 100 years was studied. The purpose of this study is to provide hypothetical scenarios, whose inputs can be easily adjusted to examine various circumstances. In this particular study, the three types of land studied were degraded land and cropland in the United States, and the temperate forest in Canada. In each case, the annual ethanol production goal was equal to the amount of ethanol that could be produced if the entire 50 mile radius were planted with an energy crop with assumed crop and ethanol yields.

In the case of energy crop production on forested land, it was assumed that native biomass would be harvested and used to produce the entire annual ethanol quota in the first year. The cleared land would then be replanted with energy crops. In the following years, the entire available energy crop is harvested. If the available energy crop is insufficient to meet the annual ethanol quota, then sufficient native biomass will be harvested to make up the difference. Poplar was assumed to be harvested on a 4 year rotation. In the case of production on American marginal and crop lands, mixed grasses are planted on the entire plot in year 1 and then harvested in full each of the following years. For poplar, due to the selected 4-year crop rotation, it was assumed that a quarter of the study plot was planted in each of the first four years and harvested accordingly.

It was assumed that the processing of the cellulosic feedstock to ethanol would yield a lignin residue and that the combustion of this residue would provide process steam and electricity as well as a small amount of electricity to export to the grid. System carbon
dioxide emissions were also determined for the sequestration of carbon dioxide from fermentation and lignin combustion. The factors used to determine CO$_2$ emissions are organized by process blocks in Figure 11.1 and summarized in Table 11.1. Based on Wyman (2004), the density and energy content of ethanol were assumed to be 6.56 lb/gal and 83,957 BTU/gal respectively.

For stream A2, Canadian forest was assumed to no longer be emitting or sequestering CO$_2$, i.e. the land is in carbon equilibrium with environment (Miller et al., 2004). It was also assumed that when existing biomass was cleared the CO$_2$ emissions were due to the loss of soil organic carbon (SOC), and that 60% of the original SOC in Canadian forest is lost due to clearing (Lal, 2004).

Streams A4 and A10 account for carbon dioxide emissions from the production of the bioenergy crop. The overall biomass production emission factors are estimated from the values presented for degraded and fertile lands by Tilman et al. (2006). American cropland and Canadian forest are treated as fertile land. The factors for streams A4 and A10 include emissions from seed production, planting, fertilizer production and application, harvesting, and biomass transportation from the field to refinery. However, Tilman et al.’s (2006) estimate of the emissions associated with farm capital, machinery and the sustaining the farm household are not included in this analysis as the emission factors for gasoline production did not consider these CO$_2$ sources. It was also assumed that biomass production emissions from poplar would be 5 times higher than that from mixed grasses. This assumption was based on Zhu’s (2009) data showing that the size reduction of woody biomass requires between 4-14 times more energy than the size
reduction of grasses. In addition, it was assumed that harvesting woody biomass in the field would require more energy, and therefore have higher emissions compared to grasses. This assumption is based primarily off data presented on the higher energy requirements for size reduction of woody versus herbaceous crops, which suggests that woody biomass requires between 3-13 more energy to process than do grasses (Zhu, 2009). Finally, it is also important to note that the CO$_2$ emissions from farming were based on application of sound agricultural practices such as no till farming.

In stream A6, it was assumed that energy crop yields on degraded land were half of the yields achieved in non-degraded soil (Tilman et al., 2006). For stream A7, the carbon content of poplar and mixed prairie grass were assumed to be 50% and 46% (Lemus and Lal, 2005), respectively. The SOC rate for the bioenergy crops recorded in stream A8 was assumed to stay constant for 50 years (Lal, 2004; Lemus and Lal, 2005) on American degraded land, or until 50% of the SOC lost due to clearing has been regained (Lemus and Lal, 2005), as for Canadian forest. Depending on the biomass crop, this period ranges from 44 to 61 years. For American cropland, it is assumed that the soil can add an additional 4.46 tons C/acre when switched from previous agricultural crops to a perennial bioenergy crop (Ranney and Mann, 1994). This is equal to 9 and 13 years of soil sequestration for poplar and mixed grasses, respectively, with the assumed SOC rates.
Figure 11.1. Illustration of factors used to determine mass flows of CO₂.
Table 11.1. Factors used to determine mass flows of CO₂ for 6 bioethanol cases.

