MISCANTHUS CONVERSION TO ETHANOL: EFFECT OF PARTICLE SIZE AND PRETREATMENT CONDITIONS FOR HOT WATER

BY

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DISSERTATION

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Abstract

Cellulosic biomass is a promising feedstock for ethanol production because it is plentiful and enriched in carbohydrates. While the basic technology for converting biomass into ethanol has been developed, processing biomass still remains relatively expensive, despite lower feedstock costs. The high cost stems in part from the recalcitrance of biomass to enzymatic hydrolysis, which necessitates an expensive pretreatment in combination with a heavy enzyme dosage. The objective of this study was to develop an efficient process for conversion of *Miscanthus x giganteus* to ethanol using hammer milling for reduction of particle size followed by a hydrothermal pretreatment.

Particle size reduction is crucial for transportation logistics as well as cellulosic conversion. *Miscanthus* was ground using a hammer mill equipped with screens having 0.08, 2.0 or 6.0 mm sieve openings. Ground samples were subjected to hot water, dilute acid or dilute ammonium hydroxide pretreatments. Sugar yields from enzyme hydrolysis was used to measure pretreatment efficiency. Geometric mean diameters decreased with screen size: 0.08 mm sieve screen (56 μm) followed by 2.0 mm (301 μm) and 6.0 mm (695 μm) screens. Enzymatic sugar yields increased ineversely with mean particle size with the best results observed for all pretreatments, using the 0.08 mm sieve screen. Enzyme hydrolysis of unpretreated biomass samples also increased total conversions as particle size decreased, although mean conversions (10 to 20%) were much lower than for pretreated biomass samples (40 to 70%), indicating the need for chemical pretreatments in biomass conversion. Samples ground using the 0.08 mm sieve was used for hot water optimization studies.

Hot water pretreatment of *Miscanthus* was evaluated with respect to pretreatment temperature and retention time. Hot water pretreatments do not require addition of chemicals, lessen the need for expensive reactors, avoid catalyst recycle and overcome neutralization costs. *Miscanthus* was pretreated at three temperatures (160, 180 and 200°C) for four reaction times (0, 10, 20 and 30 min); the solids loading was kept constant at 15%. Reactions were conducted in mini tubular batch reactors using a fluidized heating bath. Glucose and xylose yields following enzyme hydrolysis of washed pretreated solids were used as a measure of pretreatment efficacy. Best conditions, among those evaluated, for hot water pretreatment of Miscanthus were 200°C for 10 min. At optimal conditions, 6% glucose and 44% xylose were released into the pretreatment liquor. Enzyme hydrolysis of washed pretreated solids resulted in 77% glucan, 12% xylan and 62% total conversion based upon beginning carbohydrate contents. Pretreated conditions were further evaluated for conversion to ethanol in simultaneous saccharification and fermentations (SSF) using native industrial *Saccharomyces cerevisiae* strain D5A. Ethanol yields were 70% of theoretical based upon beginning glucan content following 72 hr fermentation.

Image analysis of solids from three hot water pretreatment conditions resulting in lowest (160°C, 0 min), intermediate (180°C, 10 min) and highest total polysaccharide conversion (200°C, 10 min) were conducted. Pretreated and enzyme hydrolyzed samples were imaged using thick sections for light microscopy, which allowed various plant tissues to be identified. The samples were determined to be unsuitable for imaging using atomic force microscopy or negative staining techniques for electron microscopy. Thick sections showed that pretreated and enzymatically hydrolyzed solids from the optimized pretreatment conditions were primarily disintegrated with few intact cell walls. In contrast, at milder pretreatment conditions, cell wall structure was easily identifiable even following enzymatic hydrolysis. As such thick section light microscopy can be used to qualitatively judge the success of a pretreatment for *Miscanthus*.

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Chapter 1

Introduction

Overcoming dependence on foreign oil, reducing fossil fuel consumption and decreasing green house gas emissions necessitate research for alternative and renewable sources of liquid fuels. Ethanol is a renewable transportation fuel that reduces fossil fuel consumption, mitigates greenhouse gas emissions and promotes economic growth especially in rural areas (Perlack et al. 2005). Currently, corn grain is the major feedstock used for ethanol production in US. In 2011, 209 corn production facilities produced 13.9 billion gallons of ethanol consuming 5.0 billion bushels of corn (RFA 2012). The main coproduct, distillers dried grains with solubles (DDGS), was used as a food ingredient in ruminant and nonruminant diets. In 2011, 35.7 million metric tons of DDGS were produced; 80% of which was to feed ruminant (32% dairy, 48% beef) and the remaining 19% for nonruminant animals (11% swine, 8% poultry) diets (RFA 2012).

In 2011, 40% of US corn was used for ethanol production (RFA 2012). Further siphoning of corn into ethanol market would have several undesirable effects (Yacobucci and Capehart 2008). These effects include reduced production of other grains, less frequent crop rotation, increased cost for animal food, increased production acreage and a reduced U.S. role in the global corn market. Concerns have also been expressed regarding the carbon footprint of corn ethanol because of its heavy energy inputs for planting, fertilizer application and harvesting; albeit most of this energy is derived from natural gas, a domestic energy source. Concerns led to a cap on corn ethanol production under the Renewable Fuels Standard (RFS) provision of the Energy Independence and Security Act (EISA 2007). EISA mandated an RFS with a goal to reach 36 billion gal/yr (136 billion

L/yr) of renewable fuel by 2022, with 60% of the RFS to be met by advanced biofuels, including cellulosic ethanol (RFA 2012). To encourage the commercialization of cellulosic ethanol, the Federal tax credit is limited to the first 15 billion gallons of grain ethanol production (Yacobucci and Capehart 2008).

Lignocellulosic wastes from forest products, agricultural residues and municipal wastes are available for conversion to ethanol. Dedicated energy crops cultivated on marginal farm land, including herbaceous perennials and trees are an additional source of biomass. For billion ton study, Perlack et al. (2005) outlined potential availability of 1.3 billion tons of biomass; 368 million dry tons from forestlands and 998 million dry tons from agricultural lands (including crop residues, perennial crops, grains and animal manure). Potential benefits included improved energy balance due to low fossil energy inputs, decreased greenhouse gas emissions, and lesser detrimental effects on agricultural markets and food prices (FAO 2008, Yacobucci and Capehart 2008).

There are no commercial sized cellulosic ethanol plants. However, presently 26 cellulosic ethanol projects are under development or construction in the US. Commercial success will require meeting major challenges such as feedstock costs, production of new energy crops, feedstock logistics including transport and preprocessing along with developing new technologies with increased efficiency for cellulose conversion (Lynd 1996, Vertes et al. 2008, Wyman 2007). Uncertainties regarding establishment of biomass crops, species selection, yield productivities with low fertilizer applications, seasonal feedstock availability, economic viability and time it will take to develop an efficient conversion processes have raised concerns about cellulosic biofuels.

Lignocellulosic biomass can be converted to fuels by thermochemical, biochemical or a combination of two conversion technologies (Faaij 2006) (Figure 1.1). Thermochemical conversion technologies include combustion (steam and heat generation), gasification (syngas production) and pyrolysis (oil production). Advantages of thermochemical conversions include insensitivity to biomass composition and availability of existing process

methodologies; disadvantages are high volume of biomass required (large economy of scale) and harsh process conditions (high temperature and pressure requirements) (Caroll and Somerville 2009).

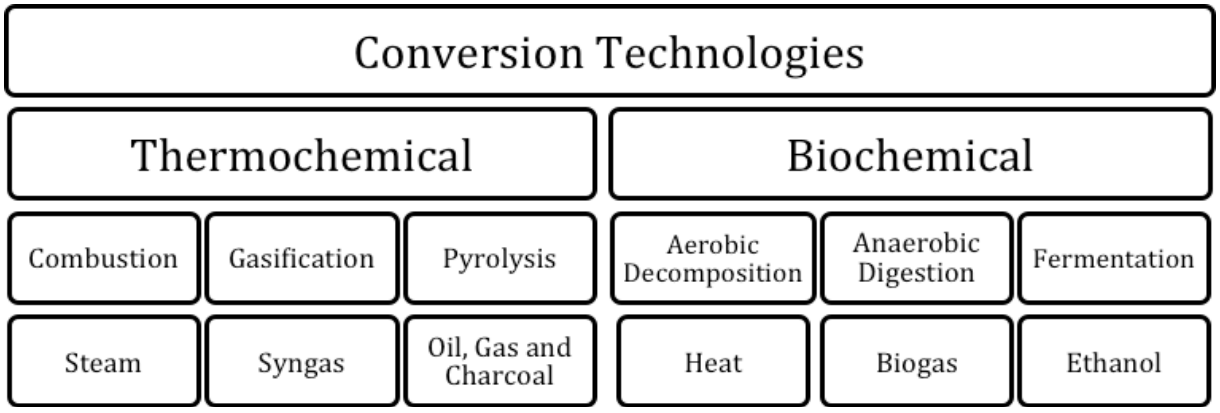

Figure 1.1. Conversion technologies.

Biochemical conversion technologies are aerobic decomposition (heat generation), anaerobic digestion (biogas production) and fermentation (ethanol and other liquid biofuel production). Ethanol production from cellulosic feedstocks requires size reduction, pretreatment, enzymatic hydrolysis and fermentation (Caroll and Somerville 2009). Biochemical conversions are scale neutral and have lower capital investments than thermochemical processes. Disadvantages include high enzyme dosages in digestions and need for efficient ethanol producing microorganisms with capabilities for mixed sugar utilization.

Preprocessing steps including size reduction and their effects on different pretreatments have not been studied. A greater fundamental understanding of chemical and physical mechanisms that occur during pretreatment and the effect of lignocellulosic biomass chemical structure on subsequent enzymatic hydrolysis and fermentation is required to develop an optimum pretreatment technology. The objective was to identify conditions that could be used for ethanol production by the biochemical conversion route using *Miscanthus x giganteus*, a perennial grass. Specific objectives were to:

1. Determine effects of particle size on liquid hot water, dilute ammonium hydroxide and dilute acid pretreatments.

2. Evaluation of different pretreatment conditions using hot water.

3. Investigate changes in cell wall structure following pretreatments using imaging techniques.

Rationale for this proposed research is by understanding changes in chemical structure of lignocellulosic biomass an effective pretreatment technology can be designed.

Chapter 2

Literature Review

Lignocellulosic feedstocks consist of three major components: cellulose, hemicellulose and lignin (Ding and Himmel 2008). Plant cell wall contains 40 to 50% cellulose, 25 to 35% hemicellulose, 15 to 20% lignin and minor constituents such as structural proteins, minerals, oils and soluble sugars (Pauly and Keegstra 2008).

2.1 Lignocellulose Structure

Cellulose is the most abundant polymer on earth and consists of glucose linked by β-1,4-glycosidic bonds where the fundamental unit is cellobiose, a glucose dimer. The average degree of polymerization ranges from 6,000 in primary cell walls to 14,000 in secondary cell walls and varies with source. Glucan chains bond via intra and interchain hydrogen bonds to form microfibrils (Ha et al. 1998). Microfibrils typically contain 36 glucan chains with degree of polymerization varying from 500 to 14,000 (Somerville 2010). The number of chains could be higher depending on plant source; cellulosic algae have as many as 200 chains (Delmer and Amor 1995).

