The Effect of Biological and Chemical Pretreatments during Storage on Corn Stover Physiochemical Properties and Reactivity

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Presented in Partial Fulfillment of the Requirements for the
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Lynn M. Wendt

Major Professor: Haiyan Zhao, Ph.D.
Committee Members: John Russell, Ph.D.; Seth Snyder, Ph.D.; Vivek Utgikar, Ph.D.
Department Administrator: Lee Vierling, Ph.D.

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Authorization to Submit Dissertation

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Major Professor: _____ Date: _____
Haiyan Zhao, Ph.D.

Committee Members: _____ Date: _____
John Russell, Ph.D.

_____ Date: _____
Seth Snyder, Ph.D.

_____ Date: _____
Vivek Utgikar, Ph.D.

Department Administrator: _____ Date: _____
Lee Vierling, Ph.D.

Abstract

Corn stover is one of the primary agricultural residues available for bioenergy production, but its cellular and tissue level complexity make it challenging to reduce to monomers that can then be converted to fuel and chemical precursors. The goal of this research is to improve the performance of corn stover for biochemical conversion to fuels and chemicals by reducing recalcitrance to deconstruction. The overarching aim of this research is to overcome the physiochemical barriers in corn stover that necessitate increased severity in conversion in terms of chemical loading, temperature, and time, during the residence time of long- and short-term storage operations. The hypothesis of this research is that low severity chemical and microbial treatment during long-term storage will reduce the degree of polymerization through saponification of ester-linked side chains or glycosidic bonds in hemicellulose or through oxidation of phenolic or non-phenolic components of lignin. These treatments will increase extractable components of corn stover, facilitate increased chemical impregnation, porosity, and solubilization of structural components, and lead to increased reactivity during downstream pretreatment. This novel approach focuses on moving recalcitrance reduction upstream in the feedstock supply chain, thus this passive operation that only preserves biomass will become an active environment that can positively impact conversion performance. Biological and chemical treatments applied during storage, one of the key unit operations in the feedstock logistics supply chain, were explored in this study with the goal of integrating these treatments into bioenergy logistics and conversion systems. Meanwhile, the mechanistic understanding of the biological and chemical reactions that can reduce biomass recalcitrance was obtained. The fundamental understanding on how these reactions change the biomass structure provided the scientific community with insight that can lead to new areas of exploration in bioenergy conversion.

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Dedication

This dissertation would not have been possible without unwavering support from my husband, Daniel Wendt, who has supported me unconditionally in every life endeavor. I am grateful for the love and encouragement from my sons Alexander and William and hope that this experience teaches them to excel in education and life experiences. Further, I am thankful for my network of core family, including Krista, Ryan, Bill, Wendy, David, Olivia, Emily, Lilli, Mary, and James.

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Statement of Contribution

This dissertation represents the research of the author, Lynn M. Wendt. Several collaborators contributed respective areas of subject matter expertise to this effort to further the mechanistic-level understanding of the physiochemical impacts of biological and chemical treatments during storage of corn stover. The following statements describe these contributions. Numerous scientists and engineers in the Energy and Environment Science and Technology Directorate at Idaho National Laboratory contributed to this research. Dr. Bradley Wahlen advised on experimental design, implementation, and results interpretation for chapters 2, 3, 4, and 5. Michelle Walton performed experimental support for sample preparation and compositional- and conversion-related performance assessments described in chapters 2, 3, 4, and 5. Rebecca Brown provided expertise in microscopy highlighted in chapters 2 and 4. Dr. Corey Pilgrim provided subject matter expertise in nuclear magnetic resonance experimentation. Drs. Gary Groenewold and Brittany Hodges contributed expertise in analytical pyrolysis described in chapters 3 and 6. Austin Murphy and William Smith designed and operated the aerobic storage experiments described in chapter 3. Dr. Yingqian Li provided techno-economic assessment described in chapters 2 and 4. Jason Nguyen executed storage experimentation detailed in chapters 2 and 4. Additional contributions on surface area and surface energy described in Chapter 5 were received from Drs. Troy Semelsberger and Juan Leal of Los Alamos National Laboratory. Overall guidance on the dissertation content was provided by my advisor Dr. Haiyan Zhao at the University of Idaho.

Chapter 1: Review on Bioenergy Storage Systems for Preserving and Improving Feedstock Value

"Review on Bioenergy Storage Systems for Preserving and Improving Feedstock Value." *Frontiers in Bioengineering and Biotechnology,* vol. 8, article 370, 2020, DOI: 10.3389/fbioe.2020.00370.

Abstract

Long-term storage is a necessary unit operation in the biomass feedstock logistics supply chain, enabling biorefineries to run year-round despite daily, monthly, and seasonal variations in feedstock availability. At a minimum, effective storage approaches must preserve biomass. Uncontrolled loss of biomass due to microbial degradation is common when storage conditions are not optimized. This can lead to physical and mechanical challenges with biomass handling, size reduction, preprocessing, and ultimately conversion. This review summarizes the unit operations of dry and wet storage and how they may contribute to preserving or even improving feedstock value for biorefineries.

Introduction

The utilization of renewable biomass feedstocks for fuel and energy production offers the potential to displace a significant portion of petroleum-based transportation fuels and related greenhouse gas emissions. The transportation sector utilizes one third of all energy and 70% of all petroleum consumed in the United States [1]. Electrification of the grid with renewable energy sources, such as wind and solar power, will contribute to reducing carbon-based fuels in the light-duty vehicle fleet. However, the need for sustainably-produced, liquid transportation fuels will remain since aviation fuel use is projected to double in the next 20 years [2] and heavy-duty vehicles and marine vessels will likely require carbon-based fuels [3]. Furthermore, bio-derived fuel and chemical production can result in the carbon negative technologies that are necessary to counteract the global warming of 1.5°C above pre-industrial levels [4].

Renewable biomass feedstocks include non-food material such as corn stover, herbaceous and woody energy crops, forest product residues, algae, and municipal solid waste. Estimates suggest that over 1 billion tons of these feedstocks are available annually for sustainable utilization in bioenergy production systems [5]. This bioeconomy has the potential to create over 1 million jobs and $260 billion in U.S. revenue, displace 30% of liquid transportation fuels, and reduce 50% of greenhouse gases compared to petroleum [3].

Major unit operations in the conversion of biomass feedstocks to fuels include supply and logistics operations including harvest, collection, transport, storage, and formatting followed by biochemical conversion of carbohydrates to fuels and chemicals (Figure 1.1 Unit operations in the conversion of lignocellulosic biomass to fuels and chemicals through a biochemical conversion approach. This review will describe the impact of long-term storage (gray box) on conversion operations.(Figure 1.1). Feedstock supply and logistics unit operations generally begin with the harvest of a crop or a portion of the crop that is cultivated either on an annual basis, (e.g., corn, wheat, sorghum, etc.), on a perennial basis (e.g., switchgrass, miscanthus, etc.), or a multi-year basis (e.g., willow, pine, etc.). In the case of agricultural residues including corn stover, commonly accepted practices are based on dry, baled logistics systems. Harvesting of the grain fraction of the plant is performed simultaneously or just preceding harvesting of the biomass residue [6]. Formation of windrows occurs either during harvest or by a windrower followed by drying in-field to facilitate stable storage conditions and collection of the biomass from windrows into bales [7, 8]. Bales are stored either field-side or at a centralized location until further use [9]. Size reduction to meet biorefinery particle size specifications is performed either at the biorefinery gate [10] or at a biomass feedstock depot [11]. Depot concepts have been proposed to facilitate densification of biomass into low-moisture pellets for stable storage and low transportation costs. The cost and performance of these logistics systems and associated unit operations have been well-documented [7, 10, 11], and estimates in 2018 suggest that delivered cost of corn stover to a refinery is estimated at $84/US ton depending on the harvest method and the draw ratio of the biorefinery [12]. These costs are low compared to the forage industry but are necessary to be competitive with fossil-based fuels of approximately $3/gallon.

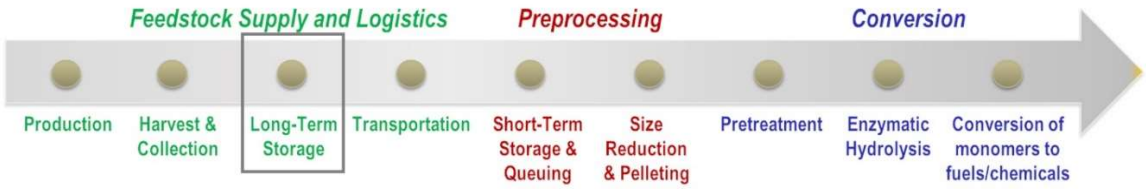

Figure 1.1 Unit operations in the conversion of lignocellulosic biomass to fuels and chemicals through a biochemical conversion approach. This review will describe the impact of long-term storage (gray box) on conversion operations.

Multiple approaches to convert biomass resources to energy sources exist and are generally characterized as either biochemical or thermochemical. Each conversion technology has advantages and disadvantages in terms of their flexibility to feedstock source and related chemical composition as well as regarding the product generated from that feedstock. These diverse conversion approaches facilitate utilization of geographically localized biomass feedstocks. For example, agricultural residues are concentrated in the middle and eastern portion of the U.S., while woody biomass and forest thinnings are concentrated in the southeast and western portions of the U.S. [5]. All these conversion approaches have a role in the formation of a stable bioeconomy and reducing the dependence on fossil-fuel based resources [13].

Biochemical conversion of lignocellulosic biomass including corn stover has been facing technical challenges during scale up despite significant investment by three commercial-scale integrated cellulosic-based biorefineries in the U.S. last decade. All these biorefineries have struggled to make biofuels a reality. Dale summarized two primary challenges that were faced including the lack of understanding of how to stably store biomass for long durations and the difficulty to chemical deconstruction in biomass during pretreatment operations [14]. The first challenge is a result of the susceptibility of biomass to microbial or physical loss when not stored in a stable manner, and the later issue stems from the variations and complexities in corn stover and associated challenges of converting this feedstock into fuels [14, 15]. Understanding lignocellulosic biomass and overcoming the associated recalcitrance is key to addressing the challenges for biochemical conversion. Therefore, the focus of this review article is aligned closely with biochemical conversion approaches for corn stover but may have applicability towards thermochemical conversion and other lignocellulosic biomass as well. This review will highlight the impact of long-term storage on conversion operations with the focus of how storage systems may be used to overcome both the challenge of stable storage for bioenergy systems and be complementary to pretreatment systems.

Lignocellulosic Biomass Structure and Associated Recalcitrance

A fundamental understanding of the structure of lignocellulosic biomass is necessary for the prediction of how biomass may be affected during each unit operation between harvest and conversion. Lignocellulosic biomass, such as corn stover, consists of an intricate combination of cellulose, hemicellulose, and lignin, that provide strength to the plant cell walls [16, 17]. These basic building blocks are in both monocots and dicots. Monocots have one cotyledon whereas dicots have two, and other key features include the arrangement of vascular bundles in the stem and vein orientation in the leaves. Plant walls (Figure 1.2) consist of a primary wall, which is composed of

cellulose, xyloglucans, and pectin as well as 10-20% protein [18]. Secondary walls contain cellulose, xylans, glucomannans and lignin and are separated into S1, S2, and S3 layers [19]. A thin layer, termed the middle lamella, connects plant cells to each other and is rich is pectin [20]. These cell wall components are multi-functional, supporting nutrient transport during growth while providing strength to the plant such that it can withstand environmental factors including wind, moisture, and physical impact. However, the complex nature of biomass tissues and their chemical makeup presents a challenge for a biorefinery. The term recalcitrance describes the resistance of lignocellulosic biomass to biological, chemical, and thermal methods of deconstruction. Each plant tissue and cell wall layer are built of unique chemical signatures increasing this recalcitrance to deconstruction, and an understanding of the chemical makeup and bonds holding them together is essential to effectively deconstruct and depolymerize lignocellulosic biomass.

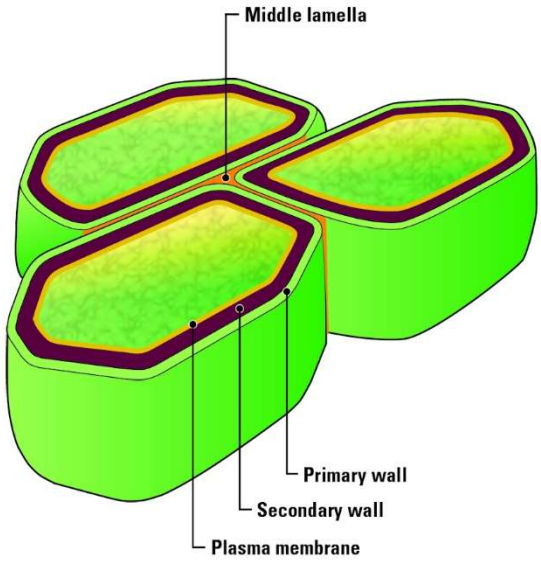

Figure 1.2 Biomass plant cell wall structure. Photo credit: U.S. Department of Energy Genomic Science Program. https://genomicscience.energy.gov

Cellulose microfibrils are the main component of the primary and secondary cell wall in plants. Microfibrils are composed of multiple glucose chains arranged in parallel in a crystalline fashion, with individual glucose chains linked internally and to each other through hydrogen bonds[18]. Individual glucan chains and are comprised of 500-14,000 repeating D-glucose units; two D-glucose molecules are linked in the β-1,4 position and rotated 180 degrees from each other, forming a cellobiose unit as shown in Figure 1.3[21]. Himmel has proposed that cellulose microfibrils are arranged

into 36 glucan chains arranged in a radial fashion[18], whereas Fernandes has proposed 18-24 glucan chains in sheets are present in each microfibril[22]. Primary cell walls contain only three to four layers of the microfibrils, while the secondary cell walls are thought to contain hundreds of microfibrils[23]. One distinct attribute of secondary cells walls is the varied orientation of cellulose microfibrils in the S1, S2, and S3 layers, which contributes to the strength of the plant tissues [24].

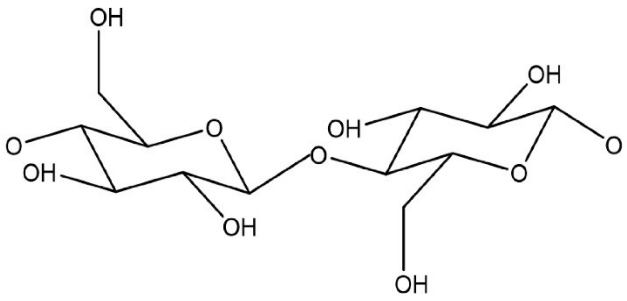

Figure 1.3 Cellulose backbone consisting of D-glucose molecules linked in the β-1,4 position and rotated 180 degrees from each other.

Hemicellulose is comprised of a complex matrix of polysaccharides generally consisting of long chains with a β-1,4-backbone and multiple side chains. Hemicellulose surrounds cellulose microfibrils and associates with them through hydrogen bonds[25], helping to strengthen the plant's primary and secondary cell walls[26]. The composition and complexity of hemicellulose has been extensively reviewed[21, 26]. Xyloglucan has a -1,4-glucan backbone with xylose side chains. Xylans have a -1,4-xylose backbone and can contain other polysaccharide side chains including arabinan and glucuronic acid. Mixed-linkage glucans are linked at both -1,3 and -1,4 positions. Gluco- and galactomannans consist of a -1,4 mannan backbone that can be substituted with glucan and galactan, respectfully. Acetyls and phenolic acids, such as ferulic acid, are common side chains linked to the hemicellulose[27], and these have been shown to reduce the accessibility of cellulose to enzymatic attack[28]. Therefore, the association of hemicellulose and cellulose is a key factor in reducing biomass recalcitrance.

Pectin is a 1,4-linked galacturonic acid-based polysaccharide that is principally located in the middle lamella and primary cell wall of lignocellulosic biomass[21, 29]. Pectin is generally not located in the secondary cell wall but can be present in the outer secondary cell wall layers. Pectin is proposed to form covalent bonds with hemicellulose and increases the strength of the cell wall[30]. A graphical depiction of the interactions between pectin (red) with hemicellulose (blue) and cellulose (brown) is shown in Figure 1.4. Pectin content is generally highest in dicots but is also present in monocots[31].

Pectin can act as a barrier against enzymatic attack and therefore is an important component when considering the conversion of lignocellulosic biomass to biofuels.

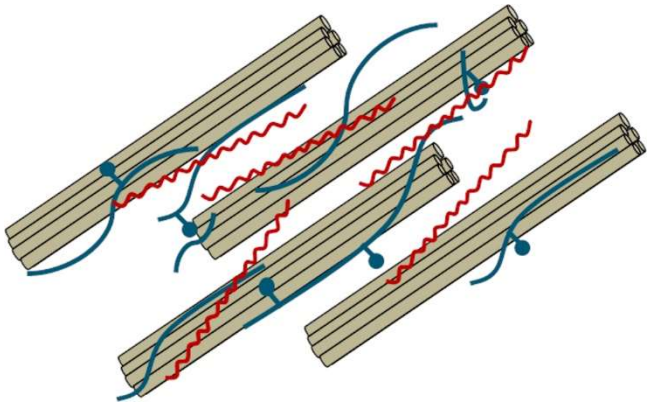

Figure 1.4 Graphical description of cellulose microfibrils (brown) surrounded by hemicellulose (blue) and pectin (red). Adapted from Cosgrove, 2005.

Lignin is a complex molecule that is made up of hundreds of monomers. Lignin concentrations are highest in middle lamella and primary cell walls [32], yet these components are of low concentration in the cell wall compared to secondary cell walls. Lignin is also present in the cellulose microfibril-rich secondary cells walls [33] of which the S2 layer is the largest fraction [19]. Lignin fills the space between cellulose, hemicellulose, and pectin and thus serves to strengthen the cell wall. Lignin is hydrophobic and can protect the cells from enzymatic attack and resulting degradation. Monolignins are the building blocks of lignin; they are synthesized from phenylalanine in the cytosol through a complex set of enzymatic reactions and are characterized by their number of methoxy side chains[34]. *p*-coumaryl, coniferyl, and sinapyl alcohols have zero, one, and two methoxy side chains, respectfully[33]. These monolignins are transported into the cell wall, where they are then polymerized oxidatively to another monolignin or a growing lignin chain, likely because of a peroxidase or laccase that results in the formation of a free radical[34, 35]. Therefore, *p*-coumaryl, coniferyl, and sinapyl alcohols result in the formation of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units within a lignin molecule (Figure 1.5). Linkages between cellulose, hemicellulose, pectin, and lignin can be ester or ether and can either directly link these molecules or use acid bridges such as ferulic acid or hydroxycinnamic acid[26, 27].

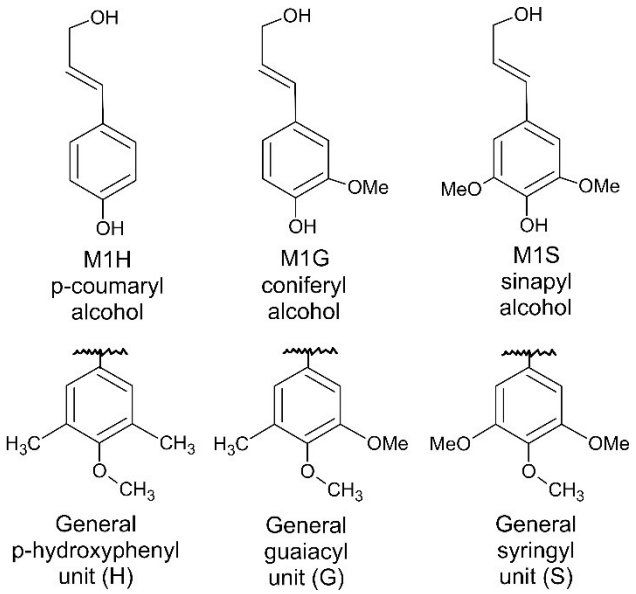

Figure 1.5 Lignin alcohol precursors and resulting monolignins.

The complex nature of the composition and associated bonds between cellulose microfibrils, hemicellulose, pectin, and lignin and resulting heterogeneity of plant tissues present a challenge for conversion of their respective monomers to fuels and chemicals[18, 26]. Additionally, factors such as the presence of waxes, the abundance of sclerenchyma and associated tissue strength, and inhibitors to fermentation (i.e. acetic acid, ferulic acid) contribute to biomass recalcitrance[18].

Lignocellulosic Biomass Conversion Approaches

Effective biochemical-based strategies for converting the biomass into fuels and chemicals generally involve the utilization of chemicals, heat, and enzymes to break down the lignocellulosic biomass into monomers followed by conversion to fuels through approaches including fermentation[36]. Recalcitrance is a significant challenge for biochemical-based conversion approaches as the cellulose microfibril is not accessible to enzymatic attack until hemicellulose and lignin have been decoupled from the matrix[37]. Enzymatic action on cellulose microfibrils is further complicated by the strong hydrogen bonding within cellulose sheets in the microfibrils[38] as well as the hydrophobic layer on the outside of the sheets that reduce the effectiveness of acid attack[39].

Biochemical approaches to the conversion of lignocellulose begin to overcome this recalcitrance in a pretreatment step that utilizes the combination of temperature, caustic, and time to increase the digestibility of lignocellulosic biomass. The particle size necessary for biochemical conversion depends on pretreatment chemistry[40], but a nominal 6 mm size is often recommended to minimize the

cost of size reduction while increasing the surface area for pretreatment[36, 41]. Dilute acid pretreatment generally occurs between temperatures of 140 °C and 200 °C, and hemicellulose hydrolysis is the primary mode in which this pretreatment chemistry makes cellulose more accessible to enzymatic attack[42]. Alkali treatments include applying sodium hydroxide[43] as well as lime[44] to remove acetyl groups from xylan and remove lignin through oxidation[45]. Steam explosion can be used to increase the surface area though defibrillation and is catalyzed by the removal of acetyl groups from hemicellulose[46]. Ammonia-based pretreatment such as ammonia fiber explosion (AFEX) impregnates plant cells during a pressure change, which results in both deacetylation as well as reduced crystallinity of cellulose[47]. Ionic liquids solubilize cellulose, hemicellulose, and lignin, which are then selectively precipitated to isolate these components[48, 49]. The commonality of these pretreatment methods is that they target specific biomass components with the goal to make others more accessible to subsequent enzymatic attack.

Enzymatic hydrolysis succeeding pretreatment is performed using glycosidases including cellulases or mixtures of enzymes that attack components in hemicellulose (e.g., xylanases, mannanases, arabionfuranosidases, and pectin lyases)[50]. Upon release of carbohydrate monomers, fermentation can proceed by yeast or bacteria. Ethanol fermentation was one of the first commercialized approaches for fuel generation from lignocellulosic biomass[41] and is based on the technology of the grain ethanol industry. Additional fermentation approaches that have gained recent attention include production of carboxylic acids including butyric acid[51, 52] or propionic acid[53] that can be upgraded catalytically to hydrocarbon fuels[54]. Succinic acid is also a produced through fermentation[55, 56] and is a valuable chemical building block[56, 57]. The commonality between all these approaches is the production and subsequent utilization of carbohydrate monomers to higher-value fuels and chemicals.

Recent attention has also been focused on lignin utilization to increase the economics of biorefineries. Combustion for process heat was the original use of lignin in cellulosic biorefinery models[41]. However, the pressure for lignocellulose-derived fuels to be cost competitive with fossil-based transportation fuels require either lower conversion costs or higher value end uses of the conversion products. Lignin can be depolymerized by chemicals and enzymes and utilized for high value products[58]. Multiple fermentation pathways exist for lignin monomers including adipic acid[59] and muconic acid[60]. Improvements in biomass recalcitrance reduction are also necessary to further advance this field given the complexity of lignin molecules.

Thermochemical approaches for biomass conversion utilize heat and/or catalysts to create either heat through combustion, into liquids such as bio-oils through pyrolysis of liquefaction, or into combustible gases through gasification[61]. Thermochemical conversion approaches have been

extensively reviewed elsewhere. Thermochemical approaches require biomass to be at a small particle size to increase surface area, typically less than 2 mm. Thermochemical conversion is often favorable for soft and hardwood biomass feedstock due to their elevated lignin level compared to herbaceous biomass feedstocks since lignin has a higher calorific value compared to carbohydrates. Thermochemical conversion approaches also can be used to generate combustible gases from low value feedstocks such as municipal solid wastes. Biomass recalcitrance in relation to thermochemical conversion is gaining attention in order to understand mechanisms that improve fuel yield[62]. For example, Kim et al. reported on the application of partial-oxidative pyrolysis to depolymerize lignin and thus allow for increased conversion of cellulose to levoglucosan in bio-oil[63]. Similarly, a low temperature pyrolysis method combined with two-dimensional gas chromatography coupled with mass spectrometry has been shown to identify storage related changes in cellulose, hemicellulose, and lignin-based pyrolysis products[64]. Advancements in the understanding of biomass recalcitrance and related yield in thermochemical conversion systems is necessary to further predict approaches to increase fuel yield.

Biomass Storage Systems

Seasonal variation is a challenge for most agricultural products, necessitating storage to provide a biorefinery with year-round access to the product. Agricultural residues, such as corn stover, are typically available during a 1- to 2-month window and are dependent on the harvest of the primary product. Energy crops are also harvested seasonally but have a more flexible harvest window since it is the primary product as opposed to residues that are reliant on a commodity crop. Engineered storage systems offer the opportunity to minimize the seasonal variation of biomass availability and allow a biorefinery to operate year-round with a consistent feedstock supply. Long term storage also allows for a biorefinery to be sized at the appropriate scale such that down-time is minimized, and this reduces costly capital expenditures.

Dry Storage Systems

The primary goal in storage is to preserve the reducing equivalents in biomass, and dry storage systems are one solution for stably storing biomass over long periods of time. Bale stacks are the state of technology for field-side storage of agricultural residues[65], and a corn stover bale stack is shown in Figure 1.6. Bales are generally covered with tarps to reduce moisture accumulation from precipitation, while improved surfaces are recommended to prevent wicking of soil moisture by the bottom bales. Smith et al. described the moisture distribution of tarped and untarped corn stover bales entering storage at the same moisture content (22% wet basis); after 5 and 9 months moisture had redistributed to levels up to 65% just under the surface of the tarp as well as in the bottom bales

where moisture adequate drainage was not present[66]. Overall, bale-based storage can effectively preserve biomass when under ideal conditions but must be managed carefully to maintain stable conditions.

Figure 1.6 Corn stover bale stack at a satellite storage location.

Biomass stored in dry systems is particularly susceptible to microbial degradation if conditions conducive to enzymatic activity or microorganism growth are present. Water activity (a_w), which describes the ability of water to react chemically and biologically, drives the storage stability of a range of industrially-relevant nutritional products for human and livestock consumption[67]. Water activity ranges between 0 and 1 and corresponds with no water being available for utilization and all water being available, respectfully. Water activity can be calculated by determining the relative humidity of air in a sample in equilibrium, and moisture sorption isotherms are used to determine the relationship of water activity and moisture content for a given material[67]. Water activity is also impacted by temperature, which is one reason refrigeration is an effective preservation method. The relationship between water activity and microbial stability is well-documented, with bacteria growth prevalent when $a_w > 0.85$, yeasts prevalent between a_w values of 0.80-0.90, and mold growth dominant when a_w value is 0.85 to 0.60. Only enzymes are considered active at $a_w < 0.60$. Athmanathan et al. related water activity to dry matter loss in switchgrass and demonstrated no appreciable loss at $a_w > 0.85$, which corresponded to a moisture content of approximately 16% (wet basis)[68]. The relationship between biomass source, chemical composition, free versus bound water, and environmental conditions such as temperature can be used relate moisture content and water activity, and an enhanced understanding of these parameters can be used to positively impact biomass storage stability.

A recent study suggests that an average of only 36% of corn stover harvested in the U.S. is capable of entering long-term bale storage at moisture levels that result in stable storage[69], which makes corn stover a particularly challenging feedstock to store using dry approaches. Similarly, a moisture content of 20% or less has been recommended for stable corn stover in baled storage[9]. Significant losses of dry matter have been reported in field-side storage of corn stover that exceeds this moisture threshold due to microbial degradation[66, 70]. Microbial degradation of aerobically stored biomass materials can be characterized in terms of CO_2 production, microbial heat generation and resulting temperature increase, and dry matter loss[71, 72]. Aerobic microbial degradation by bacteria, yeast, and fungi consumes valuable carbohydrates and produces CO_2 as a byproduct, leaving behind material enriched in non-fermentable biomass components such as ash. This degradation has been documented to begin with hydrolysis of acetyl groups and reduction in hemicellulose, which has been measured by wet chemical analysis, such that the microorganisms can access cellulose[71, 73]. Hemicellulose modification has also been documented in corn stover that suffered severe aerobic degradation during storage using a pyrolysis/two-dimensional gas chromatography/mass spectrometry (Py-GCxGC-MS) approach [64]. In this study, formation of acetic acid and furfural, which correlate to acetyl and C5 sugar degradation, was increased in corn stover samples that suffered severe degradation compared to samples that suffered only mild degradation. Understanding how microbial degradation might be used as a partial pretreatment is a topic that has not been widely reported, and this moisture management approach may have applicability in bioenergy systems that rely on dry storage approaches.

Bale storage systems can be cost prohibitive in many industrial settings because the shear amount of combustible material present must be managed safely. Corn stover bales are at risk of loss due to fires[74], necessitating significant land use to create a physical barrier to protect a burning stack from igniting other stacks. Additional insight into how dry storage systems can be managed and/or configured to reduce this risk in a cost-effective manner will support bioconversion designs by protecting the asset of biomass in the logistics supply chain.

Wet Storage Systems

An alternative approach to feedstock supply logistics systems that rely on baling biomass is to adopt the commonplace practices of the forage industry. Wet, anaerobic storage systems (i.e., ensiling) are an alternative to dry storage and have consistently and successfully demonstrated biomass preservation in long term storage for livestock feed and forage. Wet biomass logistics systems have been proposed for corn stover, primarily to address the concern of catastrophic loss of corn stover stacks to fires[73, 75]. Wet logistics systems are based on forage chopping herbaceous biomass in the field at moisture contents between 40 and 65% (wet basis), transporting the chopped biomass in

silage trucks, and utilizing anaerobic storage systems including silage bags, bunkers, or drive-over piles to limit oxygen and preserve biomass[76]. Figure 1.7, Figure 1.8, and Figure 1.9 show the harvest, transport and unloading, and resulting anaerobic storage pile described in Wendt et al., 2018a. Ensiling is a common practice for corn and grasses in humid climates of the world including parts of the United States and in Europe[77]. Over 121 million tons of corn silage were harvested in 2018 in the United States and stored for livestock forage using this approach[78]. Ensiled biomass can be stable for months to years if anaerobic conditions are maintained. Expected dry matter losses under best management practices range from 6-15% depending on storage structure, with losses as low as 3% possible[77, 79].

Figure 1.7 Collection of corn stover with forage chopper into a walking floor trailer.

Figure 1.8 Simultaneous formation and compaction of a drive over storage pile with corn stover unloaded from walking floor trailers.

Figure 1.9 Covered drive over storage pile.

The success of ensiling relies on mechanical exclusion of air through compaction, utilization of oxygen present through respiration, and fermentation to produce organic acids and a corresponding reduction in pH[80]. Obligate aerobic microorganisms are primarily responsible for the initial consumption of oxygen through respiration, although plant respiration also plays a role[81]. Once this oxygen has been consumed then lactic acid bacteria proliferate and produce organic acids[82]. Soluble sugars, which are commonly referred to as water soluble carbohydrates in the forage literature, serve as the energy and carbon source for the initial fermentation as well as sustained but reduced growth of lactic acid bacteria during the stable storage stage[81]. The combination of anaerobic conditions and the presence of organic acids and corresponding low pH serve to reduce overall microbial activity in

ensiled systems, and Leistner described this combination of factors to promote stability as the hurdle concept[83].

The soluble sugars in biomass can constitute a significant portion of biomass, and their presence is important for successful ensiling. These sugars are transported through actively growing plants, forming structural sugars as the plant grows[17]. Corn stover can contain between 4-12% of these soluble carbohydrates depending on the growth phase of the plant[84]. Forages grasses can have a wide range of soluble carbohydrates with anywhere from 5% to 30%[80], and up to 16.3% soluble carbohydrates have been documented in switchgrass[85]. Sweet sorghum can contain up to 20% soluble carbohydrates[86]. The stage of growth often determines the level of soluble carbohydrate reserves in the plant, with the levels decreasing after anthesis and as the plant sends carbohydrate reserves to the roots for wintering. Soluble carbohydrate levels in grasses have been shown to vary between 10% to 35% depending on the environmental conditions and the stage of growth[87]. Similarly, soluble carbohydrate levels in corn stover as low as 2.5% of total mass have been present at the time of harvest and still resulted in successful preservation in ensiling[73]. Ensuring that sufficient fermentable soluble sugars are present at the time of ensiling is necessary to support organic acid production and pH reduction. Low-cost additives such as molasses or chemicals can be applied when sufficient soluble sugars are not available, as discussed in the following sections.

Dry matter loss and final pH during the ensiling process is related to the type of lactic acid bacteria present and their fermentation pathway. Lactic acid bacteria are recognized as either homo- or heterofermentative depending on their reaction mechanism. Homofermentative lactic acid bacteria growth during ensiling results in the direct conversion of glucose to lactic acid, whereas heterofermentative lactic acid bacteria convert glucose to lactic acid, acetic acid, ethanol, and CO_2[80]. Therefore, homolactic acid fermentation results in lowest losses of carbon and associated dry matter and is preferred during ensiling. However, acetic and propionic acids have been shown to inhibit spoilage microorganisms during aerobic exposure at the time of utilization of silage[88]. Therefore, a mixture of acids is commonly desirable in ensiled biomass.

The protective effect of organic acids during preservation is based on inhibition of unwanted microorganisms. Lambert and Stratford describe the mechanism by which undissociated weak acids permeate across microbial plasma membranes and then dissociate into protonated hydrogen molecules and deprotonated hydroxyl groups[89]. This is followed by proton pumping out of the cell, which leaves the hydroxyl group in the cytochrome and thus lowers the internal cell pH[89]. The low pK_a of lactic acid (3.78) makes this the preferred organic acid for stability compared to acetic acid (pK_a=4.75) or butyric acid (pK_a=4.82). Lactic acid dominated silages tend to have a pH near 3.7-3.9,

and thus there is an overall increase in the level of undissociated acids outside of cell walls at lower pH values.

Degradation because of oxygen exposure in ensiling is a significant risk for these storage systems. Oxygen exposure is present during the formation and deconstruction of anaerobic storage piles. Delayed sealing or covering in ensiling has been shown to encourage the consumption of soluble carbohydrates by aerobic bacteria, yeast, and fungi[81, 90]. This results in not only less of this carbon source being available for lactic acid bacteria but also competition between lactic acid bacteria and clostridia. *Clostridia* produce butyric acid in silage, which is associated with higher dry matter loss in storage and lower consumption of the forage by ruminants[77]. *Clostridia* spores can be passed into milk and can lead to contamination in milk and the products that are made from milk including cheese[91]. This issue is of lower concern for bioenergy systems because pretreatment generally occurs at temperatures that can deactivate spores such that they are not passed into the fermentation process. However, the higher dry matter loss because of oxygen exposure is a concern for bioenergy systems due to the loss of convertible carbon to the atmosphere.

Corn stover for bioenergy production is available at the time of grain harvest and accordingly contains lower initial moisture contents and lower soluble sugars compared to feedstock dedicated for forage[92, 93]. This presents a challenge when ensiling corn stover because the reduction of water corresponding increases the interstitial oxygen that must be either mechanically removed or biologically consumed to establish conditions that favor fermentation. Similarly, insufficient soluble carbohydrates for fermentation ultimately result in lower organic acid production. Despite these challenges, Shinners et al. and Wendt et al. both demonstrated that low-moisture ensiling (~40% moisture, wet basis) was possible, with <5% loss was experienced over 6 months in covered, drive-over storage piles[73, 94]. Similarly, ensiled corn stover has demonstrated slight pretreatment in ensiled storage conditions[95, 96]. Therefore, ensiling provides a solution for biomass to be stored in a stable format and utilized in bioenergy conversion systems throughout a calendar year notwithstanding the biomass being seasonally available.

Long-term wet, anaerobic storage has been shown not only to stabilize biomass but can also provide an environment to begin depolymerization of structural components, such as lignin and hemicellulose, a benefit that could help to lower conversion costs for high moisture feedstock. The high moisture environment provides an environment that enables biological and chemical reactions to occur. The pH of typical ensiled material is in the range of 3.5-4.5, depending on the fermentation pathway. This pH range inhibits most growth by obligate aerobic bacteria, yeast, and fungi, and even lactic acid bacteria have reduced activity at pH levels below 4[97]. However, organisms that are active

may be producing enzymes that can liberate the carbohydrates from the biomass and support their growth. Fructan hydrolases produced from the ensiled plants themselves[98] or by select lactic acid bacteria strains that can create fermentable sugar monomers from polysaccharides[77, 99]. This may occur in anaerobic storage systems even with low degradation rates. Gusovius et al. correlated the reduction of fiber size in hemp to dissolution of the middle lamella by microbial activity in anaerobic storage[100]. Similarly, delamination in the middle lamella in pine has also been documented as a result of fungal treatment[101]. Further investigation is necessary to understand the role of long-term storage to influence cell walls and related structural integrity of biomass.

Despite the multiple benefits of wet anaerobic systems for corn stover in promoting stability in long term storage, prior research has been unable to close the cost gap between wet systems and their lower cost dry counterparts. The primary drawback of wet systems for corn stover is that the moisture in the biomass as well as the bulk, chopped format makes handling this biomass more costly than handling dry, baled biomass. For example, prior research has shown that transportation costs double for chopped corn stover compared to baled stover as a result of reduced bulk density compared to baled biomass [75]. However, the size reduction that can be accomplished during forage chopping that is used in wet logistics systems can reduce both harvest and collection costs as well as the cost of further size reduction during preprocessing. Harvest and collection costs were reduced from \$21 Mg^{-1} in a bale-based logistics system to less than \$16 Mg^{-1} in a wet logistics system [75]. Likewise, size reduction during forage chopping is capable of reducing particle size geometric mean to 5-10 mm [102], whereas baled logistics systems for corn stover rely on one to two steps of size reduction with a 6 mm screen during preprocessing. However, wet anaerobic storage costs are higher more than its baled counterpart. Field-side storage costs for baled corn stover are estimated to range between \$5 and \$18 Mg^{-1}, while anaerobic storage of corn stover in piles is estimated to cost between \$15 to \$22 Mg^{-1} (2015 US dollars, [75, 103, 104]. Additional research is necessary to identify approaches that will address the cost barrier of wet anaerobic storage compared to baled storage.

Storage Selection Based on Feedstock Type

Feedstock type and harvest scenario both impact the most suitable long-term storage approach. Table 1.1 lists the herbaceous crop residues and energy crops identified in the Billion Ton report [5] and the most common storage approach utilized for them. Residues that are harvested based on timing of the grain harvest are generally lower moisture content and compatible with baled storage; these include the straws and grain sorghum stubble. Energy crops including switchgrass and miscanthus are generally harvested after senescence and subsequently stored in baled formats. However, harvest of these plants is not dependent on a primary commodity crop and the timing can be flexible such that

anaerobic wet storage could be compatible with these crops. Crops that are high moisture at the time of storage including energy cane and sugarcane bagasse are best suited for wet storage systems. As discussed previously, corn stover is often stored in dry, baled formats, but challenges with achieving the desired moisture content for stability are inherent to this crop and provide an opportunity for wet storage to address this challenge. However, long-term wet storage operation is one of the unit operations in the feedstock logistics operations that can be used to improve the quality of the corn stover with the aim of reducing downstream processing requirements for conversion to fuels and chemicals. The following sections describe approaches that have or could be used to facilitate this reduction in recalcitrance.

Table 1.1 Herbaceous crop residues and energy crops identified in the Billion Ton study linked to their common storage method.

Biomass Type	Dry Storage	Wet Storage
Barley straw	x	
Corn stover	x	x
Energy cane	x	x
Grain sorghum stubble	x	
Miscanthus	x	x
Rice straw	x	
Sugarcane bagasse		x
Switchgrass	x	x
Wheat straw	x	

Storage Amendments

The application of amendments to biomass to promote stability prior to anaerobic storage is commonplace in the forage industry. The goal of these amendments is to promote the fastidious formation of a low pH environment that result in stable storage and maintain desirable qualities for forage[105]. Amendments may include acids or alkali applied directly to the biomass or microbial amendments to encourage a specific fermentation pathway, and either of these can be effective at reducing storage losses. Storage amendments are so commonplace that forage choppers are often equipped with sprayers that can apply liquid inoculants during harvest. The following section describes some of the primary amendments that have been used over the last century for forage silage and may have applicability for bioenergy systems.

Microbial Amendments

Lactic acid bacteria are commonly added to silage during harvesting to promote the proliferation of these organisms and thus more rapid fermentation during ensiling[77]. Homofermentative lactic acid bacteria that produce primarily lactic acid have demonstrated reduced aerobic stability upon removal from storage compared to the acetic acid containing biomass produced by heterofermentative lactic acid bacteria[77, 105]. A wide range of microbial inoculants are available commercially, and they generally contain a mixture of bacterial species to improve the palatability of the feedstock for livestock[105]. Anaerobic storage with microbial inoculants has been suggested to positively influence performance in bioenergy conversion systems. The combination of high-moisture storage with bacterial inoculants have been demonstrated to increase sugar release in wheat and rice straw, corn stover and corn silage, and forage sorghum[106-108].