<table>
<thead>
<tr>
<th>Stream</th>
<th>Stream Description</th>
<th>Units</th>
<th>Mixed Prairie Grass</th>
<th>Energy Crop</th>
<th>Biorefinery</th>
</tr>
</thead>
<tbody>
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<td>Existing Biomass</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>Density of Pre-existing Biomass</td>
<td>ton biomass/acre</td>
<td>0</td>
<td>0</td>
<td>35.7³</td>
</tr>
<tr>
<td>A2</td>
<td>Carbon Stored in the Pre-existing Biomass</td>
<td>ton C/acre</td>
<td>0</td>
<td>0</td>
<td>17.8³</td>
</tr>
<tr>
<td>A3</td>
<td>Soil Organic Carbon (SOC) Under Pre-existing Biomass</td>
<td>ton C/acre</td>
<td>N.A.</td>
<td>N.A.</td>
<td>72²</td>
</tr>
<tr>
<td>A4</td>
<td>CO₂ from Harvesting of Biomass</td>
<td>lb CO₂/acre biomass</td>
<td>0</td>
<td>0</td>
<td>795³,⁴</td>
</tr>
<tr>
<td>A5</td>
<td>Ethanol Yield from Pre-existing Biomass</td>
<td>gal/ton biomass</td>
<td>N.A.</td>
<td>N.A.</td>
<td>90</td>
</tr>
<tr>
<td>Energy Crop</td>
<td></td>
<td></td>
<td>3.35³,⁵</td>
<td>6.69⁵</td>
<td>6.69³</td>
</tr>
<tr>
<td>A6</td>
<td>Energy Crop Yield</td>
<td>ton biomass/acre-yr</td>
<td>46⁵</td>
<td>46⁵</td>
<td>46⁵</td>
</tr>
<tr>
<td>A7</td>
<td>Carbon Stored in the Energy Crop</td>
<td>%</td>
<td>0.357⁵</td>
<td>0.357⁵</td>
<td>0.357⁵</td>
</tr>
<tr>
<td>A8</td>
<td>SOC Under Energy Crop</td>
<td>ton C/acre-yr</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>A9</td>
<td>Ethanol Yield from Energy Crop</td>
<td>gal/ton biomass</td>
<td>124³</td>
<td>159³</td>
<td>159³</td>
</tr>
<tr>
<td>A10</td>
<td>CO₂ from Biomass Production</td>
<td>lb CO₂/acre biomass</td>
<td>304⁷,⁸</td>
<td>304⁷,⁸</td>
<td>304⁷,⁸</td>
</tr>
<tr>
<td>Biorefinery</td>
<td></td>
<td></td>
<td>6.27</td>
<td>6.27</td>
<td>6.27</td>
</tr>
<tr>
<td>A11</td>
<td>CO₂ Credit for Displacement of Grid Electricity</td>
<td>lb grid electricity CO₂/ton biomass</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
</tr>
<tr>
<td>A12</td>
<td>CO₂ Emitted from Fermentation</td>
<td>lb CO₂/gal EtOH</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
</tr>
<tr>
<td>A13</td>
<td>CO₂ Emitted from the Combustion of Lignin</td>
<td>lb/ton biomass</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
<td>1565⁷,⁹</td>
</tr>
<tr>
<td>------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Consumer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A14</td>
<td>CO₂ Emitted During Transport of Ethanol to Fueling Station</td>
<td>lb CO₂/lb EtOH</td>
<td>0.0397⁸</td>
<td>0.0397⁸</td>
<td>0.0358⁸</td>
</tr>
<tr>
<td>A15</td>
<td>CO₂ Emitted During Combustion of Ethanol</td>
<td>lb CO₂/lb EtOH</td>
<td>1.91</td>
<td>1.91</td>
<td>1.91</td>
</tr>
</tbody>
</table>

Sequestration

<table>
<thead>
<tr>
<th>Stream</th>
<th>Amount of CO₂ Sequestered by CO₂ Emitted from Compression of CO₂ from fermentation to 60 bars</th>
<th>% of available</th>
<th>80</th>
<th>80</th>
<th>80</th>
<th>80</th>
<th>80</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>A16</td>
<td></td>
<td></td>
<td>0.209¹⁰,¹¹</td>
<td>0.209¹⁰,¹¹</td>
<td>0.209¹⁰,¹¹</td>
<td>0.209¹⁰,¹¹</td>
<td>0.209¹⁰,¹¹</td>
<td>0.209¹⁰,¹¹</td>
</tr>
<tr>
<td>A17</td>
<td></td>
<td>lb CO₂/gal EtOH</td>
<td>2.13E-03 ¹¹</td>
<td>2.13E-03 ¹¹</td>
<td>2.13E-03 ¹¹</td>
<td>2.13E-03 ¹¹</td>
<td>2.13E-03 ¹¹</td>
<td>2.13E-03 ¹¹</td>
</tr>
<tr>
<td>A18</td>
<td></td>
<td>lb CO₂ emitted/ lb CO₂ sequestered</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
</tr>
<tr>
<td>A19</td>
<td></td>
<td>lb CO₂ sequestered</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
<td>2.28E-03⁸</td>
</tr>
</tbody>
</table>

¹ Myeni et al., 2001
² International Boreal Conservation Campaign
³ Tilman et al., 2006
⁴ Zhu, 2009
⁵ Lemus and Lal, 2005
⁶ Miller et al., 2004
⁷ Aden et al., 2002
⁸ Greenhouse Gases (GREET), 1999
⁹ Spatari et al., 2005
¹⁰ DeLuchi, 1991
¹¹ Kheshgi and Prince, 2005
The lignin combustion emissions reported in stream A11 were based on the report by Aden et al. (2002). Based on the carbon balance of Aden et al.’s (2002) report, the lignin combustion emission factor was set so that the total CO$_2$ emissions from lignin combustion represented 46% of the carbon contained in the biomass used. This resulted in emission factors of 1565 lb CO$_2$/ton mixed prairie grass and 1745 lb CO$_2$/ton poplar. It was also assumed based on Aden et al. (2002) that the combustion of lignin produce $6.91 \times 10^5$ BTU electricity/ton dry biomass and 8.92% of this electricity would be available for export to the grid. The export of lignin electricity displaces electricity generated from conventional sources such as coal thus indirectly reducing emissions.