Hemicelluloses are branched, noncrystalline polymers predominantly composed of pentoses, hexoses and/or uronic acid (Girio et al. 2010). Xylans (predominant in hardwoods) and glucomannans (predominant in softwoods) are the most abundant hemicelluloses. Arabinoxylans are predominant in grasses, but the degree of arabinosylation varies (Pauly and Keegstra 2008). Hemicelluloses form a gel matrix around cellulose and are degraded more readily. Hemicelluloses interact with cellulose microfibrils

and form ribbon like bundles called macrofibrils (Figure 2.1). During cell wall expansion, the macrofibrils split causing hemicellulose to form interactions with individual cellulose elementary fibrils by hydrogen bonding. Pectins is a complex polysaccharide that contains 1,4 linked α-D-galactouronic acid, which is found primary cell walls and served to glue various cell wall components together (Ding and Himmel 2008). Cellulose is produced by cellulase synthase complex (rosettes) in the plasma membrane (Somerville 2006); whereas, hemicelluloses and pectins are secreted from the Golgi apparatus.

Lignin is an amorphous polymer of aromatic compounds with average molecular weights ranging from 1,000 to 10,000 (Gottlieb and Pelczar 1951). The three main aromatic alcohol monomers in lignin are p-coumaryl, coniferyl and sinapyl alcohol (Boerjan et al. 2003). These monolignols form p-hydoxyphenyl (H), guaiacyl (G) and syringyl (S) phenylpropanoid units within the lignin polymer via radical coupling reactions (Figure 2.1). The amounts of each phenylpropanoid unit vary among feedstocks; hardwoods primarily contain G and S units, softwoods contain mostly G units and grasses have both G and S units (monocots) with higher H units in dicots (Boerjan et al. 2003). Lignin is insoluble in water and most organic solvents, and forms covalent bonds with hemicellulose. Lignins generally are present in secondary cell walls and absent in primary cell walls. Lignin deposition increases mechanical resistance, decreases water accessibility and retards microbial degradation. Lignins are deposited during the final stages of plant cell wall development and enclose microfibrils and polysaccharides (Ding and Himmel 2008).

Other components present in lignocellulosic substrates include proteins (up to 10% dry weight) that increase interactions with other cell wall components. Besides proteins, other components that could be present are suberin (polyaliphatic polyphenolic association), cutin (aliphatic polyester), cutan (nonhydrolysable aliphatic biopolymer) and waxes (Ding and Himmel 2008).

Due to extensive bonding and crosslinking of lignin and hemicellulose with cellulose, there is limited accessibility of enzymes to degrade polysaccharides and release fermentable

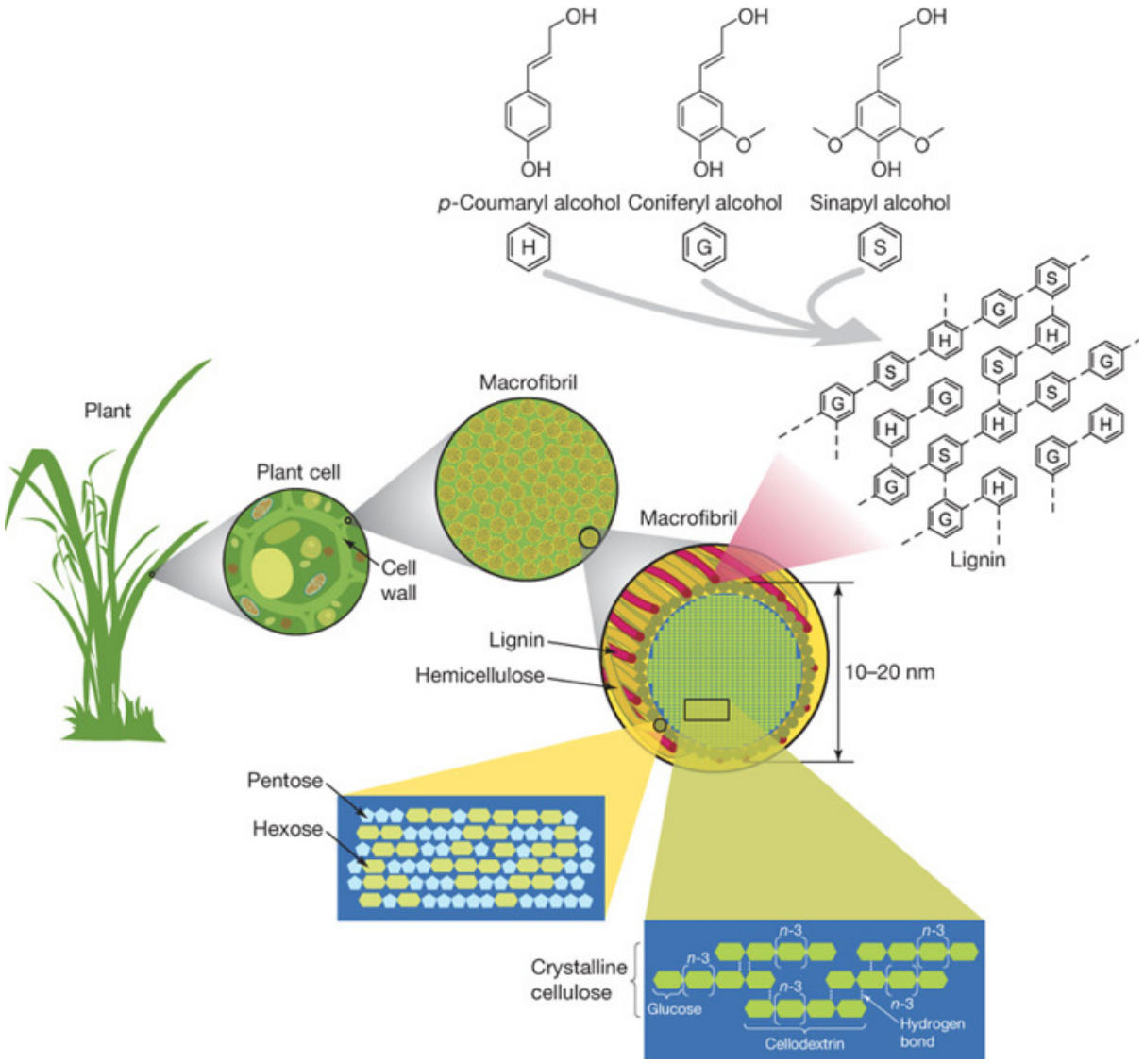

Figure 2.1. Structure of lignocellulose (reproduced from Rubin 2008).

monosaccharides. Hence, lignocellulosic material must be pretreated to disrupt the cell wall structure and allow cellulase enzymes to penetrate to the individual cellulose fibers.

2.2 Pretreatment

Ethanol production from lignocellulosic substrates involves: 1) pretreatment, 2) hydrolysis, 3) fermentation, 4) product recovery and 5) postprocessing of residues (Hendriks and Zeeman 2009). Pretreatment is the most expensive step (30¢/gal ethanol) in

conversion of biomass to ethanol (Mosier et al. 2005). Its high costs are attributable to high chemical and energy demands, and requirements for expensive corrosion resistant reactors, especially for those that use mineral acid catalysts. As an early step in the process, the choice of pretreatment influences all downstream steps and their costs. These include enzyme hydrolysis rates, fermentation toxicity, product concentrations and recovery, waste treatment demands and chemical/catalyst recycle (Wyman et al. 2005). Since processing costs account for 67% of total cost in biomass ethanol production, with pretreatment being the most expensive step, there is a need to develop low cost efficient pretreatment processes (Wyman et al. 2007).

Effective pretreatments have the following key properties (Alvira et al. 2010, Johnson and Elander 2008, Mosier et al. 2005):

1. generate high sugar yields or result in pretreated biomass that is easily hydrolyzed to sugars during enzyme hydrolysis
2. limit the formation of sugar degradation products that inhibit fermentative microorganism
3. avoid the need for biomass size reduction
4. low energy inputs and costs
5. generate high value coproducts or minimize solid waste residues
6. high yields from multiple biomass crops

Pretreatment parameters should be selected to decrease the production of toxic compounds that inhibit yeast or fermentative microorganisms downstream. Depending on pretreatment severity, polysaccharides are degraded and resulting sugars undergo decomposition reactions that produce inhibitory compounds (Alvira et al. 2010). Inhibitors generated are carboxylic acids, furan derivatives and phenolics (Palmqvist and

Hahn-Hagerdal 2000). Furan derivatives include furfural and 5-hydroxymethylfurfural (HMF) and are derived from degradation of pentose and hexose sugars, respectively. Formic acid is produced from furfural and HMF degradation; levulinic acid is formed from degradation of HMF. Phenolic compounds are generated from partial breakdown of lignin and from carbohydrate degradation. Acids inhibit cell growth and viability; undissociated weak acids diffuse through the cell membrane and dissociate within the cytosol resulting in decreased cytosolic pH and require energy to export out of the cell (Palmqvist and Hahn-Hagerdal 2000). Furfural decreases specific growth rates and specific ethanol productivity. HMF has similar inhibition mechanisms as furfural, but produce longer lag phase during growth. Phenolics interact with cell membranes leading to loss of membrane integrity and decrease their selective permeability. Pretreatments can be physical, biological and/or chemical.

2.2.1 Physical Pretreatments

Physical pretreatments involve size reduction (ball milling, attrition milling, compression milling, disk refining), extrusion and irradiation (Alvira et al. 2010, Ding and Himmel 2008). Milling methods involve size reduction of biomass, increase in specific surface area and decrease in degree of polymerization by opening up the lignocellulosic structure. Extrusion methods involve heating and shearing to produce physical and chemical changes in biomass, making it susceptible to enzymatic attack (Karunanithy et al. 2008). Mais et al. (2002) reported improved enzymatic hydrolysis of Douglas fir wood chips by adding enzymes during ball milling. Addition of additives such as ethylene glycol, glycerol and dimethyl sulfoxide during extrusion improved enzyme saccharification of woody biomass (Lee et al. 2009). Although physical pretreatment methods reduce particle size and crystallinity without producing inhibitors, their high energy requirements and inability to remove lignin reduce their feasibility (Taherzadeh and Karimi 2008).

2.2.2 Biological Pretreatments

Biological pretreatments involve microbial degradation of hemicellulose and lignin. Most biological pretreatments use white rot, brown rot and soft rot fungi (Kumar et al. 2009). The fungi degrade lignin and hemicellulose more readily than cellulose (Taherzadeh and Karimi 2008). White rot has been shown to be the most effective for biological pretreatments, which have low energy and chemical requirements, and occur in mild environmental conditions. Emerging methodologies also involve use of termite gut enzymes such as lignases and phenolic acid esterases for pretreatments (Scharf et al. 2010). However, digestion rates are slow (Taherzadeh and Karimi 2008, Sun and Cheng 2002), resulting in time consuming treatments.