Enzymes have also been added to silage in order to increase the level of soluble carbohydrates for consumption by lactic acid bacteria[109, 110]. Common enzymes include cellulases, xylanases, and pectinases, and most are applied in combination with a lactic acid bacteria inoculant that can utilize the sugars released enzymatically[105]. Organisms that produce ferulic acid esterase have also been added to silage with mixed success in improving digestibility of livestock feed[111]. Enzymes also have a role in bioenergy conversion systems, where depolymerization of structural hemicellulose in long-term storage could be utilized to reduce pretreatment severity at the biorefinery. Low-moisture corn stover (~20%, wet basis) amended with xylanase increased recovery of hemicellulose-related sugars by 10% over untreated controls during long-term storage[112]. A common concern when adding enzymes during long-term storage is the excessive hydrolysis of carbohydrates[113], which results in elevated substrate for fermentation in anaerobic storage or excessive loss upon aerobic exposure. This balance must be carefully managed based on feedstock type and utilization strategy.

Acidic Amendments

Organic and mineral acids have been used extensively in silage to rapidly decrease pH and preserve the nutrient content of the biomass. Virtanen used a blend of hydrochloric and sulfuric acids to preserve silage, and this work demonstrated that a pH of 4.0 was necessary to inhibit soluble carbohydrate and protein degradation along with butyric acid formation[114]. Virtanen received a Nobel Prize in Chemistry in 1945 for this contribution to the field[115]. Sulfuric acid is a strong acid and is used specifically to reduce pH, however Virtanen recommended that it not be applied alone due to poor digestibility by rumen. Formic acid is common silage additive that is considered to reduce pH rapidly as well as provide antimicrobial effects. Formic acid is proposed to disrupt the electron transport chain by inhibiting cytochrome oxidase[116]. While this may be desired for the suppression of

spoilage microorganisms, this same mechanism has resulted in histotoxic hypoxia in farmers exposed to vapors while making silage[117]. It has also been noted that yeasts have a higher tolerance to formic acid treated silages than lactic acid bacteria, such that the aerobic stability of formic acid treated silages is poor[118]. Formic acid is still used as a silage additive, particularly in European countries due to the ban on antibiotics in livestock feed. However, its use is limited in the United States because it traditionally is a higher cost acid. Approaches to produce lower-cost formic acid are necessary to enable additional utilization of this acid in forage and bioenergy storage systems.

Propionic acid is a low-cost additive often used in the United States, particularly in haylage[119]. Propionic acid additives have demonstrated to reduce yeast proliferation upon removal of ensiled biomass from storage, thus increasing the aerobic stability of the biomass[120]. Similarly, numerous acid and acid salt combinations have been described for their preservation effect on silage during storage and upon exposure to oxygen[105]. Nadaeu et al. demonstrated an improvement in aerobic stability of corn silage from 5.7 days to 11.8 days for biomass that entered storage after treatment with a combination of formic, propionic, benzoic, and sorbic acids[121]. Acid salt combinations including potassium sorbate, sodium benzoate, and sodium nitrite have also shown to increase aerobic stability in corn silage[122]. Perennial grasses, including switchgrass, have been successfully preserved in high-moisture storage amended with mineral acid and experienced up to 17% improvement in cellulose conversion to ethanol[123]. In summary, acids have demonstrated as effectiveness as a direct approach in improve ensiling performance and aerobic stability of biomass upon utilization. Further knowledge on the effect of these treatments to improve performance in bioconversion to fuels and chemicals will increase their utilization in commercial biorefineries.

Alkaline Amendments

Alkaline treatments have been used for stabilizing wet harvested biomass by creating a basic environment which can restrict unwanted fermentation. Anhydrous ammonia has been applied to forage for over 50 years to improve nitrogen levels and prevent proteolysis and deamination in forage, which improves the quality of the biomass for livestock feed[124, 125]. Anhydrous ammonia has been demonstrated to raise pH and decrease lactic acid formation during the initial days of ensiling as well as decrease protein degradation in long-term anaerobic storage[126].

Calcium oxide, or lime, has been used as an additive for biomass with the dual aim of improving storage stability as well as to impact thermochemical conversion performance[127, 128]. Calcium oxide (CaO) reacts with water to produce calcium dihydroxide ($Ca(OH)_2$), which then reacts with CO_2 to form calcium carbonate ($CaCO_3$). Calcium carbonate is understood to act as a sorbent and reacts with other inorganics including silica and sulfur during thermochemical conversion[129], which increases the

melting temperature of the resulting inorganic complex and thus reduces undesirable slagging on reactor surfaces and catalysts[128]. Calcium oxide treatment of reed canary grass was shown to increase pH to greater than 9 in biomass containing 35 to 65% moisture, which is desirable to reduce proteolytic organisms but not sufficiently high such that protein degradation occurred. In this study the 35% moisture content biomass exhibited stable aerobic storage over 90 days due to the combined effect of initial increased pH and reduction of moisture through drying[127]; however, higher moisture contents levels resulted in storage losses up to 30% and the subsequent reduction of pH levels to 8-9 likely as a result of liberation of acetyl side chains from the hemicellulose. Similarly, lime has been applied to poplar over a 12 week period to enhance the solubilization of lignin though oxidation and deacetylation of hemicellulose through hydrolysis in order to improve the digestibility of wood in enzymatic hydrolysis[130].

Sodium hydroxide has been assessed for use during storage to reduce biomass recalcitrance, and the advantage of this alkali above lime is that it is readily soluble. Sodium hydroxide has been used to improve the digestibility of wheat and barley straws for livestock feed by reducing lignin content[131, 132]. Sodium hydroxide treatment during 1-3 days of storage has also been applied to corn stover at 80% moisture content (wet basis) in order to increase biogas yields in anaerobic digestion, and these studies have indicated that hemicellulose is most susceptible to short-term sodium hydroxide exposure as a result of removal of acetyl groups[133-135]. Cui et al. investigated the use of sodium hydroxide treatment during 90-day ensiling of corn stover in plastic bags at moisture contents ranging from 45-75 % moisture (wet basis)[136]. This study showed that lignin and cellulose degradation was complete within 5 days of storage, but that xylan degradation continued over the 90-day storage period; however, significant dry matter loss of 13-21% occurred during the storage period. An increase in acetic acid levels was observed during the first 15 days of storage, and subsequent reduction of structural acetate after this period is consistent with the dry matter loss experienced. Similarly, glucose and xylose yields were reduced in samples that experienced 90 days of storage compared to 5 and 12 days of storage. This study shows the importance of maintaining stable storage conditions when combining sodium hydroxide with long term storage.

Alkali treatments have shown to reduce chemical recalcitrance of biomass to deconstruction and are the state-of-the-technology for cost-competitive biochemical conversion of carbohydrate and lignin monomers to biofuels[137, 138]. However, these high-severity treatments require significant alkali loading during short thermal residence times to be efficient at the biorefinery scale. Anaerobic storage offers the opportunity to allow the deacetylation reactions to occur over a longer residence time with the added benefit of protecting biomass from uncontrolled dry matter loss. As discussed in this section,

alkali treatment has demonstrated reduced recalcitrance in terms of improved digestibility for rumen. However, the combination of well-preserved biomass resulting from anaerobic storage and alkali treatment have not yet been applied in relation to both physical and chemical preprocessing to form convertible carbohydrate monomers for bioenergy systems.

Storage Systems Linked to Conversion

The impacts of long-term storage are an important variable to consider in the conversion of biomass resources to fuels and chemicals. The conditions experienced during storage and resulting biochemical changes in cells can positively or negatively impact conversion potential. For example, corn stover that had experienced significant aerobic degradation (30% loss of dry matter) was shown to have a significant shift in structural to soluble xylan but no change in structural glucan [73]. However, after either dilute acid or dilute alkaline treatment the efficiency of enzymatic hydrolysis to depolymerize glucan was increased in the aerobically degraded biomass; this suggests that the loss of hemicellulose in storage resulted a slight pretreatment effect. Dilute acid and dilute alkaline pretreatments have been applied to anaerobically stored biomass as well with success [73], and in this case dilute alkali treatment was effective in showing an increase in carbohydrate release after anaerobic storage. Limited data on deacetylation pretreatment is available for corn stover, but alkali groups in hemicellulose hydrolyzed during storage should positively impact deacetylation. Additionally, organic acids produced during anaerobic storage may serve as catalyzing agents during pretreatment including during steam explosion [139] or hot water extraction [96, 140]. However, ammonia fiber expansion pretreatment is primarily performed prior to long term storage because it results in a shelf-stable format. Additional insight is needed to understand how long-term storage can be used to enhance deconstruction based on each biomass type and each pretreatment chemistry.

Conclusion

Long-term storage of biomass is a reality for any agricultural system and is a key unit operation for bioenergy systems. However, the costs necessary to produce stable storage conditions are often misaligned with the pressures of producing biofuels that are competitive with their fossil counterparts. Focus on multiple research directions can address this cost disparity and should include (1) understand how baled biomass systems can provide protection from moisture and related physical and microbial losses, (2) application of how wet, anaerobic systems commonly used in forage might be used to overcome the cost barrier that currently makes them less attractive for bioenergy systems, and (3) an enhanced understanding of how these storage systems may affect biomass recalcitrance and subsequent conversion to fuels or chemicals. There is also potential to shift the focus of long-term storage from a cost center to a value-added operation such that bioconversion, energy balances, and

sustainability are positively impacted. Securing the storage operation of the feedstock logistics and supply chain will be a key component to making the bioeconomy a reality.

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Chapter 2: Fungal depolymerization of corn stover in bioenergy queuing operations to reduce pretreatment severity

Abstract

Recalcitrance of lignocellulosic feedstocks to depolymerization is a significant barrier for bioenergy production approaches that require conversion of monomeric carbohydrates to renewable energy sources. This study assesses how low-cost modifications in the feedstock supply chain can be transformed into targeted pretreatments in the context of the entire bioenergy supply chain. The aim of this research is to overcome the physiochemical barriers in corn stover that necessitate increased severity in conversion in terms of chemical loading, temperature, and residence time. Corn stover samples were inoculated with a selective (*Ceriporiopsis subvermispora*) and non-selective (*Phaenarochaete chrysosporium*) lignin degrading filamentous fungal strains, then stored aerobically to determine the working envelope for fungal pretreatment to achieve lignin degradation. Dry matter loss and gross chemical makeup of corn stover varied by the length of treatment (2 and 4 weeks) and by the moisture content of the treated corn stover samples (40 and 60%, wet basis). Dry matter loss in *P. chrysosporium* inoculated biomass was elevated compared to the *C. subvermispora* inoculated biomass; however, treatment also induced additional chemical composition changes suggestive of depolymerization. Scanning electron microscope images reveal hyphae attached within cell lumen and suggest structural changes within *P. chrysosporium* treated corn stover after 60% moisture storage. These results highlight that fungal treatment approaches must balance loss of convertible material with the potential for reduction in recalcitrance. Techno-economic assessment (TEA) of fungal pretreatment in a short-term queuing system indicated the viability of this approach compared to conventional queuing operations. The total queuing system cost was estimated at $1.65/tonne of biomass stored. After applying the credit of $1.48/tonne from energy savings in the conversion phase using fungal pretreated biomass, the total system cost was $0.80 lower than traditional biomass queueing approach. While the TEA results suggested that treating biomass with *C. subvermispora* is the most economically viable storage method in the designed fungal-assisted queuing system, future research should focus on additional fungal depolymerization such as those observed in the *P. chrysosporium* inoculated biomass.

Introduction

Producing renewable liquid vehicle fuels from agricultural residues that would otherwise be discarded is a significant opportunity to reduce the carbon footprint of the transportation sector[1]. Agricultural

residues in the United States are presently available in quantities of >130 million dry tonnes and could be used for bioenergy[2]. Whereas corn grain can be easily broken down mechanically and through enzymatic activity prior to fermentation to ethanol, the heterogeneity of lignocellulosic material is a challenge for bioenergy production due to the inherent molecular and structural complexity. The matrix of cellulose microfibrils, hemicellulose, and lignin provide strength to the plant cell walls such that plants can resist environmental challenges in the field[3, 4]. However, this intricately woven matrix results in a feedstock that is recalcitrant to physical and biological deconstruction to carbohydrate monomers that can readily be fermented to fuels[5]. To counter this recalcitrance, extensive focus has been devoted to exploring the mechanical, thermal, or chemical inputs required to deconstruct lignocellulosic material in the context of a biorefinery. For example, thermal and chemical treatment range from acid or alkali, steam explosion, or ionic liquids to liberate hemicellulose or lignin prior to further hydrolysis by glycosidases to fermentable monomers[6].

Filamentous fungi are well-known degraders of lignocellulosic biomass, thriving in aerobic, moist environments. The enzymes excreted by filamentous fungi, including laccase, lignin and manganese peroxidase, and others, allow them to degrade lignin and make cellulose more accessible[7]. Filamentous fungi have been characterized for their role in bioenergy conversion, biopulping, and biobleaching to deploymerize lignocellulosic feedstocks[8, 9]. These enzymes can oxidatively depolymerize lignin building blocks of p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units or cleave the acid bridges, such as ferulic acid or hydroxycinnamic acid, that link lignin to hemicellulose[10, 11]. The pulp and paper industry has employed lignin degradation in a short term "seasoning" step prior to processing, and this is accomplished primarily through the use of white-rot fungi that produce laccase and peroxidases that cleave lignin bonds and expose hemicellulose and cellulose[12]. The associated lignin degradation accomplished by filamentous fungi accomplished during storage effectively pretreats the biomass leading to higher yields in conversion of carbohydrate monomers to biofuels[8, 13-15].

The primary challenge with utilizing filamentous fungi for pretreatment is based on the selectivity of the strain for lignin versus cellulose breakdown. Specific and non-specific lignin degrading fungi have been classified based on their enzymatic response to degrading the complex lignocellulosic biomass structure. Non-specific lignin degraders must utilize cellulose as a carbon source and therefore possess cellulytic enzymes[16] and can result in significant total loss of dry matter during lignin degradation. Specific lignin degrading fungi generally leave cellulose intact but have slower reaction times, which can increase the cost of the treatment if fungal pretreatment is the primary or

only pretreatment approach. Many studies simply focus on enzymatic hydrolysis after storage treatments, and sugar yields of less than 50% are commonly reported[8, 13-15, 17].

Long- and short-term storage of lignocellulosic material is required to provide a year round supply of seasonally available biomass sources, and short term storage at a biorefinery gate allows for a readily available supply of feedstock to feed a reactor[18]. Innovative approaches to reduce biomass recalcitrance during storage have the potential to reduce the energy required for bioconversion and improve the sustainability of bioenergy systems. This study aims to investigate filamentous fungi assisted approaches to reduce recalcitrance. Success of this approach has the potential to transform short term storage in a queuing operation into a preprocessing step that adds value to the material by reducing the energy required to convert lignocellulosic biomass into fuel, in a step that currently is a net cost to the operation.

To date, a cost competitive filamentous fungi-assisted supply chain resulting in complete hydrolysis of cellulose and hemicellulose to sugar monomers has not been developed, which is an impediment to converting the sugars to fuels and/or chemical precursors. Techno-economic analysis of utilizing filamentous fungi pretreatment systems alone suggest the slow reaction times of the approach renders them more costly compared to chemical-based pretreatment systems[19, 20]. Vasco-Correa and Shah modeled that a 5 day residence time fungal pretreatment occurring at a biorefinery in a packed bed, aerated bioreactor was at least four-fold more costly than conventional pretreatment systems due to the high capital cost of the bioreactor[19]. Baral and Shah modeled fungal pretreatment was three times more costly than steam explosion, sulfuric acid, and ammonia fiber counterparts due to the long residence time of 23 days increasing capital costs and corresponding high losses of carbohydrates requiring twice as much feedstock for fungal pretreatment approach[20]. Similarly, sterilization of biomass during pretreatment operations can be required to remove competing microorganisms during fermentation. However, the literature is not universal in the conclusion that fungal pretreatment is more costly, and numerous studies consider how higher biofuel yields[21, 22] and reduction of inhibitors[23, 24] can increase economic viability of the approach beyond just increased enzymatic hydrolysis yields.

This study investigates a fungal pretreatment approach to initial recalcitrance reduction in corn stover. Success of this approach has the potential to transform short-term storage at a preprocessing gate, a step that currently is a net cost to the operation, to a value-added operation to the material by reducing the energy required to convert lignocellulosic biomass into fuel. The goal of this study was to explore operating window of fungal treatment on corn stover, specifically balancing dry matter loss with observed compositional changes with the goal of evaluating the impacts of these structural changes

on downstream deconstruction. Strain selectivity for lignin degradation, time, and moisture content were screened to understand ranges of viable conditions promoting depolymerization in sterilized corn stover. A techno-economic analysis was then used to understand how fungal treatment might complement existing queuing operations and an acid-based pretreatment approach to hemicellulose monomerization.

Methods and Materials

Filamentous Fungi Cultivation

Experiments were performed using *Phaenaerochaete chrysosporium* (NRRL 6370), a non-selective lignin degrader, and *Ceriporiopsis subvermispora* (ATCC 96608), a selective lignin degrader, using methodology described in Saha et al.[25]. The cultures were first grown on Yeast Mold Agar (BD Difco™, Franklin Lakes, NJ). Fungal cultures were grown on Yeast Mold Broth (BD Difco™, Franklin Lakes, NJ) with shaking at 50 rpm at 28 °C. *C. subvermispora*, was grown for six days until it reached a density of 2.3 mg dry weight/mL. *P. chrysosporium* fungus was grown for seven days where it reached a density of 8.4 mg dry weight/mL. The cultures were pelleted by centrifugation at 8,000 rpm, resuspended with 25 mL of a 10 mM phosphate buffer and homogenized in a Waring blender. The cultures were pelleted once more, resuspended in 65 mL of sterile tap water. Dry fungal weight was calculated based on OD_{600} measurements and correlated to the fungal slurry wight after drying overnight at 105 °C.

Corn Stover Source and Storage Procedure for Screening Experiment

Two-pass harvested corn stover was sourced from Boone, IA in 2015 and is available as reference material through Biomass Feedstock National User Facility Library at Idaho National Laboratory. Two-pass corn stover is collected after the combine collects the grain from the field where a tractor pulling a windrowing flail shredder cuts the stover followed by a baling operation. Corn stover was size reduced to pass through a 6 mm screen with a Wiley Mill Model 4 (Thomas Scientific, Swedesboro, NJ). 20 g (dry weight equivalent, dw) samples of corn stover were added to 500 ml flasks at 40 and 60% moisture (wet basis, wb) autoclaved at 121°C for 60 minutes to sterilize the corn stover, and flasks were allowed to cool to room temperature. The corn stover was inoculated with the fungal culture by pipetting 5 ml of either a 1.78 mg/ml loading of *C. subvermispora* or a 2.39 mg/mL loading of *P. chrysosporium*, or sterilized water to the flask. Similar mass-based approaches to loading are reported elsewhere[25]. Flasks were gently rotated by hand to ensure sufficient distribution of the fungal culture and water. Loose fitting lids were placed over flasks to allow for oxygen infiltration while preventing moisture loss to the atmosphere. Six reactors were prepared for each condition, and the flasks were stored at room temperature in the dark for 2 and 4 weeks.

Initial moisture content of the corn stover was assessed using a sample collected at three intervals during flask loading. Moisture content was determined by the weight of moisture lost after drying overnight at 105°C according to Equation 2.1:

$$\%\ Moisture\ (wb) = \frac{g\ Biomass_{wet} - g\ Biomass_{dry}}{g\ Biomass_{\ wet}}\ x\ 100 \quad \text{Equation 2.1}$$

After 2 or 4 weeks of storage, three flasks for each treatment were emptied into a plastic bag, homogenized by manual mixing, and sampled for moisture content (n=2). All moisture contents are reported on a wet basis. Dry matter loss was calculated according to Equation 2.2 using the dry weight basis of biomass:

$$\%\ Dry\ Matter\ Loss = \frac{g\ Biomass_{pre\ storage} - g\ Biomass_{post\ storage}}{g\ Biomass_{\ pre\ storage}}\ x\ 100 \quad \text{Equation 2.2}$$

After drying to stability at 40°C, all replicates for a condition (treatment, moisture content, and time) were combined into a single composite sample for analysis and size reduced to pass through a 2 mm screen using a Thomas Model 4 Wiley® Mill (Thomas Scientific, Swedesboro, NJ).

Scanning Electron Microscopy (SEM)

Dried corn stover pieces representing stalk were selected and prepared for SEM imaging using a protocol adapted for confocal imaging[26]. Polyethylene glycol (PEG) 2000 chips (Sigma Aldrich, St. Louis, MO) were heated at 60°C until completely melted. Samples were soaked in water to rehydrate at 60°C and then submerged in a 50% Polyethylene glycol (PEG) 2000 solution in a closed container at 60°C until they were permeated with the solution. Once samples sank to the bottom of the container, the lid was removed to allow for water evaporation. After approximately half of the volume of the solution was evaporated the samples were submerged in 100% PEG. A pan of water was placed at 60°C with the samples in 100% PEG to prevent the PEG from becoming dry and crumbly upon hardening. The samples were removed from the PEG, placed on a microscope slide, and allowed to harden to the slide at room temperature overnight. Samples were sectioned with a scalpel and soaked in water to remove residual PEG, and then they were mounted to a microscope slide at room temperature overnight. The sections were removed from the microscope slides with a scalpel, fixed to carbon SEM mounts with double sided copper tape, and sputter coated with gold. Sections were imaged using a JEOL JSM-6610LV (Peabody, MA) scanning electron microscope.

Compositional Analysis

Chemical compositional analysis on all samples was analyzed using duplicate samples according to standard Laboratory Analytical Procedures[27, 28]. Briefly, corn stover was extracted at 100°C using water and ethanol with an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA)[29].

Remaining biomass was subject to a two-stage acid hydrolysis to solubilize structural carbohydrates[30]. Liquors were analyzed for monomeric carbohydrates using high performance liquid chromatography and a refractive index detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 x 7.8 mm, Hercules, CA)[31]. Acid-soluble lignin was analyzed using a Varian Cary 50 ultraviolet-visible spectrophotometer (Agilent, Santa Clara, CA). Acid insoluble lignin and structural ash were determined gravimetrically on the remaining solids. Protein content was calculated as a function of total nitrogen, and total was is determined gravimetrically[32].

Techno-economic assumptions

A queuing system was designed based on a stacking and reclaiming queuing approach commonly used at pulp and paper mills in the U.S (Figure 2.1). All unit operations from a traditional two-pass corn stover harvest and collection approach were preserved including on-farm storage, transportation, preprocessing at a depot, and delivery to a biorefinery[33].

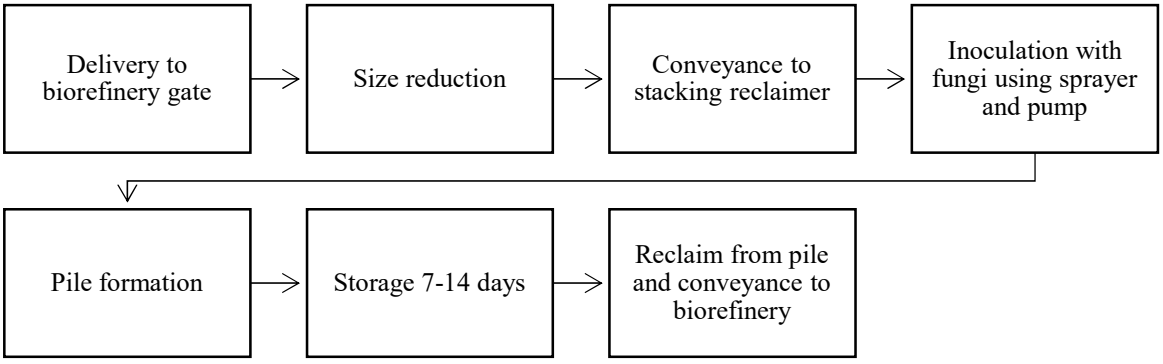

Figure 2.1. Flow diagram of queuing system added to two-pass harvest and collection approach.

Queuing operations for baled biomass at a depot co-located with a biorefinery are reported at $0.97/tonne biomass ($0.88/US ton biomass)[33]. Capital and annual operating costs were calculated for a Bruks COSR stacker reclaiming system using the Biomass Logistics Model (BLM) framework developed at Idaho National Laboratory. Model parameters were shown in Table 2.1. Equipment costs were calculated using American Society of Agricultural and Biological Engineers (ASABE) standard calculations and represented in terms of $/dry tonne biomass. The commercially available stacker reclaimer was chosen that can build up to 8 separate piles, which allows for the appropriate residence time, and then it reclaims biomass and delivers it to a conveyor that feeds the biorefinery. The biorefinery was assumed to operate 350 days a year and 24 hours a day, which can process 2,000

dry tonnes of biomass daily. Moisture content of 60% was assumed and a storage pile density of 240 wet kg/m^3 (14.98 wet lb/ft^3). Multiple dry matter loss levels were considered based on preprocessing costs at depot of a baseline of $26.25/tonne biomass[33]. Fungal inoculant was assumed to be applied during stacking using a pump for storage piles described previously[34] and assigned a cost of $0.50/tonne biomass based on previous estimates[35]. Residence times of 7 and 14 days were modeled, such that the stacker reclaimer system was able to handle the pile volume with associated, modeled residence time. All reported costs are presented in 2016 dollars.

Table 2.1. Capital and annual operating costs of a stacking reclaiming queuing pile. Processing parameters associated with a 2,000-metric ton/day biorefinery. Costs listed on a 2016 basis in U.S. dollars.

	Bruks COSR Stacker and Reclaimer	Inoculant Delivery Pump
Fuel type	Electricity	Electricity
Installed purchase price	$5,000,000.00	$3,080.00
Salvage Rate	0.1	0
Machine Life (Years)	35	15
Interest Rate	8%	8%
Insurance and Tax	2%	2%
Maintenance (% of Annual Value)	10%	10%
Energy Usage (kWh)	1475	1
Energy Unit Cost ($/kWh)	$0.07	$0.07
Labor Rate ($/hr)	$33.00	$33.00
Operators Required	1	0
Discounted Salvage Value	$33,817.27	$0.00
Capital Recovery	$63.73	$0.00
Insurance and Tax Cost	$11.90	$0.01
Maintenance and Repair	$6.37	$0.00
Energy Cost	$95.88	$0.07
Labor Cost	$33.00	$0.00
Throughput (wet tonnes/hr)	458.41	229.21
Moisture Content In (%)	60%	60%
Moisture Content Out (%)	60%	60%
Throughput (dry tonnes/hr)	183.37	91.68
Hourly Cost	$210.88	$0.07
Units	1	4
Inoculum cost ($/tonne biomass)		$0.50
Unit Operation Cost ($/tonne biomass)	$1.15	$0.503
Total Cost ($/tonne biomass)		$1.65

Cost saving for reduced temperature requirements were calculated based on the design of the dilute acid pretreatment operation as reported in the 2015 by Davis, et al[36]. This is a biochemical approach to creating fuels from carbohydrate streams originating from lignocellulosic biomass, and a 160°C dilute acid pretreatment is used to liberate hemicellulose from biomass. The design utilizes high pressure steam (Stream 220; Flow rate = 23,888 kg/hr) and hot process water (Stream 250; Flow rate = 304,369 kg/hr) to maintain the temperature. Dry biomass is fed to the reactor at a rate of 2,000 tonnes/day (83,333 kg/hr).

The total system cost of $1.65/dry tonne was calculated based on hourly throughput of storing 183.37 dry tonnes of material. Due to the dry matter loss during the storage process, preserved biomass weight will be less than 183.37 dry tonnes per hour, which caused increases in the total per dry ton storage cost. This is also true for all the operations in the depot before the storage process such as biomass handling and comminution. In order to reflect the cost of dry matter loss, dry matter loss cost was estimated using equation below[14]:

$Cost_{DML}$ = DML * (Total Cost)/(1-DML) Equation 2.3

Where $Cost_{DML}$ is the cost of dry matter loss, DML is the dry matter loss measure in %, Total Cost is the total cost to process and store the biomass at the co-located depot in $/dry tonne.

Pretreatment data (data not shown) from the present study assessed the impact on sugar yield from by reducing the temperature of reaction from 160°C to 130°C. Previous studies have shown that pretreatment at this temperature allows for differences in feedstock reactivity to be more readily discerned than at higher temperatures, such as 150-160°C[37]. While Wolfrum et al. explored the varying reactivity of different feedstocks in pretreatment, in this study it was reasoned that the lower pretreatment temperature would offer a similar advantage to the present study and enable that decreased recalcitrance to be more readily identified. Therefore, the following equation was used to account for energy savings realized by reducing the temperature of process water from 160°C to 130°C, where Cp is specific heat, q is change in energy, m is mass, and Δ T is temperature change:

$$Cp = \frac{q}{(m \, x \, \Delta \, T)}$$ Equation 2.4

The Cp of water at 130°C of 4.26 kJ/(kg) and a Δ T = 30°C were used to calculate the energy savings per kg of process water, and the flow rate of process water was used estimate total savings per hour. Total cost per ton savings were calculated based on the biomass flow rate of 83,333 kg/hr and a natural gas cost of $3.36/MMBTU, consistent with the 2019 Herbaceous Feedstock State of Technology case developed at Idaho National Laboratory[33].

Results and Discussion

This study aimed to assess the impact of fungal pretreatment in corn stover in terms of compositional, structural, and convertibility changes to gain a fundamental understanding of this potential treatment to reduce biomass recalcitrance in the context of a biorefinery. Dry matter losses and compositional changes were used as a guide to understand the working envelope of a selective (*C. subvermispora*) and non-selective (*P. chrysosporium*) lignin degrader.

Storage induced losses due a selective and non-selective lignin degrader

Loss of dry matter is an important consideration for a biorefinery for economic reasons and sustainability concerns surrounding carbon retention, and it is also an indicator of microbial activity and quality changes as a function of moisture. Screening studies were performed to understand the range of viable conditions (moisture content, strain, and duration) for fungal growth on sterilized corn stover. Dry matter loss was assessed in triplicate reactors (Table 2.2Table 2.). In the corn stover stored without an inoculant, no measurable loss occurred after 2 weeks at 40% moisture, but in the experiment conducted using 60% moisture samples resulted in over 2% loss. Results were similar after 4 weeks. These low values are likely due to sterilization of the corn stover prior to the beginning of the experiment, and Smith et al. showed that >30% dry matter loss can occur in corn stover stored aerobically at similar moisture contents[38]. The *C. subvermispora* treatment resulted in a similar loss profile to the moisture control after 4 weeks of storage at 40% moisture. These similarities between the control and *C. subvermispora* treatment indicate no statistical difference as a function of dry matter loss.

Table 2.2. Dry matter loss fungal-treated corn stover after storage for 2 or 4 weeks

	Dry Matter Loss (%, dry basis)	
Experiment	2 weeks	4 weeks
Control, 40% moisture	NA	0.1 ± 0.2
Control, 60% moisture	2.7 ± 2.4	1.4 ± 0.1
C. subvermispora, 40% moisture	0.5 ± 0.3	1.2 ± 0.4
C. subvermispora, 60% moisture	3.3 ± 1.6	2.6 ± 1.7
P. chrysosporium, 40% moisture	4.6 ± 0.7	11.9 ± 0.6
P. chrysosporium, 60% moisture	10.7 ± 1.0	21.0 ± 0.1

NA: Dry matter loss for this treatment was negligible

P. chrysosporium resulted in a significantly different dry matter loss profile. This is expected given that this strain is a non-selective lignin degrader and depolymerizes cellulose while also releasing the enzymes necessary to oxidize lignin. Losses at 40% moisture were 4.5% of total dry matter after 2 weeks and 12% after 4 weeks. In the 60% moisture corn stover the losses were accelerated, with 11% and 21% total dry matter loss after 2 and 4 weeks, respectively. Visible fungal mycelia were present

in the *P. chrysosporium* inoculated corn stover after just 2 weeks in storage while changes in the moisture control and *C. subvermispora* treatment were less evident. Figure A.1., Figure A.2., and Figure A.3. in the Appendix illustrate this vast change in fungal growth. *P. chrysosporium* inoculated corn stover stored at moisture contents of 40% and 60% also show visible differences with the naked eye, with more hyphae present.

Cross sections of the parenchyma cells and vascular bundles in the pith fraction of the stalk were visually assessed for structural impacts using SEM. Figure 2.2 shows SEM micrographs of corn stover before and after 2 weeks of *P. chrysosporium* treatment at 40% and 60% moisture. The structural variability masks many small changes with few visually discernable changes at the 100x magnification levels. However, a 500x magnification of the stover stored at 60% moisture reveal fungal hyphae attached to the secondary cell wall within the cell lumen. Fungal hyphae have also been reported to penetrate pine cell lumens[39]. This result suggests that fungal hyphae may have a greater cellular impact at the elevated moisture content. Similarly, Asgher et al. varied moisture content of corn cobs inoculated with *P. chrysosporium* and found an optimal moisture content for laccase activity occurred between 60-80% moisture, with approximately half the activity occurring at 40% moisture. SEM micrographs also show slight physical changes visually, where the 40% and 60% moisture samples appear to have increased cell wall tearing and breakage at 100X magnification levels. Overall, the changes observed in SEM micrographs can aid in interpretating other metrics of degradation, as discussed in the following section.

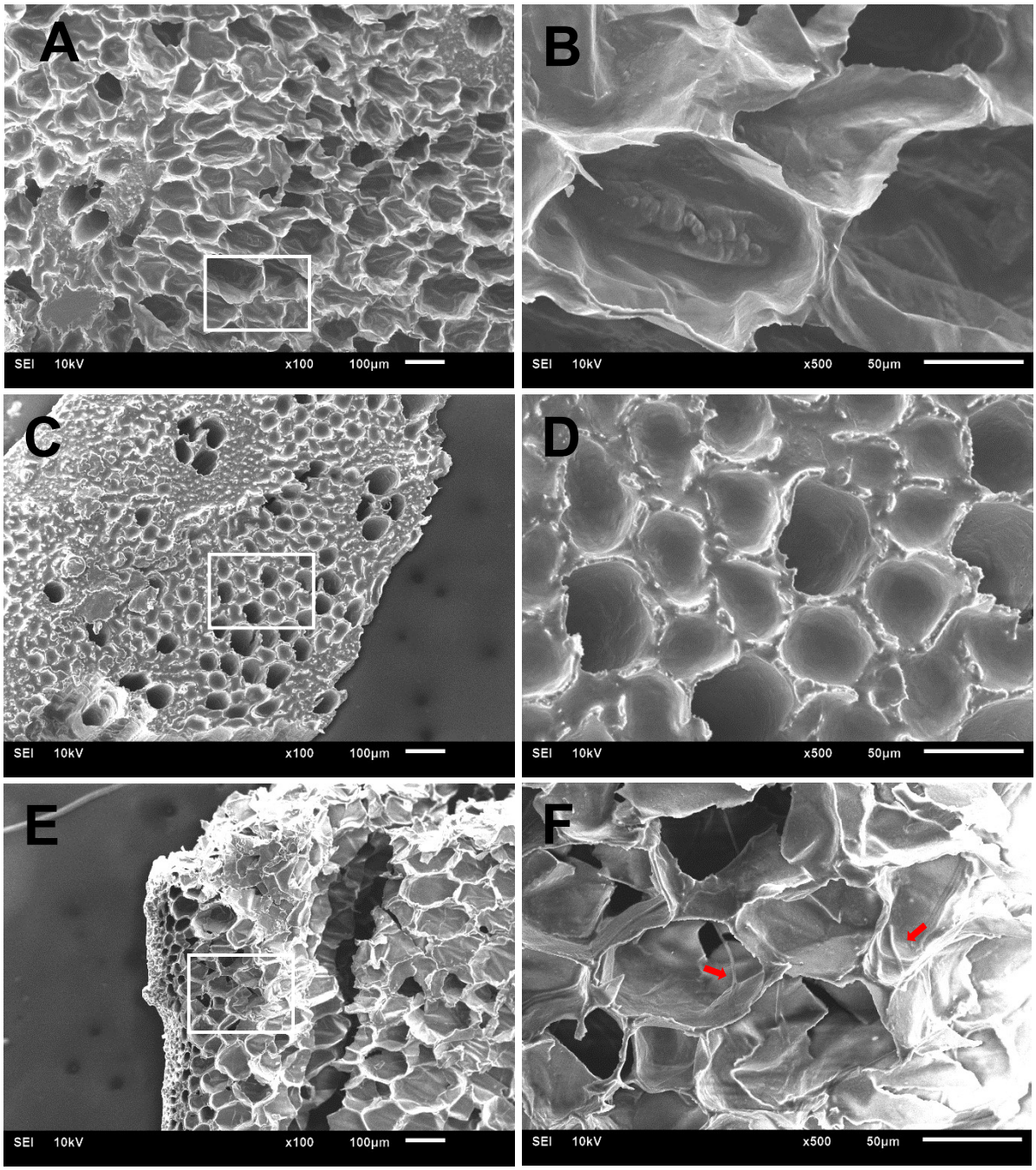

Figure 2.2. SEM micrographs of corn stover A, B: unstored; C, D: Stored 2 weeks with *P. chrysosporium* at 40% moisture; E, F: Stored 2 weeks with *P. chrysosporium* at 60% moisture. Red arrows indicate fungal hyphae attached to cell lumen.

Compositional changes provide insight into fungal mechanism

Corn stover was assessed for changes in chemical composition as a result of storage, time, moisture content, and fungal strain characterized by selective and non-selective lignin degradation. All treatments were assessed for their ability to change the soluble and structural composition of the

biomass (e.g., soluble and structural carbohydrates, acetate, lignin, and extractives). The objective was to evaluate structural components cleaved during degradation with the aim of assessing the abundance and relative proportions of these components to provide insight into mechanisms of potential recalcitrance reduction.

The unstored corn stover served as a basis for comparison, and the control that only had moisture added was used to understand the impact of moisture content and residence time as a function of compositional changes. Compositional changes in the primary components of glucan, xylan, lignin, and water extractives are shown in Figure 2.3. Results represent dry matter loss weighted composition exiting storage. Compositional analysis of the final stored mater, unweighted for dry matter loss is reported in the Appendix, Table A.1. and A.2., with the weighted composition is presented in Table A.3. and A.4.

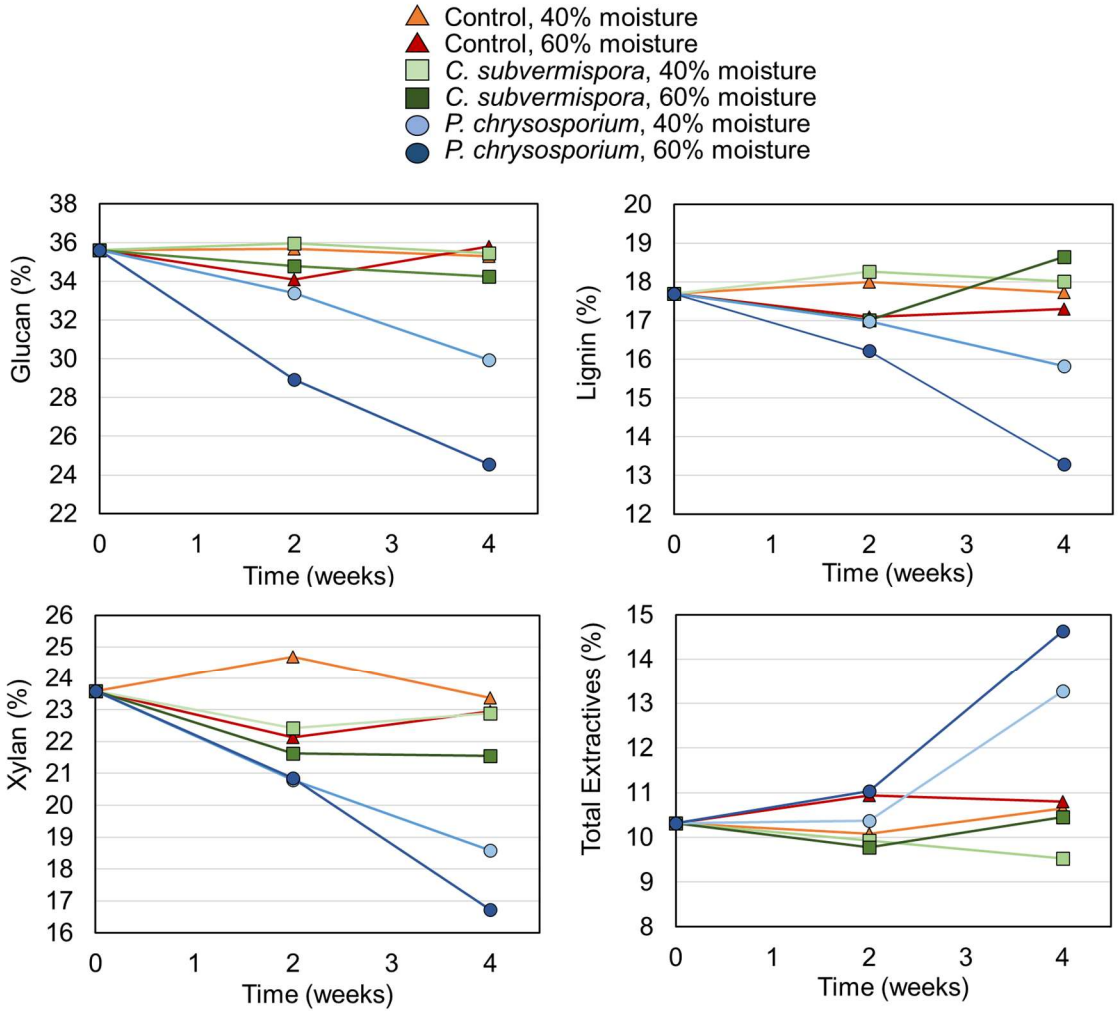

Figure 2.3. Compositional changes weighted with dry matter loss as a function of time in screening study in corn stover inoculated with *C. subvermispora* and *P. chrysosporium*.