The transportation emissions recorded in stream A14 were based on GREET 1.8. It was assumed that the CO$_2$ emitted from fermentation would be a clean stream which could be easily collected while the CO$_2$ from lignin combustion would be mixed with other gases such as N$_2$, O$_2$, CO, NO$_X$, SO$_2$ and particles. However, current technology for the separation of CO$_2$ from hydrocarbon flue gases provides the ability to capture this carbon dioxide (Conti and Sweetnam, 2007); therefore it was assumed that the CO$_2$ emissions from lignin combustion are collected and separated using similar technology. A collection efficiency of 80% was assumed in stream A16 due to lack of reference data. It was also assumed that the energy required for the separation and compression of CO$_2$ could be supplied by lignin combustion itself. It was assumed that CO$_2$ is compressed to 60 bars (Hendricks et al., 1991) and transported 65 miles (Greenhouse Gases, GREET, 1999). A sensitivity analysis of the capture efficiency and
transport emission factor was conducted to clarify their influences on final total emissions.

11.3 Development of CO₂ Emissions from Gasoline Production

Data on the carbon dioxide emitted during the production and combustion of gasoline from several feedstocks was gathered for comparison to carbon dioxide emissions from bioethanol. Based on GREET 1.8, 96% of American gasoline is produced in American refineries using a feedstock blend that is 2% Canadian oil sands, 6.86% Alaskan crude, 7.84% conventional Canadian and Mexican crude, 34.3% conventional crude from the continental United States, and 49% crude from offshore countries (Greenhouse Gases, GREET, 1999). The balance of American gasoline is imported from Canadian and Caribbean refineries. The production and transportation emissions associated with crude from Middle East were calculated using GREET 1.8 assuming that the crude oil was shipped 8336 nautical miles by tanker to an American refinery.

As discussed previously, gasoline production will likely shift from conventional oil resources to feedstocks such as oil sands, oil shale, methane or coal. Canadian oil sands were selected as the first representative alternative hydrocarbon feedstock due to the current high levels of production and potential importance to the American market. Coal was selected as the second alternative feedstock as it was the largest potential source of liquid fuels reported by Brandt and Farrell (2007). Coal to liquid (CTL) fuel production requires the gasification and reforming of coal to syngas, a mixture of CO and H₂ followed by fuel synthesis by the Fischer-Tropsch processes (Brandt and Farrell, 2007).
The carbon dioxide emissions associated with the recovery of feedstock, refining and transportation are presented in Table 11.2.

Avallone and Baumeister (2008) reported the density and energy content of gasoline as 6.15 lb/gal and 127,654 BTU/gal. The average amount of carbon dioxide emitted from combustion of gasoline is $1.52 \times 10^{-4}$ lb CO$_2$/BTU gasoline (Office of Transportation and Air Quality, 2005).

<table>
<thead>
<tr>
<th>Feedstock</th>
<th>CO$_2$ Emissions from Production and Transportation of Gasoline (lb/BTU)</th>
<th>CO$_2$ Emissions from Production, Transportation, and Combustion of Gasoline (lb/BTU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude from Continental U.S.</td>
<td>4.77E-05$^1$</td>
<td>2.00E-04$^2$</td>
</tr>
<tr>
<td>Crude from the Middle East</td>
<td>4.30 E-05$^1$</td>
<td>1.95E-04$^2$</td>
</tr>
<tr>
<td>Current Feedstock Blend</td>
<td>4.41 E-05$^1$</td>
<td>1.96 E-04$^2$</td>
</tr>
<tr>
<td>Canadian Oil Sands</td>
<td>9.50 E-05$^1$</td>
<td>2.47 E-04$^2$</td>
</tr>
<tr>
<td>Coal to Liquid</td>
<td>2.10E-04$^1$</td>
<td>3.62 E-04$^2$</td>
</tr>
</tbody>
</table>

$^1$ Values listed are based upon the average of those reported by GREET 1.8 (Greenhouse Gases, GREET, 1999) and Brandt and Farrell (2007).

$^2$ Carbon dioxide emissions from the combustion of gasoline (Office of Transportation and Air Quality, 2005)

11.4 Results and Discussion

In order to examine the long term effects of six cellulosic ethanol scenarios, Figure 11.2 shows the annual CO$_2$ emissions normalized with the energy content of ethanol produced. For comparison, emissions from conventionally sourced gasoline are also
shown in lbs CO$_2$ emitted per BTU of gasoline produced, assuming Brandt and Farrell’s (2007) gasoline CO$_2$ emission factor.