2.2.3 Chemical Pretreatments

Chemical pretreatment methods include dilute acid, dilute ammonium hydroxide, alkaline peroxide, steam explosion, hot water, alkali, organosolv, ammonia fiber expansion (AFEX), supercritical fluid, oxidative and ionic liquid (Alvira et al. 2010, Dien et al. 2005, Kumar et al. 2009, Sun and Cheng 2002, Taherzadeh and Karimi 2008). Effects of chemical pretreatment methods on cellulose, hemicellulose and lignin vary (Table 2.1).

Dilute Acid

Dilute acid pretreatments solubilize hemicellulose fractions to monosaccharides (Alvira et al. 2010). Reaction schemes include high temperature, short time (eg, 180°C, 2 to 20 min) or low temperature, longer time (120°C, 30 to 90 min). Pretreatments have been conducted in batch, mixed batch, flow through, steam heated, percolation, plug flow and shrinking bed reactors (Lee et al. 2000, Taherzadeh and Karimi 2008, Yang and Wyman 2009, Zhu et al. 2004). Dilute acid pretreatment has been studied widely using different types of acid (fumaric, maelic, sulfuric, phosphoric) on different feedstocks (several

examples include wheat straw, corn stover, corn cobs, switchgrass, prarie cord grass, yellow poplar, *Miscanthus*) (Allen et al. 2001, Duarte et al. 2009, Karunanithy et al. 2008, Kim et al. 2001, Kootstra et al. 2009, Lloyd and Wyman 2005, Um et al. 2003, Shen et al. 2008, Schell et al. 2003, Sorensen et al. 2008, Pryor et al. 2009). While more conversion studies have used dilute acid, it has two disadvantages. Once hemicellulose is hydrolyzed to xylose, under these reaction conditions xylose undergoes a further reaction to form furfural. Pretreatments with acid also require expensive reactors, constructed with low nickel steel and effective gypsum (waste product) disposing methods (Dien et al. 2005).

Table 2.1. Effect of pretreatments on cellulose, hemicellulose and lignin.

Pretreatment	**Cellulose**	**Hemicellulose**	**Lignin**	**Inhibitors**
Dilute acid	Less hydrolyzed	Completely hydrolyzed	Less removal	Many
Dilute ammonium hydroxide	Swells	Not completely hydrolyzed	Loosens	Few
Alkaline peroxide	Swells, effect on crystallinity	Removed (alkali)	Removed (peroxide)	Few
Steam explosion	Increases surface area	Removed	Altered	Many
Hot water	Swells	Dissolves	Loosens slightly	Generated during harsh operating conditions
Organosolv	Increases digestibility	Dissolves in water fraction	Dissolves in solvent	Trace amounts of solvent cause inhibition
AFEX	Reduced crystallinity	Removed	Removed	Few

Dilute acid pretreatments act by hydrolyzing hemicellulose with xylan being more susceptible than glucomannans (Hendriks and Zeeman 2009). It also disrupts lignin and displaces it (Taherzadeh and Karimi 2008). Dilute acid form nick cellulose chains increasing the number of binding sites for endocellulase (Dien et al. 2005). Neutralization

of acid following pretreatments results in formation of gypsum, a chemical waste product.

Dilute Ammonium Hydroxide

Aqueous ammonia is an effective swelling agent, has high reactivity to lignin, is noncorrosive and can be recovered easily due to volatility (Kim et al. 2003). Use of alkali (sodium hydroxide, lime or ammonia) solubilizes hemicellulose and partially removes lignin. Ammonia pretreatments have been conducted at low temperatures (room) and higher (50 to 220°C) (Kim et al. 2003, Zhu et al. 2006) using feedstocks such as corn stover (Kim et al. 2003), switchgrass (Isci et al. 2009), wastepaper and paper mill sludge (Kim et al. 2000). Alkali pretreatments break bonds among polymers more effectively than acid and oxidative methods, but may cause redistribution and condensation of lignin (Hendriks and Zeeman 2009, Taherzadeh and Karimi 2008).

Alkali pretreatments involve solvation and saponification reactions (Hendriks and Zeeman 2009). Solvation reactions cause swelling of biomass, making polysaccharides more accessible. Saponification of intermolecular ester bonds reduces cross linking among xylan hemicelluloses and lignin (Kim et al. 2003, Sun and Cheng 2002). Alkali also breaks ether bonds in lignin-carbohydrate complexes. Due to breakage of cross linking bonds, porosity of lignocellulosic substrates is increased (Kumar et al. 2009).

Alkaline peroxide

Alkaline peroxide pretreatments produce highly digestible cellulose and generate low concentrations of inhibitors. The alkaline pH removes hemicellulose and peroxide oxidizes lignin bonds (Dien et al. 2005). This pretreatment also removes waxes, silica and cutins on plant surfaces (Taherzadeh and Karimi 2008). Saha and Cotta (2006, 2007) demonstrated alkaline peroxide as an effective pretreatment for wheat straw and rice hulls. Wang et al. (2010) reported increased digestibility (95%) of *Miscanthus* when alkaline peroxide pretreatments were followed by a second pretreatment with electrolyzed water compared to

using alkaline peroxide alone (81%). Alkaline peroxide pretreatments have been conducted on corn cobs, corn husks, stalks, wheat straw and kenaf with ethanol yields between 80 and 100% of theoretical (Gould 1984). However, alkaline peroxide pretreatments are expensive and require large amounts of alkali and peroxide (Dien et al. 2005).

Hot Water

Hot water or hydrothermal pretreatment (160 to 240°C) hydrolyzes hemicellulose to soluble oligosaccharides and loosens lignin (Dien et al. 2005, Taherzadeh and Karimi 2008). Hot water pretreatments overcome requirements for corrosion resistant reactors and do not involve addition of chemicals. Buffered systems (pH 5 to 7) prevent formation of inhibitors as it minimizes formation of monosaccharides which subsequently react to form furans (Dien et al. 2005, Hendriks and Zeeman 2009). Depending on substrates, temperatures of 160 to 240°C for 20 to 40 min are utilized. Hot water pretreatments have been conducted on corn fiber (Dien et al. 2006), DDGS (Kim et al. 2008), alfalfa fiber (Sreenath et al. 1999), yellow poplar sawdust (Allen et al. 2001), prairie cord grass (Cybulska et al. 2010), sorghum bagasse (Dogaris et al. 2009), sugarcane bagasse (Boussarsar et al. 2009) and corn stover (Liu and Wyman 2005). Hot water produces results similar to dilute acid pretreatment without using chemicals, requiring neutralization of products and generating inhibitors (Taherzadeh and Karimi 2008). Hot water pretreatments also provide advantages over steam explosion because of higher pentosan yields and lower inhibitor generation but allow lower solids loadings than steam pretreatments (Dien et al. 2005).

Hot water pretreatments result in solubilization of hemicellulose (Hendriks and Zeeman 2009). The composition of hemicellulose backbone and branching groups determines the stability of hemicelluloses to thermal, acid or alkali pretreatments. At temperatures above 160°C, hemicelluloses are solubilized first, followed by lignin solubilization. Hot water disrupts hydrogen bonds among cellulose microfibers and swells the cellulose structure. At temperatures above glass transition, hemicellulose dissolves and

lignin is loosened (Dien et al. 2005). Water dissociates at high temperatures (220°C) to form a weak acid leading to deacetylation of xylan. Acetic acid produced during pretreatment further decreases pH and increases xylan hydrolysis (a process called autohydrolysis). Buffered hot water (pH 5 to 7) is used to reduce autohydrolysis and inhibitory products generated from xylan sugars.

Steam Explosion, AFEX, Organosolv and Other Pretreatments

AFEX (ammonia fiber expansion) method removes some lignin and hemicellulose, and decreases cellulose crystallinity. AFEX generates digestible cellulose fractions and less inhibitor compared to dilute acid and steam explosion. Low inhibitor concentrations result from the AFEX process as it does not solubilize hemicellulose; whereas, steam explosion and dilute acid pretreatments solubilize hemicellulose and generate compounds inhibitory to microorganisms used downstream (Sun and Cheng 2002).

Organosolv pretreatments involve the use of solvents (organic or aqueous) in the presence of mineral acid catalysts (hydrochloric or sulfuric acid) to break down the matrix of lignin and hemicellulose. Two fractions result: one has high concentrations of lignin in solvent and the second contains high concentrations of hemicellulose in water (Taherzadeh and Karimi 2008). Thus the organosolv process is capable of fractionating biomass to produce highly digestible solids and coproducts that could have other uses. Solvents used include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol (Sun and Cheng 2002). Huijgen et al. (2010) reported the use of an acetone organosolv process for wheat straw and achieved 79% delignification, 82% hemicellulose hydrolysis and 93% cellulose recovery. Temperatures ranging from 150 to 200°C have been used for organosolv pretreatments. Papatheofanous et al. (1995) incorporated a dilute acid step prior to an acid catalyzed ethanol reaction and achieved 70% lignin removal. Although lignin is extracted as a coproduct, organosolv pretreatments have limitations. Organic solvents traces must be removed completely to prevent

downstream inhibition of enzymes or fermentative microorganisms. Solvents must be recovered and reused to save costs.

Use of oxidizing agents and supercritical fluids are expensive and limit their use at a commercial scale. Steam explosion removes hemicellulose and increases cellulose fiber reactivity (Hendriks and Zeeman 2009, Mosier et al. 2005b, Taherzadeh and Karimi 2008). Production of inhibitors (furfural, hydroxyl methyl furfural (HMF) and phenolic compounds) and condensation products that increase recalcitrance result from steam explosion. Researchers have used combinations of two or more chemical pretreatment methods like dilute acid-organosolv (Brosse et al. 2009, Papatheofanous et al. 1995, Zhu et al. 2009), dilute acid-wet explosion (Sorensen et al. 2008), dilute acid-autohydrolysis (Hage et al. 2010), steam explosion-alkaline peroxide (Chen et al. 2008, Yang et al. 2010) and two step liquid hot water (Yu et al. 2010) to enhance digestibility of lignocellulosic feedstocks.

2.3 Effects of Particle Size on Biochemical Conversion of Lignocellulosics

Irrespective of methodology, the first step in biomass conversion is size reduction. Particle size may have an effect on pretreatment, mass and heat transfer (Zheng et al. 2007). However, the literature is conflicted in regards to its effect on subsequent enzyme digestibility of biomass, with varying results based on feedstock source and experimental methodology (Vidal et al. 2011). Complicating this area of study is that many studies generated various sized fractions by milling whole biomass followed by size classification, inadvertently biasing their fractions with different composition and tissue types.

A number of authors have reported little or no correlation of particle size on enzymatic hydrolysis following pretreatment (Mansfield et al. 1999, Chang and Holtzapple 2000). Ball milling of poplar wood increased enzymatic digestibility but cellulose conversion was limited to less than 50% without chemical pretreatment (Chang and

Holtzapple 2000). Rivers and Emert (1988) found bagasse conversion did not correlate with particle size but was positively correlated with increased percentage fines (sizes <53 μm). They also observed that for rice straw, hydrolysis did not correlate to average particle size or percentage of fines. Rivers and Emert (1987) reported smaller average particle sizes produced by dry milling did not produce equivalent amounts of glucose or ethanol during cellulose hydrolysis.