Few compositional changes were observed in the moisture control, likely because the corn stover was sterilized prior to storage such that the native microflora had been eliminated. However, structural galactan and arabinan decreased by a relative 11-15% decrease after 4 weeks of storage at both moisture contents. Galactan decreased from 1.14% to 1.02% and 0.97%, respectively, at 40% and 60% moisture conditions; arabinan decreased from 2.55% to 2.26% and 2.13%, respectively, at 40% and 60% moisture conditions. A corresponding slight increase in soluble arabinan occurred from 0.19% in the unstored to 0.36% and 0.26% after 4 weeks in 40% and 60% moisture conditions, respectively. While these changes are small, they are greater than error seen in the analytical control included in all compositional analyses. However, changes in galactan were not observed, suggesting those carbohydrates were consumed by microbial activity. Protein content also decreased at a similar rate over 4 weeks to structural galactan and arabinan, beginning at 3.17% and existing storage at 2.71% and 2.74% at 40% and 60% moisture, respectively; this trend supports the hypothesis that minor microbial activity was present and consuming protein to support cell growth. Lastly, slight amounts of xylan solubilization were observed at 40% an 60% moisture over 4 weeks. The corn stover entered storage with 23.58% xylan and decreased to 23.38% and 22.94% at 40% and 60% moisture, respectively. A corresponding increase in soluble xylan was observed from 0.35% to 0.49% and 0.51% at 40% and 60% moisture, respectively. These results indicate that despite sterilization there was minor microbial activity that began to depolymerize hemicellulose components over 4 weeks of storage, an important consideration when comparing to more severe fungal treatments.

The selective lignin degrader, *C. subvermispora*, incurred only minor compositional changes between 2 and 4 weeks, corresponding to the low dry matter loss levels observed in this treatment. This fungal strain exhibited enhanced xylan solubilization compared to the uninoculated moisture control. The most notable structural xylan decrease was exhibited at 60% moisture, entering storage at 23.58% and exiting at 21.63% and 21.55% after 2 and 4 weeks, respectively. Hemicellulases have been documented as one of the many enzymes produced by *C. subvermispora*[40]. However, the arabinan solubilization exhibited in the uninoculated control was not observed, and structural arabinan was enriched due to the loss of other components. Glucan content was within the same relative percentage change as the uninoculated moisture control, consistent with other reports that *C. subvermispora* targets lignin not cellulose. However, protein content decreased throughout the storage period compared to the controls, entering storage at 3.17% and exiting at 1.82% and 1.64% after 4 weeks of storage at 40% and 60% moisture, respectively. These findings suggest that *C. subvermispora* struggled with growth but was degrading the corn stover minimally to access the protein and

hemicellulose, and slight oxidation of the more soluble lignin fraction occurred. Similar results have been reported elsewhere[41].

Slight lignin solubilization was exhibited as a function of *C. subvermispora* inoculation in comparison to the native and uninoculated, stored corn stover controls. The amount of lignin that was solubilized during acid hydrolysis tended to decrease in comparison to the acid insoluble, which tended to increase due to loss of other components. This is consistent with the known laccase and peroxidase activity that is expressed in *C. subvermispora* to support depolymerization of phenolic lignin (citation in comment above). Total lignin concentration remained constant, suggesting it degraded at a rate consistent with dry matter loss. Losses of 7.7% and 13.1% have been observed in 65% moisture content corn stover inoculated with *C. subvermispora*, with losses of greater than 50% lignin by 15 days with only 3.5% and 14.7% losses in cellulose and hemicellulose, respectively[42]. However, the chemical composition of this corn stover was more concentrated in hemicellulose (33.0% vs. 27.3% in this study) and reduced in lignin (10.6% vs. 17.7% in this study), which is suggestive of a younger, less lignified corn plant that may be inherently more susceptible to fungal attack. Less mature corn plants also have a higher concentration in free sugars[43], which provide a nutrient source to encourage all microbial growth including fungal. Further studies are warranted to investigate the initial growth requirements of *C. subvermispora* on corn stover as a function of incoming attributes including free sugars, standing age, and resulting structural features including lignin and carbohydrate distributions.

Corn stover inoculated with the non-selective lignin degrader, *P. chrysosporium*, exhibited more marked changes than either the moisture controls or the *C. subvermispora* treatments. Total extractives in the *P. chrysosporium* treated stover were elevated to 10.37% and 11.04% after 2 weeks of storage at 40 and 60% moisture, respectively; this was increased to 13.28% and 14.64% after 4 weeks of storage. The greatest relative component changes, up to 300%, observed in the *P. chrysosporium* treatments were in soluble xylan and arabinan, which increased at similar rates suggesting arabinoxylan degradation. The primary hemicellulose in corn stover is arabinoxylan, a β,1-4 xylan backbone with arabinan substitutions along the chain. Likewise, structural xylan decreased from 23.58% to 16.72% in the 4-week, 60% moisture corn stover suggests cleavage of glycosidic bonds occurred in hemicellulose. However, this loss in protective hemicellulose likely exposed the cellulose and made it more susceptible to attack by *P. chrysosporium*. Cellulose degradation was evident by structural glucan decreases from 35.61% to 33.40 and 29.94% at 40% moisture and 28.94% and 24.54% at 60% moisture. Soluble glucose was unchanged, further suggesting consumption by the fungi to support respiration and lignin degradation. Lignin content in

the 40% moisture *P. chrysosporium* treatment began at 17.69% but was reduced to 16.98% and 15.83% after 2 and 4 weeks of storage, respectively, but the highest decrease in lignin was evident at 60% moisture with reduction to 15.83% and 13.29% after 2 and 4 weeks of storage. *P. chrysosporium* inoculation also resulted in a higher relative decrease in the acid soluble lignin and ethanol extractives than what occurred during *C. subvermispora* treatment. These results are consistent with the reaction mechanisms of *P. chrysosporium* when actively degrading corn stover and the combination of cellulases, hemicellulases, and lignin degrading enzymes expressed. Adav et al. characterized over 60 cellulases expressed in the fungi when corn stover was used as a growth substrate, with cellulase as a dominant contributor[44]. This is consistent with the elevated glucan degradation that occurred during the first 2 weeks of storage compared to xylan and lignin degradation. 32 actively produced hemicellulases were also characterized by Adav et al., and these play a critical role in exposing the cellulose for attack. They also found upregulation of cellobiose dehydrogenase in *P. chrysosporium*, which oxidizes cellobiose to produce the hydrogen peroxide that can further degrade lignin through the Fenton reactions that form highly reactive hydroxyl radicals.

In summary, the screening study results suggest that the combination of cellulases, hemicellulases, and lignin degrading enzymes were in action in *P. chrysosporium* treatment of corn stover. Similar trends in dry matter loss and associated compositional changes have been observed in *P. chrysosporium* after 15 and 30 days of storage at 75% moisture, with high cellulase activity occurring compared to other strains assessed[45]. *C. subvermispora*-related impacts were minor, but hemicellulase activity indicated the fungi had begun to depolymerize the corn stover. One key finding from the screening study indicates the importance of moisture content to support fungal depolymerization, with 60% moisture conditions exhibiting enhanced solubilization in this study. A higher moisture content is also aligned with outdoor storage of fungi in creating an additional barrier to uncontrolled combustion.

Cellulose and hemicellulose preservation are also a metric of importance when considering adopting a fungal pretreatment into a biorefinery model. Total structural carbohydrate targets for delivered corn stover in a biochemical conversion approach were 59%[46], and all treatments except the *P. chrysosporium* 4 week samples met that specification. Failing to meet the 59% carbohydrate target will either result in lower biofuel conversion yield or higher feedstock cost because more biomass will be needed to maintain the same conversion yield. The enhanced solubilization of hemicellulose components suggest *P. chrysosporium* treatment may be complimentary to biorefinery based treatments that utilize acid pretreatments to depolymerize hemicellulose and isolate cellulose that is then carried into fermentation. Based on these results, larger scale fungal treatment studies were

conducted with *P. chrysosporium* over a 2-week inoculation time to not only control dry matter loss but also reduce residence time and associated costs of the queuing operation.

Techno-Economic Implications of a Combined Fungal and Chemical Pretreatment

Typical biomass pretreatments necessitate a combination of high temperature, pressure, and acidic or alkaline conditions to ensure cellulose accessibility for downstream enzymatic conversion to monomers. Conversion systems require yields ideally near 100%; consequently, high severity pretreatment conditions are typically required. This can result in over-pretreatment of less recalcitrant biomass tissues, such as leaves, resulting in the formation of byproducts well known to inhibit microbial fermentation. Therefore, a combination of fungal pretreatment, non-biological pretreatment, enzymatic hydrolysis, and conversion of monomers to fuels is likely necessary for a fully integrated biomass conversion process.

The queuing operation allows for short term storage at the biorefinery gate and mitigates the risk of lack of the hourly feedstock being provided to the biorefinery. Baseline queuing residence times are 3-5 days of feedstock supply, and yet longer residence times are possible. In this study, the modeled residence time was 1-2 weeks. A logistics system was designed to utilize short term queuing at the depot to perform fungal treatment in storage piles, similar to what has been designed for wood chip piles[35]. Capital and operating costs for this system were estimated at $1.65/tonne biomass, a 1.7-fold increase over the traditional queuing pile costs of $0.97/tonne. Experimental results reported in Chapter 3 indicate that a 30°C temperature reduction was possible in pretreatment to achieve equivalent xylose yields, which is the primary goal of dilute acid pretreatment. This correlated to an energy savings of 0.44 MMBTU/tonne biomass, and thus a cost savings of $1.48/tonne was applied to the fungal-assisted queuing system that correlated to reduced natural gas consumption required heating for pretreatment at the biorefinery. After considering the cost savings from using pretreated biomass, the system cost for the fungal-assisted queuing system was about $0.17/dry tonne, which was $0.80 lower than the traditional queuing pile cost.

As discussed in the methods section, dry matter loss will impact total system cost in a negative way. Net system cost increased proportionally with dry matter loss (Figure 2.4), and therefore future research should target opportunities to prevent dry matter loss while maintaining recalcitrance reduction opportunities. Interestingly, overall dry matter loss is not a primary metric reported in literature describing fungal pretreatment for lignocellulose, and this study highlights the importance of the metric for overall cost effectiveness. This result implied that future research should target reduced dry matter loss levels in queuing or increased recalcitrance reduction, which could be possible with alternative approaches. For example, non-selective lignin degraders combined with

ferulic acid esterase active at low moisture contents could further liberate lignin and hemicellulose bonds. Additional fungal strains with unique enzyme complexes could also be explored, and Shirkavand et al. provide an extensive review of strains employed recently[17].

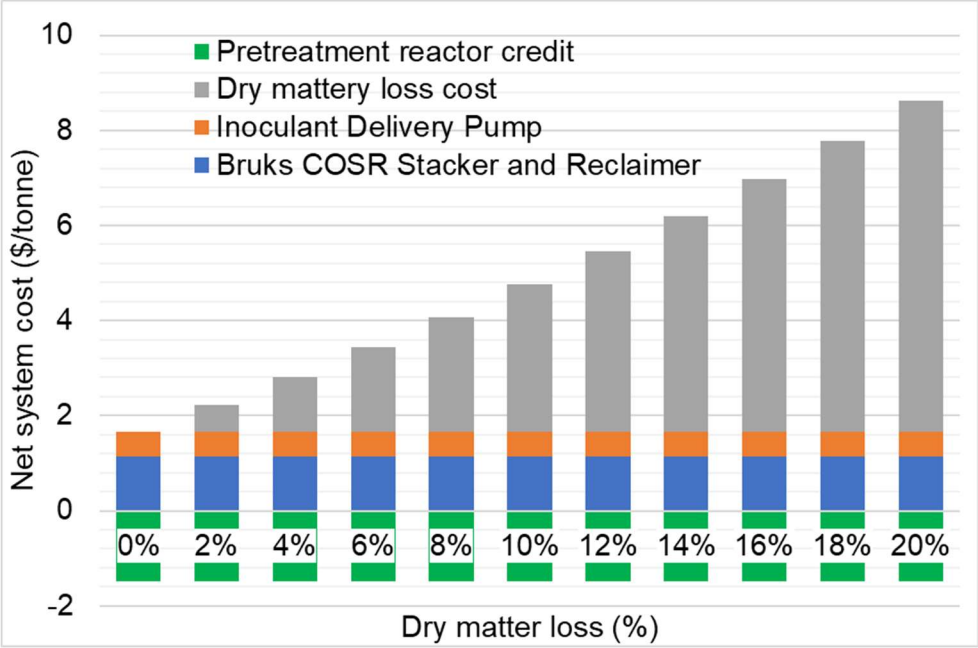

Figure 2.4. Net system cost of the fungal mediated queuing costs and corresponding cost offsets as a function of dry matter loss.

Figure 2.5 presents the total net feedstock costs for the pretreatments assessed in this study with different residence times and moisture contents. As shown in the figure, residence time of 4 weeks generally resulted in higher dry matter loss and thus high total cost than residence time of 2 weeks for all the pretreatment groups. Storing biomass with *C. subvermispora* achieved the lowest total net cost compared to the control treatment and the *P. chrysosporium* treatment in both residence times modeled, which suggested that *C. subvermispora* treatment is an economically viable way to store biomass in the fungal-assisted queuing system. Likewise, controlling dry matter loss in *P. chrysosporium* through limited storage time also reduced excessive costs due to degradation.

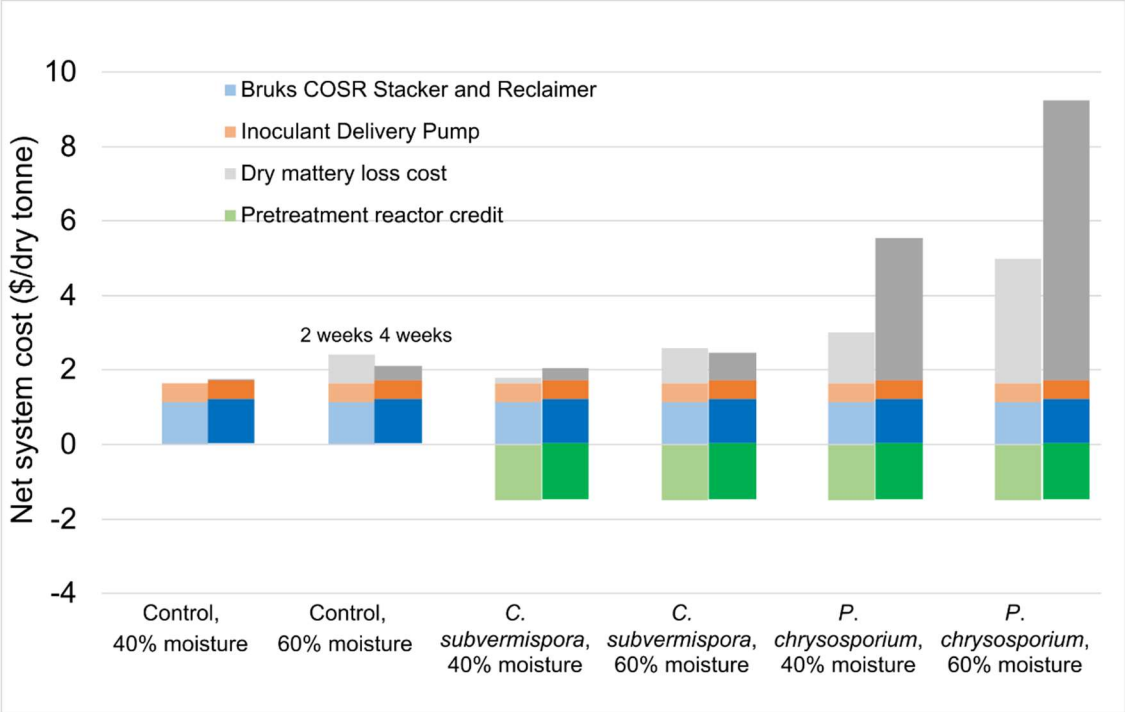

Figure 2.5. Net system cost of fungal mediated queuing costs and corresponding cost offsets in different treatments.

Conclusion

Filamentous fungi can effectively depolymerize lignocellulosic biomass through enzymatic action. This study defined the range of viable conditions, including moisture content and residence time, of both a selective and non-selective fungal strain in the context of a queuing pile at a biorefinery reactor throat. This study indicated that enhanced structural depolymerization that occurred with P. chrysosporium should be limited to 2 weeks of residence time. Slower growth and degradation of C. subvermispora allowed for tolerance of the longer residence time. Furthermore, dry matter loss, structural carbohydrate and lignin changes, and techno-economic analysis were used to suggest potential approaches for further investigation. Increased reduction in recalcitrance, which could possibly be achieved with alternative approaches, has the potential to offset costs associated with material losses and should also be explored in future research. Additionally, follow-on research should explore storage performance in larger systems to understand the range of dry matter loss correlated to not only compositional changes but also conversion-related impacts. Opportunities also exist to understand the fungal impact on a mechanistic level by following molecular changes using techniques such as analytical pyrolysis, spectroscopic characterization, x-ray diffraction, and nuclear magnetic resonance. These characterizations may reveal the physicochemical impacts of fungal

treatment on specific lignin molecules, hemicellulose linkages, and even cellulose physical state, which could open new pathways for recalcitrance reduction in corn stover.

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Chapter 3: Molecular and structural impacts of fungal depolymerization of corn stover to reduce pretreatment severity

Abstract

Filamentous fungi are known for their role in lignin degradation and have been studied to reduce recalcitrance of bioenergy feedstocks. This study explored how filamentous fungi pretreatment in a queuing operation might be applied in the working envelope of a biorefinery. Corn stover was inoculated with a non-selective lignin degrading filamentous fungal strain (*Phaenaerochaete chrysosporium*), then stored for two weeks in aerated bioreactors designed to mimic storage conditions in large-scale outdoor storage piles to determine whether the fungal pretreatment made the material more susceptible to lignin degradation. Gross composition changes resulting from *P. chrysosporium* treatment included hemicellulose and lignin degradation. Pyrolysis GCxGC/MS results suggested that cleavage of glycosidic bonds in hemicellulose resulted in enhanced sugar degradation products. Enhanced G and S lignol releases were observed. Dilute acid pretreatment and subsequent enzymatic hydrolysis indicated that lowering the reaction temperature to reduce the severity of pretreatment resulted in equivalent xylose release in unstored and fungal treated samples. These results suggest that this combined biological and thermochemical pretreatment approach can effectively augment glycosidic bond cleavage and lignin degradation in lignocellulosic biorefineries.

Introduction

Utilization of non-food lignocellulosic biomass resources to produce liquid transportation fuels is an opportunity to decarbonize the transportation sector[1]. Agricultural residues, such as corn stover, are a present-available source of this non-food biomass, with >130 million dry tonnes presently available[2]. The challenge of lignocellulosic biomass is that it is inherently complex on a molecular scale. Cellulose microfibrils protected by the complex woven hemicellulose, pectin, and lignin result in natural recalcitrance to thermal, chemical, enzymatic, or biological depolymerization[3]. Biochemical approaches to converting lignocellulosic biomass rely on the depolymerization of this complex matrix to result in monomeric carbohydrate streams, and thus significant energy is applied to the biomass using a combination of chemicals, heat, and enzymatic inputs.

Solubilization of lignin using alkali is one approach to isolating cellulose and hemicellulose and has been used in the pulp and paper industry as well as in bioenergy conversion approaches[4]. Lignin polymers consist of up to hundreds of phenolic units based on p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) subunits. These subunits are formed from oxidative polymerization of *p*-coumaryl,

coniferyl, and sinapyl alcohols, respectively. Lignin is bound to hemicellulose, pectin, and cellulose through direct ester or ether bonds, and it can also be linked by acid bridges including ferulic acid or hydroxycinnamic acid[5, 6].

An additional approach to solubilize lignin is through the mechanisms employed by filamentous fungi. Filamentous fungi are well-characterized lignin degraders, producing enzymes to depolymerize lignin and expose cellulose and hemicellulose[7]. Utilization of filamentous fungi as a biological pretreatment has been studied as a low-cost approach to depolymerizing biomass prior to biofuel production as well as in the pulp and paper industry[8, 9]. An opportunity for bioenergy systems is to employ the "seasoning" approach often used in pulp and paper mills to reduce extractives and to cleave lignin bonds [10]. This operation is synergistic with the queuing operation in a biorefinery, which enables a consistent supply of feedstock to feed a reactor[11]. Transforming the status queuing operation to an active approach for fungal pretreatment is a process intensification approach that may improve economics and life cycle metrics. A comprehensive review of studies assessing fungal-assisted pretreatments is available and highlights the opportunity space for the scientific community[12].

Three primary classifications of filamentous fungi are widely recognized: white-rot, brown-rot, and soft-rot. White-rot fungi are the most commonly studied in terms of biomass pretreatment because their targeted mode of action is lignin degradation facilitated by the secretion of enzymes including lignin peroxidase, manganese peroxidase, and laccase[13]. Brown rot fungi have evolved significantly from the white rot to utilize non-enzymatic approaches to lignin decomposition, and they are hypothesized to occur through Fenton reactions[14, 15]. Fenton reactions are based on the formation of highly reactive hydroxyl radicals (·OH) formed from the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2. The resulting hydroxyl radicals oxidize the bonds in cellulose, hemicellulose, and lignin, resulting in near complete degradation of the biomass. Soft-rot fungi lack lignin degrading enzymes and instead target cellulose as a carbon source through the utilization of cellulases and hemicellulases[9, 16]; hence, soft-rot fungi have limited applicability in biomass pretreatment but are important enzyme producers in biomanufacturing.

The most prominent lignin-modifying enzymes, lignin peroxidase, manganese peroxidase, and laccase, all result in oxidation of lignin molecules but have unique reaction mechanisms. Lignin peroxidase targets non-phenolic portions of lignin through oxidation of lignin molecules and reduction of H_2O_2 resulting in radical cation production[13]. Manganese peroxide is oxidized from Mn^{2+} to Mn^{3+}, which forms a complex with an organic acid that can then oxidize phenolics in lignin[17]. Laccase utilizes molecular oxygen to oxidize phenolics in lignin[13]. Hatakka summarized the presence of lignin-modifying enzymes and associated isoenzymes in a range of fungal species[13]. Su et al.

developed a high throughput method for evaluating fungal strains based on their levels of laccase, lignin peroxidase, and manganese peroxidase expression during biomass degradation[18]. Similarly, Sista Kameshwar and Qin characterized the genomic-level prevalence of these enzymes across white-, brown-, and soft-rot fungi[16]. The prevalence and high activity levels of these lignin degrading enzymes have resulted in broad utilization of filamentous fungi-secreted enzymes to depolymerize a range lignocellulosic-based product streams for biomanufacturing purposes.

Impacts of fungal treatment within the context of a biorefinery are often limited to metrics such as lignin loss and downstream carbohydrates released in conversion. A full understanding of storage performance and associated conversion impacts coupled with molecular characterization is necessary to further understand the mechanisms of this complex system. In the present study, corn stover was assessed for the potential impact of fungi-induced lignin degradation in a simulated outdoor environment conducted using 100L working volume aerated bioreactors. This was coupled with analytical investigations performed to better understand the mechanisms of degradation in the context of bioenergy systems. Compositional changes in macromolecular components measured using traditional methods were characterized with analytical pyrolysis coupled with two-dimensional gas chromatography mass spectrometry (GCxGC/MS) to assess molecular changes. The production of small molecules during analytical pyrolysis was correlated with ^{13}C cross-polarization magic-angle-spinning (CP/MAS) nuclear magnetic resonance (NMR) spectroscopy to understand the structural impacts on the biomass and suggest mechanisms of fungal attack. Finally, a combination of fungal pretreatment and acid-based pretreatment were assessed to show the impact of fungal treatment on hemicellulose removal and remaining cellulose accessibility to enzymatic depolymerization.

Methods and Materials

Filamentous Fungi Cultivation

Experiments were performed using *Phaenaerochaete chrysosporium* (NRRL 6370), a non-selective lignin degrader using methodology described in Saha et al.[19]. The fungi were first grown on Yeast Mold Agar (BD Difco™, Franklin Lakes, NJ) and then in Yeast Mold Broth (BD Difco™, Franklin Lakes, NJ) with shaking at 50 rpm at 28 °C. *P. chrysosporium* fungus was grown for seven days where it reached a density of 8.4 mg dry weight/mL. The cultures were pelleted by centrifugation at 8,000 rpm, resuspended with 25 mL of a 10 mM phosphate buffer and homogenized in a Waring blender. The cultures were pelleted once more, resuspended in 65 mL of sterile tap water. Dry fungal weight was calculated based on OD_{600} measurements and correlated to the fungal slurry weight after drying overnight at 105 °C.

Corn Stover Source and Storage Procedure for Large Scale Experiment

Corn stover was sourced from Hubbard, IA in 2018. Corn stover was size reduced using a Schutte Buffalo hammer mill fitted with a 6 mm screen. Duplicate 100 L storage reactors were used for *P. chrysosporium* and compared to a moisture only control, with methods similar to those described previously[20]. Briefly, the reactor design (Figure 3.1) consisted of stainless-steel chamber insulated with a temperature-controlled water jacket in stainless steel chassis surrounded by fiberglass insulation. Airflow was controlled with mass flow controllers and introduced from the bottom of the reactor and exiting the top of the reactor. Inlet air was kept at or near 100% relative humidity by sparging through a water bath that was maintained at the same temperature as the reactor.

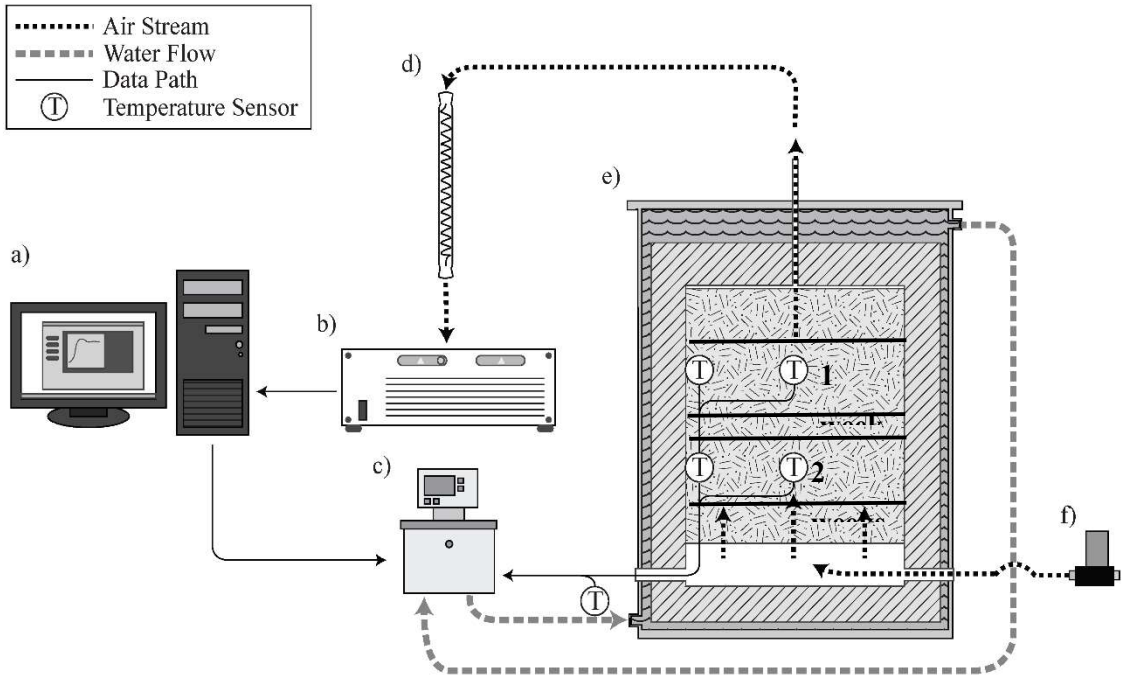

Figure 3.1. Illustration of the 100 L storage with zones for 1- and 2-week storage durations. Image modified from Bonner et al.[21] a) control terminal, b) gas chromatograph, c) heated water circulator, d) vapor condensor, e) reactor loaded with corn stover, f) mass flow controlled gas supply.

Gas exiting the reactors traveled through a vapor condensing column to remove moisture. Gas was then pumped to a MicroGC 3000 gas chromatograph (Agilent, Santa Clara, CA) with a PLOT U column and thermal conductivity detector for carbon dioxide measurement. CO_2 was a product of aerobic microbial respiration that can be used to assess degradation in real time according to what is described in McGechan (1989)[22], where $(CH_2O)_n$ relates to the carbohydrate loss calculated based on CO_2 mass measured using the molar ratio of 1:1 known for microbial respiration.

$$\% DML = \frac{\Sigma(CH_2O)_n}{g\ Biomass_{\ pre\ storage}} x100 \qquad \text{Equation 3.1}$$

The reactors were modified with stainless steel screens and spacers such that two zones for biomass were available for sampling at one and two weeks, respectively. Corn stover (10 kg) was rehydrated to 55% moisture overnight at 4°C in supersack liner and manually mixed to distribute water, and then the following day it was inoculated with 2,500 ml solution containing 3.34 g of *P. chrysosporium* to reach a moisture content of 60% wet basis (w.b.) and mixed thoroughly. Inoculated corn stover was then loaded into the duplicate reactors, and moisture content for each reactor was assessed on three samples taken throughout the loading process to account for associated moisture losses during loading. Uninoculated corn stover was also rehydrated overnight and used as a control, although inconsistent mixing resulted in moisture contents of 59.5% and 70.3% for the one- and two-week zones in Reactor 1, respectively, and 51.5% and 65.7% for the same zones in Reactor 2. Moisture content for the *P. chrysosporium* inoculated stover in Reactors 3 and 4 was between 59.0% and 59.5% for all zones. An airflow of 1 L/min filtered room air, corresponding to complete air exchange every 100 minutes, was applied to the reactors and humidification of the air at the bottom of the chamber occurred, similar to previous studies[20, 23, 24]. Temperature changes were measured at the interior of each zone within the reactor using resistance temperature detectors collecting measurements continuously over the course of the experiment.

Compositional Analysis

Chemical compositional analysis was performed using standard Laboratory Analytical Procedures on duplicate samples[25, 26]. This procedure began with corn stover undergoing exposure to 100°C water and subsequent ethanol extraction using an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA)[27]. Extracted biomass is then subject to two-stage acid hydrolysis to solubilize structural carbohydrates[28]. Monomeric carbohydrates in the liquors were quantified using high performance liquid chromatography and a refractive index detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 x 7.8 mm, Hercules, CA)[29]. Acid-soluble lignin was calculated with a Varian Cary 50 ultraviolet-visible spectrophotometer (Agilent, Santa Clara, CA). Gravimetric differences were used to quantify acid insoluble lignin, structural ash, and total ash[30]. Acetate was measured using the HPLC described above but with an HPX-87H ion exclusion column (Bio-Rad, 300 mm × 7.8 mm, Hercules, CA, USA). An additional sample was taken through all steps through the two-step acid hydrolysis to collect a lignin-enriched step used for further molecular analysis.

Molecular Characterization with Two-Dimensional Gas Chromatography and Mass Spectrometry (GCxGC/MS)

Approximately 300 μg of corn stover milled to pass a 0.2 mm screen was weighed and added into 38 mm analytical pyrolysis tubes fitted with a 19 mm quartz spacer (CDS Analytical, Oxford, PA) and a small quartz wool plug. A second quartz wool plug was added to secure the biomass followed by 3 nanomoles of the internal standard 9-(9H)-fluorenone. The fluorenone was injected into the plug as 1 μl of a 3 millimolar solution in acetonitrile. Alternatively, a 1 nanomole aliquot of biphenyl was used as an internal standard. The internal standards were used to provide quantitation for a select number of pyrolysis products, and both fluorenone and biphenyl provided comparable results. Analytical pyrolysis occurred in a CDS Analytical 5250 pyrolyzer equipped with a 36 sample autosampler. Briefly, the pyrolysis experiment occurred when the sample was lowered into the pyrolysis chamber, initially subjected to a 2 second drying time at 100°C, and then held for an additional second at 100°C. Temperature within the chamber increased at 50°C/second to the maximum pyrolysis temperature of 400°C (T_{max}) and then was held at T_{max} for 5.00 seconds followed by ejection of the sample tube. A cleaning cycle followed each run, heating the pyrolysis chamber to 1,200°C for 10 seconds to remove residual solids.

Two-dimensional gas chromatography (GC) was performed as described previously[31] using an Agilent 7890 gas chromatograph that separates compounds first on the basis of boiling point and then by polarity. GCxGC is enabled by a four-jet modulator and a secondary oven within the primary oven. The first chromatographic dimension used a 28 m L x 0.25 mm i.d. column with a 0.5 μm Rxi-5ms (Restec, Bellafonte, PA) stationary phase consisting of 5% diphenyl/95% dimethyl polysiloxane. The second chromatographic dimension used a 1 m L x 0.1 mm i.d. column with a 0.1 μm Rxi-17 (Restec, Bellafonte, PA) stationary phase consisting of 50% diphenyl/50% dimethyl polysiloxane. The addition of a second gas chromatograph dimension in the pyrolysis allowed for detection of pyrolysis products that would otherwise co-elute, such as guaiacol and anhydro sugars. A transfer capillary between the secondary oven and the mass spectrometer consists of a 21 cm section of the Rxi-17 column. He carrier gas flow was maintained at 1 mL/min occurred throughout the analysis.

The analytical pyrolysis compounds were split in the heated injector (300°C) using a split ratio of 20:1. Upon initiation of the analysis, the primary column was held at 50°C for 0.5 min, then ramped at 7.5°C/min to a target temperature of 260°C and held constant for an additional 3.00 min. The secondary oven and 4-jet modulator were maintained at 5 and 15°C, respectively, above the temperature of the primary oven. After a 3.00 sec modulation period, hot and cool pulse times were utilized as a means to efficiently trap and desorb compounds of increasing mass over the course of the

experiment, with smaller compounds eluting first. Hot pulse and cool times were 0.50 and 1.00 sec, respectively, before a retention time of 394 sec. This was repeated twice per modulation cycle by the 4-jet modulator. After 394 sec, the hot pulse and cool times were 1.00 and 0.50 sec, respectively. The transfer capillary to the mass spectrometer was maintained at 280°C.

Mass spectrometry was performed using a Leco Pegasus 4D instrument (St. Joseph, MI). An acquisition delay of 220 seconds allowed very light compounds to pass through the time-of-flight mass spectrometer before analysis was initiated. The instrument was scanned from *m/z* 43 to 300 at a rate of 200 spectra/second, a scan rate that enabled deconvolution of closely eluting compounds. The electron impact ion source was operated at 250°C with an ionization energy of 70 volts. Mass spectra for pyrolysis-generated compounds were generated using the Leco ChromaTOF software using automatic smoothing and a baseline off-set value of 2.0, which discriminated against the spectrometer noise level. Compounds were identified by library searching mass spectra against the NIST and Wiley mass spectral libraries, and identification was based on forward and reverse similarity indices, probability[32], and the judgment of the analyst.

Three dimensional chromatograms (3D chromatograms) were generated using both the total ion- and extracted ion-chromatographic data. The resulting plots provide a color-mapped qualitative assessment of the differences between the pyrolysis behavior of the unmodified and fungal-pretreated samples. The z-axes were scaled to enable pairs of 3D chromatograms to be normalized for differences in the initial sample masses. Pyrolysis efficiency data was also assessed (Appendix B).

Solid-State ^{13}C [1H] -CP/MAS NMR Spectroscopy

Solid corn stover samples milled to 0.2 mm minus were loaded into 4 mm ZrO rotors and capped with Kel-F rotor caps. The spectra were measured using a standard Bruker HX magic-angle spinning (MAS) probe as part of a Bruker Avance III spectrometer with a field strength of 9.4 T (^{1}H v = 400.03 MHz, ^{13}C v = 100.59 MHz). All samples were spun at v_R = 10 kHz. The basic Bruker cross-polarization (CP) pulse program was used for all samples.[33, 34] Proton nutation frequency was set at 92.6 kHz with a decoupling field strength of 48.1 kHz (under the SPINAL64 decoupling program).[35] The Hartmann-Hahn condition (contact time) was optimized at 1.5 msec using the unstored corn stover and used for the remaining samples. The first spectra collected were conducted on unstored corn stover and *P. chrysosporium*-inoculated corn stover that had been stored for 1 or 2 weeks in the aerated bioreactors. The relaxation delay for these experiments was set to 4 sec, the sweep width was set to 497 ppm, and the total number of transients per experiment was 16,384 (for a total experimental time of 2.85 days). Spectra were normalized to the peak at 105 ppm which coincides with the C1

bond in cellulose to allow for comparative analysis. The Crystallinity Index (CrI) was assessed by subtracting the peak area of the crystalline portion of the area under C4 bond curve from the total area of the C4 region[36]. The second set of spectra were collected from samples containing acid insoluble lignin residues collected according to the procedure outlined in the "Compositional Analysis" section above. Due to the small amount of material from these samples, the mass of the solid was enhanced by addition of sodium chloride, which provided a ^{13}C NMR invisible matrix while allowing for the rotors to be fully filled. The number of scans used in these experiments was 2048, the number of points used in the acquisition was 4,994, and the relaxation delay was set to 4 sec, which amounted to a total experimental time of 2.3 hours per sample. The spectra were normalized to the peak at 54.8 ppm that represents methoxyl groups in lignin. The total number of points for each analysis was 4,994 points but this was truncated during processing to 900 points to reduce the amount of noise in the spectra, as the free-induction decay had reached the noise level at that point.

Dilute Acid Pretreatment and Enzymatic Hydrolysis

Dilute acid pretreatment was performed in triplicate on the unstored and fungal-treated biomass from Reactor 3 using a Dionex ASE 350 Accelerated Solvent Extractor (Dionex Corporation, Sunnyvale, CA) based on a method described in Wolfrum et al.[37]. Briefly, 3 g (dry weight equivalent) of corn stover knife milled to pass a 2 mm screen was loaded manually into a Dionium extraction cell. Sulfuric acid (30 ml of 1% sulfuric acid w/w) was loaded into the cell to achieve 10% (w/w) solids loading, followed by a six-minute ramping to the desired temperature and a seven-minute incubation period. This liquor was expelled from the cell and collected in glass jars followed by neutralization of the solids with 100 ml of nanopure water and collection of this rinse water. All samples were analyzed in quadruplicate to ensure triplicates were available for statistical analysis. Residence temperatures of 110°C, 130°C, and 160°C, corresponded to a combined severity factor (CSP) of 1.31, 1.73, and 2.61, respectively. CSP quantifies in a single value the severity of the pretreatment based on reaction time, temperature (°C), and pH[38].

$$(\text{CSP}) = Log\left(t \times exp\left(\frac{T-100}{14.75}\right)\right) - pH \qquad \text{Equation 3.2}$$

Monomeric and polymeric sugars were measured using HPLC, as described above.

Pretreated solids were then subjected to enzymatic hydrolysis based on a method designed previously[39]. Size reduction was accomplished using a Wiley® Mill to pass a 2 mm screen. Hydrolysis was performed at 10% (w/w) solids loading in 50 mM citrate buffer, pH 4.8 supplemented with 0.02% of sodium azide to prevent microbial contamination. Enzyme complexes Cellic® Ctec2 and Cellic® Htec2 (Novozymes®, Franklinton, NC) were added at a loading rate at 20 mg protein/g

glucan and 2 mg protein/g glucan, respectively. Hydrolysis occurred at 50°C with mixing at 200 rpm for a 5-day period, after which the liquid was filtered and analyzed for soluble carbohydrates as described above. All samples were analyzed in triplicate. Total glucan and xylan were determined using the structural components as well as the soluble fraction of each carbohydrate and were used as the basis to estimate the maximum theoretical carbohydrate yield per the following equations:

$$\%\ Glucose\ Yield = \frac{Glucose_{Enzymatic\ Hydrolysis}}{Glucan_{Total}\ x\ (\frac{1}{0.9})} x\ 100 \quad \text{Equation 3.3}$$

$$\%\ Xylose\ Yield = \frac{Xylose_{Enzymatic\ Hydrolysis}}{Xylan_{Total}\ x\ (\frac{1}{0.88})}\ x\ 100 \quad \text{Equation 3.4}$$

Mass differences of 0.88 and 0.9 in the monomeric versus polymeric forms of glucose and xylose, respectfully, were accounted for in all calculations.

Statistical Analysis

Single-factor one-way analysis of variance (ANOVA) was performed in JMP 14.2.0 (SAS, Cary, NC) to identify significant differences, and Tukey's honest significant difference (HSD) test was performed for multiple-level comparison of statistical equivalency if the ANOVA was significant at $p < 0.05$.

Results and Discussion

This study assesses the impact of fungal pretreatment in corn stover in terms of compositional, structural, and convertibility changes on biomass recalcitrance in the context of a biorefinery. Dry matter losses and compositional changes were used as a guide to understand the working envelope of a non-selective (*P. chrysosporium*) lignin degrading fungus. This was followed by an in-depth investigation of *P. chrysosporium* degradation in a simulated outdoor storage pile combined with powerful analytical tools to understand the mechanisms of degradation and the impact on conversion performance and cost.

Storage performance and Compositional Impacts of P. chrysosporium inoculation in Aerated Bioreactors

Filamentous fungi require a sufficient supply of oxygen to maintain optimal growth, and lack of available oxygen may hinder the use of fungi as a primary pretreatment approach[40]. Aerated storage bioreactors were designed previously at Idaho National Laboratory to create a controlled environment to mimic larger storage systems while gaining crucial mass balance information that is nearly impossible to gather in outdoor settings[21]. The reactors enable real-time estimation of dry matter loss through hourly measurement of carbon dioxide emitted by bacteria and fungi as they respire carbohydrates. The reactors' exteriors are jacketed, and a circulating water bath adjusts the jacket

temperature to match the temperature at the center of the reactor, where respiratory self-heating is occurring. These reactors have been used to study corn stover degradation as a function of aeration[20, 23], initial moisture content[24], and humidity[21]. Scale up of *P. chrysosporium* as a microbial pretreatment was conducted in these reactors over a 14-day period with sampling at seven days to provide essential understanding the degradation rate of corn stover as well as any temperature changes associated with microbial decay. The experiments used non-sterile corn stover to replicate the interactions of the native microflora with the fungal amendments, as would occur in a commercial storage environment.