Carbon dioxide emissions from ethanol produced in the Canadian forest are shown to be higher than those of gasoline for the first four and five years for poplar and mixed grasses, respectively. Emissions are initially high due to the large amount of pre-existing biomass utilized and the associated release of soil organic carbon from removing pre-existing biomass. As the ratio of pre-existing biomass used to bioenergy crop used decreases, emissions from soil organic carbon will also decrease. Additionally, as more bioenergy crop is planted over time, there will be a larger amount of carbon sequestered in the soil beneath the crops. As a result, we see that as time progresses, CO$_2$ emissions continue to decrease until year 20 for both poplar and mixed grasses. At this point, the soil has regained 50% of the carbon that it lost due to clearing pre-existing biomass, but we assume that the soil will stop sequestering additional carbon. Poplar grown on both U.S. degraded and crop lands exhibit negative carbon dioxide emissions for the first 11 and 23 years, for U.S. cropland and degraded lands, respectively. For both lands, the emissions increase at one point because the soil has become saturated with organic carbon, and soil sequestration will cease. Emissions from ethanol produced from mixed grasses grown on both U.S. degraded and crop lands show similar patterns, except that their curves are shifted up and they exhibit slightly higher emissions over all 100 years.
Figure 11.2. Annual emissions of CO$_2$ per BTU of ethanol produced over 100 years for 6 cellulosic ethanol cases.

Figure 11.3 shows the total 100 year CO$_2$ emissions associated with the production and use of a fuel over the energy content of the total 100 year fuel production for the six bioethanol cases and four gasoline cases. For each land type, the use of poplar as an energy crop results in lower CO$_2$ emissions than mixed grasses. This difference can be attributed to higher soil organic carbon stored for poplar than mixed prairie grasses.

From Table 11.1, it can be seen that poplar’s SOC is about 37% higher than that of mixed prairie grasses. From figure 11.3, it can also be observed that the 100 year emissions of cellulosic ethanol are between 161 and 241 lbs CO$_2$ per MM BTU lower than the 100
year emissions of gasoline from conventional petroleum. However, as stated in the introduction, conventional petroleum will become increasingly difficult to access due to “geologic, economic, environmental, [and] political” (Brandt and Farrell, 2007) factors, therefore alternative transportation fuels will be needed. Sustainably-sourced bioethanol is one option, while gasoline sourced from hydrocarbons such as oil sands and coal to liquids is another. Figure 11.3 clearly shows that regardless of energy crop or type of land utilized, the emissions from bioethanol are much lower than the emissions associated with gasoline from oil sands or coal to liquids.

Figure 11.3. 100 year CO$_2$ emissions normalized to energy content of fuel produced (lb CO$_2$/ MM BTU fuel) for 6 cellulosic ethanol cases and 4 gasoline cases.
Figure 11.4 compares the total carbon dioxide emissions for the six scenarios from this study to that of similar studies by other authors. The study by Tilman et al. (2006) examined carbon dioxide emissions for biofuels derived from low-input high-diversity (LIHD) mixtures of native grassland perennials. This study is seen to be quite comparable to the case of mixed prairie grasses grown on U.S. degraded lands. It should be noted that Tilman et al.'s (2006) study was based on a time frame of 30 years which is considerably shorter than this study’s 100 year period. Figure 11.2 shows that the annual carbon dioxide emissions for mixed prairie grasses planted on degraded lands is very stable over the 100 year period except for a minor shift up during the 21st year which helps to explain why the difference in study period did not greatly affect the comparison.

Figure 11.4. Comparison of 100 year CO₂ emissions normalized for fuel production (lb CO₂/MM BTU) as calculated in current study to other authors.
Geological sequestration of CO$_2$ emitted from ethanol fermentation and lignin combustion was considered to show the possibility of improvement to the full carbon dioxide emission cycle of bioethanol. The CO$_2$ emissions from the separation of CO$_2$ from other gases and compression of CO$_2$ to the preferred transportation pressure were accounted for when determining the effects of sequestration. Additionally, the effects of exporting excess electricity from lignin combustion to the grid were also considered. The consequences of these activities relative to the base cases scenarios are shown in Figure 11.5. Sequestration of 80% of the CO$_2$ from fermentation, 57.3 lbs CO$_2$/MM BTU, results in negative 100 year CO$_2$ emissions per MM BTU for all six bioethanol cases indicating that CO$_2$ is being removed from the atmosphere. Sequestration of CO$_2$ from lignin combustion, assuming 80% collection efficiency, further reduces the 100 year CO$_2$ emissions by 148 to 151 lbs CO$_2$/MM BTU. The exported electricity is assumed to displace electricity generated from fossil fuels or nuclear reactors, which indirectly reduces total carbon dioxide emissions in the proposed scenarios. From Aden et al. (2002) it was determined that the electricity exported to the grid is equivalent to 8.92% of the energy content of the lignin burned; therefore 8.92% of the carbon dioxide emitted from lignin combustion should be associated with the exported electricity. As a result, depending on the cellulosic ethanol case and year, lignin-generated electricity has an emission intensity of 202-212 lb CO$_2$/MM BTU electricity. In comparison, GREET 1.8 (Greenhouse Gases, GREET, 1999) reports that the production of electricity in the United States has an average emission factor of 441 lb CO$_2$/MM BTU electricity. Accounting for this effect further reduces the 100 year emissions from the production and use of
bioethanol as seen in Figure 11.5. The consideration of sequestration and credits for electricity exports reflects the best scenario for cellulosic ethanol fuel production, and speaks to the possible improvements available to reduce carbon dioxide emissions. To test both the robustness of our model and the sensitivity of results to the assumed input values, a sensitivity analysis was performed in Section 11.5.