Other investigators have observed that smaller mean particle size did result in increased cellulose conversion. Dasari and Berson (2007) investigated the effect of four particle size fractions of sawdust on hydrolysis rates and determined the smallest particle size fraction (33 to 75 μm) produced 50 to 55% more glucose than the largest size fraction (590 to 850 μm). Using corn stover, particle sizes ranging from 75 to 152 μm had 45% conversion rates as compared to 35% for particle sizes ranging from 1680 to 2000 μm (Elshafei et al. 1991). Yeh et al. (2010) had a 60% increase in glucose concentration when average particle size of cellulose was reduced to 25.5 μm using media milling. They also observed that higher cellulose concentrations (7%) retarded particle size reduction as compared to lower cellulose concentrations (3%), hence resulting in smaller specific areas for enzyme action. Freeze milling of sunflower and palm kernel meals to size distributions of 20 to 200 μm resulted in 30 and 53% more cellulose digestions, respectively, compared to particle size distributions obtained by using <0.5 mm screen sizes (Dsterhft et al. 1993).

From an economic standpoint, chemical pretreatments are necessary for efficient enzyme hydrolysis (Mosier et al. 2005, Hendriks and Zeeman 2009). Gharpuray et al. (1983) compared hydrolysis rates following various combinations of physical and chemical pretreatments on wheat straw. They concluded that multiple pretreatments involving size reduction and chemical pretreatment were not as effective as single chemical pretreatments, but specific surface area was a critical factor. Physical pretreatments alone result in efficiencies <50% compared to chemical pretreatments that give >70% conversion efficiencies (Vidal et al. 2011). The study of effects of particle size on pretreatments has

been conducted only over a single pretreatment technology, with different methodologies used for calculating pretreatment effectiveness among researchers (Vidal et al. 2011). Hence there is a need to determine the effects of particle size reduction on different pretreatment technologies.

2.4 Elucidation of Cell Wall Structure Using Imaging Techniques

The inherent complexity of polysaccharides that constitute plant cell wall and the myriad of interactions among these polysaccharides make it difficult to elucidate cell wall structures (Hedenstrom et al. 2009). In the past, much work has been done to determine the effect of pretreatment on cell wall chemical composition such as removal of xylan and lignin. However, recent research focus has shifted to analyzing changes that occur in cell wall structure and interactions during pretreatments. Ability to detect cell wall changes provides valuable information regarding pretreatments and lay a rational basis for further improving pretreatments in terms of sugar yields.

To study changes in cell wall structures a number of methods have been used (Table 2.2). Tissue labeling techniques by antibodies have limitations of antibody availability for all kinds of polymers (Obel et al. 2009). Fourier Transform Infrared Spectroscopy (FTIR) techniques can be used to determine cell wall structure but determining and assigning linkages is not defined clearly. Oligosaccharide Mass Profiling (OLIMP) allows analysis at cell type, organelle or plant tissue levels, and when coupled with Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy (MALDI-TOF MS) can be used to generate a semiquantitative fingerprint of particular cell wall polysaccharides. Limitations of OLIMP methods are the availability of specific cell wall hydrolytic enzymes. Interpretation of X-ray diffraction data is still under discussion, and needs to be used alongside FTIR or Nuclear Magnetic Resonance (NMR) techniques

to obtain information on spatial conformation (Oh et al. 2005). Transmission Electron Microscopy (TEM) is limited due to low contrast of cellulose, Scanning Electron Microscopy (SEM) provides information only on wall surface and polarized light microscopy techniques (using dyes) only provide bulk orientation information (Anderson et al. 2010). Moreover, sample preparations required for microscopic examination introduce externalities into structure. Near Infrared Spectroscopy (NIR), FTIR and Raman spectroscopy methods, though rapid, provide limited information on chemical structure (Hedenstrom et al. 2009).

NMR techniques provide detailed information on native conformation and chemical structure of whole cell walls (Hedenstrom et al. 2009). NMR techniques provide capability to identify each glycosyl residue, in their anomeric or ring form, as well as determine glycosyl linkages (Mazumder and York 2010). Methodology involves grinding of samples using a ball mill, followed by derivatization of cell wall (without disrupting native state), dissolution and NMR (Lu and Ralph 2003). Solution state NMR provides higher sensitivity than solid state NMR (Lu and Ralph 2003). Mazumder and York (2010) reported use of NMR to determine specific arabinoxylan structures to provide detailed information on distribution, development regulation and synthesis of arabinoxylans in growing tissues. Hedenstrom et al. (2009) demonstrated the use of multivariate analysis with NMR to determine changes among *Populus* wood samples. They reported changes in composition and structure of lignin and polysaccharides. A limitation of this process is energy intensive milling of sample, which causes some degradation and reduces degree of polymerization (Lu and Ralph 2003). The method also does not provide lignin primary chain sequence data and not all structural entities present in lignin are identifiable (Jourdes et al. 2010).

Atomic force microscopy (AFM), a newer imaging technique, provides a spatial view of the surface with atom level resolution (Harris et al. 2010). Methodology involves the use of a physical tip attached to a cantilever that moves over the surface of the sample to determine topography and physical properties (Harris et al. 2010). AFM imaging

Table 2.2. Biomass imaging techniques.

Method	**Material studied**	**References**
Scanning Electron Microscopy (SEM)	Corn stover	Donohoe et al. 2008
Transmission Electron Microscopy (TEM)	Corn stover	Donohoe et al. 2008
Antibody Tissue Labelling Techniques	Corn stover	Donohoe et al. 2008, Willats et al. 2000
Fluorescent Dye Binding	*Arabidopsis* root	Anderson et al. 2010
Fourier Trasnform Infrared Spectroscopy (FTIR)	Corn stover, Kenaf fiber	Donohoe et al. 2008, Khalil et al. 2010
X Ray Diffraction (XRD)	Cellulose	Oh et al. 2005, Park et al. 2010
Oligosaccharide Mass Profiling (OLIMP), Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF MS)	*Arabidopsis*	Obel et al. 2009
Nuclear Magnetic Resonance (NMR)	*Miscanthus sinensis*, Pine wood, Aspen wood, *Miscanthus*, *Populus* wood, cellulose	Alriols et al. 2010, Lu and Ralph 2003, Mazumder and York 2010, Hage et al. 2010, Hendenstrom et al. 2009, Park et al. 2010
Atomic Force Microscopy (AFM)	Corn stover, corn leaves, green algae (*Valonia ventricosa*), banana cellulose microfibrils, parenchyma cells (from apple, water chestnut, potato, carrot)	Zeng et al. 2010, Hanley et al. 1992, Chundawat et al. 2011, Zuluaga et al. 2010, Kirby et al. 1996

techniques have been used by Ding and Himmel (2006) to describe the arrangement of the cellulose microfibrils in maize stem pith cells. Advantages with using AFM are 1) in vivo sample measurements can be made (in air or fluid) with minimal sample preparation steps, 2) high resolution capacity comparable to TEM and 3) simultaneous collection of sample topography (height) and elasticity (phase imaging) data (Ding and Himmel 2006). A limitation with AFM imaging is the introduction of artifacts leading to image broadening. Use of sharper tips and accurate calibrations minimize artifacts due to AFM. Modes of operation have been developed that improve surface and subsurface imaging (Tetard et al. 2009).

2.5 *Miscanthus*

Miscanthus x giganteus (*Miscanthus*) was chosen as the feedstock for this study. *Miscanthus*, a perennial grass, yields high amounts of biomass, requires lower nutrient and is good for carbon sequestration (Ha et al. 1998). *Miscanthus* can grow to over 3 m tall and produce 20 to 25 tons of dry matter per hectare (Brosse et al. 2009). *Miscanthus* undergoes an annual cycle of senescence leading to low removal of nutrients during harvesting (Somerville 2010). *Miscanthus* contains 40% cellulose and 20% hemicellulose, which is higher than most other warm season grasses.

Pretreatment studies on *Miscanthus* are scarce. Vrije et al. (2002) employed a mechanical-chemical pretreatment method and achieved 69% cellulose and 38% hemicellulose conversions to sugars. AFEX process conditions at 160°C, 5 min reaction time and 2:1 w/w ammonia:biomass loading resulted in 96% cellulose conversion (Murnen et al. 2007). Dilute acid presoaking also has been shown to improve delignification and increase glucose recovery when used prior to pretreatments such as wet explosion (Sorensen et al. 2008), organosolv (Brosse et al. 2009) and autohydrolysis (Hage et al. 2010). Huyen et al. (2010) reported increased maturity led to higher lignin deposition and ether cross

linkages, hence decreasing disruption capabilities compared to early harvest crop. Wang et al. (2010) reported 95% cellulose digestibility, with 63% hemicellulose and 64% lignin removal from combined pretreatment involving alkaline peroxide and electrolyzed water. Villaverde et al. (2010) examined fractionation of *Miscanthus* bark for production of valuable phenolic byproducts and found that using an acetosolv process (acetic acid:water:hydrochloric acid in ratios of 90:9.85:0.15) removed lipophilic compounds and resulted in highest yields of phenolic byproducts.

2.6 Technical Challenges with Biochemical Conversion of Lignocellulosics

The progress of commercialization of ethanol plants has been much slower than first predicted by researchers in the field. This in part reflects the economic risk inherent in an industry associated with high capital costs, a commodity product, and requirement for inclusion of multiple novel unit operations. Other barriers are technical and unique to this industry (Figure 2.2). Size reduction of incoming material is needed prior to pretreatments, but grinding to very fine sizes has high energy requirements (Caroll and Somerville 2009).