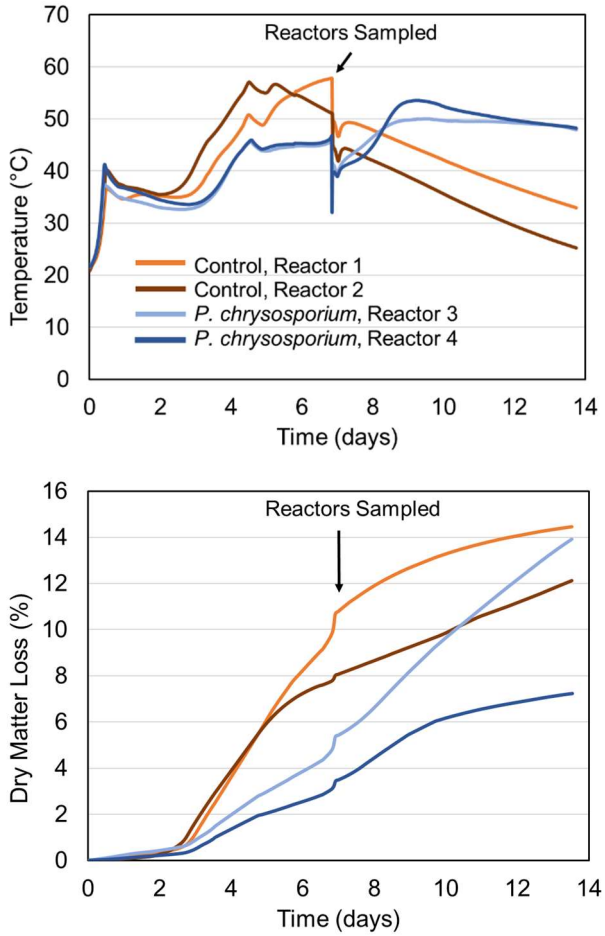

Figure 3.2. Temperature (top) and dry matter loss (bottom) profiles of corn stover inoculated with *P. chrysosporium* and stored in aerobic bioreactors at 60% moisture for two weeks.

Storage induced losses and temperature changes due to fungal treatment

P. chrysosporium-inoculated corn stover rehydrated to 60% moisture content was stored in duplicate highly controlled and instrumented aerobic bioreactors and compared to control samples with no fungal treatment. Mean interior temperature in each reactor increased due to microbial heating and

caused all reactors to heat from 20°C to 40°C within the first hours of storage (Figure 3.2). A lag then occurred until 3 days of storage after which temperature spiked to 51°C and 57°C in the control reactors by 4.5 days. The variation in maximum temperature was likely attributed to differing initial moisture contents as demonstrated previously in corn stover trials using these reactors[24]. Elevated internal temperature correlated with elevated dry matter loss rates calculated based on CO_2 evolution; dry matter losses for Reactors 1 and 2 after 1 week were 9.2% and 7.6%, respectively. Reactors with *P. chrysosporium*-inoculated corn stover heated at a slower rate, only reaching 46°C, which suggested that fungal growth was preventing other microorganisms from thriving. Sulej et al. (2019) showed an increase in antimicrobial production against gram-positive bacteria by *P. chrysosporium*, which may explain the decreased respiratory self-heating seen in the fungal-amended corn stover[41]. Similarly, dry matter loss in the corn stover were reduced in these reactors, which only experienced 4.4% and 2.8% in Reactors 3 and 4 after 1 week, respectively. All reactors cooled slightly during the 1-week sampling, but temperatures increased again once the reactors were sealed and aerated. However, the control reactors steadily cooled, an indication that the readily accessible carbohydrates had been consumed and the microbial community could no longer sustain the same growth rate. Approximately 5% additional dry matter loss was incurred in the second week of storage. In contrast, the *P. chrysosporium*-inoculated corn stover resumed heating, a sign of increased respiratory activity, likely due to fungal-based depolymerization of the corn stover to release additional carbon sources. Temperature increases of up to 42°C have been measured in fungal inoculated, unventilated wood chip piles[42]. The rate of dry matter loss in the fungal inoculated stover increased dramatically between 1 and 2 weeks of storage; 2-week dry matter loss was 13.9% and 7.2% in Reactors 3 and 4, respectively. These trends indicate that once *P. chrysosporium* had depolymerized the corn stover to a certain extent, fungal growth rates increased dramatically. These results are consistent with the findings of Adav et al.[43] that suggested the lignin degrading peroxidase activity of *P. chrysosporium* only increased after initial hemicellulase and cellobiose dehydrogenase activity had liberated sufficient hemicellulose and cellulose for consumable carbohydrates to facilitate further growth and lignin breakdown.

Compositional changes due to fungal treatment

Macromolecular changes in composition were assessed on corn stover stored in aerated bioreactors with and without *P. chrysosporium* inoculation (Figure 3.3, Appendix B Figures B.1-B.4). Xylan was reduced from 20.9% to as low as 16.8% after 2 weeks of storage with *P. chrysosporium* (Reactor 3). Lignin content was reduced slightly from 18.2% to 17.2% in this reactor after 2 weeks. However, lignin content was increased to 19.1% in the duplicate Reactor 4, indicating this reactor had less fungal induced degradation, a conclusion supported by the reduced dry matter loss rates and extents.

Total extractives in the *P. chrysosporium* inoculated corn stover in the aerated bioreactors stayed relatively constant (13.07% initially and after 2 weeks only 11.68% and 12.70% in Reactors 3 and 4, respectively). It is possible that microorganisms originally present on the corn stover at the time of harvest consumed these soluble products to support respiration. Total extractives decreased from 13.07% to 8.8% in both control reactors in the aerated bioreactors, although soluble arabinan did increase slightly. However, the fungal inoculated aerated reactors contained a higher ratio of non-quantified (unknown) to quantified (e.g., soluble sugars) extractives, which presumably indicates that soluble lignin degradation products were produced as a function of inoculation and storage. Acid soluble lignin and ethanol extractives decreased to a greater extent than acid insoluble lignin. Based on these cumulative results the *P. chrysosporium*-inoculated corn stover from Reactor 3 was selected for further analysis of changes on molecular compositional and conversion yields given that it experienced the greatest hemicellulose and lignin loss, a significant indicator of the intended mode of degradation.

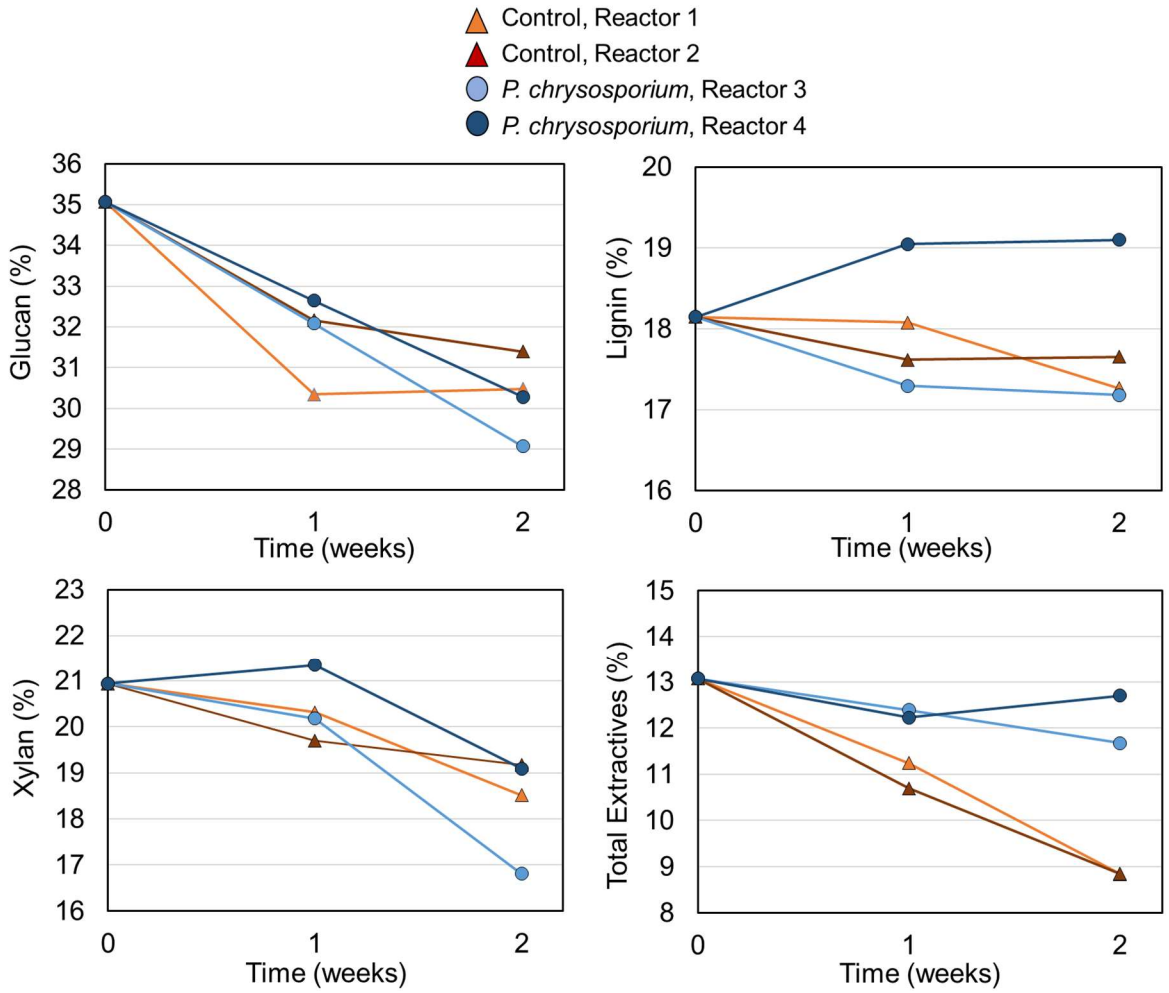

Figure 3.3. Compositional changes weighted with dry matter loss as a function of time. Corn stover inoculated with *P. chrysosporium* was compared alongside uninoculated stover. Both fungal-inoculated and control experiments were conducted in aerobic bioreactors at 60% moisture for two weeks.

Structural Impacts of P. chrysosporium inoculation in Corn stover

Fungal Degradation Products Measured by Pyrolysis GCxGC/MS

Traditional chemical compositional analysis methods, such as those used in this work, fractionate biomass into soluble and structural components. However, high solids storage treatments that aim to depolymerize structural lignocellulose components may cleave structural bonds without fully solubilizing a carbohydrate molecule or creating a measurable lignin oxidation product. Pyrolysis coupled with two dimensional GC/MS has been used to measure the abundance of the compositional subunits released from lignocellulose by means of thermal energy[31, 44]. The goal of this analysis was to understand if pyrolysis at 400°C could be used to assess partial cleavage of the backbone of cellulose, hemicellulose and associated side chains or substitutions. A pyrolysis temperature of 400°C

is lower than that typically used for biomass characterization; however, in previous studies lower temperatures enhanced differences between biomass samples from different pretreatment environments.[31] Quantitative analysis of pyrolysis production efficiency from S, G, and H lignols released as a function of fungal treatment also provide insight into the mechanisms of degradation given the complexity of native corn stover.

Comparative 3D chromatograms from early eluting compounds (220 to 920 s retention time in the first chromatographic dimension, rt1) indicate that 2-oxopropanal, acetic acid, and acetic anhydrate are present in greatest abundance in the unstored corn stover with several additional oxygenates present in lower concentrations (Figure 3.4). In comparison, *P. chrysosporium* treated corn stover showed markedly enhanced acetol and anhydro sugar compared to the unstored corn stover, corresponding to the reduction of acetate binding to hemicellulose as well as the increase in soluble hemicellulose components. Anhydro sugar production in pyrolysis of the fungal treatment could result from partially hydrolyzed hemicellulose or cellulose with enhanced dangling ends that have a greater propensity for producing pyrolysis products at 400°C. Xylan degradation in pyrolysis begins to occur at 200°C whereas cellulose degradation has been reported at 300-375°C in thermogravimetric analysis[45]. Furfural, furyl alcohol, and 2(5H)-furanone were also produced in pyrolysis of corn stover, and these 5-carbon monomers are all xylose or arabinose degradation products. Quantitative analysis of the pyrolysis production efficiencies of select oxygenates indicated slightly enhanced production of 2,3-butanedione, acetoxyacetone, and 2(5H)-furanone in *P. chrysosporium* treated corn stover (Appendix Figure B.2.). Elevated pyrolysis efficiency of 5-methyl furfural, a degradation product of 5-hydroxymethyl furfural[46] thermally produced from glucose degradation[47], correlates to the enhanced soluble glucose content in the unstored corn stover, whereas consumption of this soluble sugar occurred during fungal attack. Overall, the changes in these early eluting compounds in pyrolysis indicate changes consistent with chemical composition results suggesting glucose consumption and hemicellulose degradation seen in *P. chrysosporium* treated corn stover.

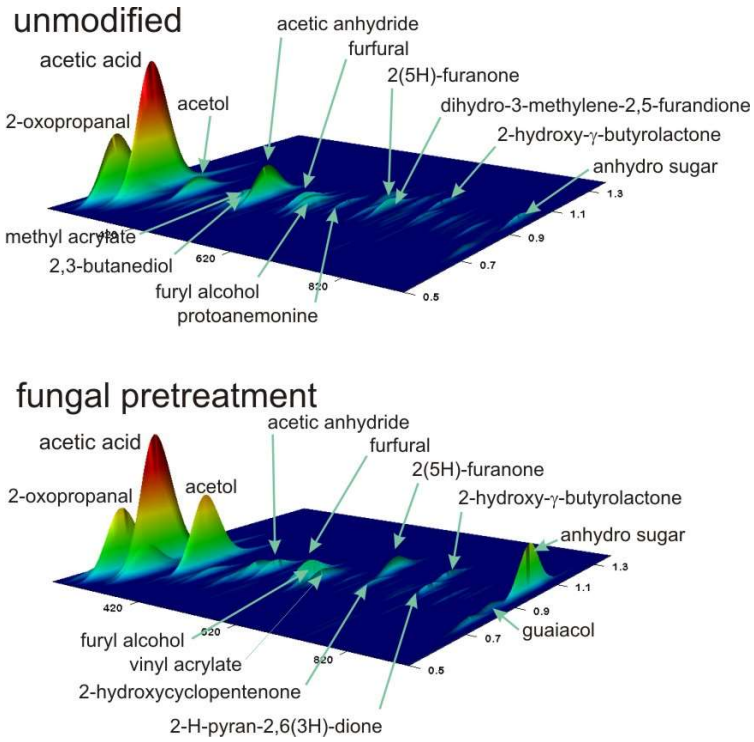

Figure 3.4. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the early eluting peaks (220 – 920 s in the first chromatographic dimension). Top - unmodified; bottom – fungal pretreatment

Lignin-degradation products formed during pyrolysis are observed as later-eluting compounds (920 to 1620 s rt1) in comparative 3D chromatograms (Figure 3.5). 4-vinyl phenol, a decarboxylation product of p-coumaric acid and a common pyrolysis product from herbaceous biomass such as corn stover[48], is the most prominent peak along with the internal pyrolysis standard of 9(9H)-fluorenone. Visualizing the 3D chromatographs in the range of 1051 to 1600 s (Figure 3.6) shows the differences in the lower concentration lignin pyrolysis products. Unstored corn stover has elevated 4-formyl phenol from H lignols, whereas production of 4-vinyl-guaiacol and 4-formyl-guaiacol are enhanced after *P. chrysosporium* treatment. This suggests that G-residues in the lignin polymers produced an enhanced number of G-lignols, which would be consistent with lignin degradation forming dangling guaiacyl moieties that are precursors for the formation of the G lignols seen in the pyrolysis experiments. Zeng et al. also documented lignin degradation and G lignol changes due to *P. chrysosporium* treatment as indicated pyrolysis 610 °C coupled with GG/MS detection[49].

Pyrolysis efficiencies of lignols confirm enhanced production of guaiacol, 4-vinyl guaiacol, 4-formyl guaiacol, and phenol in fungal treated biomass but reduced 4-formyl phenol (Appendix B Figure

B.3.). Pyrolysis efficiency measurements also indicate enhanced syringol after *P. chrysosporium* treatment, the only pyrolysis product observed originated from an S-lignin. Corn stover has been noted to contain less than 10 wt. % S lignols and near equal distribution of H and G lignols[50].

In summary, these findings confirm that G and S lignols in corn stover were targeted by *P. chrysosporium* but that H lignols were impacted at a lesser extent. Changes in the relative H, G and S lignol distributions have been shown to be feedstock dependent; herbaceous crops have different lignin and cellulose ratios compared to softwood and hardwoods therefore fungal degradation impacts on lignin are often feedstock specific[40]. Assessing the pyrolysis products that are produced at 400°C allows for enhanced observation of the mechanisms of *P. chrysosporium* attack, which would be more difficult to observe using higher temperature pyrolysis, where fast pyrolysis kinetics would overshadow more subtle differences originating from fungal degradation.

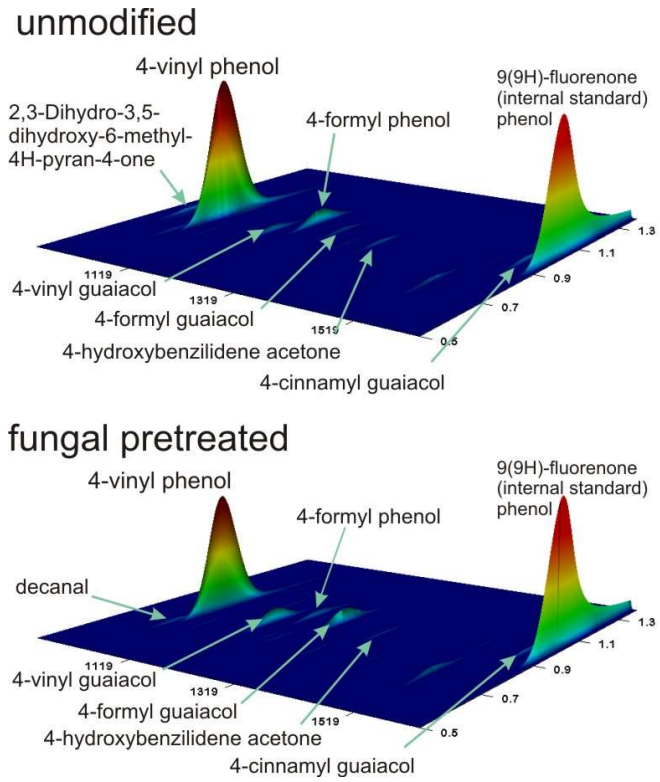

Figure 3.5. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the late eluting peaks (920 – 1620 s in the first chromatographic dimension). Top - unmodified; bottom – fungal pretreatment

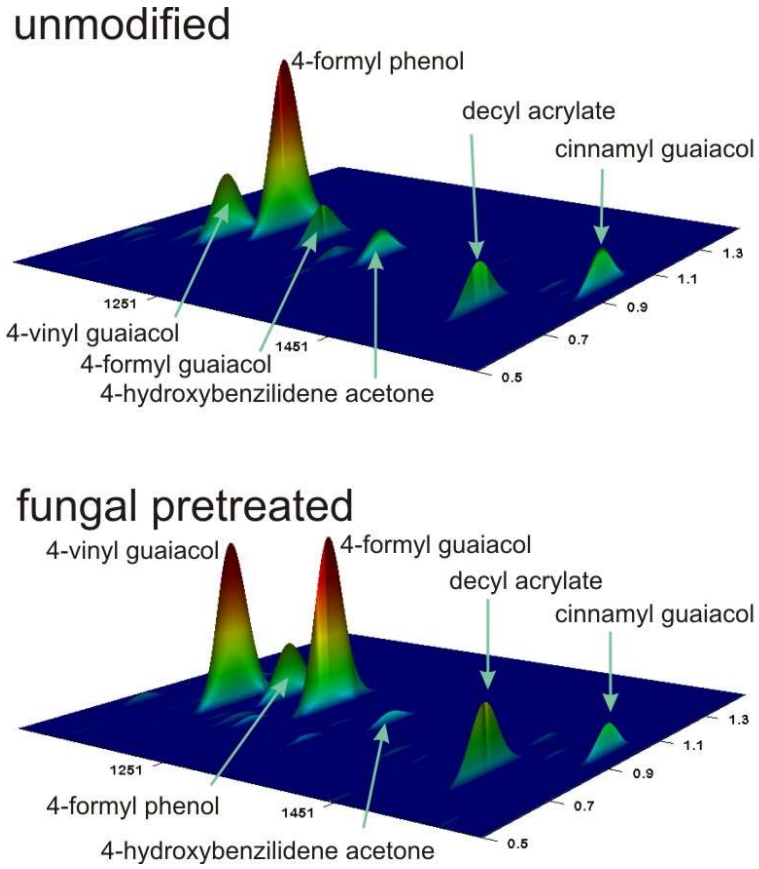

Figure 3.6. 3D total ion chromatograms generated from pyrolysis/GCxGC/MS of corn stover samples, using maximum pyrolysis temperature of 400°C. Chromatograms display the region form 1150 – 1550 s (first chromatographic dimension) where the majority of the lignols elute. Z axis is expanded compared to Figure 3.4 and Figure 3.5. Top - unmodified; bottom – fungal pretreatment.

Structural binding profiles observed with Solid-State ^{13}C-CP/MAS NMR Spectroscopy

^{13}C cross-polarization magic-angle-spinning (CP-MAS) NMR is a solid-state technique that uses the connected hydrogens in the structure to increase the signal of the carbon backbone, and this technique is useful for analyzing biomass samples that are rich in cellulose and hemicellulose. Analysis of the unstored, one- and two-week fungal inoculated samples from aerated bioreactors indicate slight changes in the NMR spectra (

Figure 3.7). One notable difference is in the cellulose peaks at ~70-80 ppm, which correspond to the C2, C3 and C5 positions. Differences appear in the signal intensity, yet they are unlikely to represent changes in the cellulose monomer given the strong hydrogen bonding in this polymer. On the other hand, differences are likely due to the reduction of hemicellulose and lignin in the fungal treated samples; these carbon atoms are observed at the same ^{13}C chemical shift in hemicellulose and cellulose. This is supported by the gross chemical composition data for these samples. Similarly,

significant differences in the peak intensities are observed at 21.1 and 171.5 ppm, which represent methyl (CH_3) and carboxyl (COOH) groups, respectively. The peak height at both these locations was enhanced in fungal inoculated corn stover after two weeks of storage compared to both the unstored and 1-week stored samples, likely a function of the increased degradation rate between one and two weeks of storage. It is likely that the methyl and carboxyl groups are formed after fungal degradation (including acetate hydrolysis) of the hemicellulose, which is reflected in gross compositional changes from 3.6% to 3.3% and 2.1% after one and two weeks of storage, respectively.

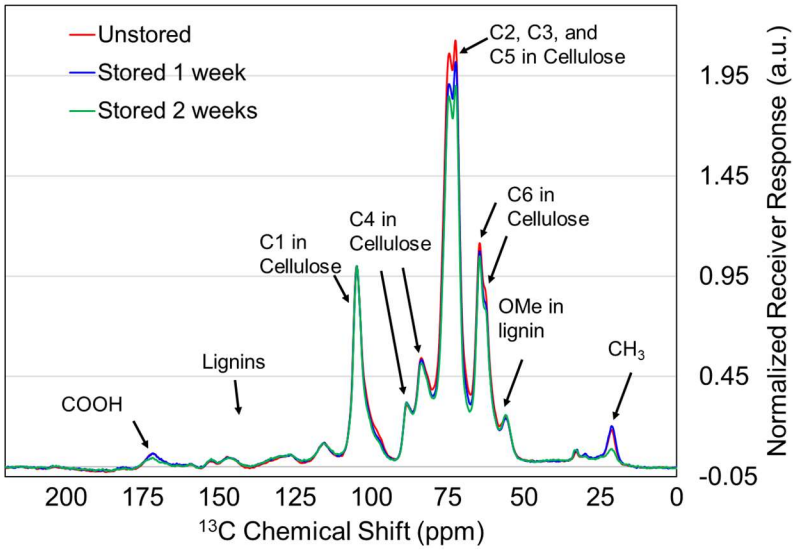

Figure 3.7. Solid-State ^{13}C-CP/MAS NMR profiles of unstored and *P. chrysosporium*-inoculated corn stover.

Lignin isolation in the unstored and *P. chrysosporium*-inoculated was accomplished with a two-stage hydrolysis aiming to solubilize all carbohydrates and leave lignin in the solid phase. ^{13}C-CP/MAS NMR spectra from the lignin isolated samples provide dual functions of providing higher resolution spectra of the lignin and insight into the structural changes in corn stover as a function of *P. chrysosporium* inoculation over the two-week storage period (Figure 3.8). Comparison of the spectra of the unstored sample and fungal treated corn stover reveal additional molecules in the lignin-rich fungal treated sample. Enhanced signals of the C1-C6 carbons in cellulose after fungal treatment suggest irreversible binding between lignin and cellulose occurred as a function of treatment. Lignin coalescence has been demonstrated in during pretreatment previously in low solids pretreatment[51]. Carboxyl groups at 171.5 ppm were present in the lignin-rich sample, and like the unstored corn stover the fungal treated sample, this peak was slightly reduced in intensity, suggesting fungal treatment impacted these functional groups in lignin. One other notable change was a peak at 32.2

ppm, which is prominent in the fungal treated sample. It is hypothesized that it is a CH_2 group based on NMR peak libraries[52], potentially an aliphatic hydrocarbon that is a lignin degradation product or a product formerly attached to lignin. Methylene present in dangling phenylpropanoid moieties formed from lignin degradation is another possible explanation. The slight increased signal in the fungal treated sample at 29.5 ppm, which corresponds to the methyl group, suggests that perhaps lignin degradation product is contributing to this intensity. Overall, the use of ^{13}C CP/MAS NMR analysis demonstrated that the lignin rich fungal sample had enhanced cellulose contamination, a key data point that may be useful to predict potential responses in downstream conversion operations.

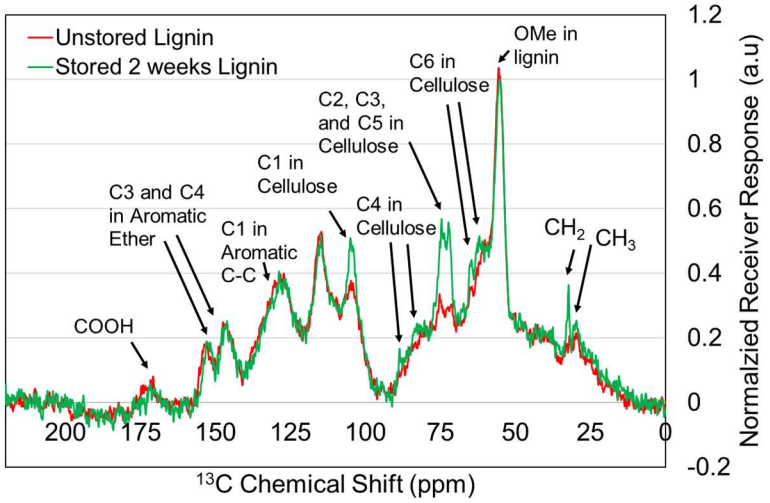

Figure 3.8. Solid-State ^{13}C-CP/MAS NMR profiles of a lignin rich sample of unstored and *P. chrysosporium*-inoculated corn stover.

Carbohydrate Yield in Hydrolysis as a Function of Thermochemical Severity

The goal of enzymatic hydrolysis in the context of a biorefinery is to provide the maximum theoretical free sugar levels for the next unit operation, in this case microbial fermentation to fuels or fuel precursors. Low glucose and xylose yields using only mechanical treatment prior to enzymatic hydrolysis, reported in the Appendix, suggest a more severe pretreatment method than mechanical processing alone is necessary to achieve a high solubilization rate of the corn stover for downstream fermentation. Corn stover inoculated with *P. chrysosporium* and stored for one and two weeks were assessed using a dilute acid pretreatment and enzymatic hydrolysis method. Dilute acid is a pretreatment that targets hemicellulose hydrolysis so that cellulose can be exposed to cellulytic attack, and it was used in this study to investigate any synergies in fungal depolymerization that solubilized hemicellulose. Washing is typically performed after dilute acid pretreatments to create a neutral pH

for hydrolysis, and it would have the combined effect of removing any competing fungal enzymes. Dilute acid pretreatment combined with enzymatic hydrolysis has been proposed by Wolfrum et al. as a means to screen biomass for reduced recalcitrance[37], and these conditions have been used previously to assess the impact of corn stover storage on carbohydrate release[23, 24]. Three severity levels with varied temperatures were chosen to determine if fungal pretreatments could reduce the energy requirements of the dilute acid pretreatment. *P. chrysosporium* treatment showed a reduction in hemicellulose recalcitrance during dilute acid pretreatment and subsequent enzymatic hydrolysis compared to unstored corn stover. Figure 3.9 shows that more xylose is released from the fungal-stored corn stover at all pretreatment temperatures tested. Fungal inoculated samples stored for one week displayed ~ 30% more xylose released compared to unstored samples for the experiments conducted at 110°C (severity factor of 1.31), but an additional week of storage had no further impact. Storage duration resulted in enhanced xylose release for the two experiments conducted at higher temperatures of 130°C and 160°C, (severity factor of 1.73 and 2.61, respectfully). Additionally, this experiment indicated that similar xylose yields (82.9% ± 1.4%) were achieved after two-week fungal treatment with a pretreatment temperature of 130°C, as compared to the xylose yields for untreated corn stover subjected to a pretreatment temperature of 160°C (80.2% ± 0.5%). These results suggest the combined fungal treatment to liberate hemicellulose and oxidize lignin increased the ability of dilute acid pretreatment to depolymerize hemicellulose to a greater extent than in the untreated corn stover.

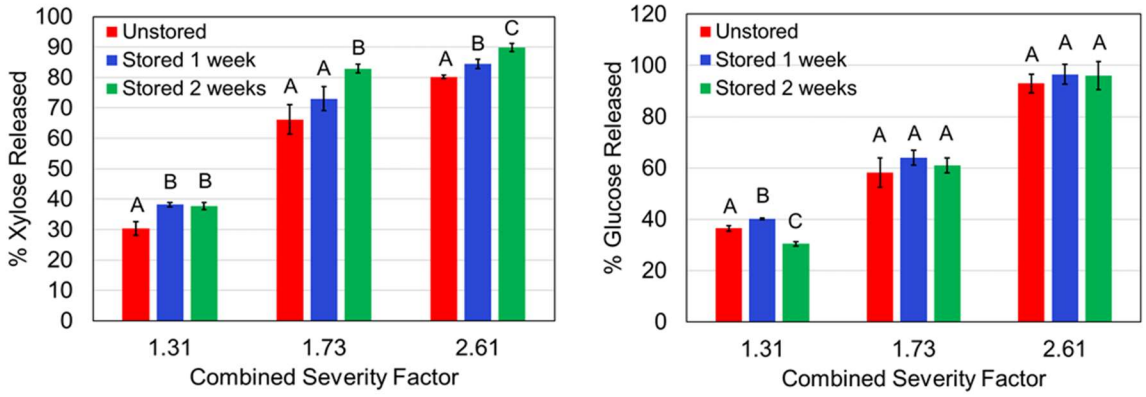

Figure 3.9. Combined dilute acid and enzymatic hydrolysis yields corn stover subject to *P. chrysosporium* treatment over a two-week period. Error bars represent standard deviation, n=3 or 4. Letters represent significant differences based on a Tukey Kramer test.

Glucose yield corresponded to the reaction temperature increases associated with increased combined severity factor. Yields of 37% glucose released were measured in experiments conducted at the lowest temperature (110°C, severity factor 1.31), significantly higher at 40% after 1 week of *P. chrysosporium* treatment, but then decreased to 30% after 2- weeks of treatment. Glucose yields increased to approximately 60% and 95% at the 1.73 and 2.61 severity factors, but no significant difference was observed as a result of treatment. This study did not explore changing enzyme loading or concentrations, but it is hypothesized that increasing the cellulase loading during hydrolysis would increase the glucose release from what was observed in the 1.73 severity treatment.

Others have shown that reduced severity pretreatment was possible, for example Kuhar et al. indicated sulfuric acid loading could be reduced from 3.5% to 2.5% in fungal pretreated wheat straw[53]. Energy cane biomass subjected to *P. chrysosporium* treatment combined with reduced sulfuric acid pretreatment maintained high yields during ethanol fermentation[54]. Combined fungal and phosphoric acid pretreatment has been shown to result in nearly 90% conversion of glucose to ethanol in residues from oil palm[55]. Future opportunities to optimize fungal pretreatment with existing and emerging thermal and chemical treatments have the possibility to dramatically change the operating conditions in lignocellulosic biorefineries by reducing the costs associated with higher severity pretreatment conditions.

Conclusion

Filamentous fungi effectively reduce the recalcitrance of lignocellulosic biomass to thermal and caustic pretreatments, resulting in increased access of cellulose to enzymes and subsequent hydrolysis to fermentable carbohydrate monomers. This study explored a combination of fungal pretreatment, dilute acid pretreatment and enzymatic hydrolysis to create monomeric carbohydrates that could be fermented to fuels in a fully integrated biomass conversion process. These results point to the promise of incorporating fungal treatment in the short-term storage operation of queuing at the biorefinery. Key findings in this study indicate that *P. chrysosporium* degradation on corn stover increased the release of hemicellulose and G and S lignols in a low temperature pyrolysis, that condensed lignin may be cross-linking the cellulose as indicated by ^{13}C-CP/MAS NMR, and the fungal treatment over a two-week period resulted in the reduction of pretreatment temperature from 160°C to 130°C while achieving a similar xylose yield. Additional investigation is warranted to explore techniques to reduce dry matter loss or further improve performance of fungal pretreatment feedstocks in mechanical, thermal, or enzymatic depolymerization approaches.

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Chapter 4: Screening of alkali assisted storage conditions to define the operational window of deacetylation within storage systems in the bioenergy supply chain

Abstract

Overcoming biomass heterogeneity and associated recalcitrance to thermal, chemical, and enzymatic depolymerization is a necessary but challenging aspect of valorizing lignocellulosic biomass to fuels and chemicals. This study explores how this recalcitrance can be reduced during the supply chain unit operation of long-term storage, which is required for seasonally harvested agricultural residues to maintain constant throughput at a biorefinery. In this work partial alkali pretreatment was performed just prior to corn stover entering storage, which had the benefit of providing a high pH environment entering storage such that soluble sugars were preserved as well as saponifying acetyl groups in hemicellulose. This work investigated a range of viable conditions where saponification and preservation occur simultaneously by varying the moisture content (40% and 60%) and concentrations of sodium hydroxide (low and high) during aerobic and anaerobic storage. Anaerobic conditions preserved overall dry matter below 5% in three scenarios evaluated, and the highest alkali loading solubilized up to 15% lignin, 18% xylan, and 50% of acetate meanwhile doubling the extractable components. Scanning electron microscopy images highlighted potential physical impacts including cell wall disruption near vascular bundles, pitting within parenchyma cells and cell wall distortion. Techno-economic assessment indicated that this storage approach and associated logistics system is economically competitive with a conventional approach using low-moisture bales.

Introduction

Corn stover has the unique advantage over other biomass crops of being a widely produced, commercially available feedstock for bioenergy conversion, and yet it and other lignocellulosic crops are inherently a challenging feedstock for conversion due to the molecular and physical complexity of the lignocellulosic matrix. Catalysts for depolymerization can include heat, caustic chemicals, and enzymes, and a combination of these approaches is generally used in low-temperature conversion approaches that utilize fermentation to produce fuels or chemicals. The residence time of these treatments is generally on the order of hours in order to minimize capital equipment costs at a biorefinery. However, additional opportunities for residence times become an opportunity for investigation when the entire feedstock logistics supply chain for agricultural residues, such as corn

stover, are considered. For example, long term storage can be on the order of weeks to months. Corn stover is harvested within a seasonal window, and yet a biorefinery must have a consistent feedstock supply to maximize use of capital investment and ensure consistent fuel production. Thus, long term storage of biomass feedstocks must be accounted for to reduce the risk of biorefinery down time due to lack of feedstock. The focus of this study is to investigate approaches to reduce recalcitrance within the long-term storage operation in the supply chain such that more readily convertible biomass is delivered to a biorefinery.

Long term storage operations for herbaceous biomass typically are either performed in aerobic conditions using stacked bales or in the anaerobic approach of silage. Field-side storage of bale stacks is commonly utilized in feedstock logistics supply designs due to its low cost and to facilitate transportation to the biorefinery[1, 2]. Moisture content of less than 15% wet basis (w.b.) is required to reduce the risk of loss to microbial degradation[3]. However, significant degradation has been documented due to a number of factors including entering storage at high moisture contents[4, 5], wicking moisture from the ground[6], or from moisture condensation under tarps[6]. The alternative approach of using wet, anaerobic storage has been documented for bioenergy systems[7, 8]. These systems leverage forage chopping harvest and collection approaches to reduce particle size and facilitate increased packing density in compacted storage. High moisture contents (40-70%, w.b.) facilitate both mechanical oxygen removal by occupying void space in biomass pores and enable fermentation of soluble carbohydrates to organic acids. The resulting low pH environment has a stabilizing effect on biomass and has also shown promise to reduce biomass recalcitrance[9, 10]. However, designs using ensiled storage for agricultural residues have been shown to be 10% more costly than their baled counterparts primarily due to low density of chopped biomass in transportation[11]. However, storage and transportation in silage tubes has been documented to improve the economics by 24% compared to a baled scenario[12]. Therefore, anaerobic storage can be an economically viable approach to consider for opportunities to reduce biomass recalcitrance to downstream preprocessing.

Deacetylation is a frequently used pretreatment method for biochemical conversion of lignocellulosic fuels[13, 14]. This pretreatment chemistry has a dual purpose in saponifying acetyl and glycosidic bonds in hemicellulose and saponifying ester linkages between lignin and hemicellulose. Alkali extraction is followed by mechanical milling to improve surface area for enzymatic hydrolysis to monomerize carbohydrates[14]. This method allows conversion of both a carbohydrate stream as well as the lignin stream, resulting in multiple product streams and additional co-products beyond just fuels. Current challenges in this approach include problems with alkali impregnation due to the short residence time,

alkali neutralization due to saponification of ferulic acid and acetyl groups, and additional alkali requirements for more recalcitrant biomass. 4.8-7.0 wt% sodium hydroxide is currently added to the biomass during deacetylation to accomplish all desired impacts within just a 2-hour window[13, 14]. Long term storage has the potential to address these challenges with the range of residence times that are possible in this unit operation. Sodium hydroxide addition to straws has been shown to increase digestibility for rumen[15, 16]. Others have shown that sodium hydroxide loading combined with anaerobic storage increased glucose and xylose yields after 5 days of storage, yet high dry matter loss after 10 days due to unstable storage conditions eliminated any further gains in digestibility[17]. However, no published studies exist to our knowledge that explore how treatment conditions in mechanical preprocessing and deacetylation could be reduced as a result of long-term anaerobic storage. The aim of this work was to assess if alkali could be added to corn stover prior to storage for the dual purpose of preserving biomass from unwanted microbial degradation along with providing a longer reaction time that can aid in depolymerization to reduce biomass recalcitrance. This approach is a process intensification strategy that has significant promise to impact how biorefineries manage their feedstock supply chain.

This work focused on designing a supply system that can facilitate sodium hydroxide treatment during storage to increase alkali impregnation prior to deacetylation at the biorefinery. Storage performance results are also reported for two concentrations of alkali-based treatment for 4 weeks at varied moisture content (40 and 60%, w.b.) in aerobic and anaerobic conditions. Characterization of dry matter preservation and extractable material was performed on all samples in order to select samples for full compositional analysis. Full compositional characterization of the anaerobic conditions enabled the assessment of changes in extractives, carbohydrates, lignin, and acetate. Integration of this approach into the feedstock logistics supply chain was explored to understand the potential for cost savings with this approach. A logistics system was designed that included partial alkali pretreatment prior to corn stover entering storage providing a high pH environment entering storage to (1) preserve soluble sugars, (2) saponify the ester linkages between lignin and hemicellulose and (3) to weaken the hydrogen bonding network between cellulose and hemicellulose.

Methods and Materials

Corn Stover Source and Storage Procedures

Corn stover was sourced from Boone, IA using a two-pass harvest and collection configuration following grain harvest; this biomass is available as reference material through Biomass Feedstock National User Facility Library at Idaho National Laboratory. Corn stover was knife milled using a

Wiley Mill Model 4 (Thomas Scientific, Swedesboro, NJ) to pass through a 6 mm screen to prepare it for storage experimentation. Four moisture and alkali concentrations were utilized as described in Table 4.1 along with controls for 10 experimental conditions each performed in triplicate.

Table 4.1. Corn stover sample nomenclature and corresponding experimental treatment for this study.

Sample ID	**Experimental Treatment**	**Alkali concentration (%, d.b.)**	**Alkali concentration (Molar, w.b.)**
Native	As-received corn stover	NA	NA
Control, 40% Moisture	Aerobic	NA	NA
Control, 60% Moisture	Aerobic	NA	NA
Low NaOH, 40% Moisture	Aerobic, Anaerobic	0.5	0.18 M
Low NaOH, 60% Moisture	Aerobic, Anaerobic	2.4	0.08 M
High NaOH, 40% Moisture	Aerobic, Anaerobic	0.5	0.92 M
High NaOH, 60% Moisture	Aerobic, Anaerobic	2.4	0.40 M

Concentrated NaOH (50 w/w%, Sigma Aldrich) was added to water for dilution, and then the mixture was applied to the corn stover such that a final moisture content of 40% or 60% w.b. was achieved alongside an alkali loading of either 0.5 % or 2.4% (w/w dry basis [d.b.] biomass). Concentrations were chosen to reflect overall alkali reduction of one tenth and one half of what has been reported previously[14, 18]. Aerobic storage was conducted in triplicate 250 ml flasks containing 20 g biomass d.b. with a loose fitted cap to prevent excessive moisture loss. Anaerobic storage conditions were formed using 125 mL glass reactors based on a configuration reported previously using 0.1M NaOH loading at 8% solids content[19]. Alkali-treated corn stover was manually compacted in jars and sealed with airtight lids fitted with an S-shaped fermentation airlock to limit exchange with the atmosphere. Jars were purged with nitrogen immediately after sealing to rapidly establish an anaerobic atmosphere that would be likely observed in a large-scale silage pile that was rapidly compacted and sealed[20]. All samples were stored at room temperature in the dark. After four weeks of storage the corn stover was mixed thoroughly to create representative subsamples and assessed for moisture and mass changes. Dry matter loss was calculated gravimetrically:

$$\%\ Dry\ Matter\ Loss = \frac{g\ Biomass_{pre\ storage} - g\ Biomass_{post\ storage}}{g\ Biomass_{\ pre\ storage}} x\ 100 \quad \text{Equation 4.1}$$

Replicates for each storage condition (treatment, moisture content, alkali loading) were combined into a single composite sample for follow-on analysis, and a subsample was size reduced to pass through a 2 mm screen using a Thomas Model 4 Wiley® Mill (Thomas Scientific, Swedesboro, NJ) to prepare for compositional analysis.