Figure 11.5. 100 year CO\(_2\) emissions from cellulosic ethanol normalized for fuel production (lb CO\(_2\)/MM BTU fuel) implementing geologic sequestration.

### 11.5 Sensitivity analysis

To test both the robustness of our model as well as the sensitivity of results to the assumed input values, a sensitivity analysis was performed. The x-axis of all sensitivity plots shows the relative percent change in the input value, while the y-axis is the relative percent change in the 100-year total CO\(_2\) emissions from ethanol, minus the total 100-year total CO\(_2\) emissions avoided from replacing gasoline, all divided by the 100-year
total of BTU energy produced. Thus, any value that lies above the x-axis represents a decrease in CO$_2$ emissions for EtOH as compared to gasoline, while any value that lies below the x-axis represents an increase in CO$_2$ emissions for EtOH as compared to gasoline.

Figure 11.6. Sensitivity analysis for mixed grass grown in the Canadian forest.

Figure 11.7. Sensitivity analysis for poplar grown in the Canadian forest.
Figure 11.8. Sensitivity analysis for mixed grass grown on American degraded land.

Figure 11.9. Sensitivity for poplar grown on American degraded land.
Figure 11.10. Sensitivity analysis for mixed grass grown on American cropland.

Figure 11.11. Sensitivity analysis for poplar grown on American cropland.
By examining Figures 11.6 through 11.11, it can be observed that for all scenarios, the carbon dioxide emissions are most sensitive to the ethanol yield from the bioenergy crop and the lignin combustion emission factor. When both inputs increase, the resulting CO$_2$ emissions for ethanol also increase. Larger emissions associated with an increasing lignin combustion emission factor are intuitive. Less apparent however is why ethanol CO$_2$ emissions on a per BTU basis from ethanol increase with a higher ethanol yield. When the ethanol yield from bioenergy crop is changed, four individual CO$_2$ flux outputs change in terms of 100 year CO$_2$ flux per BTU. These four outputs include the following: CO$_2$ absorbed by biomass, excluding soil; CO$_2$ absorbed by bioenergy crop SOC; CO$_2$ released from harvest; and CO$_2$ released from lignin combustion. All four of these outputs increase with decreasing ethanol yield from bioenergy crop; however, the first two represent CO$_2$ sequestration, so they result in decreasing overall emissions for ethanol, while the latter two are in the form of emissions, and thus increase overall emissions. Total 100 year process CO$_2$ emissions decrease with decreasing ethanol yield from bioenergy crop because the two emission fluxes CO$_2$ released from harvest and CO$_2$ released from lignin combustion, change more strongly than the two sequestration fluxes CO$_2$ absorbed by biomass, excluding soil and CO$_2$ absorbed by bioenergy crop SOC.

A sensitivity analysis was also performed to examine all the inputs used for geological sequestration of ethanol fermentation and lignin combustion CO$_2$. Grasses grown in a Canadian forest are shown in Figure 11.12 as a representative scenario since all scenarios display similar trends when varying the inputs. By examining all of the sequestration related inputs, the collection efficiency is found to be the most dominant
factor that affects the final output, as shown in Figure 11.12, while change to the other inputs has negligible impact on the output.

![Graph showing sensitivity analysis of CO₂ sequestration inputs for grasses grown in a Canadian forest.](image)

Figure 11.12. Sensitivity analysis of CO₂ sequestration inputs for grasses grown in a Canadian forest.

### 11.6 Conclusions

Because there is a need for a sustainable and lasting solution to petroleum usage, the long term effects of ethanol must be considered. To this end, this study was developed to examine the carbon dioxide emissions associated with producing ethanol from poplar or mixed grasses grown on American degraded land or crop land, or in Canadian temperate forest for 100 years. Key to the development of this study was the assumption that cellulosic ethanol would be produced intelligently through the successful utilization of pre-existing biomass, the adoption of good farming practices, and energy efficient biorefinery design. To provide perspective, the emissions were compared to the carbon dioxide emissions from gasoline production from various petroleum sources.
For poplar production on both American land types, carbon dioxide was sequestered each year during the 100 year period, while the growth of mixed grasses on these lands resulted in some years of net sequestration and some years of net emissions within the study period. Replacing the natural vegetation of the Canadian forest with energy crops resulted in carbon dioxide emissions higher than those from gasoline for approximately the first five years after energy crop establishment. However after this point, ethanol emissions decreased below gasoline emissions until the environment was sequestering carbon dioxide each year.

When comparing among the 6 different cellulosic ethanol scenarios, it was shown that poplar results in lower CO$_2$ emissions than for grasses grown on the same land type. It was shown that over 100 years, with the exception of the production of mixed grasses on American cropland or in Canadian forests, the ethanol systems have negative CO$_2$ emissions. The growth of poplar on both types of American land result in highly negative emissions indicating that both environments would act as strong carbon sinks. It has also been shown that emissions from all 6 ethanol scenarios can be further decreased if sequestration of CO$_2$ from fermentation and from lignin combustion were employed. For all ethanol production cases, carbon dioxide emissions over a 100 year period are significantly lower than emissions from the production and use of gasoline from conventional crude oil and alternative fossil fuels such as coal.