Pretreatment is the most expensive step in bioconversion of lignocellulosics to ethanol (Mosier et al. 2005). Pretreatments also play an important role in downstream processes. Inhibitors like furfural, hydroxymethylfurfural (HMF) and acetic acid generated in pretreatment processes result in low productivities of fermenting microorganisms. Biomass recalcitrance due to lignin presence also interferes with cellulose hydrolysis. Enzymes used in hydrolysis are inhibited by reaction intermediates and end products (cellobiose and glucose). Lignin also binds irreversibly to cellulase and imposes higher enzyme dosage. Catabolite repression decreases the fermentative capacity of genetically engineered microorganisms. New enzymes capable of withstanding high product concentrations, microbes with higher inhibitor tolerance and ethanol producing capabilities

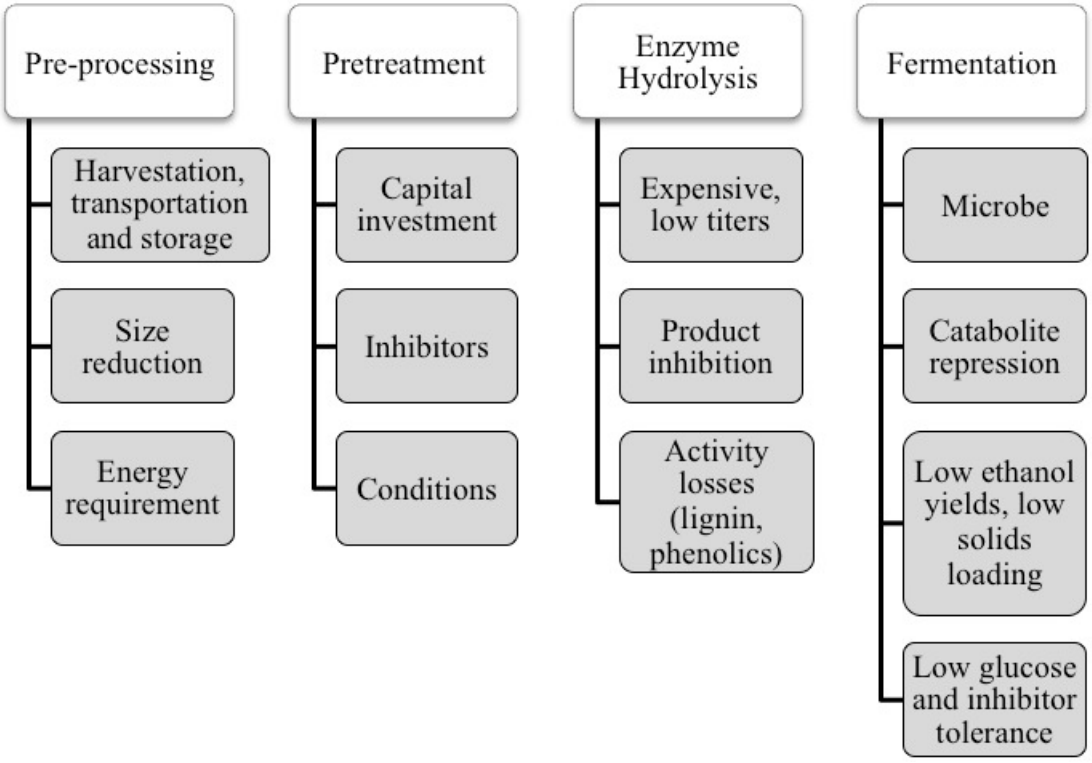

Figure 2.2. Challenges with biochemical conversion of biomass to ethanol.

could facilitate high solids fermentations (Huang et al. 2010). Advantages with high solids fermentations include lower water inputs and lower energy requirements for recovering ethanol and drying fermented solids. Biomass source and variability are issues that need to be examined further especially with respect to pretreatment and enzyme dosing, which might vary depending on biomass type and composition (Lamsal et al. 2010).

Chapter 3

Effect of Particle Size on Enzymatic Hydrolysis of Pretreated *Miscanthus*

3.1 Introduction

Prior to pretreatment, biomass is milled to reduce mean particle size. Particle size reduction is a crucial factor in transportation logistics as well as cellulosic conversion. Particle size effects transportation logistics; smaller sized biomass is easier to transport. Despite its importance, relatively little has been published in regard to the effect of different milling regimes on pretreatment and prior studies are limited to studying a single type of pretreatment. However, as discussed above, different chemical pretreatments have very different mechanisms. Furthermore, most prior studies are flawed as they generate different sized fractions by classifying a single whole milled biomass sample. In this study, whole biomass samples were milled to different mean particle sizes and each milled sample was pretreated using three different methods.

Miscanthus was ground using a hammer mill equipped with screens having 0.08, 2.0 or 6.0 mm sieve openings. Ground samples were subjected to hot water, dilute acid or dilute ammonium hydroxide pretreatment. Enzyme hydrolysis was conducted on washed pretreated solids; sugar generation was used as a measure for pretreatment efficiency. Glucan, xylan and total conversion yields were determined by comparing final sugar concentrations obtained to theoretical amounts present in raw biomass.

3.2 Materials and Methods

3.2.1 Materials

Lignocellulosic Substrate

Miscanthus x giganteus used in this study was cultivated in 2004 at the Energy Farm, University of Illinois at Urbana-Champaign. It was harvested, baled and stored in 2007. The baled grass was ground using a knife mill equipped with an 8 mm screen. Samples from the knife mill were processed further for this study. Compositional analysis of *Miscanthus* was conducted using a two step acid hydrolysis procedure developed by Ruiz and Ehrman (1996). *Miscanthus* (moisture content of 5.1%) contained 41.6% glucan, 20.6% xylan, 20.8% acid insoluble lignin, 0.7% acid soluble lignin and 5.8% extractives. Extractives contained sucrose (1.52 mg/g biomass), glucose (3.04 mg/g biomass), mannose (5.79 mg/g biomass) and fructose (3.66 mg/g biomass).

Enzymes and Chemicals

Enzymes used for hydrolysis were Accellerase 1500, Accellerase BG, Accellerase XY and Accellerase XC, which were obtained from Genencor International (Palo Alto, CA). Accellerase 1500 enzyme complex has the ability to hydrolyze lignocellulosic carbohydrates into fermentable monosaccharides. Accellerase 1500 contains exoglucanase, endoglucanase and β-glucosidase activities produced from a genetically modified strain of *Trichoderma reesei*. The endoglucanase activity was from 2200 to 2800 CMC U/g (carboxymethylcellulose activity units) and beta-glucosidase activity ranged from 450 to 775 pNPG U/g (p-nitrophenyl-B-D-glucopyranoside units). Accellerase BG, XY and XC are accessory enzymes that support Accellerase 1500 activity. Accellerase BG, a betaglucosidase is produced from a genetically modified strain of *Trichoderma reesei*, and has an activity of 3000 pNPG U/g. Accellerase XY, a hemicellulase enzyme complex, has

an activity of 20,000 to 30,000 ABX U/g (acid birchwood xylanase units) and also is produced from a genetically modified strain of *Trichoderma reesei*. Accellerase XC is produced from a selected strain of *Penicillium funiculosum*, and contains both xylan and glucan degrading enzymes. Accellerase XC has endoglucanase activities ranging from 1000 to 1400 CMC U/g and xylanase activities from 2500 to 3800 ABX U/g. Ammonium hydroxide (28%) and sulfuric acid (72%) used for pretreatments were obtained from Sigma-Aldrich (St. Louis, MO). Citric acid monohydrate and sodium hydroxide used for preparation of 1 M citrate buffer (pH 4.8) and sodium azide used as a preservative were obtained from Sigma-Aldrich (St. Louis, MO).

Pretreatment

Samples were pretreated in steel pipe reactors using a fluidized sand bath similar to methods described by Dien et al. (2004). Pretreatments were conducted in batch tubular reactors (20 mL total volume) using 0.75 inch O.D. x 0.065 inch wall thickness 316 stainless steel tubing (SS-T12-S-065-20, Swagelok, Chicago Fluid System Technologies, Chicago, IL). Tubing was cut to 4.125 inch lengths and capped on both ends by 0.75 inch 316 stainless steel Swagelok caps (SS-1210-C, Swagelok, Chicago Fluid System Technologies, Chicago, IL). One tube reactor was fitted with a 0.75 inch to 0.25 inch reducing union (SS-1210-6-4BT, Swagelok, Chicago Fluid System Technologies, Chicago, IL) at one end to accommodate a thermocouple (39105K212, Penetration/Immersion Thermocouple Probe Mini Conn (Pointed-Tip, Type K, -418 to 1652°F), McMaster-Carr, Robbinsville, NJ) for internal temperature measurements. Data from the thermocouple were recorded using a datalogger (HH306/306A, Datalogger Thermometer, Omega, Stamford, CT).

3.2.2 Methods

Particle Size Reduction and Distributions

Miscanthus samples ground to a sieve size of 8 mm using a knife mill were used as starting material. Samples were further ground using 6.0, 2.0 or 0.08 mm screen sizes using a hammer mill (1100W, model MHM4, Glen Mills, Clifton, NJ) (Figure 3.1). After grinding, moisture content of ground samples was determined using standard procedure NREL LAP-001 (Ehrman 1994). Particle size distributions were determined for 0.08 mm sieve size samples using a particle size analyzer (LA-300, Horiba, Kyoto, Japan). The analyzer uses angular light scattering techniques to determine particle size distributions of the sample suspended in liquid.

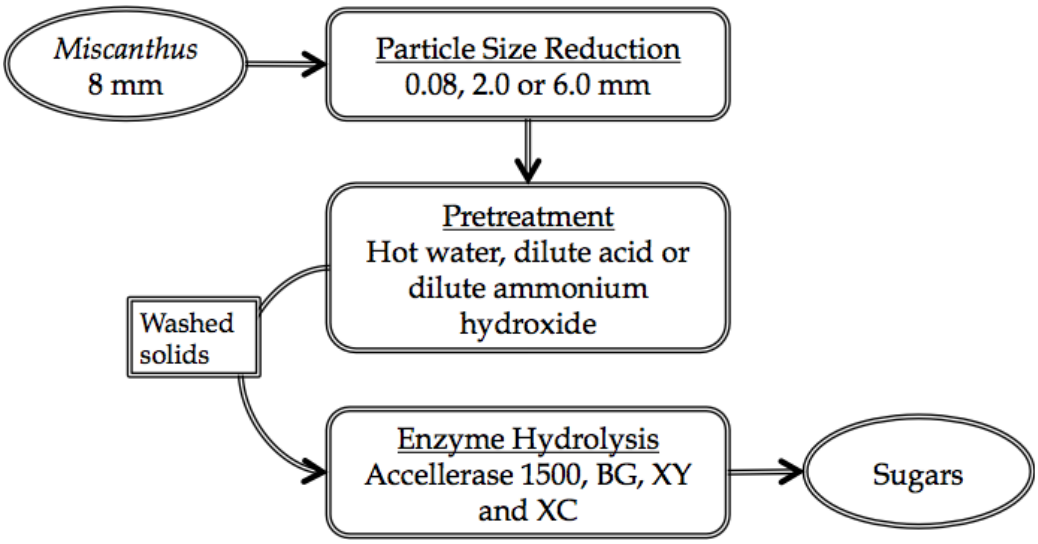

Figure 3.1. Experimental design.

Samples ground using the 6 and 2 mm screen were large and could not be analyzed using the particle analyzer. The larger sample particle size distribution was obtained using a sonic sifter (ATM model LP3, AdvanTech, New Berlin, WI) equipped with U.S. no. 30, 40, 60, 120, 325 and 400 screens (600, 425, 250, 125, 45 and 38 μm openings). Fractions retained on screens and undersize fractions were weighed to determine particle size distributions and geometric mean diameters. Particle size analyses were conducted in

triplicates. Particle size distributions were plotted and geometric mean diameters were compared.

Pretreatments

Each pretreatment was conducted at fixed conditions; parameters were selected from previously reported studies. Pretreatments were conducted at 10% solids (d.b.) with a loading of 1 g dry solids per tube, in duplicates.

Hot Water

Pretreatment parameters for hot water pretreatment were 200°C for 30 min, an intermediate operating condition for temperature (160 to 240°C) and time (0 to 60 min) as described by Dien et al. (2005). For hot water pretreatments, 9 mL water was added to dry solids.

Dilute Acid

Dilute acid pretreatments were performed at 160°C for 10 min with 1% w/w sulfuric acid based on standard procedure NREL LAP-007 (Hsu et al. 1995). For dilute acid pretreatment, 9 mL of 1% w/w sulfuric acid solution (prepared from 72% sulfuric acid solution) was added to solids in tube reactors.