Compositional Analysis

Corn stover samples were assessed for gross compositional changes using methods establish by the National Renewable Energy Laboratory (NREL) according to Laboratory Analysis Procedures (LAPs)[21, 22]. Total extractives were quantified with a 100°C water and subsequent ethanol extraction accomplished using an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA)[23]. Further analysis of select samples involved a two-stage acid hydrolysis to solubilize structural carbohydrates from the lignin and ash stream[24]. Carbohydrate monomers glucose, xylose, arabinan, and galactan were quantified using high performance liquid chromatography (HPLC) with a refractive index (RI) detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 x 7.8 mm, Hercules, CA)[25]. Acetate was also analyzed with HPLC equipped with an RI detector (Waters, Milford, MA) and an Aminex HPX 87H ion exclusion column (Bio-Rad, 300 × 7.8 mm, Hercules, CA). Acid insoluble lignin[24] and ash[26] were determined based on gravimetric difference, and acid soluble lignin was quantified using a Varian Cary 50 ultraviolet-visible spectrophotometer (Agilent, Santa Clara, CA)[24].

Scanning Electron Microscopy

Dried corn stover samples were sectioned using a protocol adapted for confocal imaging[27]. The corn stover pieces were rehydrated and slowly embedded in polyethylene glycol (PEG) 2000 (Sigma Aldrich, St. Louis, MO) at 60°C. The rehydrated samples were submerged in 50% PEG solution at 60°C in a closed container until completely permeated. The lids were removed from the sample containers to allow water to evaporate from the solution until the volume was reduced by half. The samples were then submerged in 100% PEG at 60°C overnight. A humid environment was created by placing a pan of water with the samples at 60°C to prevent the PEG from becoming too dry upon hardening. The samples were removed from the PEG and placed directly on a microscope slide in the desired orientation and allowed to harden to the microscope slide overnight at room temperature. The samples were hand sectioned with a scalpel, rinsed twice with water to remove PEG, and placed on a new microscope slide to dry overnight at room temperature. Sections were removed from the slide with a scalpel and mounted to SEM pin stub mounts with double sided copper tape. The sections were sputter coated with gold and imaged using a JEOL JSM-6610LV (Peabody, MA) scanning electron microscope.

Techno-economic assumptions

Chopped, Silage Tube Logistics Design

A logistics system compatible with alkali addition in an anaerobic environment was designed that included forage chopping, long-term storage in silage tubes stored fieldside, and transportation to a

depot co-located with a biorefinery. This case was designed around the 40% moisture anaerobic conditions explored experimentally in this study and is consistent with previous field results for corn stover[8]. An overview of the equipment list enabling the logistics design is shown in Table 4..

Table 4.2. Equipment list for chopped, silage tube logistics systems including quantity, capacity, purchase price and installation factor. All costs are shown in 2016 US dollars.

Operation	Description	Quantity	Design capacity	Purchase price	Installation Factor
Harvesting and collection	New Holland FR9060	7	40 tons/hr	$366,433	0
	Case IH Puma 180 HP Tractor	7	40 tons/hr	$129,090	0
	High Dump Wagon	7	40 tons/hr	$45,789	0
Field storage	John Deere 110 Gallon Standard Sprayer w/ 18' Boom, LP40782	53	-	$2,595	0
	Kelly Ryan Centerline silage tube bagger 5X12	53	8 tons/hr	$12,524	0
Transportation	Peterbilt 367 Conventional-Day Cab	37	11.27 tons/hr	$131,640	0
	IMCO 45' 2 axle aluminum loader	37	11.27 tons/hr	$57,966	0
Receiving site/Depot	JCB Wheel Loader, 457ZX	3	126 tons/hr	$179,990	0
	Magnetic Separator Conveyor	1	2577 m^3/hr	$30,000	0.5
	Magnetic Separator	1	-	$117,000	0.5
	30,000 BPH Pit Hopper	2	37333 m^3/hr	$16,473	0.5

Grain harvest was assumed a separate operation, consistent with previous designs[11, 28, 29]. Corn stover harvest was accomplished using a self-propelled forage harvester that pneumatically delivered chopped stover into a high dump wagon pulled by a tractor. Forage chopping of the corn stover has been shown to achieve a 11-17 mm particle size[30], which met the 18 mm size requirement entering deacetylation at the pretreatment reactor[18]. The high dump wagon transported corn stover to the designated field edge for silage tube formation. A Kelly Ryan Centerline bagger was used to form the silage tubes, and 40 ft lengths were used such that single units could be loaded onto a trailer for transportation. Sodium hydroxide was assumed to be sprayed on the stover during silage tube filling with a John Deere 110-gallon sprayer. Storage density in the silage tubes was assumed to be 8.7 lbs/ft^3 (dry weight basis), which has been previously reported for forage chopped, 40% moisture corn stover stored in silage tubes[7]. The overarching premise of this research is that alkali incorporated during pretreatment at a biorefinery can be partially integrated within the window of long-term storage occurring in the supply chain. As such, alkali costs accounted for in biochemical biofuel

conversion designs[13] were assumed to be costed downstream of logistics. This would result in no net increase of alkali in the supply system and a net zero impact on final fuel production costs.

The transportation design was modeled after what was reported previously for chopped corn stover stored in silage tubes[12]. Each 40 ft silage tube was assumed to be recovered from storage by manually loading with a 45 ft long self-loading/unloading trailer with a walking floor bottom at a rate of 5.1 dry tons/hr. This was pulled by a conventional semi-truck day cab and transported 36 miles to a biorefinery gate. The transportation distance assumed was similar to what was used in a two-pass harvest baled logistics system[28]. On-site preprocessing at the biorefinery involved unloading the silage tube using the walking floor trailer, and plastic wrap removal that was facilitated by a high tonnage knuckle boom. The used wrap was assumed to be collected and disposed of in a local landfill. The receiving system for chopped corn stover described previously was used to provide a 24-hour supply to the biorefinery[11]. Corn stover was fed through a magnetic separator conveyance system to remove any rocks. Then a day pile was created using a stacking reclaimer and associated conveyance system, and the final conveyor delivered stover to the biorefinery throat as reported in a previously modeled logistics case that delivered ensiled biomass to a biorefinery reactor throat[11].

Cost basis

The cost-year of 2016 was chosen to be consistent with current feedstock logistics supply chain[28] and conversion models[13] to facilitate comparison. Capital costs provided for other years were adjusted to the basis year of 2016 using Consumer Price Index (CPI) from the U.S. Bureau of Labor Statistics CPI Databases[31]. The equation for year-dollar back-casting is shown below:

$$2016\ cost = Base\ year\ cost \times \left(\frac{CPI\ in\ base\ year}{CPI\ in\ 2016}\right) \quad \text{Equation 4.2}$$

Grower payment for corn stover residues assumed was based on 2-pass corn stover harvest and collection approach representing the state of technology within the context of bioenergy production[28]. Fixed costs or all unit operations included capital recovery, insurance, and taxes, and were estimated following the guideline published by American Society of Agricultural and Biological Engineers (ASABE). A list of all equipment used in this scenario is presented in Table 4., along with machine purchase price, machine life, and salvage value. The equipment prices used were either obtained from local dealerships or online webpages and were used to estimate discounted salvage value and capital recovery based on machine lifespan. Annual discounted salvage value was calculated as:

$$Discounted\ salvage\ value = \frac{Purchase\ price \times Salvage\ rate}{(1+Interest\ rate)^{lifetime(yr)}} \quad \text{Equation 4.3}$$

Capital recovery was calculated as:

$$Capital\ recovery = \frac{[(Purchase\ price - Discounte\quad salvage\ value) * (Interest\ rate/(1-(1+Interest\ rate)^{-lifetime(yr)}) + (Discounted\ salvage\ value * Interest\ rate)]}{Operating\ hrs\ per\ year} \quad \text{Equation 4.4}$$

The ownership costs included interest and depreciation, insurance, and taxes. This scenario assumed an annual interest rate of 8% and insurance and tax rate of 2%, similar to previous analysis of a chopped logistics system for corn stover[11]. For the equipment in the receiving site such as magnetic separator, conveyor, and hopper, an installation factor of 0.5 was included to estimate additional installation-related costs including electrical connections, instrumentation, and safety control.

Operating costs (2016 US$ h^{-1}) accounted in this scenario included the costs of repair and maintenance, fuel, labor, and material and land for storing silage tubes. Repair and maintenance costs were calculated as 1% of capital recovery, which referred to the value of initial capital investment over the equipment lifespan. Fuel cost was estimated based on hourly energy usage of each equipment, and energy unit cost. Cost for off-road diesel and electricity were assumed at 2.13 US$/gal[32] and 0.065 $/kWh[33]. The assumed labor rate in this scenario was US$33 h^{-1} consistent with previous designs[11]. Material cost included cost of silage tube bags, which was assumed to be about 106.4 2016 US$ for each 40 ft silage tube used. Land cost for storing silage tubes was estimated based on assumed land cost of 105.0 2016 US$/acre[28].

Results and Discussion

Storage Performance of Alkali-Treated Corn Stover

Ten experimental conditions were screened as a function of both dry matter loss and the ability to shift structural components into soluble forms after 4 weeks of aerobic or anaerobic storage. The results in Figure 4.1 represent dry matter loss experienced over 4 weeks along with trends in the changes of extractives (1.8-2.2%), where water extractable and ethanol extractable components were assessed for their ability to suggest reduced downstream recalcitrance. No differences in ethanol extractives were observed across the ten treatments of corn stover, but significant changes in water extractives were present. Retention of water extractable components was observed in experimental controls stored aerobically; however, corn stover stored for 4 weeks at 40% and 60% moisture w.b. under aerobic conditions exhibited 8.1% and 10.6% total dry matter loss, respectively. Additionally, the water extractable component of the 60% moisture experimental control decreased by a relative 11%, likely the result of microbial respiration of soluble carbohydrates. This result is consistent with

previous studies that correlated aerobic microbial degradation with moisture contents over 20% w.b.[34]. A full characterization of water extractable components in native corn stover has shown that monomeric carbohydrates are primary constituents in the extractable fraction (ranging from 14-27% on a dry weight basis) along with alditols, aliphatic acids, inorganic ions, and other soluble oligomers[35]. Monomeric carbohydrates provide the energy source for microbial respiration and are well-known to be consumed during aerobic storage[4, 36].

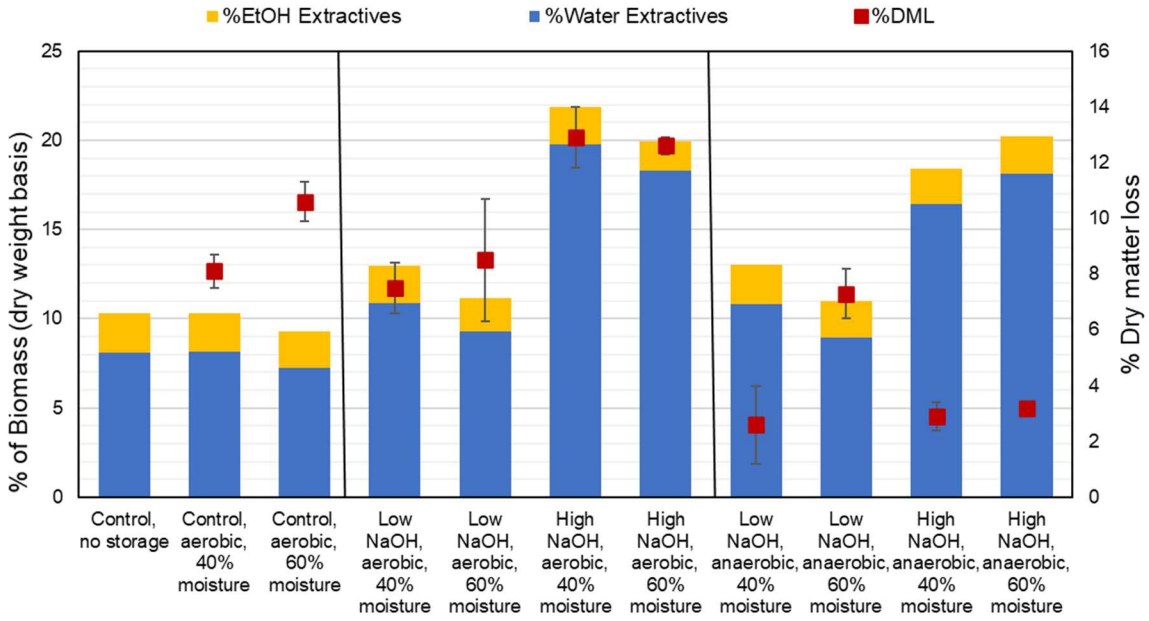

Figure 4.1. Changes in extractable components and associated dry matter loss alkali-treated corn stover over 4 weeks of storage.

Corn stover stored under aerobic conditions at both low and high alkali loadings exhibited varied losses of dry matter (Figure 4.1). Both Low NaOH, aerobic corn stover samples stored at 40% and 60% moisture exhibited approximately 8% dry matter loss, consistent with the control samples that received no alkali addition. Of note, water extractives in the Low NaOH aerobic, 40% moisture stover were elevated (10.6%, d.w. basis) over the corresponding moisture control sample, signaling the impact of alkali addition on solubilization of structural components. Following the Aerobic, High NaOH loading and storage, approximately 20% and 18% of components were extractable in water after 40% and 60% moisture storage, respectively. However, losses over the 4-week period resulted in nearly 13% of total mass loss; solubilization of structural components that were microbial accessible are likely the cause of such substantial dry matter losses over the control samples.

Anaerobically stored, High NaOH 60% moisture stover and both Low NaOH samples exhibited nearly equivalent changes in water extractable components compared to the corresponding treatment in aerobically stored corn stover. A slight difference in extractives was present (16.4% vs 19.7%) between the High NaOH, 40% moisture stored corn stover, but the trend of elevated extractives after alkali loading was consistent in both aerobic and anaerobically stored samples. However, dry matter losses over the 4-week storage scenario were dramatically different in three of the four anaerobically stored samples. Dry matter losses were reduced from the 8-13% exhibited in the aerobic samples to less than 4%, apart from the Low NaOH, 60% moisture sample where equivalent losses of 7.3% were experienced in aerobic and anaerobic storage. This result can be understood by assessing the molar concentration of NaOH in the different treatments at the time of storage. Molar equivalents reported in Table 4.1. Corn stover sample nomenclature and corresponding experimental treatment for this study. demonstrate that the Low NaOH, 60% moisture corn stover alkali loading was the lowest (0.08 M), suggesting that minimal alkali-induced effects and that higher alkali conditions (0.18-0.92 M) were required for preservation during anaerobic storage. While anaerobic conditions can preserve corn stover in storage, silage inoculants containing lactic acid bacteria are commonly applied to forage crops prior to entering long term storage[37, 38]. This controls against proliferation of microorganisms associated with silage spoilage including *Clostridia* species[39] that produce butyric and acetic acid, resulting in greater dry matter losses. In summary, the combined effect of alkali-treatments >0.18M and anaerobic storage were able to preserve corn stover over 4 weeks.

Further investigation of the structural composition was undertaken to probe for further impacts to the cellulose, hemicellulose, and lignin due to combined alkali treatment and anaerobic storage (Table 4.3). In comparison to the native corn stover, Low NaOH treatment had minimal impacts on composition after 4 weeks of storage. Slight lignin loss from 17.69% to 17.11% was observed in the Low NaOH, 40% moisture stover; likely the higher molar concentration of the alkali encouraged additional saponification of ester linkages in lignin and hemicellulose. No lignin changes were observed in the Low NaOH, 60% moisture stover. However, xylan was reduced in both Low NaOH samples (22.3%) compared to the native sample (23.58%). Previous results assessing anaerobic storage of corn stover showed that no xylan reduction occurred[8], and it is hypothesized that the alkali addition prior to storage in the present study destabilized hemicellulose sufficiently that a fraction was solubilized. However, no changes in acetate were observed highlighting that the alkali concentration was not sufficient to promote deacetylation. Additionally, the glucan and arabinan content were increased as a function of Low NaOH storage, likely a proportional enhancement due to loss of other components. Overall, these results show minimal compositional impacts as a function of the Low NaOH storage.

Table 4.3. Composition of native and alkali-treated corn stover after anaerobic storage for 4 weeks.

% Component (d.b.)	Native	Low NaOH, 40% moisture	Low NaOH, 60% moisture	High NaOH, 40% moisture	High NaOH, 60% moisture
Lignin	17.69	17.11	17.83	15.59	14.96
Glucan	35.61	37.17	38.52	35.84	35.07
Xylan	23.58	22.26	22.30	21.32	19.41
Galactan	1.14	1.18	1.18	1.12	1.09
Arabinan	2.55	2.96	2.98	2.77	2.61
Acetate	1.77	1.76	1.68	0.89	0.87
Protein	3.17	1.99	1.51	1.72	1.98
Structural Ash	2.66	2.43	2.38	1.91	2.39
Extractable Inorganics	2.60	2.91	3.06	5.48	4.84
Unquantified Extractives	5.48	7.90	5.88	10.93	13.27
Ethanol Extractives	2.60	2.91	3.06	5.48	4.84
Total	98.50	99.91	99.36	99.56	98.61

Differences in compositional components were observed in the High NaOH stored corn stover compared to the native corn stover as well as a function of moisture content. In general, the 60% moisture storage environment resulted in higher solubilization of structural components. Lignin was reduced from 17.7% in the native sample to 15.6% and 14.9%; xylan was reduced from 23.6% in the native to 21.3% and 19.4% in 40% and 60% moisture samples, respectively. Modest glucan changes were observed from 35.6% in the native to 35.1% in the 60% moisture sample. Acetate was reduced by half in both alkali treatments, highlighting the deacetylation occurred due to alkali treatment. These changes corresponded to extractable component increases highlighted in Figure 4.1 and designated in Table 4. as Unquantified Extractives. In summary, these changes show that initial saponification of acetyl bonds was accomplished with alkali treatment but that the moisture content during storage a greater impact on depolymerization of structural components than did alkali alone. Despite the 40% moisture conditions having twice the molarity of the alkali solution, the increased moisture content of 60% promoted additional depolymerization.

The one notable exception to moisture influenced structural components being released at 60% compared to 40% moisture is in the case of structural ash content, where a reduction from 2.6% in the native stover to 1.9% in the High NaOH, 40% moisture sample was observed, and little difference was seen in other samples. Alkali can solubilize some ash components such as silica at high pH (>10.5)[40], and it is possible that the 0.92 M NaOH concentration enabled that whereas the 0.4M

NaOH concentration of the 60% moisture sample did not. This more than an issue of just pH shift in lignocellulosic but also of competing mechanisms of acetyl groups in hemicellulose being released and neutralizing the alkali as well as the overall buffering capacity of inorganics contained in lignocellulosic biomass. Regardless, the impact of silica on wear and abrasion as well as slagging in reactors has been characterized elsewhere[41, 42]. Further analysis of ash speciation would provide additional insights into how this approach may be leveraged in the future, perhaps to reduce the abrasiveness of specific biomass fractions.

In summary, low dry matter losses in both anaerobic High NaOH treatments suggest either approaches could be viable depending on factors such as moisture content at the time of harvest, moisture specification at the pretreatment reactor, and logistical concerns such as transportation of high-moisture material. While the Low NaOH treatment at 40% also preserved dry matter in anaerobic conditions, the Low NaOH 60% moisture treatment showed little utility.

One additional characterization approach, SEM, was used to assess for any structural changes as a function of treatment and storage. Native corn stover pith cells are pictured in Figure 4.2, A and B, with parenchyma and vascular bundles readily observable and visually bound. SEM images of High NaOH, 40% moisture scenario pith shown in Figure 4.2, C and D show that parenchyma cells are visually similar in the two samples. Perhaps some changes occurred in the rupture of cells on the outside of the vascular bundle (shown in red), but this observation is clearly speculative. Overall, observed structural changes are relatively mild.

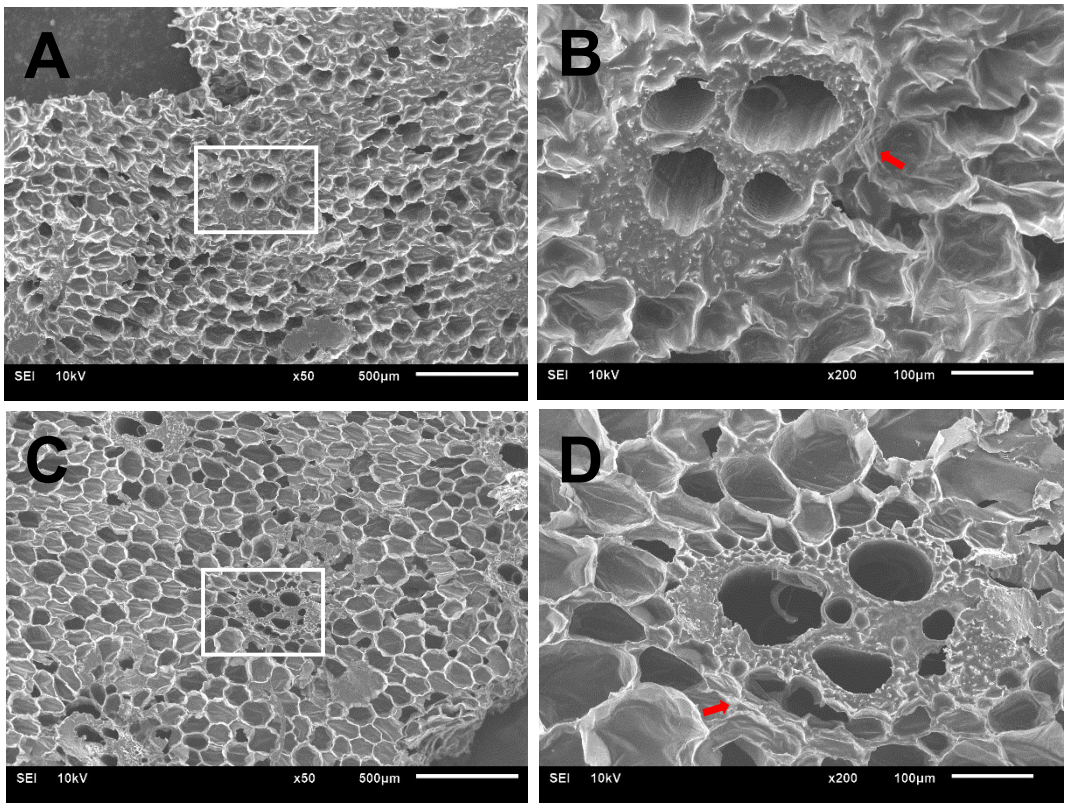

Figure 4.2. SEM micrographs at 50X (left) and 200X (right) of corn stover A, B: native; C, D: High NaOH, 40% moisture stored anaerobically for 4 weeks. Red arrows indicate linkages near vascular bundle.

Techno-economic Assessment of an Alkali-assisted anaerobic storage method

Experimental results highlight how anaerobic storage conditions can be utilized to preserve corn stover total dry matter in combination with alkali-assisted treatments. Techno-economic analysis was utilized to compare this approach with current designs that utilize baled biomass as the common format to facilitate storage, transportation, and handling. A chopped logistics system was designed that encompassed corn stover stored and transported in a silage tube at 40% moisture, similar to a design reported previously for transportable silage tubes[12]. This process uses forage chopping of corn stover to meet biorefinery size specifications and increase the packing density in anaerobic, long term storage and subsequent transportation to the biorefinery gate. The design also leveraged receiving and handling unit operations that had been designed for a logistics system that transported chopped corn stover in bulk form to a biorefinery gate for storage in 50,000 ton piles[11].

The costs of the chopped silage tube logistics system were compared to the bale-based logistics system using two-pass harvest and collection, storage of four bale high stacks, and transportation to a depot co-located with a biorefinery (Table 4.4). Grower payment was costed equally for both

approaches at $20.13/ton. The anaerobic silage tube approach incurred $17.28/ton cost for harvest and collection; however, efficiencies were experienced in this system since size reduction occurred during the one-pass forage chopping operation. Harvest and collection in the baled logistics system include two-pass harvest and collection into square bales, and hence costs were slightly higher ($18.79/ton) compared to the chopped system. Storage costs were increased three-fold in the silage tube scenario ($21.70/ton) compared to the baled approach ($6.53/ton) due to cost of the silage tube bagging operation, which included costs for plastic utilization as well as the increased land use requirement compared to stacked bales. Likewise, transportation costs were higher in the silage tube scenario ($17.65/ton vs. $14.98/ton) because of the reduced total mass and increased moisture in each truckload delivered. In the baled logistics system, preprocessing at the depot required two-stage grinding operations to achieve biorefinery particle size targets and cost ($20.84/ton). However, in-plant preprocessing costs dropped dramatically for the silage tube scenario ($1.10/ton) because size reduction was incorporated during harvest. A dockage of $0.89/ton was also applied to the Bale Logistics Scenario to account for soil incorporated during the baling process, whereas corn stover harvested during the forage chopping process does not encounter the ground. The resulting total cost of the silage tube approach were calculated to be $77.86/ton compared to $82.37/ton baled logistics system.

Table 4.4. Cost comparison of a baled system using two-pass harvest compared to the chopped harvest utilizing silage tube storage. All costs are in US 2016 dollars per ton deliver corn stover.

Unit Operation	**Baled Logistics System ($/ton)**	**Chopped, Silage Tube Logistics System ($/ton)**
Grower payment	$20.13	$20.13
Harvest and collection	$18.79	$17.28
Storage and queuing	$6.53	$21.70
Transportation and handling	$14.97	$17.65
In-plant receiving and preprocessing	$19.43	$1.10
Dockage	$0.89	-
Total Feedstock Cost	**$79.92**	**$77.89**

In the silage tube logistics system, storage and queuing, transportation and handling, and harvest and collection each contributed about 25% to the total cost. To understand the sensitivity of the total cost of the silage tube logistics system to important factors such as harvesting productivity, silage tube density, and transportation distance, a sensitivity analysis was carried out and presented in Figure 4.3. This shows that the impacts of the three evaluated factors on the total system cost were nonlinear. Decreases in silage tube density and harvesting productivity and increase in transportation distances caused increases in the total logistics cost. Harvest productivity and bulk density in transportation have been identified as the major cost drivers for chopped corn stover logistics systems[11]. Among the three factors, decrease in silage tube density increased the total cost the most. This is attributed to the fact that silage tube density directly impacted both the number of silage tube units used and the transportation weight per truckload. This result implies that to avoid system cost increase, it is most important to control the silage tube density. Bulk density in transportation also was identified as a key cost driver in previous chopped corn stover logistics designs[11].

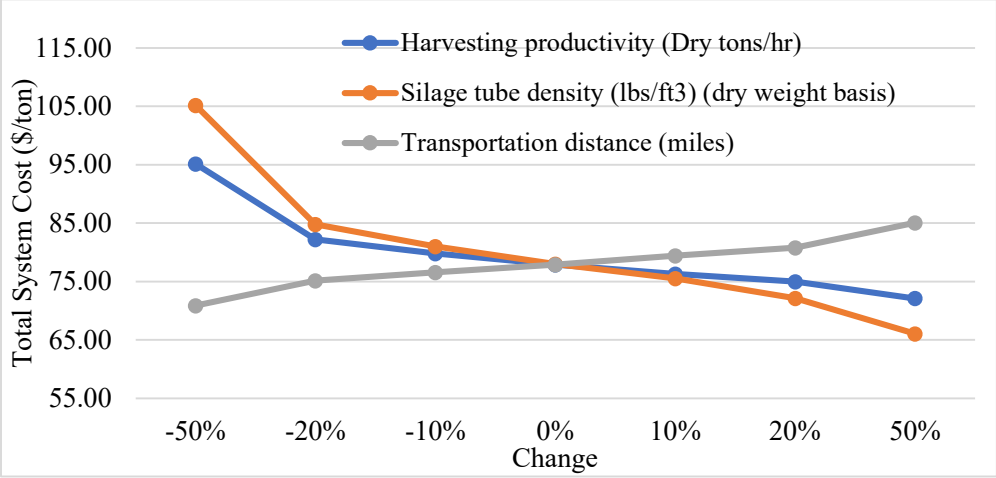

Figure 4.3. Changes in the total system cost for the silage tube logistics system corresponding to changes in harvesting productivity, silage tube density, and transportation distance.

The sensitivity analysis results also suggested further cost reductions should reduce the transportation distance, which is directly related to harvest yield and biorefinery size. This may not be possible with a crop such as corn stover that is limited to 1-2 ton/acre harvest yields. However, other energy crops such as switchgrass, miscanthus, or biomass sorghum[43-46] produce higher harvest yields and could benefit from the approach described in this study.

In conclusion, the technoeconomic analysis results showed that the designed silage tube logistics system incurred lower costs than a conventional two-pass baled logistic system, mainly due to moving biomass comminution upstream in the feedstock logistics supply chain and using an

innovative approach to increase bulk density during transportation of silage. Future efforts should be focused on improving silage tube density, reducing silage tube costs, and alternate approaches to compact forage chopped corn stover.

Conclusion

The primary goal of this study was to explore the potential for partial deacetylation and saponification of intermolecular bonds between lignin and hemicellulose prior to long term storage to reduce recalcitrance in corn stover. This study explored the working envelope of this approach to explore the compatibility of varying storage conditions in the supply chain, such as moisture content, oxygen availability, and alkali loading. Extractives were monitored as an indicator for effectiveness and results indicate that there is no difference in extractives between aerobic and anaerobic storage after four weeks of storage. However, significant dry matter loss occurred in aerobic storage (7.5-13%) compared to anaerobic storage (3-7%). Gross compositional changes in the anaerobically stored corn stover verify lignin, xylan, and acetate reduction consistent with low-severity structural sugar and lignin depolymerization. Three anaerobic storage scenarios were recommended for future investigation. A logistics supply chain configuration was designed around the anaerobic storage unit operation, and the modeled approach was found to be cost competitive to a bale logistics system traditionally used for corn stover. In summary, this work provides foundational data for the scientific community such that further optimization and understanding of the underlying physiochemical impacts can be explored.

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Chapter 5: Combined alkali treatment and anaerobic storage in corn stover enhances reactivity and surface energy properties

Abstract

The inherent recalcitrance of lignocellulosic biomass to physical and chemical depolymerization necessitates high severity thermal and chemical treatments to liberate carbohydrates and lignin for production of fuels and chemicals. The aim of this study was to examine the potential for process intensification within the feedstock supply chain operation of long-term storage. The aim was to use anaerobic storage to provide an environment conducive to slow transformations in corn stover following saponification of ester bonds in lignin and hemicellulose brought about by alkali addition. Results indicate the alkali-treated corn stover was well preserved in anaerobic storage over 2 weeks, showing only 4% dry matter loss. Monomerization of glucose and xylose in the presence of glycosidases confirmed the alkali-treated, stored biomass was more susceptible to enzymatic cleavage, suggesting reduced recalcitrance. Washing at room temperature was employed to remove any molecules that could be blocking enzyme assess. Washing increased enzymatic accessibility in terms of carbohydrate monomerization in the native sample and increased surface area in both native and alkali-stored samples. Surface energy confirmed the unwashed samples contained equivalent basic features, which primarily are attributed to lignin, yet washing removed the coalesced lignin in the alkali-treated stored samples. The alkali-stored, washed stover exhibited increased surface area, hydrophilicity, and work of cohesion and adhesion compared to the native, washed samples. This research drives the fundamental understanding that adding alkali prior to anaerobic storage not only improves enzyme accessibility to corn stover but, when combined with a room temperature wash, will result in a product that has favorable physical properties in downstream preprocessing.

Introduction

Renewable lignocellulosic feedstocks offer the potential to provide carbon-rich materials to produce liquid transportation fuels. In 2020 alone the transportation sector utilized >75% of petroleum consumed in the U.S[1]. The need for renewable liquid fuels will continue to grow, especially for transportation modes that are not likely to be electrified, including heavy-duty vehicles, marine vessels, and the aviation fleet. Biological pathways to enable renewable fuel production are often centered around microbial upgrading of carbohydrates, which is consistent with the corn grain to ethanol approach of bio-based transportation fuel production. However, a key barrier to this approach

for lignocellulosic crops compared to the corn grain model is the highly ordered, intertwined, and recalcitrant structure of lignocellulosic biomass that must be deconstructed such that macromolecules (primary cellulose, hemicellulose, and lignin) are solubilized into microbial-accessible streams, such as monomeric glucose[2]. A combination of thermal energy, water, caustic, and enzymes are used to accomplish this deconstruction, resulting in significant cost and energy inputs. This challenge is countered with biofuel efficiency goals of reducing greenhouse gas emissions by >50% compared to petroleum-based fuels[3]. The research in the present study is focused on approaches to address the lignocellulosic recalcitrance barrier through innovative solutions within the feedstock supply chain operational window.

Feedstock logistics supply operations include all points between production and utilization, including harvest, collection, storage, transportation, and size reduction. Storage is the unit operation with the longest residence time, especially for agricultural residues and energy crops that rely on seasonal harvest but must provide a biorefinery with consistent supply year-round. Dry, baled[4] and wet, ensiled[5] storage approaches have been investigated and are well characterized for their roles in bioenergy supply chains. Recent studies have highlighted the risk of feedstock loss in baled systems due to microbial degradation when low-moisture requirements are not met[6, 7]. Therefore, wet, ensiled storage has been explored as an approach to reduce loss to uncontrolled degradation[8] and possible depolymerization[9].

Ensiling principles are based on mechanical oxygen removal followed by microbial respiration of remaining oxygen using soluble carbohydrates, and finally production of organic acids through fermentation[10]. Previous studies have explored how anaerobic storage of corn stover could be compatible with both dilute acid and dilute alkali pretreatment and subsequent enzymatic hydrolysis to fermentable monomeric carbohydrates[8], and ensiled stover was shown to be compatible with both approaches. The alkali pretreatment approach is attractive because downstream microbial fermentation inhibitors are not produced from carbohydrate degradation, specifically ferulic acid or hydroxymethylfurfural[11]. Additionally, alkali treatment saponifies acetyl and glycosidic bonds in hemicellulose and ester linkages between lignin and xylan, which results in deacetylation and lignin liberation[12, 13]. In recent designs this deacetylation approach is conducted at a solids content as low as 8% (w/w) at 80°C, allowing the isolation of both a solid carbohydrate stream as well as a soluble lignin stream, resulting in multiple product streams and additional co-products beyond just fuels[13-16]. Further defibrillation through mechanical refining, a form of shear and compression based comminution, aids alkali extraction in improving carbohydrate surface area for enzyme access during follow-on hydrolysis of complex carbohydrates to fermentable monomers[13]. Diffusion-limited alkali

impregnation challenges in lignocellulosic biomass necessitate the low solids content. Hence, opportunities to address chemical impregnation challenges of alkali-assisted pretreatments are prime for further investigation.

The overarching aim of this study was to explore approaches to positively improve physical and chemical properties of corn stover during the long-term storage operation necessary to provide a biorefinery with a year-round stable supply of feedstock annually. A potential mechanism of reduced recalcitrance enabled by alkali-assisted anaerobic storage was correlated to carbohydrate release following glycosidic enzyme hydrolysis, and a room temperature washing was further explored to improve material performance. Gross chemical composition analysis was aided by molecular chemical composition analysis using ^{13}C cross-polarization magic-angle-spinning (CP-MAS) nuclear magnetic resonance (NMR) spectroscopy. Surface area and surface energy measurements were used to quantify the changes in porosity as well as the changes in surface chemistry that impact the overall work of cohesion and adhesion as well as the wettability of solid biomass.

Methods and Materials

Alkali Assisted Anaerobic Storage

Corn stover harvested in Hubbard, IA in October 2018 was packed into 55-gallon, sealed drums and transported to Idaho National Laboratory. Stover was subject to initial size reduction using a Schutte Buffalo hammer mill until passing through a screen with 6 mm openings. Concentrated NaOH (50 w/w%, Sigma Aldrich) was added to water for dilution, and then the mixture was applied to 10 kg of the 6 mm minus corn stover such that a final moisture content of 60% wet basis (wb) and alkali loading of 2.3% (w/w dry basis biomass) was achieved. The treated corn stover was mixed thoroughly then manually compacted into 20 L reactors, each sealed with a gasket-lined lid fitted with gas sampling ports and an aluminized gas collection bag. Reactors were purged with nitrogen and stored at room temperature for 2 weeks. Upon exiting storage, the corn stover was mixed thoroughly to create representative subsamples, assessed for moisture and mass changes, and prepared for further analytical assessment. Table 5. details the preparations and corresponding sample nomenclature.

Table 5.1. Sample nomenclature and corresponding experimental treatment for this study.

Sample ID	Experimental Treatment
Native	As-received corn stover
Native, washed	Native sample subject to 1X volume water wash
Alkali-stored	Corn stover with 2.3% NaOH (w/w dry biomass) stored anaerobically
Alkali-stored, washed	Corn stover with 2.3% NaOH (w/w dry biomass) stored anaerobically subject to 1X volume water wash
Alkali-unstored	Corn stover with 2.3% NaOH (w/w dry biomass) not stored
Alkali-unstored, washed	Corn stover with 2.3% NaOH (w/w dry biomass) not stored but subject to 1X volume water wash

Washing was conducted using a 1:1 ratio of wet biomass to 18MΩ-cm nanopure water. Approximately 10 g of wet biomass was placed into a 60 mL syringe. The end of the syringe was covered with Parafilm to ensure no water leakage, an equivalent volume of nanopure water was added to the biomass, and the plunger was inserted to create an air-tight seal. The biomass was incubated at room temperature for one hour with a rotation after 30 minutes. A bench top manual hydraulic press (Carver Inc., Wabash, IN) was then used to expel free liquid from the syringe into a scintillation vial. Liquid was concentrated using a TurboVap® evaporator (Biotage, Uppsala, Sweden).

Multiple size reduction methods were utilized to prepare samples for analytical assessment (Table 5.). Mechanical refining was accomplished with a PFI mill (Rycolab, Deerlijk, Belgium) prior to enzymatic hydrolysis. Corn stover was knife milled in a Wiley Mill Model 4 (Thomas Scientific, Swedesboro, NJ) until it passed through a 2 mm screen. A Retsch ZM 100 (Haan, Germany) centrifugal mill was fitted with a 200 µm screen.

Table 5.2. Size reduction performed and corresponding analytical method.

Size reduction performed after hammer-milling	Analytical method
None	Organic acids
Mechanical refiner	Enzymatic hydrolysis after 2,000 revolutions (Appendix C), 4,000 revolutions, or 8,000 revolutions (Appendix C)
Knife mill, 2 mm screen	Chemical composition, ^{13}C-NMR, surface area and energy, enzymatic hydrolysis (Appendix C)
Centrifugal mill, 200 µm screen	X-ray diffraction

Chemical Composition and Lignin isolation

Organic acids extracted from alkali-stored biomass were assessed according to the method previously described[17] where 1:10 ratio of wet biomass to 18MΩ-cm nanopure water was equilibrated at 4°C for

24 hr. Liquid was filtered (0.2 μm) and acidified to a pH of 4 with sulfuric acid. Acids were analyzed using high-performance liquid chromatography (HPLC) with a refractive index (RI) detector (Waters, Milford, MA) and quantified based on a five-point analytical standard with succinic, lactic, acetic, butyric and isovaleric acids. Compositional analysis was conducted on duplicate samples following the Laboratory Analysis Procedures (LAPs) established by the National Renewable Energy Laboratory (NREL)[18, 19]. A 100°C water and subsequent ethanol extraction using an automated solvent extractor ASE 350 (Dionex, Sunnyvale, CA)[20] was followed by a two-stage acid hydrolysis to solubilize structural carbohydrates[21]. Carbohydrate monomers were quantified using HPLC with a RI detector (Agilent, Santa Clara, CA) and Aminex HPX 87P column (Bio-Rad, 300 x 7.8 mm, Hercules, CA)[22]. The remaining acid insoluble solids were assessed for acid-soluble lignin and ash components after heating at 100°C and 550°C, respectively. Acid soluble lignin was analyzed using an ultraviolet-visible spectrophotometer (Varian Cary 50, Agilent, Santa Clara, CA). Acid insoluble lignin and structural ash are determined gravimetrically on the remaining solids. Protein content was calculated as a function of total nitrogen measured[23].

Enzymatic hydrolysis

Enzymatic hydrolysis was conducted in triplicate for all samples at 10% w/w solids in 50 mM citrate buffer, pH 4.8, supplemented with 0.02% of sodium azide to prevent biological contamination. Cellic® CTec2 and HTec2 enzymes (Novozymes®, Franklinton, NC) were used at loading rates of 20mg/g and 2 mg/g glucan, respectively for samples that were mechanically refined at 4,000 revolutions. Flasks were wrapped with Teflon tape and placed into a New Brunswick Scientific Excella E25 Incubator Shaker series at 50°C for five days. After incubating, samples were then vacuum filtered to pass 0.2 μm, and a 1:20 dilution was assessed for soluble carbohydrates using HPLC as described above.