For both environmental and political reasons, there is an urgent need to seek alternatives to gasoline usage. This study has shown that ethanol can be an effective alternative to gasoline from the perspective of reducing carbon dioxide emissions. When
ethanol is produced on domestic degraded or crop lands, or in Canadian forest, it has
either low or negative CO₂ emissions over a 100 year period. However, these benefits
will take time to accrue, so the sooner the technology is implemented, the sooner we will
see positive results.

11.7 References

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Chapter 12. Overview and Significance of Major Findings
12.1 Summary of key developments and findings

The main motivation for this thesis was to develop a better understanding of biomass recalcitrance so that future pretreatment strategies, enzymes, and modified plants can be more intelligently designed to improve biological deconstruction. A significant amount of research has been directed at gaining a better understanding of why the hydrolysis of native lignocellulosic biomass is slow and incomplete, but the answers remain elusive. Unfortunately, the literature published to date on biomass recalcitrance is often conflicting, and as a result, there is no clear picture about what plant characteristics do and do not play a role. Due to this limited understanding, it is difficult to rationally select superior plant species, devise low cost pretreatments, or select enzymes for sugar deconstruction without prior sugar release testing.

To address this limitation, this thesis first focused on developing downscaled and high throughput (HT) tools that have revolutionized biomass analysis. As a result, measuring biomass composition, as well as sugar release from pretreatment and enzymatic hydrolysis now require significantly less materials than conventional methods and can be run at much greater throughput. This opened the door to an entirely new set of research in both this thesis and outside studies that was previously not possible.

In addition to enabling the analysis of small amounts of materials, the HT systems facilitated screening of larger samples sets than was previously feasible. Using conventional approaches, evaluating many biomass samples for sugar release from pretreatment and enzymatic hydrolysis is very time- and energy-intensive, which limited the number of biomass samples that could be analyzed. However, in this thesis, a large
population of natural *Populus* variants was screened in combination with a variety of pretreatment conditions. As a result, it was discovered that lignin content correlates negatively with glucose release in poplar only for samples with certain lignin compositions (low syringyl to guaiacyl, S/G, ratio).

Due to the small amounts of material available, the downscaled and HT systems were also perfectly suited to study the ring-by-ring composition and sugar release patterns within Aspen trees. In this study, we found that although there was significant within tree radial variation in composition and sugar mass release, the digestibility remained almost constant, suggesting that wood maturity does not influence recalcitrance. In a similar study, prairie grasses and legumes were fractionated into their anatomical components and analyzed with the HT tools developed in this thesis. As such, significant variation was found in the composition and sugar release of the different species and their components, and anatomical composition was identified as a key factor in both grass and legume recalcitrance. A third study that was also made possible by the development of the downscaled HT systems was the analysis of compositional and digestibility variation within a single pretreated wood chip. It was discovered that effective hydrothermal steam pretreatment was limited in industrially-sized wood chips at short reaction times due in part to the inability of the chip to reach the target temperature uniformly throughout its thickness.

HT screening studies in this thesis provided key pieces of information about the recalcitrance of a wide variety of biomass species. Furthermore, biomass outliers and trends in sugar release behavior were identified. However, another central goal of this
thesis was to focus in on select biomass species, namely poplar and switchgrass, and
conduct more detailed analyses in order to gain insight into what specific cell wall
components may contribute to biomass recalcitrance and how these recalcitrant features
are overcome with pretreatment processes. As such, by studying the detailed effects of
pretreatment-induced deconstruction in Chapter 9, it was discovered that there is a
sequence of structural changes that occur in the plant cell walls of poplar. There is an
initial disruption of lignin-polysaccharide interactions in concert with a loss of pectins
and arabinogalactans. This is followed by significant removal of xylans and xyloglucans,
with certain fractions of each polysaccharide being more labile than others. These
observations significantly improved the resolution and scope of our understanding of
pretreatment processes, and how they overcome biomass recalcitrance. Using these
results, additional studies were undertaken in Chapter 10 to evaluate the effect of more
specific and targeted cell wall changes in both poplar and switchgrass. Significant
differences were observed between the two species, presumably due in large part to cell
wall structural differences between dicots and monocots. Whereas the removal of certain
classes of polysaccharides reduced the recalcitrance of switchgrass, their effect was less
beneficial in poplar.

12.2 The causes of recalcitrance are diverse

The results from this thesis fully support the hypothesis that recalcitrance is a multi-
scale phenomenon (Himmel et al., 2007; Chundawat et al., 2010). Plant anatomical
composition, which dictates tissue presence and distribution, as well as cell type, size,
and cell wall thickness, was demonstrated to control the recalcitrance of grasses and legumes. Within the cell wall, specific chemistries and structures, including those of both hemicellulose and lignin, were also found to directly impact the digestibility of poplar and switchgrass. The causes of recalcitrance identified in this thesis were diverse, which matches well with diversity of the plants studied and the complexity of their cell walls.