Dilute Ammonium Hydroxide

Conditions for dilute ammonium hydroxide pretreatments were 160°C for 5 min with 5% ammonium hydroxide, modified from results reported by Murnen et al. (2007) for AFEX pretreatment of *Miscanthus*. For dilute ammonium hydroxide pretreatments, 9 mL of 5% ammonium hydroxide solution (prepared from 28% ammonium hydroxide) was added to solids.

After addition of reaction contents, tubes were capped and placed in the fluidized sand bath (IFB-51 Industrial Fluidized Bath, Techne Inc., Burlington, NJ) along with the tube reactor fitted with thermocouple. The sand bath was set 20°C higher than desired temperature to achieve quick heat up times. The thermocouple was used to determine when the desired internal temperature in the tubes was achieved. Once reactions were completed, tubes were cooled by quenching in cold water (4°C). Following pretreatment, liquid portions of samples were used for estimation of total sugars using dilute acid hydrolysis, NREL LAP-014 (Ruiz and Ehrman 1996a). Solids were collected, washed and moisture contents were determined. Pretreated solids were washed using centrifugation procedures described by Edy et al. (1998). Solids were emptied into pre-weighed 50 mL centrifuge tubes (Corning Inc., NY). Distilled water was added to tubes, vortexed and centrifuged at 1500 x g (IEC CL30, Thermo Scientific, Asheville, NC) for 10 min. Supernatant was discarded carefully to avoid loss of solids. Washing steps were repeated till final pH of wash water was between 5 and 7.

Enzymatic Hydrolysis

Following pretreatment with hot water, dilute acid or dilute ammonium hydroxide, enzyme hydrolysis was conducted similar to procedure discussed in NREL LAP-009 (Brown and Torget 1996), with modifications. Hydrolysis was conducted at 10% solids content (d.b.) in 50 mL centrifuge tubes (Corning Inc., NY). Washed pretreated solids with known moisture content were added to pre-weighed tubes. Citrate buffer (1 M) was added to obtain a final concentration of 0.05 M. Sodium azide was added to a final concentration of 0.005% to prevent microbial contamination. Enzyme dosages were based on dry solids content of washed pretreated biomass and highest dosage levels were selected from manufacturers recommended range. Accellerase 1500 was added at 0.25 mL/g biomass, Accellerase XY at 0.05 mL/g biomass, Acellerase XC at 0.125 mL/g biomass and Accellerase BG at 0.09 mL/g biomass. Distilled water was added to bring the volume of

reaction to 10% solids content. Substrate blank flasks were prepared for each sample similar to reaction flasks with the exception of enzyme addition. An enzyme blank flask was also prepared consisting of all reaction constituents except substrate. Hydrolysis was performed on all reaction, substrate blank and enzyme blank flasks in a water bath (Gyromax 939XL, Amerex Instruments, Inc., Lafayette, CA) set at 50°C and 75 rpm. Aliquot samples (0.5 mL) were taken at 3, 12, 24, 48 and 72 hr for glucose and xylose determinations. Each sample was centrifuged at 11,230 x g (Model 5415 D, Brinkmann-Eppendorf, Hamburg, Germany) and supernatant analyzed using HPLC.

HPLC Analyses

Liquid samples were filtered through a 0.2 μm syringe filter into 200 μL HPLC vials. Filtered liquid was injected into an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C. Glucose and xylose concentrations were measured using HPLC with a refractive index detector (model 2414, Waters Corporation, Milford, MA). Data were processed using HPLC software (Waters).

Data Analysis

Particle size analyses were conducted in triplicates. Particle size distributions were generated and geometric mean diameters compared among samples. Each pretreatment and enzymatic hydrolysis was carried out in triplicates. Glucose and xylose profiles were generated for all samples. Hydrolysis rates were calculated from linear portions (0 to 24 hr) of glucose profiles. Glucose and xylose concentrations from HPLC and *Miscanthus* compositional analysis results were used to determine conversion yields as follows:

$$Glucose_{theoretical} = \frac{W \times Glucan}{0.9} \tag{3.1}$$

$$Xylose_{theoretical} = \frac{W \times Xylan}{0.88} \tag{3.2}$$

$$Glucan\ Conversion(\%) = \frac{Glucose_{72} \times Volume}{Glucose_{theoretical}} \tag{3.3}$$

$$Xylan\ Conversion(\%) = \frac{Xylose_{72} \times Volume}{Xylose_{theoretical}} \tag{3.4}$$

$$Total\ Conversion(\%) = \frac{(Glucose + Xylose)_{72} \times Volume}{(Glucose + Xylose)_{theoretical}} \tag{3.5}$$

where;

Glucan	glucan content in *Miscanthus* (41.6%)
Xylan	xylan content in *Miscanthus* (20.6%)
W	sample weight (d.b.) added to pretreatment tubes
0.9	conversion factor of glucose to equivalent glucan
0.88	conversion factor of xylose to equivalent xylan
$Glucose_{72}$	glucose concentration (% w/v) at 72 hr
$Xylose_{72}$	xylose concentration (% w/v) at 72 hr
Volume	volume of enzymatic hydrolysis reaction (mL)

Experimental setup was randomized, with analysis of variance (ANOVA) and Fishers least significant difference test (SAS) used to compare geometric mean diameters, final sugar concentrations, hydrolysis rates and conversions. Level of statistical significance was set at 5% ($P<0.05$).

3.3 Results and Discussion

3.3.1 Particle Size Distributions

Particle size distributions were determined and plotted as histograms (Figure 3.2). Geometric mean diameters also were calculated to allow for comparisons among samples (Table 3.1). Mean particle diameter varied depending upon the screen size. Sample ground

using the smallest screen (0.08 mm) produced particles with the smallest geometric mean diameter (56 μm). Samples ground using 2 and 6 mm screens resulted in higher geometric mean diameters (300 and 695 μm, respectively). Standard deviations for geometric mean diameters for samples ground using 2 and 6 mm screen sizes were higher than for samples from 0.08 mm screen size (Table 3.1). Sieving was used on dry samples to broadly classify particle size distributions (Dibble et al. 2011, Zhu et al. 2009).

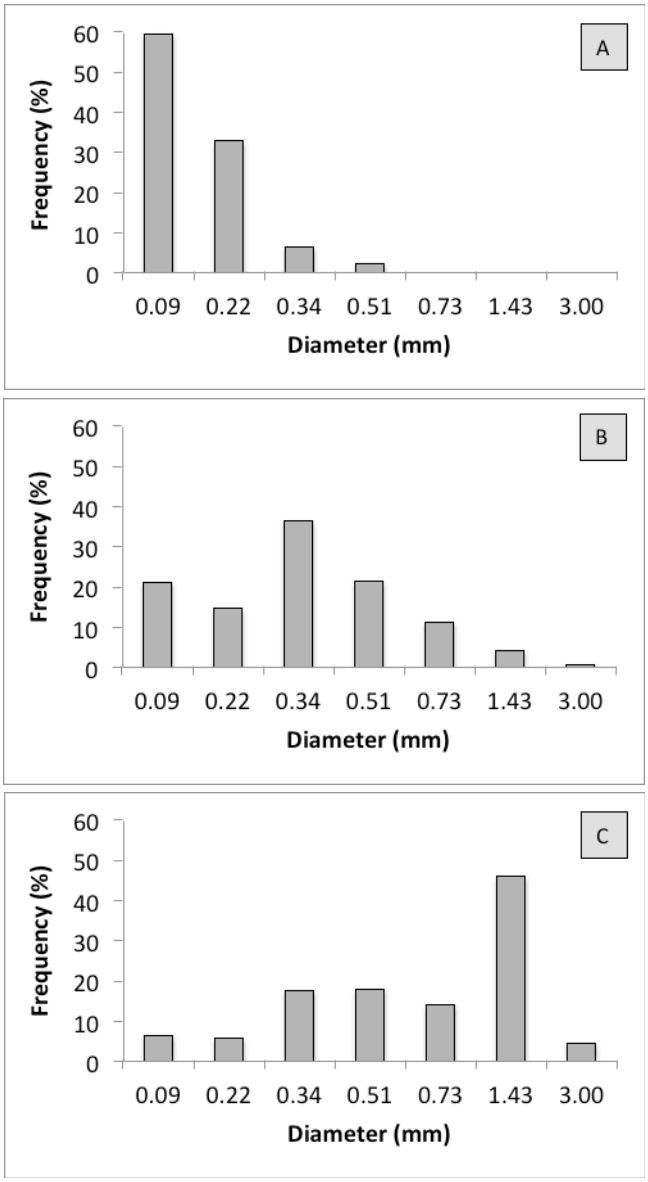

Figure 3.2. Particle size distribution for *Miscanthus* ground using different hammer mill sieve sizes A. 0.08 mm B. 2.0 mm and C. 6.0 mm.

Table 3.1. Geometric mean diameters (μm) for samples ground using different sieve sizes

Sieve Size (mm)	Geometric Mean Diameter[1] (μm)
0.08	56.00 ± 0.54 C
2.00	300.5 ± 4.10 B
6.00	695.3 ± 69.1 A

[1]Mean diameters followed by the same letter in a column are not different ($P < 0.05$)

3.3.2 Effect of Particle Size on Pretreatments

Hot Water

Mean particle size influenced most of the properties measured for enzymatic saccharification including final glucose concentration, rate, and final conversion efficiency (e.g. % glucan present extracted as monosaccharides) (Tables 3.2 and 3.3). The effect on xylose saccharification was less apparent, because much less xylose was released compared to glucose. Samples ground using the smallest screen (0.08 mm) resulted in highest glucose concentrations compared to 2 and 6 mm screen sizes. Glucose release rates were highest from 0.08 mm (0.143% w/v/hr) compared to 2 mm (0.122% w/v), which was higher than 6 mm (0.107% w/v). Glucan conversion (defined as the amount of glucose released compared to theoretical glucose in biomass) was highest from 0.08 mm screen (80.5%), followed by 2 mm (70.9%) and 6 mm (60.7%) (Table 3.3). No differences were observed in xylose release rate, xylose yield and xylan conversion (Tables 3.2 and 3.3). Low xylose concentrations could be due to hemicellulose extraction during pretreatments, and hence low xylan content for enzyme hydrolysis or inefficient enzymes (Hendriks and Zeeman 2009; Negro et al. 2003). Successful enzymatic conversion requires adequate debranching activities to release side groups from xylan as well as sufficient β-xylosidase activity to avoid end product inhibition. Total conversions were highest from 0.08 mm screen size (55.4%) compared to 2 mm (49.0%), which was higher than 6 mm (42.1%).