Total theoretical glucose and xylose yield was determined using the structural glucan and xylan composition as well as the soluble fraction of each carbohydrate released in hydrolysis per the following equations:

$$\%\ Glucose\ Yield = \frac{Glucose_{Enzymatic\ Hydrolysis}}{Glucan_{Total}\ x\ (\frac{1}{0.9})} x\ 100 \quad \text{Equation 5.1}$$

$$\%\ Xylose\ Yield = \frac{Xylose_{Enzymatic\ Hydrolysis}}{Xylan_{Total}\ x\ (\frac{1}{0.88})}\ x\ 100 \quad \text{Equation 5.2}$$

Mass differences of 1:0.9 and 1:0.88 between the monomeric versus polymeric forms of glucan and xylan, respectfully, were accounted for in all calculations.

Solid-State ^{13}C-CP/MAS NMR Spectroscopy

Corn stover samples milled to 0.2 mm minus were placed into 4 mm ZrO rotors for solid-state NMR analysis. Samples analyzed included the native and alkali-stored corn stover as well as the washed solids from each sample. After packing all samples and capping with Kel-F rotor caps, the filled rotors were spun at ν_R = 10 kHz. ^{13}C spectra were obtained using a Bruker Avance III spectrometer with a field strength of 9.4 T ν = 100.59 MHz) and a standard HX magic-angle spinning (MAS) probe. The basic Bruker cross-polarization (CP) pulse program was used for all samples[24, 25]. The proton nutation frequency was 92.6 kHz with a decoupling field strength of 48.1 kHz using the SPINAL64 decoupling program.[3] The Hartmann-Hahn condition was optimized at 1.5 msec. The sweep width of the experiment was set to 497 ppm and the relaxation delay was set at 4 sec. To improve resolution of the native and alkali-stored stover samples, the total number of transients in these experiments was increased to 16,384, which led to an experimental duration of 2.85 days. In processing, the free-induction decay was truncated from 4,994 points to 900 points to limit the amount of noise in the transformed spectra while still maintaining the integrity of the measured peaks.

BET Surface Area

The corn stover samples were dried to less than 2% moisture by passing dry argon through the sample for 24 hours at 45°C. Multipoint Brunauer-Emmett-Teller (BET) specific surface area (SSA) was estimated using a Micromeritics 3Flex surface area analyzer, with nitrogen gas as the adsorbate. The samples (masses ranging from 1.3 to 1.6 grams) were further conditioned under vacuum at 45°C for 20 hours in the 3Flex outgassing setup prior to measurements. Measured mass loss during outgassing was less than 0.0005 g for all samples. Sample porosity was estimated using the Barrett, Joyner and Halenda (BJH) method (Harkins and Jura t-curve), and Faas correction. This method limits the data collection to pores of diameters from 1 nm to 50 nm (micropore region; < 2nm, mesopore region; between 2 nm and 50 nm).

Inverse Gas Chromatography Surface Energy

Surface energy measurements were carried out with inverse gas chromatography (IGC) using a surface energy analyzer (SEA) (Surface Measurement Systems; London, United Kingdom). The analyzer was equipped with a flame ionization detector (FID) with helium as the carrier gas (10 SCCM). Dry corn stover samples (after surface area measurements) were packed in silanized glass columns (4 mm ID, 6 mm OD x 300 mm L). Sigma-Aldrich HPLC grade *n*-alkanes (C_7–C_{10}) were used to interrogate the corn stover surface to measure the dispersive surface energy (γ_s^d) component. The specific surface energy (γ_s^{ab}) component was quantified using a monopolar Lewis acid and base,

trichloromethane and ethyl acetate. All IGC solvent probes were HPLC grade from Sigma-Aldrich. The densities of the packed columns were maintained in the range of 0.31–0.40 g/cm^3.

The IGC measurements were performed at infinite dilution (0.005 n/n$_m$ or 0.5% mono- layer coverage) under isothermal conditions at 30°C. Dispersive surface energy was evaluated using the Dorris-Gray method using $\gamma sd= \quad RTln \;\; V⍰N,Cn+1H2n+4VN,CnH2n+224NA2aCH22\gamma CH2d$ Equation 5.3[26, 27], where a_{CH_2} is the surface area of a methylene group (6 Å^2), $\gamma^d_{CH_2}$ its surface tension (35.6 mJ/m^2), N_A is Avogadro's constant, and V_N is the retention volume of the alkanes.

$$\gamma_s^d = \frac{\left[RTln\left(\frac{V_{N,C_{n+1}H_{2n+4}}}{V_{N,C_nH_{2n+2}}}\right)\right]^2}{4N_A^2 a_{CH_2}^2 \gamma_{CH_2}^d}$$ Equation 5.3

The specific surface energies were calculated based on the polarization method with the van Oss-Chaudhury-Good (vOCG) approach on the Della Volpe scale (DV). Works of cohesion, adhesion were determined using SEA Analysis software on the DV scale (water surface energy values, γ_w^d; 26.25 mJ/m^2, γ_w^a; 48.50 mJ/m^2, γ_w^b; 11.20 mJ/m^2), using the Dorris-Gray, polarization methods.

Statistical Analysis

Significant differences were determined using a one-way analysis of variance (ANOVA) performed in JMP 14.2.0 (SAS, Cary, NC) for enzymatic hydrolysis results. Where $p < 0.05$ was observed in ANOVA, indicating statistical significance, Tukey's honest significant difference (HSD) test was performed to obtain multiple-level comparison of statistical equivalency.

Results and Discussion

The overarching aim of this research was to explore approaches to positively improve physical and chemical properties of corn stover during a long-term storage operation, which is necessary to provide a biorefinery with a year-round stable supply of feedstock annually. The present study was undertaken to explore the mechanisms of reduced recalcitrance to glycosidic enzymes in alkali treated, anaerobically stored corn stover that aimed to (1) saponify the ester linkages between lignin and hemicellulose, (2) weaken the hydrogen bonding network between cellulose and hemicellulose, and (3) preserve solubilized material and total dry matter using anaerobic conditions. The result of the combined treatment was enhanced carbohydrate yield in hydrolysis with glycosidases.

Storage Performance

The combined impact of alkali treatment and anaerobic storage in corn stover was assessed to probe the impact of a high moisture environment to further reduce recalcitrance following saponification of

ester linkages between lignin and hemicellulose. Dry matter loss over 2 weeks was 4.3% ± 1.6%, which is consistent with previous anaerobic storage of corn stover[8].

Macromolecular compositional analysis (Table 5.3) indicated that the alkali-assisted anaerobic storage enhanced depolymerization of structural components, with total structural components decreasing from 86.1% to 79.2%. Total lignin decreased from 18.1 ± 0.3% to 17.3 ± 0.5%, which highlights that the alkali saponification attacked the ester linkages holding lignin to hemicellulose and cellulose. A higher relative proportion of acid soluble lignin (23%) was released compared to acid insoluble lignin (3%). Previous reports suggest that syringyl moieties dominate the acid soluble lignin fraction[28,29]. Tsutsumi et al. demonstrated that during alkali treatment syringyl β-aryl ether bonds exhibited higher cleavage rate compared to guaiacyl β-aryl ether bonds[30]. Further characterization of lignin moieties using methods such as heteronuclear two-dimensional NMR would further elucidate the structural impacts of alkali-storage on corn stover and should be investigated in future work.

Glucan decreased from 34.8% to 33.1% after alkali-storage (5% relative change), and while this primarily reflects changes in cellulose, glucose substitutions within hemicellulose chains may have also been impacted in alkali treatments. However, alkali-assisted storage impacted major hemicellulose components at a higher relative percentage compared to lignin or glucan. Xylan decreased from 22.8% to 20.1% (12% relative change), arabinan decreased from 3.6% to 2.9% (19% relative change), acetate decreased from 3.7% to 2.4% (35% relative change), and galactan levels were unchanged. While the acetate release is well-known based on saponification of acetyl bonds during alkali treatment, the changes in xylan and arabinan are unexpected when considering other anaerobic storage studies. In prior studies, hemicellulose components were well preserved after anaerobic storage of freshly-harvested corn stover[8] and were enhanced proportionally after deacetylation[31]. It is theorized that the destabilization induced by alkali addition weakened the intramolecular hemicellulose bonds, and as it entered anaerobic storage it was more susceptible to further bond weakening and depolymerization.

Total extractable components increased from 10.5% to 17.3% as a function of alkali storage, with organic acids accounting for the majority of these components (6.8 ± 0.3%). These acids correlated to a reduction in soluble glucose from 1.7% to 0.3%, which is well documented as the primary energy source for microbial respiration and subsequent fermentation after establishing anaerobic conditions[32]. Ethanol extractable components increased significantly from 2.6% to 3.2%. The change in extractable inorganics (2.0% to 5.1%) is expected based on the addition of alkali. Unquantified extractives represent 3.6% of the native corn stover and 1.2% in the alkali-stored corn stover. In total, these changes are consistent with the reduction of structural components.

Table 5.3. Chemical composition changes as a function of alkali storage in 60% moisture corn stover. Results represent native and alkali-stored corn stover from triplicate reactors.

% Component (w/w, dry basis)	Native	Alkali-stored
Structural Components	**86.1 ± 0.2**	**79.2 ± 0.4**
Acid-Insoluble Lignin	16.5 ± 0.4	16.1 ± 0.5
Acid-Soluble Lignin	1.6 ± 0.0	1.2 ± 0.0
Glucan	34.8 ± 0.3	33.1 ± 0.3
Xylan	22.8 ± 0.2	20.1 ± 0.6
Galactan	1.3 ± 0.1	1.2 ± 0.0
Arabinan	3.6 ± 0.1	2.9 ± 0.2
Acetate	3.7 ± 0.0	2.4 ± 0.1
Structural Ash	1.9 ± 0.2	2.2 ± 0.2
Extractable Components	**10.5 ± 0.5**	**17.3 ± 0.6**
Soluble Glucan	1.7 ± 0.2	0.3 ± 0.1
Soluble Xylan	0.3 ± 0.1	0.4 ± 0.0
Soluble Galactan	0.2 ± 0.0	0.2 ± 0.0
Soluble Arabinan	0.1 ± 0.0	0.1 ± 0.0
Organic Acids	NA	6.8 ± 0.3
Extractable Inorganics	2.0 ± 0.5	5.1 ± 0.2
Unquantified Extractives	3.6 ± 0.8	1.2 ± 0.2
Ethanol Extractives	2.6 ± 0.0	3.2 ± 0.2
Total	**96.7 ± 0.5**	**97.0 ± 1.2**

Reduction of structurally bound acetate described above is expected based on what is known of the mechanism of alkali treatment; however, the quantity of extractable acetic acid exceeds the amount anticipated by the observed decrease in hemicellulose-bound acetate, indicating that fermentation contributed to the total acetate measured in the water extraction. Quantification of the organic acids indicated acetic acid concentrations of 3.3 ± 0.3%, which is the combined result of acetyl released from hemicelluloses and acetate formed through microbial fermentation. Lactic acid was present at 2.6 ± 0.2%, further suggesting fermentative microorganisms were active despite the high initial pH occurring with alkali addition. Succinic acid (0.5 ± 0.1%), butyric acid (0.1 ± 0.0), and isovaleric acid (0.3 ± 0.0) were also formed during anaerobic storage. These results suggest that the high pH conditions that were initially present in the corn stover were likely neutralized by the acetic acid released during saponification of the acetyl bonds, producing a neutral pH environment where microorganisms could proliferate. Homo- and hetero-fermentation by lactic acid bacteria are well documented as the mechanism for silage preservation in anaerobic storage[33], producing the organic acids that reduce pH and inhibit proliferation of other microorganisms. Other examples of the combined impact of alkali-assisted depolymerization with anaerobic storage are present in the

literature in limited numbers. Calcium oxide impregnation prior to anaerobic storage was recently documented in *Hippophae rhamnoides*, with lactic and acetic acid prevalent as fermentation by-products resulting in a pH of 4.2[34]. Similarly, in the present study, despite the alkaline treatment prior to storage, the combination of deacetylation, corn stover buffering capacity, and metabolic activity of the native microorganisms reduced the pH to 5.6.

Enzymatic Hydrolysis Reveals Impact of Washing and Storage on Carbohydrate Yield

State-of-the-art approaches to preparing carbohydrate monomers from biomass for fermentation to fuels and chemicals employ deacetylation with sodium hydroxide at 80°C for 2 hours at 8 wt% solids followed by mechanical milling and enzymatic hydrolysis, which monomerizes over 85% of glucan and xylan[13, 16]. The low solids content results in significant water consumption and requires large reactor volumes that increase capital costs at a modeled biorefinery, and high alkali loading increases operating costs[12]. The present study investigated the potential for achieving preliminary depolymerization during the residence time in storage at a moisture contents consistent with ensiled storage (60%, w.b.) to promote chemical impregnation.

Native and alkali-stored biomass was subjected to mechanical refining, resulting in dramatically different carbohydrate yield in enzymatic hydrolysis. Alkali-stored corn stover exhibited significantly increased glucose (44.5 ± 0.3%) and xylose (23.1 ± 0.1%) release compared to native feedstock (28.3 ± 0.3% and 13.4 ± 0.3%, respectively) (Figure 5.1). Additional experiments performed using either reduced enzyme loading and varying revolution number in mechanical milling are described in Appendix C and generally follow this same trend. Native corn stover with alkali addition (alkali-unstored) was also subjected to mechanical refining and enzymatic hydrolysis in order to understand the effect of alkali addition alone. However, washing of the alkali-unstored sample prior to mechanical refining was necessary to obtain repeatable hydrolysis results, likely from the reactive alkali causing enzyme inhibition during hydrolysis (data not shown). This allowed for additional comparison of unwashed and washed material during mechanical refining and subsequent hydrolysis. Native, washed stover released 34.5% of the available glucose and 17.1% of xylose, a relative increase of 22% and 27% respectively, compared to the native sample. These results indicate that the washing alone prior to mechanical refining improved defibrillation as measured in glucose and xylose release in subsequent hydrolysis. It is anticipated that the one-hour hydration time during washing allowed the hemicellulose and cellulose to swell slightly, making them more susceptible to the compressive and shear forces during the milling step. Olejnik demonstrated that a 70 min period of free swelling was sufficient to hydrate cellulose fiber, also known as internal fibrillation[35]. Similar

effects of swelling and enhanced enzyme accessibility and subsequent monomer release have been demonstrated in grasses[36].

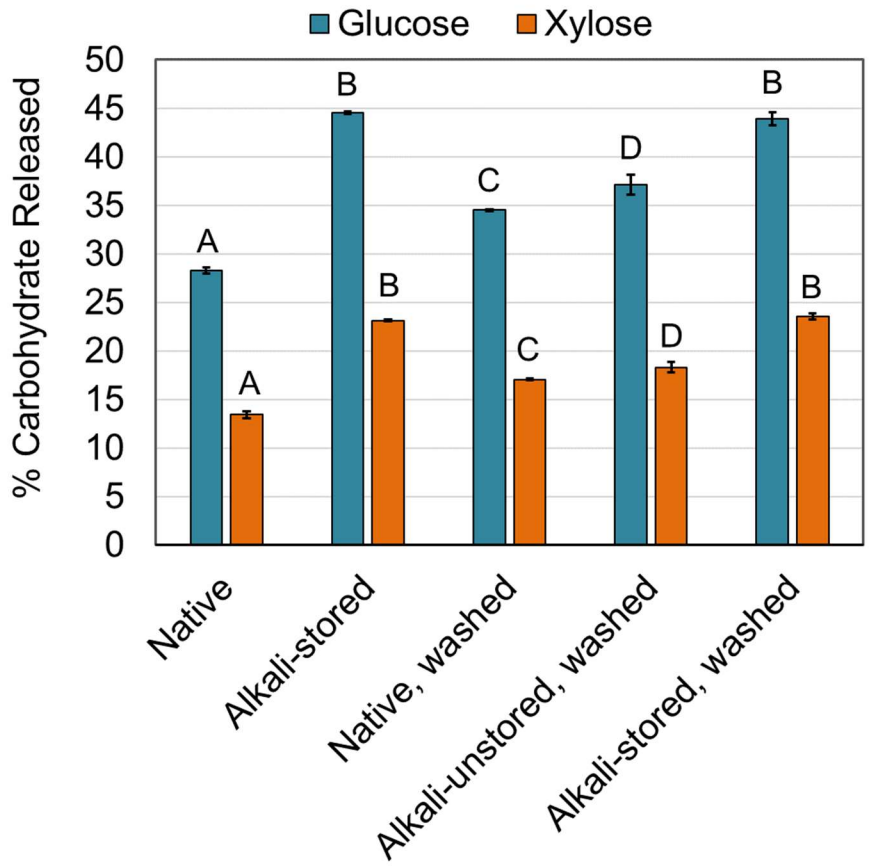

Figure 5.1. Carbohydrate yields during enzymatic hydrolysis. Samples represent native, alkali-stored, and alkali-unstored corn stover subject to first to mechanical refining and subsequent hydrolysis. Washing was preformed prior to mechanical refining. Error bars represent standard deviation, n=3.

The alkali-unstored, washed stover exhibited 37.1% glucose release and 18.3% xylose release, a slight but statistically significant increase compared to the native, washed stover (34.5% glucose and 17.1% xylose released). However, compared the alkali-unstored, washed stover to the glucose and xylose yields in the alkali-stored, washed sample were enhanced by a relative 18% and 29% (43.9% and 23.6% release, respectively). These results indicate that the residence time during anaerobic storage was conducive to further recalcitrance reduction in hemicellulose and cellulose beyond initial saponification reactions induced by alkali addition. Youssefian et al. demonstrated that lignin interacts more rapidly with water compared to hemicellulose; all hydrogen bonds in the lignin nanostructure were saturated at 10% moisture, whereas saturation of hemicellulose hydrogen bonds with water did not occur rapidly until higher moisture contents were provided[37]. It is hypothesized that the two-week residence time in storage resulted in swelling of hemicellulose due to weakening of

internal hydrogen bonding during this time, as well as increased interactions with hydroxide ion and both hemicellulose and cellulose. No significant difference in carbohydrate yield was observed in alkali-stored corn stover as a function of washing, and this is expected since any likely swelling had already occurring during the two-week residence time.

^{13}C-CP/MAS NMR

^{13}C cross-polarization magic-angle spinning NMR was utilized to relate nuclear interactions in the corn stover to structural changes as a result of alkali treatment and anerobic storage (Figure 5.2). Native corn stover exhibited an NMR spectrum typical for lignocellulosic material, normalized based on the peak intensity of C1 group in cellulose, with the C1-C6 carbons of cellulose exhibiting the predominant peaks given their high hydrogen content[38]. Hemicellulose signals in NMR are generally prevalent underneath the primary cellulose peak and not easily discernable in a cellulose containing sample[39]. CH_3 groups generally attributed to acetate were present around 24 ppm and lignin is widely shown in the chemical shift area above 110ppm. Alkali-stored corn stover displayed a similar NMR profile with minor differences. Peak intensity was reduced in the C2, C3, C5 and C6 carbons in cellulose, likely due to alkali treatment that solubilized hemicellulose followed by microbial consumption. This is expected based on the reduction of xylan and arabinan in the sample. However, additional experiments subjecting isolated hemicellulose to alkali treatment and anaerobic storage would further elucidate these physiochemical impacts on associated molecular bonds. Alkali storage also resulted in NMR chemical shifts at 25 ppm and 180 ppm, the latter which can be attributed to the C=O group of carboxylates commonly produced in anaerobic storage. However, changes in lignin are not easily observable using cross-polarization methodology due to the low hydrogen content in lignin.

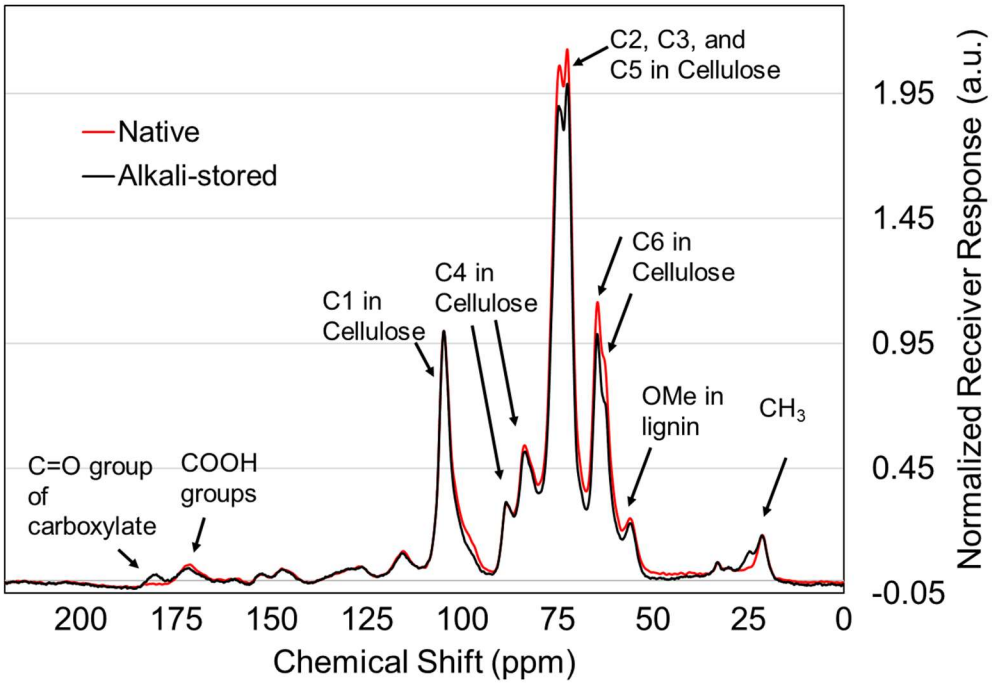

Figure 5.2. Solid-State ^{13}C-CP/MAS NMR profiles of native and alkali-stored corn stover. Spectra were normalized to the C1 atom of cellulose.

Both native, washed and alkali-stored, washed corn stover samples were analyzed by solid state NMR (Figure 5.3). Nearly equivalent peaks in the C1-C5 atoms of cellulose were observed with very minor changes to the C6 carbon atom, which is involved in linking one cellulose chain to an adjoining chain through hydrogen bonding of its CH_2OH moiety, the matrix of which forms the microfibril structure. The C6 atom has a slightly reduced intensity compared to the C1-C5 carbon atoms, and it is hypothesized the mechanism of swelling in alkali-stored cellulose could target this bond first. It is generally accepted that the strong hydrogen bonding of glucan in cellulose prevents structural changes of this molecule such that changes in peak intensity observable by NMR associated with rehydration, but cellulose swelling and associated C6 atom changes have been observed at aqueous NaOH solutions concentrations > 9 w/w%[40]. Further investigation of this phenomenon should be explored with and without anaerobic storage using increased alkali concentrations (up to 30 w/w% NaOH) in ^{13}C-NMR as described elsewhere[40 41], following the alkali-induced swelling of cellulose as a function of concentration and chemical shift of cellulose C1-C6 atoms.

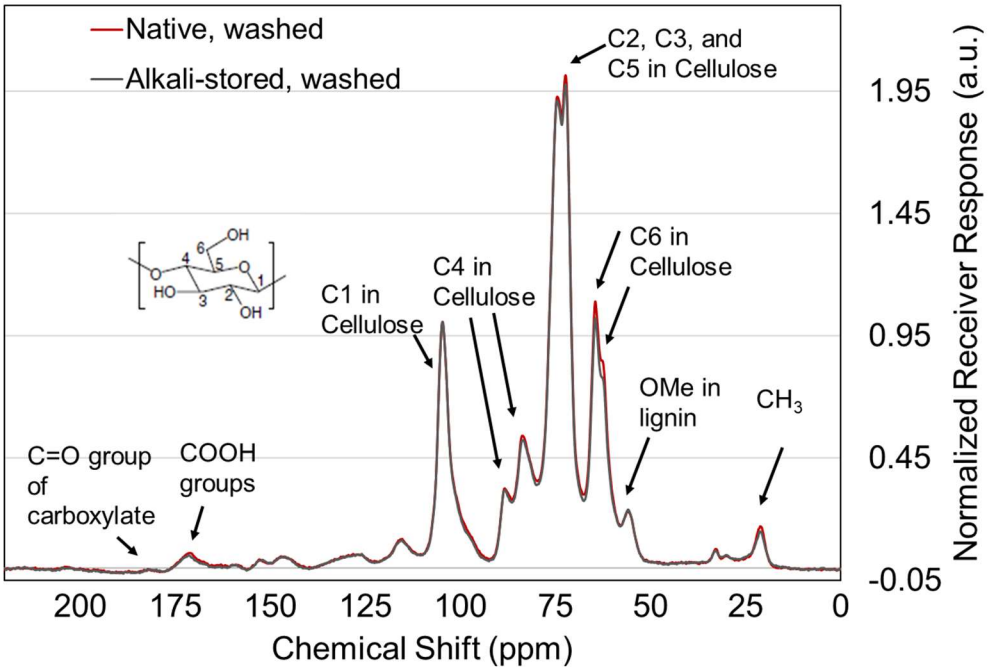

Figure 5.3. Solid-State ^{13}C-CP/MAS NMR profiles of native and alkali-stored corn stover subject to water washing. Spectra were normalized to the C1 atom of cellulose.

Surface area and porosity

Total surface area and porosity metrics were assessed in the native and alkali-stored corn stover to explore the relationship with enhanced enzymatic hydrolysis yields as a function of both alkali treatment and washing. Figure 5.4 depicts the relative differences of the native sample to the treated samples with full results reported in the Appendix. Total surface area and pore surface area reveal that the alkali-stored, unwashed sample exhibited equivalent properties to the native sample, and the largest impact was observed in the native, washed (19.2% and 83.9% increase over native, respectively) and alkali, washed stover (52.4% and 138.7% increase over native, respectively). This observation is expected in the native sample based on the soluble glucose present. It is also expected that the physiochemical changes occurring with alkali treatment solubilized lignin and acetyl groups during saponification reactions, and that these components would still be associated with the lignocellulosic matrix in the 60% moisture content environment of anaerobic storage. Moreover, organic acids produced during anaerobic storage that would further block surfaces. However, a one-hour water wash was able to liberate these soluble components and restore any pores or sites that had been blocked. Of note, the measured surface areas are in agreement with previous measurements of corn stover[42, 43] and variability (in terms of standard deviation) was below 0.002 m^2/g for all samples,

whereas the surface areas of native corn stover have been observed to be more variable due to the inherent heterogeneity of corn stover, ranging up to ~2 m^2/g [2].

Pore volume and pore diameter assessments confirm the largest impact across the treatment are due to washing, with ~500% and >100% relative increases, respectively, in both washed samples. Changes in average pore volume and average pore diameter were statistically equivalent among the washed samples. Based on the pore distribution data, it is suggested that ~ 97 % of the porosity and the relative changes were in the mesopore range of 2-50 nm. Pore diameter was calculated at 13.6 nm and 16.1 nm in the native, washed and alkali-stored, washed samples, respectively. Bubner et al. describe pore volume limitations for cellulase enzymes, with typical diameters of 4-6.5 nm and lengths of 18-21.5 nm[44]. It is therefore hypothesized that the pore size increases experienced during washing facilitated additional enzymatic access in the cell.

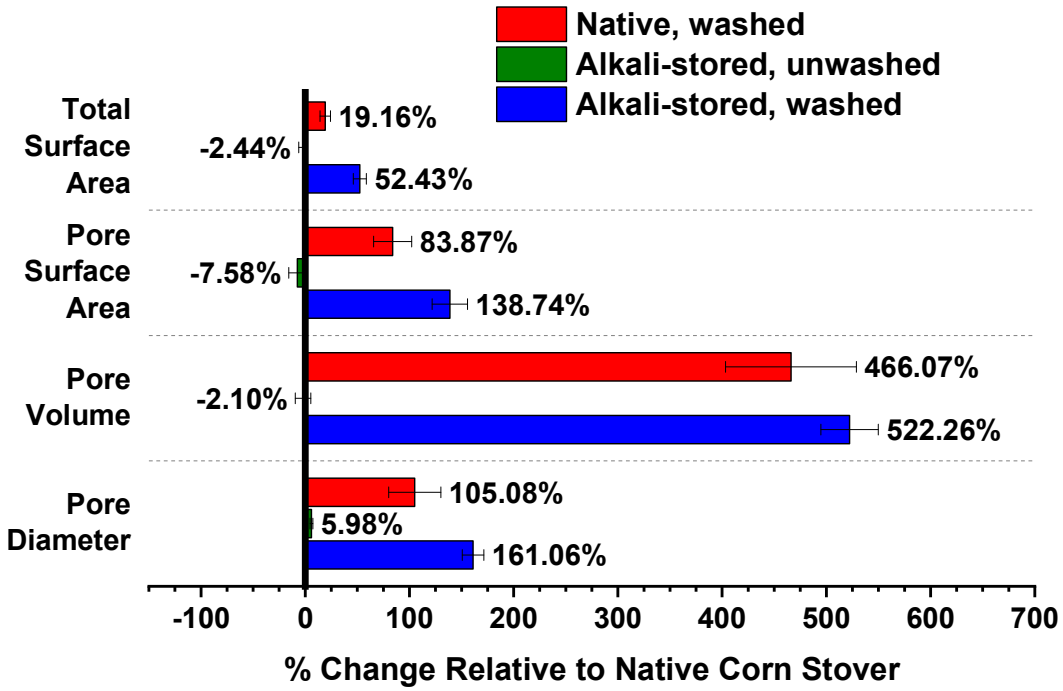

Figure 5.4. Measured changes in porosity and surface area of the alkali-stored corn stover and washed corn stover relative to the native corn stover samples.

Surface Energy

Fundamental thermodynamic insights related to the intermolecular forces of van der Waals forces, polar bonds, and non-polar bonds can be elucidated through surface energy characterization. These measurements give rise to several key properties that include wettability, hydrophilicity, adhesion, cohesion, and adsorption capacity. In additional to these forces, the surfaces of lignocellulosic

material are complex, where the constituents (cellulose, hemicellulose, and lignin) will undergo surface reconstruction in response to changes in the thermochemical environment[45-47]. Total surface energy is the aggregate of both the non-polar (dispersive) and polar (specific) surface energies. Figure 5.5 depicts the total surface energy, both dispersive and specific, for native and alkali-stored corn stover as a function of washing. In general, each sequential treatment increases the surface energy of the corn stover, and the following section will probe each measurement in the context of a lignocellulosic biorefinery.

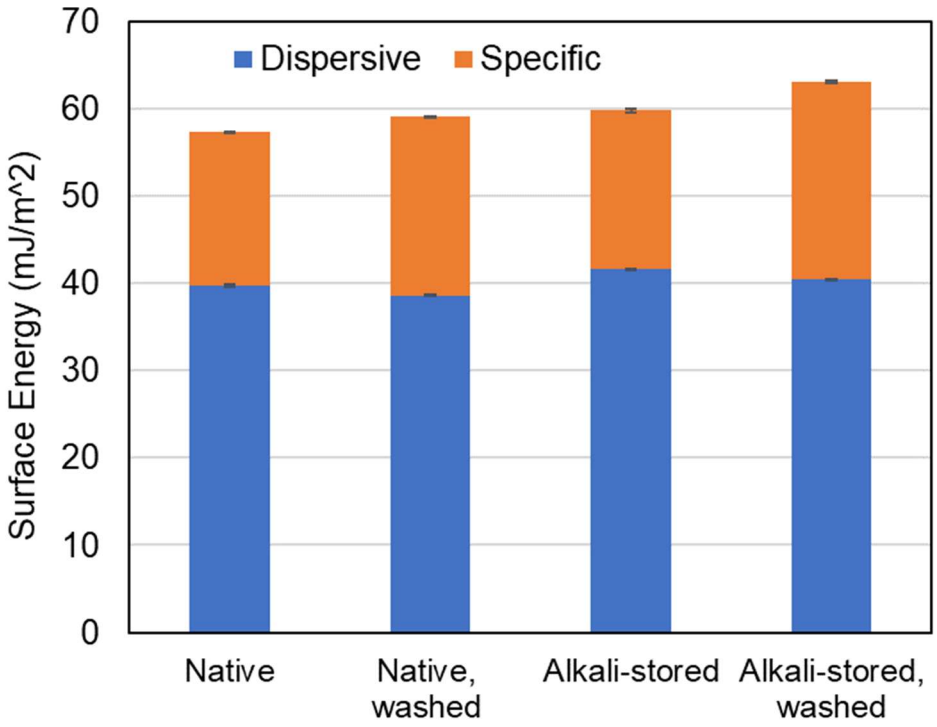

Figure 5.5. Total contributions of dispersive and specific surface energy as a function of native and alkali-stored corn stover as received and washed.

Dispersive Surface Energy

Dispersive energy is a measure of non-polar interactions such as the Van der Waals forces between lignin and carbohydrates. The dispersive surface energy (γ_s^d) is determined from the retention volumes of *n*-alkane (heptane, octane, nonane and decane) probes. Figure 5.6 shows a relative 4.6% increase in dispersive energy in alkali-stored corn stover over the native corn stover (39.8 to 41.6 mJ/m^2, Appendix D Table 1). The increase in dispersive surface energy can be attributed to the saponification of ester linkages between lignin and hemicellulose structures during alkaline storage conditions, thus exposing more lignin and cellulose or hemicellulose surfaces and increasing the

dispersive surface energy. Washing reduced the dispersive surface energy in both the native and alkali-stored samples, possibly attributed to the removal of lignin oxidation or degradation products, fermentation acids or other soluble components. Observations of a decrease in dispersive surface energy after alkali treatment and washing are in agreement with those of Cordeiro et al.[48] and is consistent with lignin removal[49].

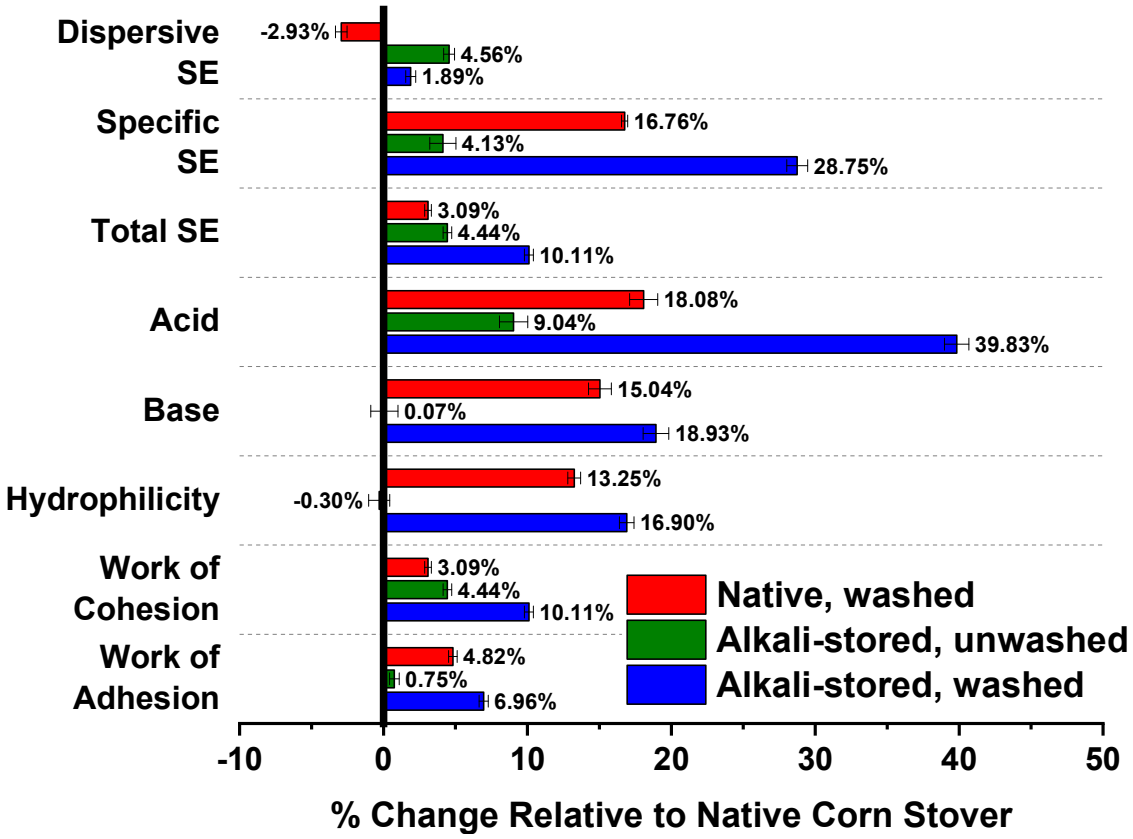

Figure 5.6. Measured increases in surface energy of the alkali-stored corn stover and washed corn stover relative to the native corn stover samples.

Specific Surface Energy

Whereas washing was shown to decrease dispersive surface energy due to removal of a highly dispersive component, washing exposed new binding sites for the Lewis acid and base IGC probes in both native and alkali-stored corn stover. Prior to washing there was no significant change in specific surface energy in the native and alkali-stored corn stover (17.5 and 18.25 mJ/m^2, respectively). Soluble components in unwashed stover, such as soluble carbohydrates in the native sample or solubilized lignin degradation products and fermentation byproducts in that alkali-stored sample,

likely were blocking the polar and hydrogen bonding sites of the IGC probes. Once washed, the alkali-stored, specific surface energy exhibited a 28.7% increase from the native, unwashed, and a 23.6% increase from the alkali-stored, unwashed corn stover (Appendix C). Additionally, alkali-stored, washed stover demonstrated a 10.3% increase in specific surface energy as compared to the native, washed stover.

This is further understood through evaluation of the binding of the Lewis acid and base probes and the respective changes in acid and base surface energies. Lewis acids are electron-pair acceptors, while Lewis bases are electron donors[49]. The acid component of the specific surface energy of the alkali-stored sample increased on average of 9.0% as compared to the native sample. The alkali-stored, washed sample increased of the acidic component of surface energy by 39.8% compared to the native and a ~30% increase over the alkali-stored, unwashed sample. Washing also increased the acid binding in the native sample by 18%. These results demonstrate that the act of washing removed surface moieties blocking accessibility of IGC Lewis acid probes.

The Lewis base component of surface energy showed no statistically significant difference between the unwashed native and alkali-stored samples, indicating that the alkali treatment did not expose measurable changes in electron donors. However, washing increased the basic component of the surface energy in both the native, washed and alkali-stored, washed samples by a relative 15 and 19% compared to the native sample. Lignin is predominantly a Lewis base; however, cellulose is amphoteric, but predominantly a Lewis acid[50]. These results suggest that the act of washing was key in exposing new binding sites in the lignocellulose matrix, and this was further facilitated by alkali treatment. In other words, substitution, addition, or removal of atomic and structural contributors to the specific surface energy resulted in a net gain of specific surface energy in the case of the native, washed and alkali-stored, washed corn stover. This result confirms the washed sample had an increased concentration of polar groups on the surface, contributing to increases in measured specific surface energy. Similar impacts of washing on Lewis acid and base surface energies have been demonstrated previously[49]. Furthermore, these results correlate with the increased yields of glucose and xylose in enzymatic hydrolysis, likely due an increase in the number of accessible active sites for the enzyme as a function of alkali-assisted anaerobic storage. Similar correlations of corn stover surface energy and carbohydrate enzymatic have not been previously documented in the peer-reviewed literature.

Hydrophilicity

Hydrophilicity, or wettability, indicates the ability of a sample to wet and retain water, and it can be expressed quantitatively using the ratio of the specific surface energy to the total surface energy.

Higher values represent more wettable materials[51, 52]. Enhancing the wettability of lignocellulosic feedstock is desired in the context of a biorefinery as it may aid in higher rates during enzymatic or aqueous chemical processing. Figure 5.6 shows no difference in hydrophilicity in the native and alkali-stored samples. However, washing with water resulted in an increase in hydrophilicity between the native corn stover and the native, washed sample (13.25%) and to a greater extent (16.9%) in the alkali-stored, washed sample. Lignin removal is suspected to account for the increase in hydrophilicity, as lignin is predominantly hydrophobic. Previous studies assessing corn stover stored in bale form exhibited enhanced hydrophilicity as a function of combined ash and moisture content entering storage[43], as a function of harvest location[46], and as a function of preprocessing operations[42].

Work of Cohesion/Adhesion

Changes of works of cohesion and adhesion for the native and alkali-stored corn stover and as a function of water washing are represented in Figure 5.6, and these provide insight into physical flowability properties including agglomeration and bulk solids handling of corn stover[43]. Work of cohesion is calculated as double the total surface energy for a material, and it represents the work required for two solid particles to be separated. As a result of the increases in both dispersive and specific surface energies, the work of cohesion increased by 4.4% from the native to the alkali-stored corn stover. An additional 7.8% increase was observed as result of washing the alkali-stored samples. Elevated work of cohesion may lead to flowability challenges including bridging and plugging in bulk handling systems and increases in work of cohesion must be balanced with changes in the lignocellulose structure that improve wettability and enzyme access.

The work of adhesion represents how easily a liquid will wet a solid surface, where a higher work of adhesion will wet more easily. Work of adhesion was statistically equivalent in native and alkali-stored corn stover. Washing the alkali-stored sample resulted in a 6.9% increase in the work of adhesion. These results suggest the washed particles may separate into the water phase more easily during rewetting activities. One key question remains as to whether the particles would be more likely to clump together due to a higher work of cohesion or if the work of adhesion would be sufficiently strong to act as a binder between the particles to bridge that gap between particles that otherwise.