Although significant research is ongoing in the field of biological conversion of biomass to find a single factor that controls the recalcitrance of plants, this thesis suggests that this is unlikely. Instead, these results demonstrate that a multitude of interrelated factors contribute to recalcitrance, with some being more important than others. Furthermore, factors that are important in one plant may not play the same role in another, as demonstrated in Chapter 10. This is due to the incredible diversity in plant cell wall chemistry and structure, as exemplified by the Glycome Profiles of poplar and switchgrass in Figure 10.1, in which the interactions of different cell wall components, as well as their resulting integration within the cell wall differed substantially. As a result, we must be careful in generalizing the causes of recalcitrance for all biomass feedstocks. Consequently, more time and effort must be invested to evaluate species or subsets of species individually. Pretreatment processes and enzyme cocktails will likely have to be tailored to batches of feedstock, dependent not only on species type, but also anatomical fraction, harvest time, and possibly other factors.

In the past, lignin and hemicellulose contents were two features that were commonly proposed to govern the enzymatic digestibility of a wide range of plants, regardless of species, anatomical fraction, or harvest time. While the removal of various cell wall
components, including fractions of both lignin and hemicellulose, have been shown to improve enzymatic digestibility, the above statement is likely too broad. Work from this thesis clearly demonstrates that not all polysaccharides of a given class respond in the same manner to deconstruction, with some being more recalcitrant than others. For example, fucosylated xyloglucan was removed more easily by hydrothermal pretreatment of poplar than non-fucosylated xyloglucan. Observations such as these can often be related to the cell wall structure of the plant in question. As such, it has been reported that hemicellulose that is less substituted, such as non-fucosylated xyloglucan, has greater binding potential with cellulose (Albersheiem et al., 2011). As a result, it may be that the closer interaction between non-fucosylated xyloglucan and cellulose imparts added strength and is more difficult to remove by pretreatment and enzymatic hydrolysis than fucosylated xyloglucan. Thus, it is important to recognize that hemicellulose and lignin content as a whole do not tell the entire story.

To test this hypothesis, Figure 12.1 plots the glucose yield from enzymatic hydrolysis versus acid insoluble residue (AcIR) (Figure 12.1A) and xylan (Figure 12.1B) content for over 130 samples tested throughout the course of this thesis. Although the enzymatic hydrolysis conditions, as well as the treatment methods to prepare the biomass prior to enzymatic hydrolysis, varied widely, it is still interesting to note that there are no clear trends within or among all species to suggest that AcIR or xylan content are factors that directly control the recalcitrance of all biomass samples. Instead of hemicellulose or lignin content alone, the composition, structure, and the integration of these individual
components within the cell wall likely provides a much more detailed and accurate account.

![Figure 12.1](image)

**Figure 12.1.** Glucose yields from enzymatic hydrolysis versus AcIR content (A) and xylan content (B). Data is shown for the following: pretreated mixed prairie species (MPS), including the legume *L. perennis*, the C4 grass *S. scoparium*, and the C3 grass *P. Pratensis* (Chapter 8, 72 hour incubation); pretreated *P. tremuloides* wood chip sections and milled materials (Chapter 5; 168 hour incubation); pretreated annual rings from a 28-year old *P. tremuloides* cross section (Chapter 6; 72 hour incubation); *P. trichocarpa* and *P. virgatum* samples produced by enzymatic and chemical extraction at both low and high enzyme loading (Chapter 10, 168 hour incubation).

### 12.3 Recalcitrance in poplar and switchgrass

As discussed above, this thesis cannot identify single cell wall features that cause recalcitrance in all species; however, the detailed studies conducted in Chapters 9 and 10 provide insights into factors that influence (and do not influence) the recalcitrance of poplar and switchgrass. In Chapter 9 for example, it was demonstrated that the recalcitrance of poplar could be overcome without the removal of lignin and/or hemicellulose. For the first time, the collective changes of pectin/arabinogalactan
removal, xylan loosening, and lignin-polysaccharide disruption, was found to play a large
role in the preliminary stages of overcoming biomass recalcitrance. However, it was also
demonstrated that the removal of xylans and xyloglucans was required to further improve
enzymatic digestibility to approach theoretical 100% glucose yields.

In Chapter 10, more targeted studies provided further insight into these observations.
For example, to follow up on the possible importance of pectins and arabinogalactans,
enzymatic removal of these components was performed in both poplar and switchgrass.
The resulting digestibility of these samples did not result in improved digestibility,
suggesting that pectins and arabinogalactans do not significantly contribute to the
recalcitrance of either species despite their reported role in regulating the pore size of cell
walls (Albersheim et al., 2011). Additionally, to further probe the influence of lignin,
chemical extractions were performed to remove approximately 50% of the lignin present
in poplar and switchgrass. As such, it was discovered that the removal of lignin had more
beneficial effects on the final digestibility of poplar (75% glucose yield) than it did on
switchgrass (66% glucose yield). The exact cause of this is unclear, but lignin
composition (ratio of monomer units) and content vary significantly between monocots
and dicots, which likely results in differences in cell wall integration and influence of
lignin on the recalcitrance of the two tested species. And as demonstrated in Chapter 7,
lignin composition and resulting cell wall integration can be strongly correlated with
sugar yields. In addition to lignin, the removal of methyl glucuronoxylan, arabinoxylan,
and unsubstituted straight chain xylan also had a significant effect on enzymatic
digestibility. The removal of these components improved the glucose yields by 67% in
poplar and 100% in switchgrass. Removal of lignin and xyloglucan was not required to
produce these highly digestible materials. Xylans account for a larger fraction of
hemicellulose in switchgrass than they do in poplar (Vogel, 2008). As a result, cellulose
may be more highly coated by xylans in the cell wall of switchgrass than it is in the cell
wall of poplar. This potential difference may explain why the removal of xylans in
switchgrass resulted in glucose yields that were 40% higher than those in poplar.