Most investigators reported no effect of particle size reduction on sugar or ethanol yields of hot water pretreated biomass (Vidal et al. 2011). Zheng et al. (2007) reported no

differences in corn stover glucose conversion (%) for two particle sizes, 53 to 75 μm and 425 to 710 μm, when pretreated at 190°C for 10 min using hot water. However, different mean particle sizes were generated by sifting corn stover previously ground using 2 mm sieve screens. Sieving ground biomass samples has been shown to fractionate into sizes with different physical and chemical compositions (Chundawat et al. 2006, Lamsal et al. 2010). Large particle size fractions for corn stover were more recalcitrant than smaller sizes (Chundawat et al. 2006). Lamsal et al. (2010) reported a decrease in hemicellulose content in wheat bran from 47% to 32% in sieve fraction with particle size <132 μm. They showed similar trends for soybean hulls and wheat straw, with a lignin decrease in finer fractions for wheat straw. Negro et al. (2003) using two size ranges (2 to 5 mm and 12 to 15 mm) showed no differences in ethanol yields, but again this experiment was conducted by sifting ground biomass into two sample sizes.

Table 3.2. Final sugar concentrations (% w/v) following enzymatic saccharification and hydrolysis rates (% w/v/hr)

Pretreatment	**Sieve Size** (mm)	**Glucose**[1] (% w/v)	**Xylose**[2] (% w/v)	**Glucose Release Rate**[3] (% w/v/hr)	**Xylose Release Rate**[4] (% w/v/hr)
Hot Water	0.08	6.30 ± 0.18 A	0.19 ± 0.01 B	0.143 ± 0.004 A	0.004 ± 0.000 A
	2.00	5.55 ± 0.22 B	0.19 ± 0.01 AB	0.122 ± 0.005 B	0.004 ± 0.001 A
	6.00	5.31 ± 0.30 B	0.20 ± 0.01 A	0.107 ± 0.006 C	0.004 ± 0.001 A
Dilute Acid	0.08	5.41 ± 0.63 A	0.20 ± 0.03 A	0.098 ± 0.006 A	0.016 ± 0.021 A
	2.00	5.06 ± 0.56 A	0.24 ± 0.04 A	0.096 ± 0.004 AB	0.003 ± 0.004 A
	6.00	4.56 ± 0.66 A	0.22 ± 0.01 A	0.085 ± 0.007 B	0.005 ± 0.003 A
Dilute Ammonium Hydroxide	0.08	4.30 ± 0.16 A	1.89 ± 0.11 A	0.061 ± 0.004 A	0.020 ± 0.001 A
	2.00	2.81 ± 0.19 B	1.57 ± 0.12 B	0.040 ± 0.003 B	0.021 ± 0.002 A
	6.00	3.01 ± 0.37 B	1.71 ± 0.18 AB	0.036 ± 0.002 B	0.022 ± 0.002 A
No Pretreatment	0.08	1.04 ± 0.05 A	0.34 ± 0.02 A	0.014 ± 0.002 A	0.006 ± 0.001 A
	2.00	0.59 ± 0.02 B	0.21 ± 0.01 B	0.005 ± 0.001 B	0.002 ± 0.0001B
	6.00	0.53 ± 0.04 B	0.16 ± 0.01 C	0.004 ± 0.001 B	0.002 ± 0.0001B

Mean ± Standard Deviation

[1]Mean glucose concentrations followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[2]Mean xylose concentrations followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[3]Mean glucose release rates followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[4]Mean xylose release rates followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

Dilute Acid

An increase in glucan conversion and total polysaccharide conversion was observed when biomass size was reduced prior to dilute acid pretreatment. No differences were observed in glucose and xylose concentrations from size reduction (Table 3.2). Glucose release rate was higher from 0.08 mm (0.098% w/v/hr) compared to 6 mm (0.085% w/v/hr), but was not different from 2 mm (0.095% w/v/hr). Glucan conversions for 0.08 mm sample were higher (70.2%) than 2 and 6 mm (56.0 and 52.1%, respectively) (Table 3.3). Total conversions also were highest from 0.08 mm sample (48.3%). Xylose concentrations and xylan conversions observed was low due to hydrolysis of hemicellulose during pretreatment (Hendriks and Zeeman 2009).

A number of studies have been conducted to determine the effect of particle size on dilute acid pretreatments using various size ranges and biomass types (Vidal et al. 2011). In recent studies, Dibble et al. (2011) showed improved enzyme digestibility when size reduction was a result of dilute acid pretreatment severity over mechanical methods for size reduction. Hsu et al. (1996) demonstrated increased switchgrass digestibility from 60 to 80% when particle size was decreased from 10 to 3 mm.

However, no differences were observed when dilute acid pretreated biomass of different particle sizes was homogenized prior to enzyme hydrolysis, showing that particle size did not have an effect on pretreatment efficacy. Lamsal et al. (2010) conducted dilute acid pretreatment on different sieved fractions of ground soybean hulls, wheat straw and wheat bran. They observed highest sugar release from particle size fraction <132 μm across all biomass types. However fractionating biomass changed the chemical composition; particle size fraction <132 μm had reduced lignin (20 to 5%) and hemicellulose content.

Table 3.3. Conversion (%) for pretreatments using different particle sizes

Pretreatment	**Sieve Size** (mm)	**Glucan Conversion**[1] (%)	**Xylan Conversion**[2] (%)	**Total Conversion**[3] (%)
Hot Water	0.08	80.5 ± 2.34 A	4.70 ± 0.30 B	55.4 ± 1.65 A
	2.00	70.9 ± 2.88 B	4.70 ± 0.17 A	49.0 ± 1.97 B
	6.00	60.7 ± 3.40 C	4.50 ± 0.20 A	42.1 ± 2.35 C
Dilute Acid	0.08	70.2 ± 4.40 A	5.07 ± 0.35 A	48.3 ± 3.00 A
	2.00	56.0 ± 7.22 B	5.30 ± 0.75 A	39.0 ± 4.56 B
	6.00	52.1 ± 6.60 B	4.97 ± 0.21 A	36.3 ± 4.45 B
Dilute Ammonium Hydroxide	0.08	71.4 ± 2.75 A	62.0 ± 3.49 A	68.2 ± 3.00 A
	2.00	37.8 ± 0.69 B	41.8 ± 0.81 B	39.1 ± 0.60 B
	6.00	40.8 ± 2.22 B	45.7 ± 2.11 B	42.4 ± 2.16 B
No Pretreatment	0.08	22.5 ± 1.13 A	14.5 ± 0.73 A	19.8 ± 0.99 A
	2.00	12.8 ± 0.47 B	9.00 ± 0.17 B	11.6 ± 0.37 B
	6.00	11.4 ± 0.96 B	7.00 ± 0.52 B	9.92 ± 0.81 C

Mean ± Standard Deviation

[1]Mean glucan conversions followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[2]Mean xylan conversions followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[3]Mean total conversions followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

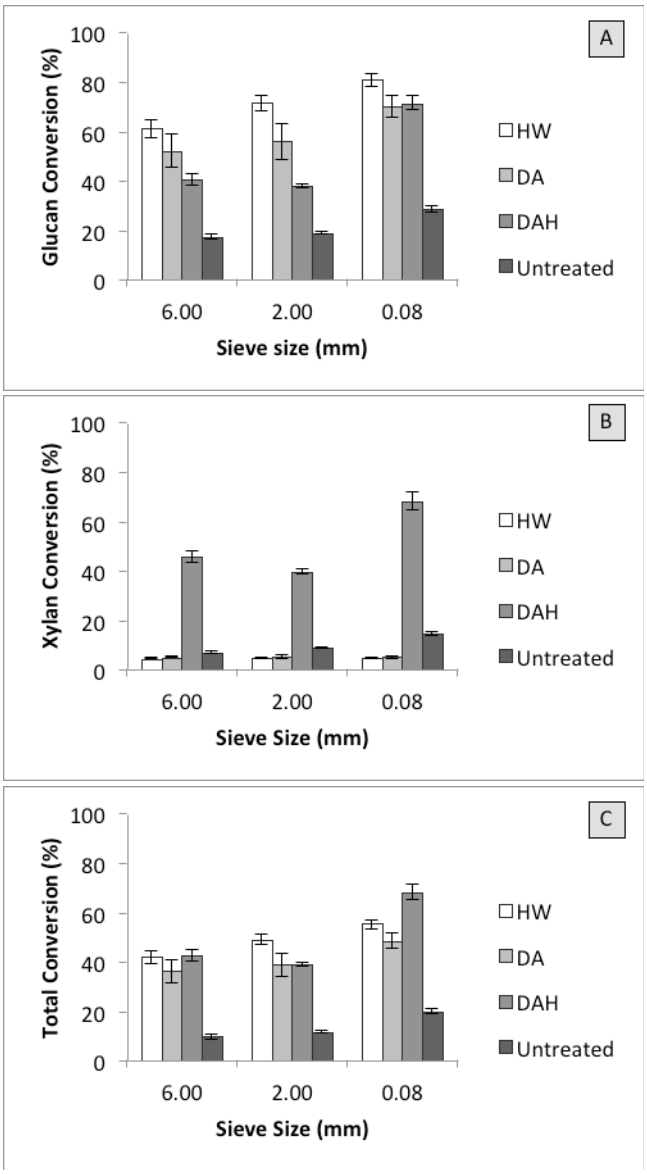

Figure 3.3. Comparison of conversion (%) using hot water (HW), dilute acid (DA) and dilute ammonium hydroxide (DAH) with untreated raw *Miscanthus* A. Glucan conversion (%) B. Xylan conversion (%) C. Total sugar conversion (%).

Dilute Ammonium Hydroxide

Size reduction only had an effect on sugar yields for the smallest versus two larger sized fractions (Figure 3.3). Glucose concentrations were higher from 0.08 mm screen samples (4.30% w/v) compared to 2 and 6 mm (2.81 and 3.01% w/v, respectively)

(Table 3.2). Glucose release rates were also higher from 0.08 mm (0.061% w/v/hr) compared to 2 and 6 mm samples (0.040 and 0.036% w/v/hr, respectively). Xylose concentrations from 0.08 mm (1.89% w/v) were higher than 2 mm (1.57% w/v), but were not different from 6 mm screen size sample (1.71% w/v). No differences were observed in xylose release rates across all particle sizes. Glucan conversions for samples from 0.08 mm screen were 30.6 to 33.6% higher than 6 and 2 mm screen; xylan conversion for 0.08 mm samples were 16.3 to 20.2% higher than 6 and 2 mm samples (Table 3.3). Overall, 0.08 mm samples gave higher total polysaccharide conversion (68.2%) compared to 2 and 6 mm samples (39.1 and 42.4%, respectively). Xylose concentrations and xylan conversions were higher in dilute ammonium hydroxide pretreatment compared to hot water and dilute acid pretreatments since it resulted in lower hemicellulose solubilization than acid and hydrothermal pretreatments (Alvira et al. 2010).

Li et al. (2004) observed similar results with dilute alkali pretreatment of corn stover ground to three different sizes (2.00, 0.707 and 0.25 mm). Decreasing size increased glucose yields; they concluded 0.707 mm was a sufficient size reduction because the increase from 0.707 to 0.25 mm was 9.6% compared to 30% from 2 mm to 0.707 mm. Other studies conducted on effect of particle size on pretreatments using alkaline agents include work reported by Chundawat et al. (2006) where particle size reduction increased glucose yields from AFEX (Ammonia Fiber Expansion). Chang et al. (1997) reported increase in sugar yields with decreasing particle size up to 20 mesh. Sizes below 20 mesh did not increase sugar yields.