Conclusion

The approach explored in this research was to combine alkali treatment, which saponifies acetyl groups from hemicellulose as well as ester linkages between lignin and carbohydrates, with anaerobic storage to both preserve biomass during extended storage and facilitate additional depolymerization

enabled by alkali treatment. The conditions explored in this study are compatible with the unit operation and associated residence time of long-term storage that mitigates seasonal influxes of lignocellulosic crops and enables consistent supply to a biorefinery. This study correlated alkali-assisted anaerobic storage of corn stover with enhanced release of glucose and xylose during enzymatic hydrolysis as well as enhanced porosity and surface energy characteristics. Likewise, increases in hydrogen bonding sites after alkali treatment was consistent with predicted swelling and enhanced hemicellulose and cellulose conversion measured in enzymatic hydrolysis after 2 weeks of storage, resulting in calculated hydrophilicity changes. In addition, a room temperature water wash improved carbohydrate yields during enzymatic hydrolysis and was correlated with increased pore surface area, volume, and diameter. Future research should investigate the role of mild washing to not only improve downstream preprocessing and conversion, but also to capture the value of extractable carbohydrates, soluble nutrients, and reduce undesirable components including soil-borne ash. Future work should further probe the opportunities of the combined impact of alkali addition and anaerobic storage in terms in preprocessing operations including in moisture-tolerant comminution methods such as mechanical refining.

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Chapter 6: Concluding Remarks

The overarching goal of this research was to explore innovative approaches to transform storage to a value-add operation by taking advantage of the residence time to overcome the physiochemical barriers in corn stover to depolymerization. Low severity chemical and microbial treatments were explored for their ability to reduce the degree of polymerization through saponification of ester-linked side chains or glycosidic bonds in hemicellulose or through oxidation of phenolic or non-phenolic components of lignin. Screening experiments in Chapters 2 and 4 were used to identify the range of viable conditions and supported by techno-economic analysis to highlight the validity of the approach. Chapters 3 and 5 represent a fundamental study to understand the mechanisms of fungal and alkali assisted treatment within the context of anaerobic storage. The following concluding remarks will (1) frame the dissertation goals in the context of the state of technology for lignocellulosic based logistics supply chains, (2) describe the novelty of the alkali-assisted anaerobic storage based on findings in this dissertation, and (3) discuss how fungal-assisted pretreatment in storage and the approach used in this work addressed many gaps in the literature, and (4) provide next steps recommended for future investigation throughout these remarks.

State of Technology for Lignocellulosic-based Supply Chains

Deep decarbonization approaches are necessary to reverse the impact of climate change induced by the industrialization of modern society. Electrification of the grid using low carbon generation sources of wind, solar, or hydropower are a promising opportunity to reduce the carbon intensity overall and for light duty vehicles, but all efforts will fall short for aviation fuels, heavy duty vehicles, and marine vessels that all rely on liquid transportation fuels. Efforts in the bioenergy community have identified plant-based biomass as a promising feedstock for liquid transportation fuels, and when combined with wastes and algae, projected estimates of this resource exceed 1 billion tons. There are opportunities for this biomass to replace coal as a biopower source, but the simple fact is that biomass is a low-density energy source in its natural state and cannot compete economically with fossil-based alternatives such as coal. However, the selling point for biomass is that it is an incredible carbon source. In the context of deep decarbonization, biomass has the unique aspect of being able to support carbon neutral or even carbon negative approaches if combined with capture.

The United States currently produces approximately 16 billion gallons of ethanol through fermentation of corn grain. Approximately 40% of the corn grain produced in this country supports that industry, providing income to farmers across the Midwest. The logistics system that supports this

first generation (1G) ethanol conversion is well understood. Corn grain is harvested at maturity, dried to stability, transported in its natural dense and flowable format to a biorefinery year-round. At the biorefinery gate the corn is conveyed to a hammer mill for comminution, treated with amylases to easily digest the simple carbohydrates, and fermented to ethanol. Recent advances of corn fiber utilization, which is primarily arabinoxylan, are considered as 1.5 Generation (1.5G) bring some complexity to the refinery.

In the context of 1G and 1.5G ethanol biorefineries, second generation (2G) lignocellulosic based biorefineries are extremely complicated on many levels. First, the inherent complexity of lignocellulosic biomass with the interwoven cellulose microfibrils within cell walls as well as the protective hemicellulose and lignin complexes is a depolymerization challenge. Chapter 1 of this dissertation highlights this complexity on the molecular level. Second, the logistical aspects of the supply chain present practical challenges including securing biomass, timing harvests based on crop and geographical region, delays including weather, insufficient infrastructure including roads, equipment, and land for storage. Third, environmental factors including nutrient and water management and carbon retention are important to design systems that are sustainable for society. At a high level, this dissertation was focused on addressing these three high level challenges. Of note, many more challenges for the industry exist in the context of requiring robust conversions, aligning fuel produced with end users, and gaining widespread public adoption.

The concept that one billion tons of biomass will be available for bioenergy conversion overshadows the logistical challenges currently hindering the industry. The failure of the first integrated cellulosic biorefineries, which were built at a nameplate capacity to convert 350,000 tons of biomass utilized per year, highlights this challenge. The extension of the basic approach used for 1G biorefineries to lignocellulosic based, 2G refineries highlights the overestimation of the complexity on many fronts. All the integrated biorefineries struggled due to an underestimation of the complexity, variability, and inherent recalcitrance of lignocellulosic biomass and the challenges these issues posed for downstream feeding and handling, processing, and conversion. Each biorefinery relied on baled logistics systems to supply all their biomass to the refineries. Issues were apparent around degradation resulting from poor moisture management resulting in mishappen bales that could not be handled, in twine that could not be cut on the preprocessing line due to loss of bale structure, and in clogged preprocessing lines due to size reduction challenges. However, fire was one item that caused the most public contention. Each of the three integrated biorefineries suffered massive bale yard fires during 2013-2015 from causes ranging from lightning to arson. Storage stacks each consisting of over 1,000 4’x4x’8’ square bales (Chapter 1, Figure 1.6) represented a daily allotment of biomass for the

biorefinery, and large satellite storage facilities were set up within miles of the biorefineries. Once a bale within a stack ignited the entire stack would be engulfed in flames, and like wildfires the fire would jump if stacks were located to closely or if high winds carried sparks. In these scenarios, the amount of combustible material within each of these storage systems could not be extinguished manually, and fires continued to burn and then smolder for weeks until only ashes remained. Large land spacing, security cameras, and physical security led to unanticipated costs for the biorefinery.

Baled biomass storage challenges were only one issue that stalled the integrated biorefineries. Variability in biomass, challenges with creating flowable material following impact-based comminution methods, and soil-borne ash contamination all created challenges upstream of pretreatment reactors. Impact milling used for corn grain caused challenges for corn stover, where leaves where shattered, husks and other stringy material passed the grinder unimpacted and created balls of material representing bird's nests, and high moisture material in wet bales caused clogging and delays in preprocessing lines when it took longer to be size reduced compared to dry bale counterparts.

These issues bring to light a question of why baled logistics have taken such a strong prominence in lignocellulosic biorefineries. An alternative logistics approach, ensiling, is practiced across the world to preserve forage for livestock feed. Ensiling is based on forage chopping in the field for combined size reduction and collection, and manual compaction and oxygen barriers assist the microbial community to create anaerobic environments in storage structures. These structures are easily recognized across the U.S. as silos, plastic covered drive over piles, or silage tubes. Ensiled biomass can be well-preserved in a stable form for months to years, and risk of loss to fire or degradation is minimal if anaerobic conditions are maintained. Roadmaps published by the Department of Energy prior to 2010 highlight baled logistics systems could be complimented with high moisture logistics systems based on forage chopping and ensiling, but these designs were replaced with logistics systems based on bales to support the bale-centric integrated biorefinery approach. Flowability challenges were addressed by designs that added densification into stable, uniform pellets. High moisture corn stover logistics systems did not fit into that design.

The fires at the integrated biorefinery bale yards sparked new interest in more stable logistics systems. Anaerobic storage was once again investigated as a means to provide a stable, fire resistant alternative to dry bales. A logistics design centered around forage chopping and anaerobic storage in large, industrial scale piles was designed by Wendt et al. to supply a biorefinery with a size reduced, stable alternative to bale storage[1]. Not allowing the stover to touch the ground eliminated soil contamination, forage chopping in the field eliminated most issues centered around hammer milling

and provided initial size reduction, separation of particles meeting the size requirements of the biorefinery reduced the capacity requirements of second stage size reduction of larger particles. Stacking and reclaiming equipment facilitated formation of four 50,000-ton storage piles and located 500 ft of the biorefinery gate, with daily reclaim and conveyance supporting year-round operations. However, the wet storage logistics system was not cost competitive with baled logistics system under n^{th} plant cost scenarios, where it is assumed that industry has solved emerging problems supply chain problems such storage degradation and fire prevention. Key reasons are obvious. The wet logistics system struggled due to low harvest yield of agricultural resides including corn stover where 0.75 to 2 tons per acre are harvested at best. The system also struggled with low bulk density during transportation from the field to the storage location. Density of chopped biomass in forage wagon ranges from 2 to 5 pounds per cubic feet, whereas high density bales can easily reach 10-12 pounds per cubic feet. These two factors are compounded in cost models where though harvest yield necessitates long transportation distances and low bulk density in transportation further increases costs. As the industry grows, storage insurance may help equilibrate the economic difference between aerobic and anaerobic storage scenarios.

One underlying objective of this dissertation was to understand approaches to mitigate the challenges resulting from bale-based logistics systems and design a cost-competitive approach that could be compatible with forage chopping and anaerobic storage. Prior knowledge and a survey of the literature made it clear that increasing the bulk density in transportation was just one factor that needed to be addressed for the logistics systems. The Wendt et al., 2018 design required all corn stover harvested to immediately be transferred to a central location, creating a logistical challenge of needed to design multiple entry points and receiving stations for unloading trucks. Moreover, the preprocessing operation was designed to handle the annual surge of biomass in just a 3-month window, and as such it was not utilized for the entire year. Bale based logistics designs utilize field-side storage to manage the influx of material in the fall and only transport the necessary supply daily such that logistical considerations such as traffic congestion are minimized. Field-side storage also eliminate infrastructure costs for the biorefinery, where the farmer carries the risk of feedstock loss not the biorefinery. This decentralization approach can also help support concepts such as on farm utilization of wet resources, for example anaerobic digestion to support bio-gas production. Additionally, such concepts can be most useful for valorizing materials not meeting the quality specification of biorefineries.

A significant gap in the industry that still will need to be addressed is how to compensate farmers fairly for the service they provide of decentralizing risk and infrastructure requirements at the

biorefinery. Regardless, Chapter 4 in this dissertation explored an approach to utilize both on-farm storage while maintaining bulk density in storage. Silage tubes were used as the storage mechanism for corn stover, stored field-side, and daily transportation of intact tubes to the biorefinery was used to distribute costs evenly on an annual basis. This approach was shown to be cost competitive with the baled logistics approach at the transportation distance modeled. This represents a significant gap filled in the feedstock logistics and supply chain area, demonstrating how high-moisture storage systems could be economically viable.

Defining the Opportunity Space for Alkali-assisted Storage

One issue that the high moisture anaerobic storage approach described above does not address is recalcitrance in the corn stover. It is well-known that lignocellulosic biomass is a challenge to depolymerize, and a survey of the pretreatment approaches being explored is presented in Chapter 1. Only a few studies have looked to the residence time in long-term storage to address this recalcitrance[2-4]. The forage chopped anaerobic storage approach for corn stover was assessed for recalcitrance reduction at high severity conditions[3], and no significant differences were shown at these levels. To understand the potential for anaerobic storage to reduce recalcitrance, low severity conditions are needed to observe measurable changes. This is consistent with the overall goal of reducing severity levels required to depolymerize biomass. The research in this dissertation explored how one might utilize anaerobic storage in the context of current biochemical approaches to pre-treatments and fermentation. Alkali pretreatment is utilized in designs supported by the Department of Energy[5], and it is favorable due to the potential reduction of fermentation inhibitors created as well as the ability to isolate a lignin stream that could be upgraded to co-products. An innovative approach was explored in the dissertation where alkali was partially impregnated into the corn stover prior to anaerobic storage to improve chemical impregnation rates as well as deacetylation such that it could begin to attack the physiochemical barriers in corn stover during the residence time of storage.

Alkali treatment prior to entering long-term storage of one month was explored in Chapter 4 to understand the working envelope this approach may have within the context of a supply chain. The approach explored applying alkali prior to storage to couple the saponification of acetyl groups from hemicellulose and ester linkages between lignin and hemicellulose with an increased reaction residence time possible in storage. Alkali levels, moisture content, and anaerobic or aerobic storage conditions highlighted the changes due to treatment in terms of losses of total matter and change in extract of all components. Anaerobic conditions were shown to improve alkali treatments and maintain losses below 4%, whereas aerobic storage conditions resulted in losses exceeding 10%. The highest alkali loading in anaerobically stored corn stover solubilized up to 15% lignin, 18% xylan,

and 50% of acetate meanwhile doubling the extractable components. This research paved the approach utilized in Chapter 5 to further understand the mechanism of this reaction in the context of anaerobic storage. However, findings in Chapter 4 also point to future research approaches. Scanning electron microscope images (Figure 4.2) highlight that most alkali induced changes are not physically identifiable to the eye, although slight dislocation of the vascular bundle in alkali-treated corn stover stored at 40% moisture was visualized. Ongoing research efforts should investigate approaches to map physical and chemical changes simultaneously, and Ramen microspectroscopy is one approach to accomplish this. Raman Spectroscopy coupled with microscopy has been shown to reveal chemical changes in middle lamella and primary and secondary cell walls in woody biomass[6]. It is likely that the loss of lignin, xylan, and potentially acetate that was measured in gross chemical composition analysis of the alkali-treated corn stover could be linked to specific cell walls and cell layers using Raman approaches, and this should be investigated in future work.

Additionally, future treatments should consider the role of the middle lamella of cells as an approach to further target recalcitrance reduction. This layer is rich in lignin and pectin and connects adjacent plant cell walls. Pectin has generally been overlooked as a research target in the biorefining process because of its low concentrations. However, it is one of the key chemical components that hold biomass cells together and is an important consideration in mechanical preprocessing of biomass. Pectin is a 1,4-linked galacturonic acid-based polysaccharide that is concentrated in the middle lamella and primary cell wall of lignocellulosic biomass. Pectin forms covalent bonds with hemicellulose and increases the strength of the cell wall. Future efforts should consider how targeted pectin degradation can improve mechanical and chemical preprocessing. Screening studies such as those presented in Chapter 4 would be an approach for defining the working envelope of pectin degradation in the context of the residence time of long-term storage.

Alkali treatments and anaerobic storage was further explored to characterize additional impacts on a molecular and physical scale. 20 L anaerobic reactors allowed for a higher fidelity storage study to verify that low dry matter loss was possible with this approach, meanwhile the production of large quantities of alkali-stored corn stover enabled substantial research exploration using multiple approaches. Traditional approaches to assessing recalcitrance reduction include methods such as gross compositional analysis of macromolecules, x-ray diffraction to assess crystallinity impacts, and enzymatic hydrolysis to assess conversion potential. While these methods were assessed in this dissertation, they fall short of allowing researchers to understand the full impact of alkali treatment and long-term storage to impact the physiochemical barriers in lignocellulosic biomass. A novel approach of coupling enzymatic hydrolysis testing results with surface area and surface volume

measurements was key to uncovering a relationship with high moisture conditions to pore volume changes and enhanced carbohydrate monomerization in the presence of enzymes. Surface energy treatments also highlighted additional binding sites for probes highlighting the small chemical reactions that had been facilitated by the alkali-assisted storage approach. However, assessments of Lewis basic sites that soluble compounds on the pore surface were inhibiting probe blockage. A water wash was shown to release these contaminants and restore surface energy in both the native and unstored sample. Even the unstored sample improved from the mild water washing of one hour and similarly enzymatic hydrolysis yields improved even as a function of washing. This finding is a great significance to the bioenergy industry, where the role of washing biomass is not generally considered. However, washing can reduce soil contamination, remove any inhibitors or soluble nutrients that could sent to the field, or capture and valorize co-products produced during anaerobic storage.

Anaerobic storage has been explored previously to create valuable co-products in a high solids low-cost operation. Henk explored the conversion of soluble sugars into ethanol in a sweet sorghum showing that up to 10 w/w% ethanol could be produced by yeast in anaerobic storage[7]. Similar results have been reported in freshly cut grasses, energy cane, and in sweet sorghum[8-10]. This approach was explored previously in corn stover (unpublished data by Wendt et al., 2007); however; high yield of ethanol was not able to be reproduced in corn stover due to the low starting soluble carbohydrate content. However, the approach still has validity in crops that enter storage with sufficient soluble sugars. Shell has invested in this approach extensively with multiple patents in the space. Additional products that could be considered for valorization include lactic acid, which is a common fermentation product and a precursor to polylactic acid-based plastics. Succinic acid is also an important commodity chemical that has been shown to be produced in anaerobic storage, although the high concentrations (greater than 12%) produced during anaerobic storage of algae are necessary to improve economics[11].

One impressive finding resulting from the alkali-assisted anaerobic storage study in Chapter 5 points the direction for further exploration. Analytical pyrolysis at 500°C coupled with detection of thermalized compounds with two-dimensional gas chromatography and mass spectrometry identified production of 2,3-butanediol, a value-added compound that has received extensive attention as a potential product from lignocellulosic biomass[12-14]. The production of 2,3-butanediol in the alkali-stored corn stover compared to the native sample and the enhancement of this molecule after storage is of notable interest (Figure 6.1). 2,3-butanediol elutes at 475 and at 487 s, which is accounted for by the fact that there are multiple stereoisomers with the elution order S,S ≈ R,R < R,S)[15]. Two possible explanations of this production are possible, one is that it was produced during the alkali-anaerobic

storage operation. The other is that the alkali treatment partially hydrolyzed cellulose and hemicellulose polymers that then resulted in enhanced 2,3-butanediol production during pyrolysis. 2,3-butanediol production in pyrolysis bio-oil has shown to be enhanced at 450 °C[16]. Further investigation of the mechanism of production of this of 2,3-butanediol will be the focus of future research efforts.

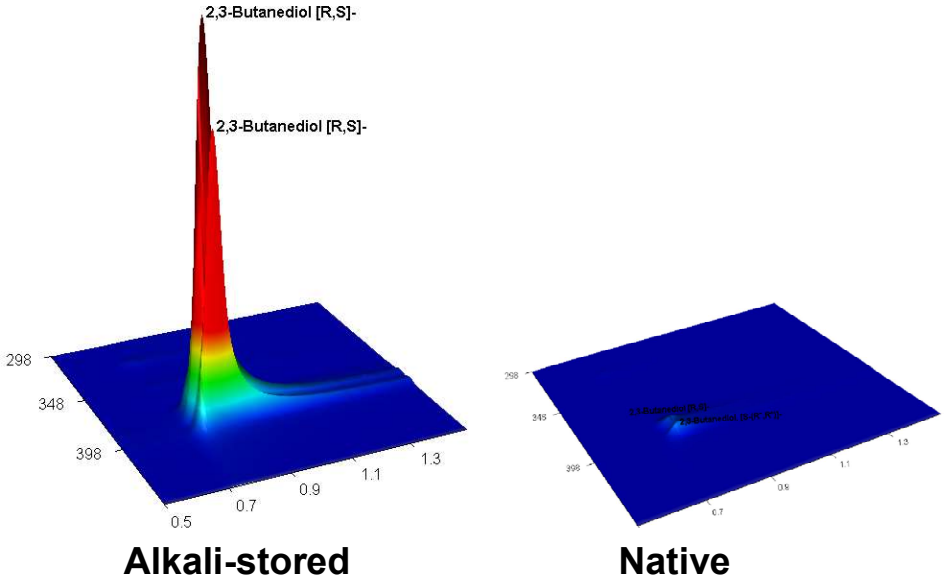

Figure 6.1. 3D extracted ion chromatogram generated from pyrolysis/GCxGC/MS at 500°C. Peak shown represents m/z 45, which correlates to 2,3-butanediol and was confirmed using a pure sample of 2,3-butanediol. Left: alkali-stored corn stover; right: native corn stover.

Fungal Pretreatment R&D requirements

Fungal treatment was explored as an additional approach to reducing corn stover recalcitrance in Chapter 2 and 3 of this dissertation. There is a vast body of literature that shows the validity of fungal pretreatment approaches and review articles summarizing it[17, 18], but one thing that is not apparent is how when might work this into a working logistics system and in the context of a functional by refinery. This research explored a fungal assisted pretreatment approach that could function at the biorefinery and be cost competitive. An experimental screening approach was used to understand the range of viable conditions for such a design. Storage duration ended up being one of the crucial contributors in the system. Where long residence times are possible in anaerobic storage, the results in Chapter 2 showed that excessive time was detrimental to quality. Therefore, a queuing system was

designed that could utilize short term storage at the biorefinery gate to reduce recalcitrance through fungal inoculation. Techno-economic analysis verified that, when losses exceeded 3% of total dry matter, the system was not cost-competitive despite considering slight reductions in thermal pretreatment processing requirements facilitated by fungal pretreatment. Further studies are warranted to investigate the alternative approaches such as combined fungal enzymatic treatment, new strains, or different pretreatment approaches for corn stover for other bioenergy crops. Quality factors for further investigation should be expanded to incoming biomass attributes including free sugars, crop standing age, and structural features including inherent lignin and carbohydrate distributions.

A surprising finding in surveying the fungal pretreatment literature was that total degradation is reported only in a few cases, which is significant gap in the literature given the importance of delivering biomass to a refinery with low losses. Real time fungal degradation profiles were explored in Chapter 3 to provide a quantitative measure of the impact of dry matter loss on compositional changes. One interesting finding was that the fungal inoculation inhibited dry matter loss in the first five days of storage compared to a corn stover sample without inoculation, highlighting that the inoculated fungi initially impacted the microbial community of indigenous bacteria, yeasts, and fungi. Additional research should consider metagenomic analysis to further understand these population dynamics on best to take advantage of them to reduce recalcitrance.

Additionally, the corn stover collected from the fungal inoculated corn stover after 1 and 2 weeks were assessed for impact using the traditional approaches of gross compositional changes, changes in carbohydrates released during enzymatic hydrolysis, x-ray diffraction. These methods are often reported to show the impact of fungal treatment on biomass recalcitrance. Of note were x-ray diffraction patterns, which have been used widely to assess changes in crystalline versus amorphous cellulose due to fungal pretreatment. Diffraction patterns comparing native and stored corn stover show no clear indication of the changes in crystalline versus amorphous states of the cellulose reported elsewhere, leading to the conclusion that x-ray diffraction is poorly suited to look at changes in biomass that might be observed with low severity treatments such as any of those used in fungal treatment. Similarly, no changes in crystallinity were observed in this study using nuclear magnetic resonance. While changes in crystallinity could be expected in treatments that completely isolate cellulose, such as in nanocellulose, its utilization as an indicator of microstructure changes in lignocellulose is highly overutilized in the field. It is recommended that the field abandon this method for analyzing lignocellulosic biomass unless methods for assessing soil contamination are required. X-ray diffraction is an excellent approach for analyzing silica content in corn stover exhibiting contamination of soil, and the results highlight this.

One of the key novelties of the study presented in Chapter 3 was that the fungal treatment was combined with a dilute acid approach to convert the entire carbohydrate fraction to fermentable sugars, and it was shown that fungal treatment impacted hemicellulose such that a less severe pretreatment condition could be utilized. Analysis of macromolecular changes in hemicellulose and lignin by analytical pyrolysis confirmed this was the case, and this method has never been applied to fungal pretreated samples. Combining these approaches with alternative fungal pretreatment approaches aimed at lower total degradation is recommended for future efforts such that the impacts on macromolecular structure can be rapidly predicted, thus driving the pace of research. Additionally, utilizing fungal pretreatment for only the most recalcitrant fractions of biomass to reduce the pretreatment severity has promise. Physical fractionation, such as rind and pith in corn stover stalk, can help isolate recalcitrant fractions such as the rind. Similar approaches are used to isolate hemp fibers. Targeted fungal pretreatment of this material could balance the total dry matter loss experienced in this study with only the loss of a fraction of biomass.

Summary

The broader impact of this research is to design more efficient and sustainable biomass conversion systems that integrate feedstock supply and logistics and conversion operations to improve overall economics and sustainability. While reducing biomass recalcitrance in the supply chain has been documented in the literature, it has not been integrated into existing commercial feedstock supply chain logistics scenarios to date, and this research provides the knowledge base to support that necessary link. By understanding the biological, chemical, and physical changes that can occur during long-term storage and queuing operations, realization of how storage systems can be used to not only preserve the carbon in biomass but also how they can add value to biomass through recalcitrance reduction. The shift in cost-centered biomass storage systems to value-add systems represents a novel approach that could positively impact all unit operations in the biomass feedstock logistics supply chain and ultimately the conversion to fuels and chemicals.

Future investment in this research is critical to advancing the state of technology for feedstock supply scenarios to enable industry to use biomass effectively and safely for fuel production. Successful development of robust logistics systems for bioenergy not only ensures that the United States will maintain leadership in biofuel development but also could mobilize the existing forage industry across the rural landscape. The research questions addressed in this dissertation and in follow-on studies will be used to facilitate additional industrial interest and expanded utilization of preprocessing in feedstock logistics systems.

The research proposed embodies the intent of the Environmental Science Program at University of Idaho by combining knowledge of biological and chemical impacts to biomass during storage to understand the mechanisms of biomass recalcitrance reduction. Relevant biological science specialties include both microbiology and biochemistry. Physical science is represented by the inclusion of analytical chemistry as well as engineering approaches and techno-economic analysis. Social science aspects will include incorporation of sustainability of the proposed systems in relation to the overall bioeconomy.

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Appendix A - Chapter 2: Fungal depolymerization of corn stover in bioenergy queuing operations to reduce pretreatment severity

Fungal strain screening to compare a selective and non-selective lignin degrader

Visual and compositional changes observed in the fungal screening study are reported in Figures A1, A2, and A3 below along with Tables A1-A4.

Figure A.2. Corn stover with no inoculum after 2 weeks of storage. 40% moisture content samples are on the left, and 60% moisture content samples are on the right.

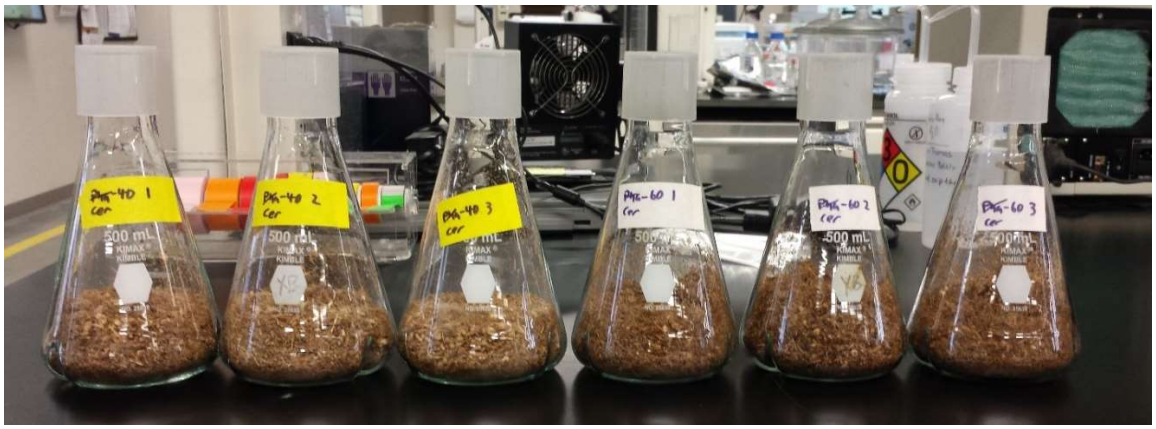

Figure A.3. Corn stover inoculated with *C. subvermispora* after 2 weeks of storage. 40% moisture content samples are on the left, and 60% moisture content samples are on the right.

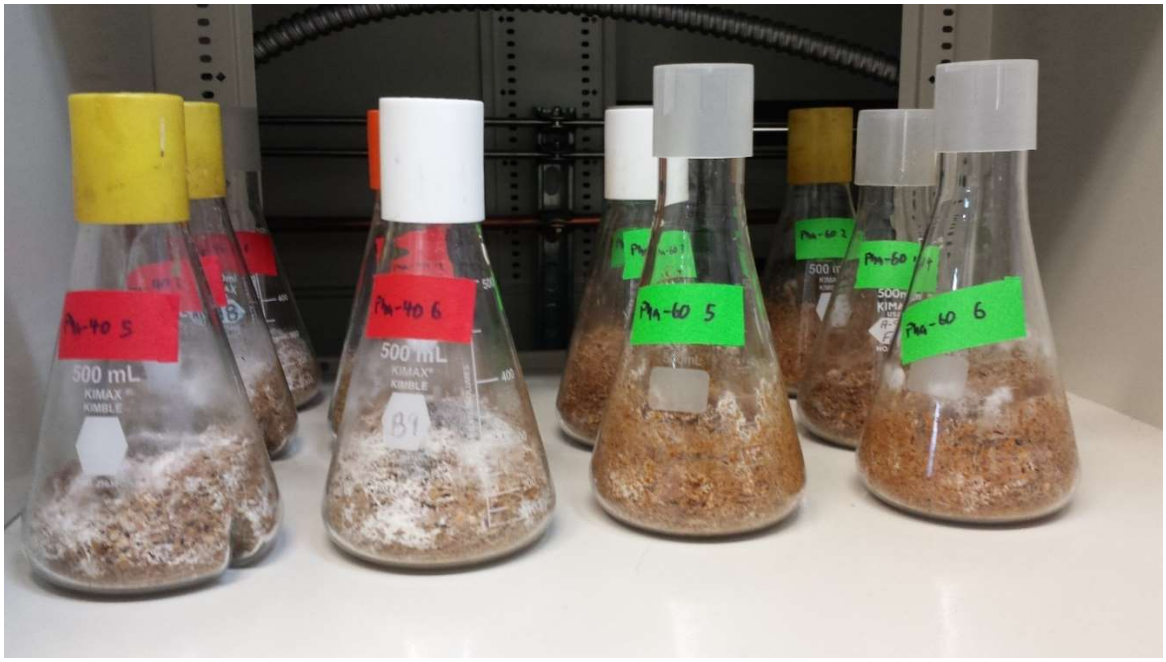

Figure A.4. Corn stover inoculated with *P. chrysosporium* after 2 weeks of storage. 40% moisture content samples are on the left, and 60% moisture content samples are on the right.

Table A.1. Distribution and composition of extractives in the screening study for fungal-treated corn stover after storage for 2 or 4 weeks (Top). Percent change in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom). Dry matter loss for the control sample at 40% moisture stored for 2 weeks was negligible and as such was excluded from comparative analysis.

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	8.08	0.52	0.35	0.35	0.19	4.06	2.24	10.32
Control, 40% moisture, 2 wk	NA	7.78	0.47	0.44	0.24	0.24	3.64	2.30	10.08
Control, 40% moisture, 4 wk	0.1±0.2	8.34	0.53	0.49	0.26	0.36	5.14	2.31	10.65
Control, 60% moisture, 2 wk	2.7±2.4	8.95	0.56	0.53	0.27	0.31	5.23	2.29	11.24
Control, 60% moisture, 4 wk	1.4±0.1	8.64	0.53	0.51	0.30	0.27	5.26	2.31	10.95
C. subvermispora, 40% moisture, 2 wk	0.5±0.3	7.70	0.64	0.49	0.28	0.28	3.62	2.28	9.98
C. subvermispora, 40% moisture, 4 wk	1.2±0.4	7.46	0.38	0.45	0.31	0.23	4.24	2.19	9.65
C. subvermispora, 60% moisture, 2 wk	3.3±1.6	7.86	0.67	0.52	0.29	0.29	3.62	2.25	10.11
C. subvermispora, 60% moisture, 4 wk	2.6±1.7	8.80	0.47	0.54	0.38	0.27	4.71	1.93	10.73
P. chrysosporium, 40% moisture, 2 wk	4.6±0.7	8.82	0.60	0.68	0.30	0.39	4.83	2.05	10.87
P. chrysosporium, 40% moisture, 4 wk	11.9±0.6	12.94	0.45	1.25	0.52	0.60	8.10	2.13	15.08
P. chrysosporium, 60% moisture, 2 wk	10.7±1.0	10.56	0.62	1.00	0.38	0.52	5.90	1.80	12.36
P. chrysosporium, 60% moisture, 4 wk	21.0±0.1	16.73	0.58	1.85	0.70	0.87	10.31	1.81	18.54

NA: Dry matter loss for this treatment was negligible

	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%EtOH Extractives	%Total Extractives
Dry, unstored	-	-	-	-	-	-	-	-
Control, 40% moisture, 2 wk	-	-	-	-	-	-	-	-
Control, 40% moisture, 4 wk	-3%	-2%	-38%	25%	-90%	-27%	-3%	-3%
Control, 60% moisture, 2 wk	-11%	-9%	-51%	23%	-67%	-29%	-2%	-9%
Control, 60% moisture, 4 wk	-7%	-2%	-45%	15%	-42%	-29%	-3%	-6%
C. subvermispora, 40% moisture, 2 wk	5%	-25%	-39%	20%	-47%	11%	-2%	3%
C. subvermispora, 40% moisture, 4 wk	8%	26%	-28%	14%	-21%	-4%	2%	7%
C. subvermispora, 60% moisture, 2 wk	3%	-30%	-46%	18%	-56%	11%	-1%	2%
C. subvermispora, 60% moisture, 4 wk	-9%	9%	-52%	-8%	-46%	-16%	14%	-4%
P. chrysosporium, 40% moisture, 2 wk	-9%	-16%	-91%	15%	-107%	-19%	8%	-5%
P. chrysosporium, 40% moisture, 4 wk	-60%	14%	-252%	-46%	-217%	-99%	5%	-46%
P. chrysosporium, 60% moisture, 2 wk	-31%	-21%	-183%	-7%	-178%	-45%	20%	-20%
P. chrysosporium, 60% moisture, 4 wk	-107%	-12%	-424%	-97%	-364%	-154%	19%	-80%

Table A.2. Composition of structural features in the screening study for fungal-treated corn stover after storage for 2 or 4 weeks (Top). Percent change in structural changes in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom). Dry matter loss for the control sample at 40% moisture stored for 2 weeks was negligible and as such was excluded from comparative analysis.

	% Dry Matter Loss	% Ash	% Protein	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate	% Mass Closure
Dry, unstored	-	5.26	3.17	16.14	1.55	35.61	23.58	1.14	2.55	1.77	97.37
Control, 40% moisture, 2 wk	NA	4.20	2.86	16.47	1.53	35.69	24.69	0.97	2.46	1.97	97.20
Control, 40% moisture, 4 wk	0.1±0.2	3.91	2.71	16.21	1.52	35.33	23.39	1.02	2.26	1.79	96.21
Control, 60% moisture, 2 wk	2.7±2.4	4.58	3.16	16.09	1.48	35.05	22.75	1.39	2.33	1.70	96.34
Control, 60% moisture, 4 wk	1.4±0.1	4.12	2.77	16.01	1.52	36.31	23.26	0.98	2.16	1.78	97.13
C. subvermispora, 40% moisture, 2 wk	0.5±0.3	4.58	2.17	16.91	1.45	36.15	22.54	1.30	2.90	2.39	97.97
C. subvermispora, 40% moisture, 4 wk	1.2±0.4	4.82	1.84	16.75	1.48	35.89	23.17	1.00	2.97	1.88	97.60
C. subvermispora, 60% moisture, 2 wk	3.3±1.6	4.47	1.70	16.14	1.46	35.98	22.37	1.17	2.81	2.58	96.32
C. subvermispora, 60% moisture, 4 wk	2.6±1.7	5.65	1.68	17.74	1.41	35.18	22.13	1.40	3.06	1.79	98.36
P. chrysosporium, 40% moisture, 2 wk	4.6±0.7	4.92	2.45	16.38	1.41	35.01	21.80	1.24	2.64	2.53	97.23
P. chrysosporium, 40% moisture, 4 wk	11.9±0.6	5.79	2.03	16.66	1.32	34.00	21.10	1.20	2.62	1.81	99.57
P. chrysosporium, 60% moisture, 2 wk	10.7±1.0	4.83	1.95	16.71	1.45	32.41	23.36	1.10	2.51	2.75	97.30
P. chrysosporium, 60% moisture, 4 wk	21.0±0.1	5.54	2.20	15.42	1.42	31.08	21.18	0.91	2.36	1.86	98.10

NA: Dry matter loss for this treatment was negligible

	%Protein	%Lignin (Acid Insoluble)	%Lignin (Acid Soluble)	%Glucan	%Xylan	%Galactan	%Arabinan	%Acetate
Dry, unstored	-	-	-	-	-	-	-	-
Control, 40% moisture, 2 wk	-	-	-	-	-	-	-	-
Control, 40% moisture, 4 wk	15%	0%	2%	1%	1%	11%	11%	-1%
Control, 60% moisture, 2 wk	1%	0%	5%	2%	4%	-22%	9%	4%
Control, 60% moisture, 4 wk	13%	1%	2%	-2%	1%	14%	15%	-1%
C. subvermispora, 40% moisture, 2 wk	32%	-5%	7%	-1%	4%	-14%	-14%	-35%
C. subvermispora, 40% moisture, 4 wk	42%	-4%	5%	-1%	2%	12%	-17%	-6%
C. subvermispora, 60% moisture, 2 wk	46%	0%	6%	-1%	5%	-3%	-10%	-46%
C. subvermispora, 60% moisture, 4 wk	47%	-10%	9%	1%	6%	-23%	-20%	-1%
P. chrysosporium, 40% moisture, 2 wk	23%	-1%	9%	2%	8%	-9%	-4%	-43%
P. chrysosporium, 40% moisture, 4 wk	36%	-3%	15%	5%	11%	-6%	-3%	-2%
P. chrysosporium, 60% moisture, 2 wk	38%	-4%	7%	9%	1%	4%	2%	-56%
P. chrysosporium, 60% moisture, 4 wk	31%	4%	9%	13%	10%	20%	7%	-5%

Table A.3. Dry matter loss weighted distribution and composition of extractives in the screening study for fungal-treated corn stover after storage for 2 or 4 weeks (Top). Percent change in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom). Dry matter loss in the control sample at 40% moisture stored for 2 weeks was negligible and was excluded from comparative analysis.

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	8.08	0.52	0.35	0.35	0.19	4.06	2.24	10.32
Control, 40% moisture, 2 wk	NA	7.78	0.47	0.44	0.24	0.24	3.64	2.30	10.08
Control, 40% moisture, 4 wk	0.1±0.2	8.33	0.53	0.49	0.26	0.36	5.14	2.31	10.65
Control, 60% moisture, 2 wk	2.7±2.4	8.71	0.55	0.52	0.27	0.31	5.09	2.23	10.94
Control, 60% moisture, 4 wk	1.4±0.1	8.52	0.52	0.51	0.30	0.26	5.19	2.28	10.80
C. subvermispora, 40% moisture, 2 wk	0.5±0.3	7.66	0.64	0.49	0.28	0.27	3.60	2.27	9.93
C. subvermispora, 40% moisture, 4 wk	1.2±0.4	7.37	0.38	0.45	0.30	0.23	4.19	2.16	9.53
C. subvermispora, 60% moisture, 2 wk	3.3±1.6	7.60	0.65	0.50	0.28	0.28	3.50	2.18	9.78
C. subvermispora, 60% moisture, 4 wk	2.6±1.7	8.57	0.46	0.52	0.37	0.27	4.59	1.88	10.45
P. chrysosporium, 40% moisture, 2 wk	4.6±0.7	8.41	0.57	0.64	0.29	0.37	4.61	1.96	10.37
P. chrysosporium, 40% moisture, 4 wk	11.9±0.6	11.40	0.39	1.10	0.46	0.52	7.13	1.88	13.28
P. chrysosporium, 60% moisture, 2 wk	10.7±1.0	9.43	0.56	0.90	0.34	0.47	5.27	1.61	11.04
P. chrysosporium, 60% moisture, 4 wk	21.0±0.1	13.21	0.46	1.46	0.55	0.69	8.14	1.43	14.64

NA: Dry matter loss for this treatment was negligible

	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%EtOH Extractives	%Total Extractives
Dry, unstored	-	-	-	-	-	-	-	-
Control, 40% moisture, 2 wk	-	-	-	-	-	-	-	-
Control, 40% moisture, 4 wk	-3%	-2%	-38%	25%	-90%	-26%	-3%	-3%
Control, 60% moisture, 2 wk	-8%	-6%	-47%	25%	-63%	-25%	1%	-6%
Control, 60% moisture, 4 wk	-5%	-1%	-43%	16%	-40%	-28%	-2%	-5%
C. subvermispora, 40% moisture, 2 wk	5%	-24%	-38%	20%	-46%	11%	-1%	4%
C. subvermispora, 40% moisture, 4 wk	9%	26%	-26%	15%	-20%	-3%	4%	8%
C. subvermispora, 60% moisture, 2 wk	6%	-26%	-41%	21%	-51%	14%	3%	5%
C. subvermispora, 60% moisture, 4 wk	-6%	12%	-48%	-5%	-42%	-13%	16%	-1%
P. chrysosporium, 40% moisture, 2 wk	-4%	-11%	-82%	19%	-98%	-13%	13%	0%
P. chrysosporium, 40% moisture, 4 wk	-41%	24%	-210%	-29%	-179%	-75%	16%	-29%
P. chrysosporium, 60% moisture, 2 wk	-17%	-8%	-153%	5%	-149%	-30%	28%	-7%
P. chrysosporium, 60% moisture, 4 wk	-64%	11%	-314%	-56%	-266%	-100%	36%	-42%

Table A.4. Dry matter loss weighted composition of structural features in fungal-treated corn stover after storage for 2 or 4 weeks (Top). Percent change in structural changes in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom). Dry matter loss for the control sample at 40% moisture stored for 2 weeks was negligible and as such was excluded from comparative analysis.