In summary, the removal of lignin and classes of hemicellulose had differing effects
on the enzymatic digestibility of poplar and switchgrass. In poplar, lignin was the most
significant contributor to recalcitrance, while in switchgrass, methyl glucuronoxylan,
arabinoxylan, and unsubstituted straight chain xylan were the most important factors. To
further prove or disprove the above findings, more studies involving the genetic
modification of poplar and switchgrass are needed to test the effect of single cell wall
alterations. Although there has been significant progress in the genetic modification of
lignin to alter both its content and composition, the same cannot be said about the diverse
classes of hemicellulose. While lignin content may be more important in plants that
contain higher levels of lignins, such as poplar, based on this work, subsets of
hemicellulose were identified as key recalcitrant features, particularly in switchgrass due
to their presumed role in blocking the access of enzymes to cellulose. Thus, these
findings further support the need for more genetic modification studies involving
hemicellulose.
12.4 Closing remarks

Based on the research conducted in this thesis, it can be concluded that the causes of biomass recalcitrance are numerous and diverse. Significant variation in composition and enzymatic digestibility can even exist within a single plant due to the distribution and properties of different tissue and cell types (Chapters 6 and 8). Furthermore, the diversity within a single species is equally large due to the variation present in natural populations (Chapter 7). In hardwoods such as poplar, lignin content, composition, and integration with other cell wall components was demonstrated to have a strong influence on enzymatic digestibility (Chapters 7, 9 and 10), whereas in switchgrass, the presence and structure of xylans controlled glucose yields (Chapter 10). Regardless of biomass species, it is likely safe to assume that not only the presence and/or composition of cell wall components affects biomass recalcitrance, rather the interactions between cell wall components, as well as their integration within the wall likely is equally important.

Based on the work in this thesis, Figure 12.2 was developed as a simplified illustration of the secondary cell wall. The three main components of the cell wall, cellulose, hemicellulose and lignin are shown, as well as the two main interactions between them. As reviewed in Chapter 2, hemicellulose is bound to cellulose through hydrogen bonding, whereas lignin is more closely associated with hemicellulose via covalent bonds. Although both hemicellulose and lignin limit the porosity and available surface area, a fraction of hemicellulose coats the cellulose surface to an unknown degree, and serves to directly block access of enzymes to cellulose. Thus, the breaking of hydrogen bonds that connect cellulose and hemicellulose will promote the removal of impeding hemicellulose
groups (likely xylan in grasses and xyloglucan and xylan in woody dicots). Additionally, these bonds are weaker than the covalent linkages between hemicellulose and lignin; thus the breaking of hydrogen bonds to remove hemicellulose may also promote the removal or restructuring of lignin in a way that also promotes enzymatic digestibility.

Figure 12.2. Simplified model of the plant secondary cell wall, including cellulose microfibrils, hemicellulose, and lignin. Two types of bonds are shown, including hydrogen bonds between cellulose and hemicellulose (Type 1), as well as the covalent bonds between hemicellulose and lignin (Type 2). Additionally, a water molecule (0.28 nm diameter) and the catalytic core of a celllobiohydrolase enzyme from *Trichoderma reesei* (5 x 6 x 4 nm, Mansfield et al., 1999) are also shown to demonstrate the approximate relative dimensions between the different wall components.

Although the diverse and interconnected causes complicate our ability to fully understand biomass recalcitrance, it also provides a variety of routes for overcoming limitations to sugar release. As demonstrated in this thesis, the design of pretreatment strategies to remove specific cell wall components that most severely inhibit enzymatic digestibility, as well as the development of improved enzymes that contain the proper
components and activities to break down the recalcitrant structures remaining after pretreatment are key routes toward overcoming biomass recalcitrance. Alternatively, this thesis also demonstrated that the selection of harvest age for short rotation woody trees will impact the amount of sugar produced, whereas in mixed prairie species, the time of year in which the crop is harvested will affect both composition and recalcitrance to sugar release. Finally, based on the identification of recalcitrant structures and chemistries, this thesis also provides plant engineers with targets for genetic modification of plants that have a strong likelihood of reducing biomass recalcitrance. All of these methods provide possible routes to overcoming biomass recalcitrance and realizing the high sugar yields that are crucial for achieving a commercially-viable industry based on the use of cellulosic materials for the production of renewable fuels and chemicals.

12.5 References