	% Dry Matter Loss	% Ash	% Protein	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate
Dry, unstored	-	5.26	3.17	16.14	1.55	35.61	23.58	1.14	2.55	1.77
Control, 40% moisture, 2 wk	NA	4.20	2.86	16.47	1.53	35.69	24.69	0.97	2.46	1.97
Control, 40% moisture, 4 wk	0.1±0.2	3.91	2.71	16.20	1.52	35.31	23.38	1.02	2.26	1.79
Control, 60% moisture, 2 wk	2.7±2.4	4.46	3.07	15.66	1.44	34.11	22.14	1.35	2.27	1.65
Control, 60% moisture, 4 wk	1.4±0.1	4.06	2.74	15.79	1.50	35.82	22.94	0.97	2.13	1.76
C. subvermispora, 40% moisture, 2 wk	0.5±0.3	4.56	2.15	16.82	1.44	35.96	22.42	1.29	2.88	2.38
C. subvermispora, 40% moisture, 4 wk	1.2±0.4	4.77	1.82	16.55	1.46	35.47	22.90	0.99	2.94	1.85
C. subvermispora, 60% moisture, 2 wk	3.3±1.6	4.32	1.64	15.61	1.41	34.79	21.63	1.13	2.71	2.50
C. subvermispora, 60% moisture, 4 wk	2.6±1.7	5.50	1.64	17.27	1.38	34.26	21.55	1.37	2.98	1.74
P. chrysosporium, 40% moisture, 2 wk	4.6±0.7	4.69	2.34	15.63	1.35	33.40	20.80	1.19	2.52	2.41
P. chrysosporium, 40% moisture, 4 wk	11.9±0.6	5.10	1.79	14.67	1.16	29.94	18.58	1.06	2.31	1.59
P. chrysosporium, 60% moisture, 2 wk	10.7±1.0	4.31	1.74	14.92	1.29	28.94	20.85	0.98	2.24	2.46
P. chrysosporium, 60% moisture, 4 wk	21.0±0.1	4.38	1.74	12.17	1.12	24.54	16.72	0.72	1.87	1.47

NA: Dry matter loss for this treatment was negligible

	%Protein	%Lignin (Acid Insoluble)	%Lignin (Acid Soluble)	%Glucan	%Xylan	%Galactan	%Arabinan	%Acetate
Dry, unstored	-	-	-	-	-	-	-	-
Control, 40% moisture, 2 wk	-	-	-	-	-	-	-	-
Control, 40% moisture, 4 wk	15%	0%	2%	0.9%	1%	11%	11%	-1%
Control, 60% moisture, 2 wk	3%	3%	7%	4.2%	6%	-19%	11%	6%
Control, 60% moisture, 4 wk	14%	2%	3%	-0.6%	3%	15%	16%	1%
C. subvermispora, 40% moisture, 2 wk	32%	-4%	7%	-1.0%	5%	-14%	-13%	-34%
C. subvermispora, 40% moisture, 4 wk	43%	-3%	6%	0.4%	3%	13%	-15%	-5%
C. subvermispora, 60% moisture, 2 wk	48%	3%	9%	2.3%	8%	0%	-6%	-41%
C. subvermispora, 60% moisture, 4 wk	48%	-7%	11%	3.8%	9%	-20%	-17%	1%
P. chrysosporium, 40% moisture, 2 wk	26%	3%	13%	6%	12%	-4%	1%	-36%
P. chrysosporium, 40% moisture, 4 wk	44%	9%	25%	16%	21%	7%	9%	10%
P. chrysosporium, 60% moisture, 2 wk	45%	8%	17%	19%	12%	14%	12%	-39%
P. chrysosporium, 60% moisture, 4 wk	45%	25%	28%	31%	29%	37%	27%	17%

Appendix B - Chapter 3: Molecular and structural impacts of fungal depolymerization of corn stover to reduce pretreatment severity

Appendix B contains four subsections, each with methods and results, as follows.

Fungal strain screening to compare a selective and non-selective lignin degrader

Methods

Compositional analysis methods are reported within the manuscript.

Results

Compositional changes observed in the fungal screening study are reported in Tables B.1-B.4.

Table B.1. Distribution and composition of extractives in aerated bioreactors for control and fungal-treated corn stover after storage for 1 or 2 weeks (Top). Percent change in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom).

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	9.55	1.96	0.48	0.25	0.16	3.68	3.52	13.07
Control, Reactor 1, 1 wk	9.20	9.33	0.64	0.35	0.26	0.17	5.14	3.05	12.38
Control, Reactor 1, 2 wks	14.47	7.85	0.27	0.31	0.28	0.55	1.85	2.48	10.33
Control, Reactor 2, 1 wk	7.62	8.55	0.64	0.31	0.25	0.18	4.84	3.03	11.58
Control, Reactor 2, 2 wks	12.13	7.52	0.29	0.24	0.26	0.55	3.85	2.55	10.07
P. chrysosporium, Reactor 3, 1 wk	4.38	9.79	0.67	0.29	0.27	0.18	5.50	3.16	12.96
P. chrysosporium, Reactor 3, 2 wks	13.92	11.01	0.30	0.43	0.76	0.23	6.34	2.56	13.57
P. chrysosporium, Reactor 4, 1 wk	2.87	9.33	0.59	0.29	0.25	0.17	4.99	3.26	12.59
P. chrysosporium, Reactor 4, 2 wks	7.22	11.11	0.48	0.62	0.60	0.28	6.01	2.58	13.69

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	-	-	-	-	-	-	-	-
Control, Reactor 1, 1 wk		2%	67%	27%	-4%	-6%	-39%	13%	5%
Control, Reactor 1, 2 wks		18%	86%	36%	-12%	-239%	50%	30%	21%
Control, Reactor 2, 1 wk		10%	67%	36%	1%	-14%	-31%	14%	11%
Control, Reactor 2, 2 wks		21%	85%	50%	-6%	-243%	-5%	28%	23%
P. chrysosporium, Reactor 3, 1 wk		-3%	66%	39%	-8%	-13%	-49%	10%	1%
P. chrysosporium, Reactor 3, 2 wks		-15%	85%	11%	-203%	-45%	-72%	27%	-4%
P. chrysosporium, Reactor 4, 1 wk		2%	70%	40%	0%	-8%	-35%	7%	4%
P. chrysosporium, Reactor 4, 2 wks		-16%	75%	-30%	-140%	-72%	-63%	27%	-5%

Table B.2. Distribution and composition of structural components in aerated bioreactors for control and fungal-treated corn stover after storage for 1 or 2 weeks (Top). Percent change in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom).

	% Dry Matter Loss	% Ash	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate	% Mass Closure
Dry, unstored	-	5.37	16.80	1.35	35.07	20.94	1.15	3.04	3.56	98.90
Control, Reactor 1, 1 wk	9.20	5.18	18.45	1.46	33.42	22.37	1.31	2.78	2.53	97.11
Control, Reactor 1, 2 wks	14.47	6.12	18.84	1.35	35.63	21.65	1.34	2.79	1.04	97.44
Control, Reactor 2, 1 wk	7.62	5.33	17.70	1.37	34.82	21.33	1.28	2.69	2.28	96.04
Control, Reactor 2, 2 wks	12.13	5.44	18.71	1.38	35.73	21.82	1.41	2.90	1.29	96.42
P. chrysosporium, Reactor 3, 1 wk	4.38	4.99	16.69	1.40	33.55	21.10	1.19	3.39	3.34	98.25
P. chrysosporium, Reactor 3, 2 wks	13.92	6.67	18.73	1.22	33.77	19.53	1.18	3.00	2.10	96.83
P. chrysosporium, Reactor 4, 1 wk	2.87	5.42	18.19	1.42	33.61	21.98	1.26	2.70	2.57	96.69
P. chrysosporium, Reactor 4, 2 wks	7.22	6.43	19.33	1.25	32.63	20.58	1.26	2.51	1.41	95.99

	% Dry Matter Loss	% Ash	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate
Dry, unstored	-	-	-	-	-	-	-	-	-
Control, Reactor 1, 1 wk		3%	-10%	-8%	5%	-7%	-13%	9%	29%
Control, Reactor 1, 2 wks		-14%	-12%	0%	-2%	-3%	-17%	8%	71%
Control, Reactor 2, 1 wk		1%	-5%	-1%	1%	-2%	-11%	12%	36%
Control, Reactor 2, 2 wks		-1%	-11%	-2%	-2%	-4%	-22%	5%	64%
P. chrysosporium, Reactor 3, 1 wk		7%	1%	-3%	4%	-1%	-3%	-11%	6%
P. chrysosporium, Reactor 3, 2 wks		-24%	-12%	9%	4%	7%	-3%	2%	41%
P. chrysosporium, Reactor 4, 1 wk		-1%	-8%	-5%	4%	-5%	-9%	11%	28%
P. chrysosporium, Reactor 4, 2 wks		-20%	-15%	7%	7%	2%	-9%	17%	60%

Table B.3. Dry matter loss weighted distribution and composition of extractives in aerated bioreactors for control and fungal-treated corn stover after storage for 1 or 2 weeks (Top). Percent change in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom).

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	9.55	1.96	0.48	0.25	0.16	3.68	3.52	13.07
Control, Reactor 1, 1 wk	9.20	8.47	0.58	0.32	0.24	0.16	4.67	2.77	11.24
Control, Reactor 1, 2 wks	14.47	6.72	0.23	0.26	0.24	0.47	1.58	2.12	8.84
Control, Reactor 2, 1 wk	7.62	7.90	0.59	0.28	0.23	0.17	4.47	2.80	10.70
Control, Reactor 2, 2 wks	12.13	6.61	0.26	0.21	0.23	0.49	3.38	2.24	8.85
P. chrysosporium, Reactor 3, 1 wk	4.38	9.37	0.64	0.28	0.26	0.17	5.26	3.02	12.39
P. chrysosporium, Reactor 3, 2 wks	13.92	9.47	0.26	0.37	0.65	0.20	5.45	2.20	11.68
P. chrysosporium, Reactor 4, 1 wk	2.87	9.06	0.57	0.28	0.24	0.17	4.84	3.17	12.23
P. chrysosporium, Reactor 4, 2 wks	7.22	10.31	0.45	0.58	0.56	0.26	5.58	2.39	12.70

	% Dry Matter Loss	%Water Extractives	%Soluble Glucan	%Soluble Xylan	%Soluble Galactan	%Soluble Arabinan	%Water Extractives Others	%Ethanol Extractives	%Total Extractives
Dry, unstored	-	-	-	-	-	-	-	-	-
Control, Reactor 1, 1 wk		11%	70%	33%	5%	3%	-27%	21%	14%
Control, Reactor 1, 2 wks		30%	88%	45%	4%	-190%	57%	40%	32%
Control, Reactor 2, 1 wk		17%	70%	41%	9%	-5%	-21%	21%	18%
Control, Reactor 2, 2 wks		31%	87%	56%	7%	-202%	8%	36%	32%
P. chrysosporium, Reactor 3, 1 wk		2%	67%	42%	-3%	-8%	-43%	14%	5%
P. chrysosporium, Reactor 3, 2 wks		1%	87%	23%	-161%	-25%	-48%	37%	11%
P. chrysosporium, Reactor 4, 1 wk		5%	71%	42%	3%	-5%	-31%	10%	6%
P. chrysosporium, Reactor 4, 2 wks		-8%	77%	-21%	-122%	-59%	-51%	32%	3%

Table B.4. Dry matter loss weighted composition of structural features in aerated bioreactors for control and fungal-treated corn stover after storage for 1 or 2 weeks (Top). Percent change in structural changes in stored corn stover compared to the unstored parent corn stover; red colors indicate extent of loss and green values indicate gains (Bottom).

	% Dry Matter Loss	% Ash	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate
Dry, unstored	-	5.37	16.80	1.35	35.07	20.94	1.15	3.04	3.56
Control, Reactor 1, 1 wk	9.20	4.70	16.75	1.33	30.35	20.31	1.19	2.52	2.30
Control, Reactor 1, 2 wks	14.47	5.23	16.11	1.15	30.48	18.52	1.15	2.38	0.89
Control, Reactor 2, 1 wk	7.62	4.93	16.35	1.26	32.16	19.70	1.18	2.48	2.11
Control, Reactor 2, 2 wks	12.13	4.78	16.44	1.21	31.39	19.17	1.24	2.55	1.13
P. chrysosporium, Reactor 3, 1 wk	4.38	4.77	15.96	1.33	32.08	20.18	1.13	3.24	3.19
P. chrysosporium, Reactor 3, 2 wks	13.92	5.74	16.13	1.05	29.07	16.81	1.02	2.58	1.81
P. chrysosporium, Reactor 4, 1 wk	2.87	5.26	17.67	1.38	32.64	21.35	1.22	2.62	2.49
P. chrysosporium, Reactor 4, 2 wks	7.22	5.96	17.94	1.16	30.28	19.09	1.17	2.33	1.31

	% Dry Matter Loss	% Ash	% Lignin (Acid Insoluble)	% Lignin (Acid Soluble)	% Glucan	% Xylan	% Galactan	% Arabinan	% Acetate
Dry, unstored	-	-	-	-	-	-	-	-	-
Control, Reactor 1, 1 wk		12%	0%	2%	13%	3%	-3%	17%	35%
Control, Reactor 1, 2 wks		2%	4%	15%	13%	12%	0%	22%	75%
Control, Reactor 2, 1 wk		8%	3%	6%	8%	6%	-3%	18%	41%
Control, Reactor 2, 2 wks		11%	2%	10%	10%	8%	-7%	16%	68%
P. chrysosporium, Reactor 3, 1 wk		11%	5%	1%	9%	4%	1%	-6%	10%
P. chrysosporium, Reactor 3, 2 wks		-7%	4%	22%	17%	20%	11%	15%	49%
P. chrysosporium, Reactor 4, 1 wk		2%	-5%	-2%	7%	-2%	-6%	14%	30%
P. chrysosporium, Reactor 4, 2 wks		-11%	-7%	14%	14%	9%	-1%	23%	63%

Conversion impacts of P. chrysosporium activity on corn stover

Methods:

Enzymatic hydrolysis was performed on knife milled and mechanically refined samples on unstored and fungal-treated biomass from aerated bioreactor Reactor 3 based on a method designed previously[1]. Size reduction was accomplished using a Wiley® Mill to pass a 2 mm screen or mechanically refining for 4,000 revolutions in a PFI mill (Rycolab, Deerlijk, Belgium). Conditions were as reported in the manuscript with the exception that enzyme complexes Cellic® Ctec2 and Cellic® Htec2 were added at a loading rate at 10 mg protein/g glucan and 1 mg protein/g glucan, respectively.

Results:

The *P. chrysosporium*-inoculated corn stover from Reactor 3 was size reduced using two methods to understand the impacts of depolymerization occurring during storage as a function of mechanical deconstruction. The aim of this work is to understand if fungal treatments partially hydrolyze and weaken structural components that are reflected in additional sites for enzymatic attack. Two methods were compared, a disk refining mill, which uses cutting, compression, and shear forces to defibrillate biomass, and a knife mill, which uses only the cutting mechanism. Size reduced material was subject to hydrolysis using a mixture of commercially relevant glycosidases over a 120-hour period, with samples taken at 5 additional timepoints during the hydrolysis to assess any differences in the reaction rate. Figure B. presents glucose and xylose released as a function of storage time and milling method. Glucose and xylose release were enhanced in all mechanically refined samples (compared to the knife-milled, or untreated?) suggesting that defibrillation did expose the hemicellulose and cellulose for additional enzyme access. Xylose yields in mechanically refined were not different in the fungal treated samples compared to the control. Surprisingly, glucose release was reduced compared to the unstored sample in both *P. chrysosporium*-inoculated samples. One possible explanation is that the more easily accessible cellulose was degraded by the fungi. However, it has been documented that the laccases and lignin peroxidases secreted because of fungal treatments can inhibit the glycosidases provided in the enzymatic hydrolysis, and washing has required to remove these fungal enzymes and restore glycosidase activity[2-4]. A second potential explanation of the reduced carbohydrate release after 2 weeks of storage is that the coalesced lignin resulting during oxidative conditions occurring during fungal treatment blocked the cellulases from accessing the crystalline and amorphous cellulose. Further studies are necessary to fully understand the impact of this phenomenon and how to mitigate its impact on downstream conversion.

Glucose and xylose release in the knife milled samples are reduced by approximately one third and one half, respectively, compared to the mechanically milled samples. This difference suggests that the shear mechanism is useful for severing cellulose chains to expose them to enzymatic attack. However, the defibrillation action of the mechanical refining exposes a higher amount of hemicellulose. A similar trend in the knife milled samples compared to the mechanically refined samples was observed in terms of fungal treatment resulting in decreased glucose yields. Increasing glucose and xylose release over the hydrolysis time indicates that enzymes were still actively attacking cellulose and hemicellulose.

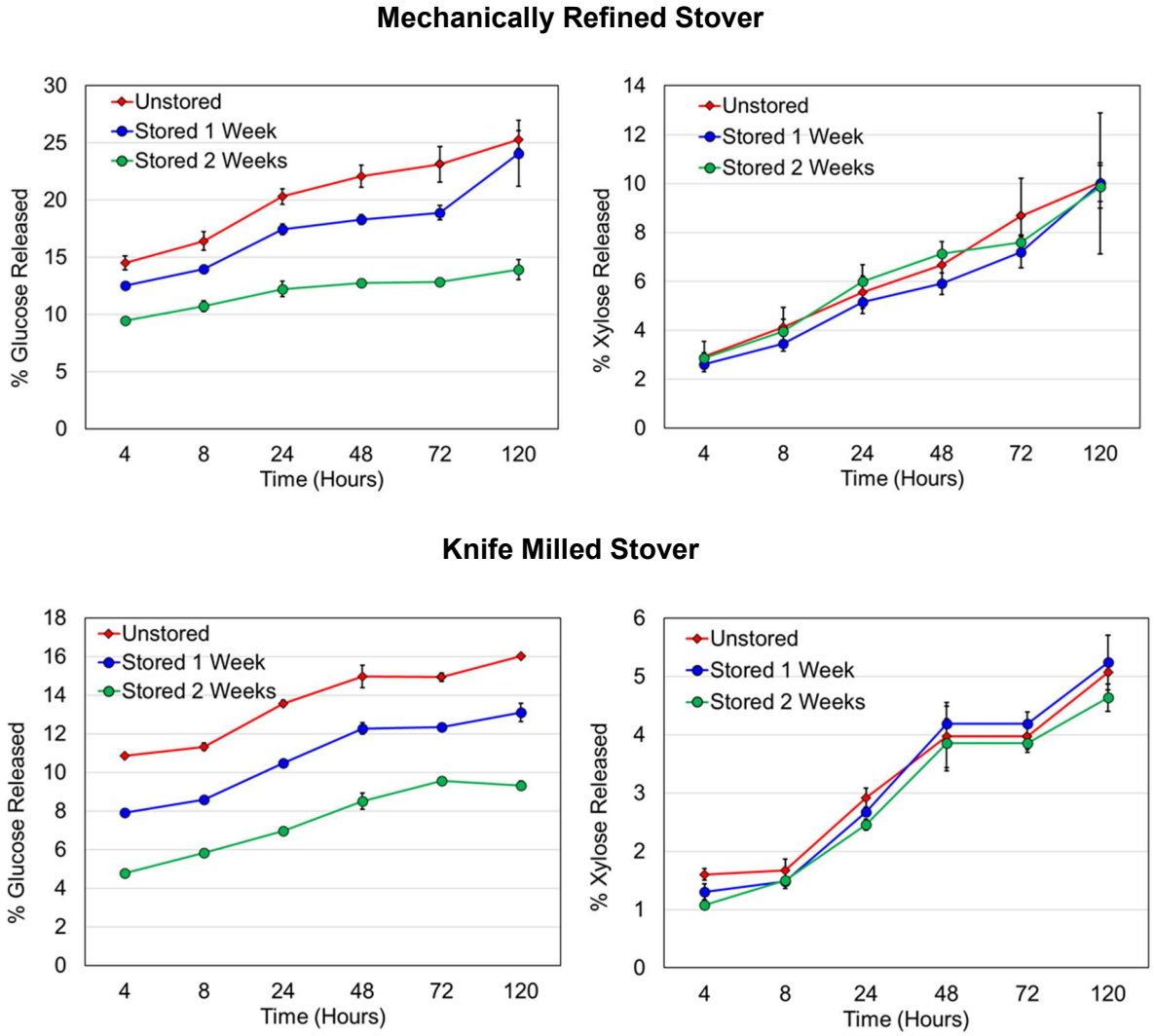

Figure B.1. Enzymatic hydrolysis yields subject to *P. chrysosporium* treatment over a two-week period followed by mechanically refining or knife-milling. Error bars represent standard deviation, n=3.

Production efficiencies of the pyrolysis-GCxGC/MS for P. chrysosporium in aerated corn stover

Methods:

Corn stover analyzed samples in pyrolysis GCxGC/MS displayed variations in pyrolysis production efficiencies, which motivated quantitative measurement for a set of oxygenates and lignol pyrolysis products listed in Table A5. Calibration curves were generated from the analyses of oxygenate and lignol mixtures by direct injection of serially diluted standards containing fluorenone as an internal standard. The calibration analyses were conducted using the rail sampler on the GCxGC/MS and not the pyrolizer. Extracted ion profiles in the GCxGC/MS analyses for ions used for quantitation of the calibrated compounds and the internal standard were integrated using the Leco ChromaTOF software. Response factors (RF) were generated by plotting the intensity ratios of the calibrated compounds-to-internal standard ($i_{compound}/i_{i.s.}$) versus the quantity ratios of the calibrated compounds-to-internal standards ($q_{compound}/q_{i.s.}$), and then calculating the slope using standard regression in Origin. Intercepts were negligible, and so regression was forced through the origin. Then $q_{compound}$ was calculated using the following formula:

$$q_{compound} = (i_{compound}/i_{i.s.})(q_{i.s.})/RF \qquad \text{Equation B.1}$$

The quantity of the pyrolysis compound generated was then normalized to the sample mass to furnish the pyrolysis production efficiency, in the units of nanomoles/μg.

Table B.5. Calibrated compounds considered for measuring efficiency in the pyrolysis-GCxGC/MS analyses.

Type	Compound	1st dimension retention time, s	*m/z* of ions used for quantitation	RF
oxygenate	Acetic acid	289	45+60	0.162
oxygenate	2,3-Butanedione	298	86	0.0744
oxygenate	Furfural	547	95+96+67	0.811
oxygenate	Acetoxyacetone	580	116+86+73+43	1.23
oxygenate	2(5H)-Furanone	652	84+55	0.696
oxygenate	5-Methyl furfural	721	110+109+81+53	0.821
oxygenate	2-Hydroxy-g-butyrolactone	751	58+57+44	0.199
lignol	Phenol	745	94+66	0.07651
lignol	Guaiacol	904	124+109+81	0.0824
lignol	Phenol, 4-vinyl	1045	65+91+120	0.18341
lignol	Guaiacol, 4-vinyl	1168	150+135+107+77	0.03403
lignol	Phenol, 4-formyl	1201	122	0.034
lignol	Syringol	1204	154+139	0.04676
lignol	Guaiacol, 4-formyl	1264	152+151	0.05746
lignol	Guaiacol, 4-(1-propenyl)-, (E)-	1312	164+149	0.03096
lignol	Guaiacol, 4-cinnamyl	1579	178+147+135	0.02666
Internal standard	9(9H)-Fluorenone	1612	180+152+151+150+76+63	n/a

Results:

Analysis of the results is provided within the manuscript.

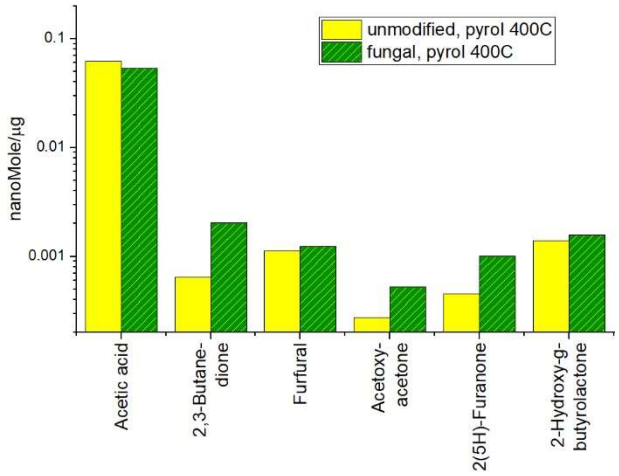

Figure B.1. Comparison of the pyrolysis production efficiency of oxygenates produced by pyrolysis at 400°C. Results are reported on a log scale.

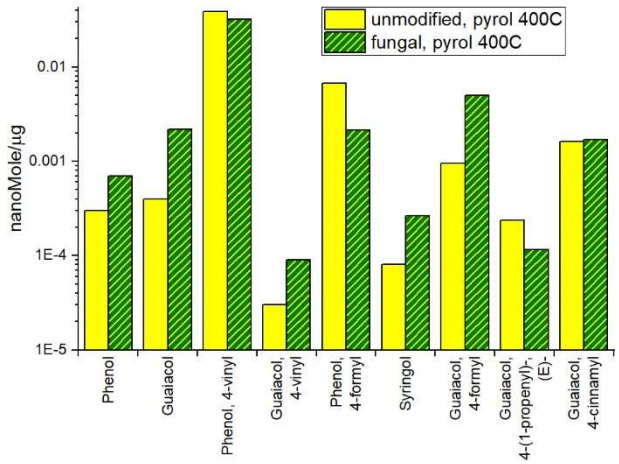

Figure B.4. Comparison of the pyrolysis production efficiency of lignols produced by pyrolysis at 400°C. Results are reported on a log scale.

Cellulose Crystallinity Remains Unaltered in Fungal Pretreatment

Methods

X-ray diffraction (XRD) profiles were collected on 0.2mm minus samples using a powder X-ray diffractometer (Brucker D8-Advance, Karlsruhe, Germany). Samples were manually compacted in the sample holder. 2θ measurements ranged from 5° to 40° two-theta with a scanning speed of 0.125°/min and step size of 0.0106° apart from the unstored sample from the aerated bioreactor experiment, which was analyzed with a scanning speed of 0.250°/min step size of 0.0205°. Crystallinity index was calculated using the subtraction of amorphous cellulose from crystalline cellulose approach[5] according to Equation B.2.:

$$Crystallinity\ Index\ (CrI) = \frac{I_{002} - I_{am}}{I_{002}} x\ 100 \qquad \text{Equation B.2.}$$

Results

Assessing changes in crystallinity index (CrI) of lignocellulosic biomass is one approach to investigate a mechanism of pretreatments that reduce biomass recalcitrance. Increases in CrI due to removal of amorphous cellulose fraction as well as amorphous hemicellulose and lignin on *P. chrysosporium* inoculated biomass has been suggested in the literature using widely used x-ray diffraction and crystallinity index calculations[5]. For, example, a slight increase in crystallinity has been stated in corn stover treated with *P. chrysosporium* ligninolytic enzyme complexes at 45°C for 24 hours[4]. Similar effects have also been reported in rice straw[6] and wheat straw[7] inoculated with *P. chrysosporium* mycelium. Using the crystallinity index calculation that subtracts the crystalline peak (I_{002}) from underlying amorphous signals (I_{am}), no measurable changes in CrI were observed in this study as evidenced by no changes in peak shape when normalized for height (Appendix Figure A6). Similarly, the CP-MAS NMR data indicates no changes between the C4 crystalline cellulose peak (90 ppm chemical shift) and the C4 amorphous peak (86 ppm chemical shift) shown in Figure 3.8. Therefore, fungal treatment resulted in no substantial microstructure changes influencing cellulose crystallinity, which is not surprising since the mechanisms of fungal attack are targeted at cleaving lignin and hemicellulose bonds. However, XRD is a powerful tool for assessing inorganic contamination in biomass. As shown in Figure A6, a sharp peak at 26.5° two theta corresponds to silica dioxide, likely from soil contamination. Ray et al. suggested the use of XRD for assessing variability in corn stover samples, and similarly they saw no changes in crystallinity index across a range of corn stover samples[8]. In summary, assessment of crystallinity indicates that fungal treatment did not impact the crystalline cellulose.

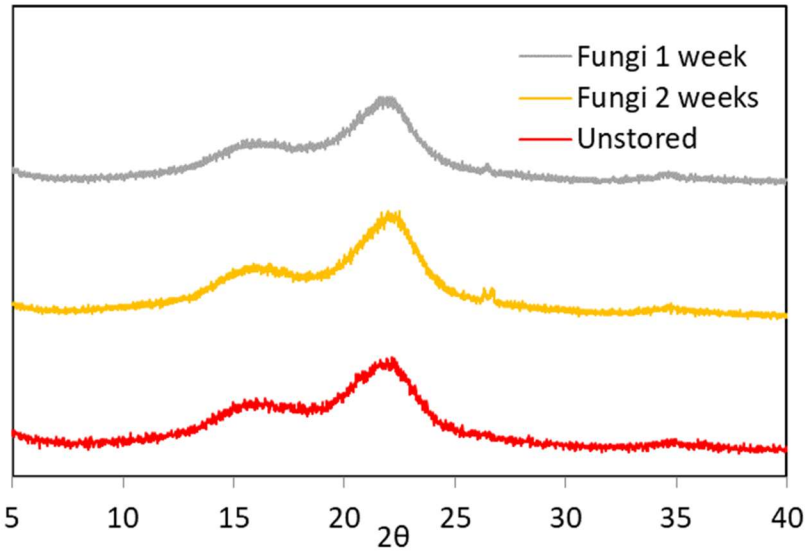

Figure B.4. Comparison of X-ray Diffraction profiles for unstored corn stover, and *P. chrysosporium* treated stover stored in aerated bioreactors for 1 or 2 weeks.

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Appendix C - Chapter 5: Combined alkali treatment and anaerobic storage in corn stover enhances reactivity and surface energy properties

Enzymatic Hydrolysis as a function of comminution type and impact

Method

Knife milled and mechanically milled (2,000 revolutions) native and alkali stored corn stover were assessed in enzymatic hydrolysis. Enzyme complexes Cellic® Ctec2 and Cellic® Htec2 (Novozymes®, Franklinton, NC) were added at a loading rate at 5 mg protein/g and 0.5 mg protein/g biomass, respectively. Hydrolysis occurred at 50°C with mixing at 200 rpm for a 5-day period, with daily sampling, after which the liquid was filtered and analyzed for soluble carbohydrates using HPLC methods described in the manuscript. All samples were analyzed in triplicate. Carbohydrate yield was calculated as described in the manuscript.

An additional experiment varied revolutions in mechanical refining (2,000, 4,000, and 8,000) and increased the enzyme content by a factor of 4. Enzymatic hydrolysis conditions were identical to what was reported above with the exception that hydrolyzed carbohydrates were only measured at the duration of the 5-day experiment.

Results

Two mechanical preprocessing strategies were utilized to explore impacts of sodium hydroxide and anaerobic storage of corn stover on enzyme accessibility and subsequent carbohydrate monomerization. Mechanical refining is a defibrillation approach to that combines cutting, shear, and compression to increase the surface exposure of cellulose microfibrils. This was compared to knife milling, which only uses the cutting approach to size reduce but can break microfibrils into smaller pieces and increase the number of sites for cellulase attack. Approximately 1/3 the enzyme dosage that has previously been reported [1] and were chosen so that the effect of the storage treatment would not be dwarfed by excess processing and enzyme loading. Glucose and xylose monomerization for these two comminution approaches were assessed over a 120-hour period in the presence of glycosidases, as shown in Figure. Mechanical refining for 2,000 revolutions exposed the samples equally, and yields were 25.3 and 24.0% for the native and alkali-stored sample stover, respectively. However, xylose release was elevated in the alkali-stored sample at 13.7% compared to 10.1% in the unstored sample, suggesting some ester linkages between lignin and xylan had been cleaved due to saponification. Glucose and xylose release were significantly increased for the caustic stored sample

after knife milling, although the yields were lower than for the mechanical refined samples. However, increasing yields throughout the 120-hour period suggested enzymes continued to depolymerize cellulose and hemicellulose. Follow-on experiments utilized a four-fold increase in enzyme loading, consistent with previous studies[2].

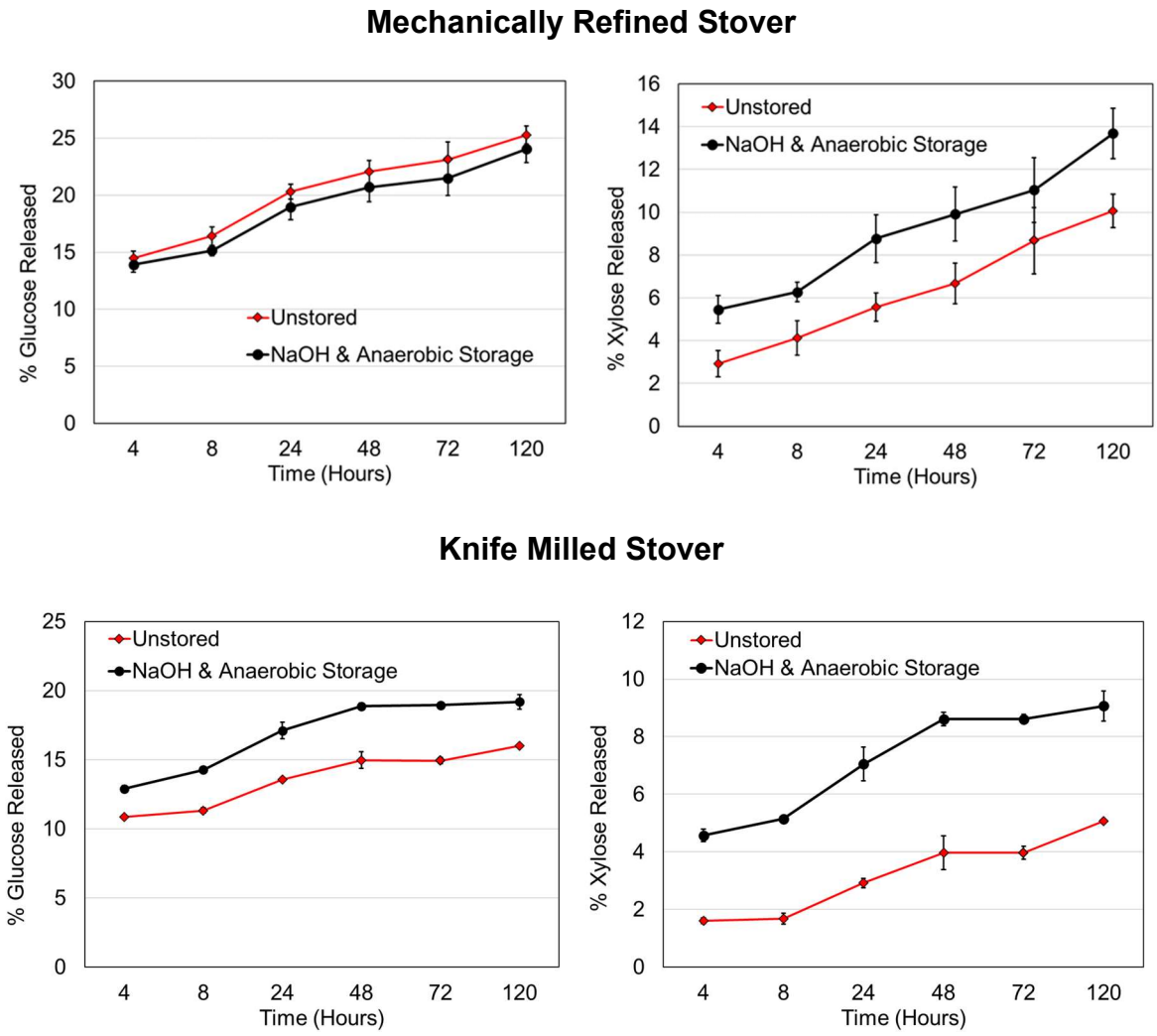

Figure C.1. Glucose and xylose yield in the presence of 5 mg Ctec2 and 0.5 mg per gram corn stover.

Increased enzyme loading and increased revolutions in milling were explored in order to uncover any additional impacts of alkali-based anaerobic storage. Increasing enzyme concentration alone with no additional size reduction revealed that the alkali stored sample had significantly higher glucose and xylose yields than the native sample. However, combining the increased enzyme loading with 2,000 revolutions of mechanical refining increased the rate of carbohydrate release in the alkali stored sample. While the increased enzyme addition in the native sample increased the glucose yield from

25.3% to 27.2% and xylose yield from 10.1% to 12.3%, the alkali stored sample demonstrated an increase in the glucose yield from 24.0% to 43.6% and xylose yield from 13.7% to 23.3%. Increasing revolutions beyond 2,000 had little impact on sugar release. Mechanical milling energy was also assessed to understand if alkali could reduce processing energy requirements for this comminution step. No difference in energy consumption was experienced in samples at 2,000 and 4,000 revolutions, but a 5.7% reduction from 0.035 kW to 0.033 kW was observed a result of combined alkali addition and anaerobic storage.

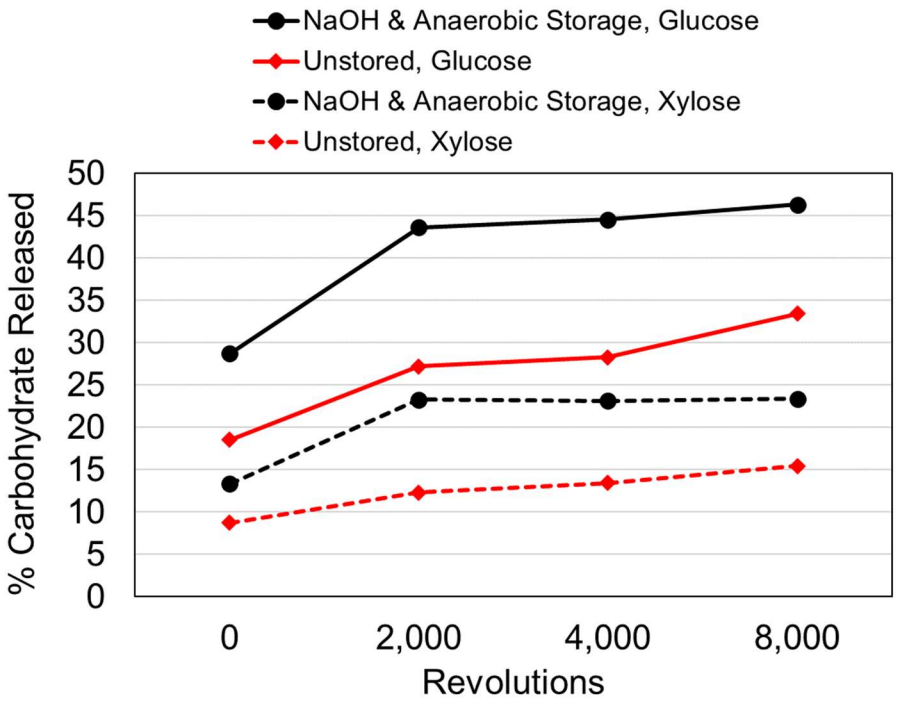

Figure C.2. Glucose and xylose yield in native and alkali-stored corn stover as a function of revolutions in mechanical milling.

Surface Energy Data

Table C.1. Surface energy data comparing the four samples in this research.

	Native	Native, washed	Alkali-stored	Alkali-stored, washed
Dispersive Surface Energy (mJ/m²)				
rep 1	39.65	38.67	41.63	40.53
rep 2	39.75	38.52	41.45	40.52
rep 3	39.89	38.60	41.65	40.50
average	39.76	38.60	41.58	40.51
standard deviation	1.21E-01	7.51E-02	1.10E-01	1.61E-02
% dev from mean	3.03E-03	1.94E-03	2.65E-03	3.97E-04
Specific Surface Energy (mJ/m²)				
rep 1	17.50	20.39	18.12	22.44
rep 2	17.48	20.44	18.12	22.65
rep 3	17.60	20.56	18.51	22.61
average	17.53	20.46	18.25	22.57
standard deviation	6.43E-02	8.74E-02	2.25E-01	1.14E-01
% dev from mean	3.67E-03	4.27E-03	1.23E-02	5.07E-03
Total Surface Energy (mJ/m²)				
rep 1	57.15	59.07	59.76	62.96
rep 2	57.23	58.97	59.58	63.17
rep 3	57.49	59.16	60.17	63.11
average	57.29	59.06	59.83	63.08
standard deviation	1.78E-01	9.50E-02	3.05E-01	1.09E-01
% dev from mean	3.10E-03	1.61E-03	5.10E-03	1.73E-03
Hydrophilicity				
rep 1	0.31	0.35	0.30	0.36
rep 2	0.31	0.35	0.30	0.36
rep 3	0.31	0.35	0.31	0.36
average	0.31	0.35	0.31	0.36
standard deviation	4.32E-04	1.18E-03	2.32E-03	1.18E-03
% dev from mean	1.41E-03	3.42E-03	7.60E-03	3.30E-03
Acid (mJ/m²)				
rep 1	0.59	0.69	0.64	0.82
rep 2	0.59	0.70	0.64	0.83
rep 3	0.59	0.70	0.65	0.83
average	0.59	0.70	0.64	0.83
standard deviation	0.00E+00	5.77E-03	5.77E-03	5.00E-03
% dev from mean	0.00E+00	8.29E-03	8.97E-03	6.06E-03
Base (mJ/m²)				
rep 1	129.53	150.18	129.27	154.30

rep 2	129.46	148.49	128.53	154.98
rep 3	130.91	149.85	132.37	154.42
average	129.97	149.51	130.06	154.56
standard deviation	8.18E-01	8.96E-01	2.04E+00	3.63E-01
% dev from mean	6.29E-03	5.99E-03	1.57E-02	2.35E-03
Work of Cohesion (mJ/m²)				
rep 1	114.30	118.13	119.51	125.92
rep 2	114.46	117.93	119.15	126.35
rep 3	114.98	118.31	120.34	126.22
average	114.58	118.12	119.67	126.16
standard deviation	3.56E-01	1.90E-01	6.10E-01	2.18E-01
% dev from mean	3.10E-03	1.61E-03	5.10E-03	1.73E-03
Work of Adhesion (mJ/m²)				
rep 1	228.19	239.98	229.81	244.29
rep 2	228.22	238.94	229.23	244.71
rep 3	229.23	239.78	231.77	244.37
average	228.55	239.57	230.27	244.46
standard deviation	5.92E-01	5.52E-01	1.33E+00	2.23E-01
% dev from mean	2.59E-03	2.30E-03	5.78E-03	9.12E-04

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