No Pretreatment

Samples ground using different sieve screens were evaluated for sugar release from enzyme hydrolysis without any chemical pretreatment, to evaluate the effect of size reduction alone. Glucose and xylose concentrations were highest from 0.08 mm screen (1.04 and 0.34% w/v) compared to 2 and 6 mm screens (0.59 and 0.21% w/v, 0.53 and 0.16%

w/v, respectively) (Table 3.2). Glucan conversions increased from 13 to 23% between 2 and 0.08 mm screens (Table 3.3). Xylose yields increased from 9 to 15% when screen size was decreased from 2 to 0.08 mm. Smallest screen sieve, 0.08 mm, resulted in highest total conversion (20%), followed by 2 mm (12%), which was higher than 6 mm (10%).

3.4 Conclusions

Across all pretreatments, an increase in total polysaccharide conversion (12 to 26%) was observed when *Miscanthus* particle size was decreased from 6.0 mm to 0.08 mm. Glucan conversion increased (18 to 31%) when particle size was decreased (6.0 to 0.08 mm) for all pretreatments. Increased xylan conversion (16%) with decreasing particle size was observed for dilute ammonium hydroxide pretreatment; dilute acid and hot water pretreatments cause xylan breakdown during the pretreatment process. Unpretreated biomass samples also had increased total conversion (10%) with decreasing particle size (from 6.0 to 0.08 mm), although total conversions were lower (20 to 60%) compared to chemical pretreatments. Increased percent conversion could be attributed to increased surface area and improved mass and heat transfer during pretreatment.

Chapter 4

Evaluation of Pretreatment Conditions Using Hot Water

4.1 Introduction

As discussed in the literature review, there are numerous chemical pretreatments available for grasses. These include dilute acid, dilute ammonium hydroxide, alkaline peroxide, steam explosion with and without catalyst, hot water, alkali, ammonia fiber expansion (AFEX), supercritical fluid, oxidation (e.g. ozone) and ionic liquids (Alvira et al. 2010, Dien et al. 2005, Kumar et al. 2009, Sun and Cheng 2002, Taherzadeh and Karimi 2008). The most widely published method is dilute acid pretreatment. Dilute acid is advantageous because it is highly effective on a wide variety of biomasses, and it chemically hydrolyzes hemicellulose to monosaccharides, avoiding the need for xylanases. However, the use of dilute acid as a catalyst has its disadvantages. Dilute acid pretreatments generate enzymatic and microbial inhibitors, require neutralization resulting in production of gypsum (low value product) if calcium hydroxide is used and increases capital costs to purchase reactors rated to withstand acid at high temperatures (Dien et al. 2005). Pretreatments utilizing alkali agents also require neutralization that adds to processing costs. Recycling of solvents and ammonia must be considered for scalability of organosolv and AFEX pretreatments, respectively (Sun and Cheng 2002, Taherzadeh and Karimi 2008). Use of oxidizing agents, supercritical fluids and ionic fluids are expensive and that limits their use at a commercial scale.

The simplest pretreatment method is hot water because no external catalyst is added for the reaction. Hot water or hydrothermal pretreatments involve reacting

lignocellulosic at high temperatures (e.g. 160 to 240°C) solely in water (Dien et al. 2005). At these high temperatures water acts as a weak acid and, furthermore, released organic acids bonded to hemicellulose that further acidifies the solution. Hot water partially hydrolyzes hemicellulose to soluble oligosaccharides and loosens the lignin network (Taherzadeh and Karimi 2008). Hot water pretreatments are advantageous because they do not require the addition of chemicals and allow for less expensive grades of steel to be used for reactor construction compared to dilute acid catalyzed pretreatments (Mosier et al. 2005). It also avoids both catalyst and neutralization costs. Hot water pretreatment conditions also can be tuned to maximize production of oligosaccharides and minimization of monosaccharides from hydrolysis of hemicellulose (Mosier et al. 2005). This in turn minimizes production of furfural, a potent inhibitor of microbial fermentation. Hot water pretreatments have been applied to many substrates (Table 4.1) but outside of this thesis not previously to *Miscanthus x giganteus*.

In Chapter 3, hot water pretreatment was observed to give superior sugar yields following enzymatic hydrolysis compared to dilute acid or alkaline pretreatment. In this chapter, hot water pretreatment conditions that result in high glucose and xylose yields after enzymatic hydrolysis of *Miscanthus* were investigated. Pretreatment parameters evaluated were temperature (160, 180 and 200°C) and reaction time (0, 10, 20 and 30 min). Pretreated solids from the best pretreatment conditions were investigated further for ethanol yield using simultaneous saccharification and fermentation.

Table 4.1. Reported reaction conditions for hot water pretreatment of various feedstocks

Feedstock	**Pretreatment Conditions**			**Enzyme Loading**	**Sugar/Ethanol Yield**	**Reference**
	Temperature	**Time**	**Solids Loading**			
Switchgrass	200°C	10 min	16.6%	30 mg protein/g glucan	70% glucose (24 hr)	Shi et al. 2011
Yellow Poplar Sawdust	220°C	2 min	5%	25 IFPU cellulase/g cellulose	97% ethanol yield (from glucan), 85% xylan recovery	Allen et al. 2001
Corn Stover	200°C	24 min	Continuous flow of water at 10 mL/min, 20% solids loading	-	96% total sugar (glucose and xylose) yield	Liu and Wyman 2005
Corn Stover	190°C	15 min	16.6%	15 FPU cellulase/g glucan	88% ethanol	Mosier et al. 2005
Eucalyptus grandis	Step 1: 180°C, step 2: 200°C	Step 1: 20 min, step 2: 20 min	-	-	96.63% total sugar recovery after two step pretreatment	Yu et al. 2010

Table 4.1. (cont.) Reported reaction conditions for hot water pretreatment of various feedstocks

Feedstock	**Pretreatment Conditions**			**Enzyme Loading**	**Sugar/Ethanol Yield**	**Reference**
	Temperature	**Time**	**Solids Loading**			
Corn Fiber	215°C	2 min	5%	15 FPU cellulase/g cellulose	86% glucan to ethanol	Allen et al. 2001
Sugarcane Bagasse	220°C	2 min	3%	15 FPU cellulase/g cellulose, 4 IU β-galactosidase/FPU cellulase	93% cellulose conversion	Laser et al. 2001
Sorghum Bagasse	210°C	20 min	15%	-	36.8% total polysaccharide conversion	Dogaris et al. 2009
Sugarcane Bagasse	220°C	120 sec	15-25 g biomass, 0.6 to 1.2 kg water	-	Fractionated biomass; 90% hemicellulose, 10% cellulose, >60% lignin solubilized after pretreatment	Allen et al. 1996

Table 4.1. (cont.) Reported reaction conditions for hot water pretreatment of various feedstocks

Feedstock	**Pretreatment Conditions**			**Enzyme Loading**	**Sugar/Ethanol Yield**	**Reference**
	Temperature	**Time**	**Solids Loading**			
Sugarcane Bagasse (for xylose recovery)	170°C	2 hr	5%	-	78% xylose recovery	Boussarsar et al. 2009
Sugarcane Bagasse, Aspen Chips, Hardwood Flour	220°C	120 sec	10 to 15 g biomass (d.b.)	15 to 30 FPU cellulase/g	90% total conversion	Peter van Walsem et al. 1996
Wheat Straw (Two Stage)	188°C	40 min	10%	15 FPU Celluclast, 15 IU β-glucosidase Novo 188	79.8% glucose and 20.5% xylose yield after enzyme hydrolysis	Perez et al. 2008
Alfalfa Fiber	220°C	2 min	30 g biomass	2 to 4% w/v cellulase	59 to 65% reducing sugar	Sreenath et al. 1999
Prarie Cord Grass	210°C	10 min	Parr reactor	15 FPU NS50013/g, 60 CBU β-glucosidase/g	94.5% hydrolysis conversion	Cybulska et al. 2010

4.2 Materials and Methods

4.2.1 Materials

Biomass

Lignocellulosic feedstock used for this study was *Miscanthus x giganteus*, cultivated in 2004 at the Energy Farms, University of Illinois, Urbana-Champaign. *Miscanthus* was harvested, baled and stored in 2007. A knife mill (1500W, Retsch Inc., Newtown, PA) equipped with an 8 mm screen was used to cut baled grass. Further size reduction was achieved using a hammer mill (1100W, model MHM4, Glen Mills, Clifton, NJ) equipped with a 0.08 mm screen. Moisture content of ground samples were determined using a standard procedure (LAP-001) developed at NREL (Ehrman, 1994).

Enzymes and Chemicals

Enzymes used for hydrolysis were Accellerase 1500, Accellerase BG, Accellerase XY and Accellerase XC obtained from Genencor International (Palo Alto, CA). Accellerase 1500 enzyme complex has the ability to hydrolyze lignocellulosic carbohydrates into fermentable monosaccharides and contains exoglucanase, endoglucanase and β-glucosidase activities produced from a genetically modified strain of *Trichoderma reesei*. Endoglucanase activity was from 2200 to 2800 CMC U/g (carboxymethylcellulose activity units) and β-glucosidase activity ranged from 450 to 775 pNPG U/g (p-nitrophenyl-B-D-glucopyranoside units). Accellerase BG, XY and XC are accessory enzymes that support Accellerase 1500 activity. Accellerase BG, a beta-glucosidase is produced from a genetically modified strain of *Trichoderma reesei*, and has an activity of 3000 pNPG U/g. Accellerase XY, a hemicellulase enzyme complex, has an activity of 20,000 to 30,000 ABX U/g (acid birchwood xylanase units) and also is produced from a genetically modified strain of *Trichoderma reesei*. Accellerase XC is produced from a

selected strain of *Penicillium funiculosum* and contains both xylan and glucan degrading enzymes. Accellerase XC has endoglucanase activities ranging from 1000 to 1400 CMC U/g and xylanase activities from 2500 to 3800 ABX U/g. Citric acid monohydrate and sodium hydroxide used for preparation of 1 M citrate buffer (pH 4.8) and sodium azide, used as a preservative, were obtained from Sigma-Aldrich (St. Louis, MO).

Pretreatment

Samples were pretreated in steel pipe reactors using a fluidized sand bath similar to methods described by Dien et al. (2004). Pretreatments were conducted in batch tubular reactors (Figure 4.1) designed using 1.00 inch O.D. x 0.065 inch (25.4 x 1.65 mm) wall thickness 316 stainless steel tubing (SS-T16-S-065-20, Swagelok, Chicago Fluid System Technologies, Chicago, IL). Tubing was cut to 7.24 inch (183.9 mm) lengths and capped on both ends by 1.00 inch (25.4 mm) 316 stainless steel Swagelok caps (SS-1610-C, Swagelok, Chicago Fluid System Technologies, Chicago, IL). One tube reactor was fitted with a 1.00 inch to 0.25 inch (25.4 to 6.35 mm) reducing union (SS-1610-6-4BT, Swagelok, Chicago Fluid System Technologies, Chicago, IL) at one end to accommodate a thermocouple (39105K212, Penetration/Immersion Thermocouple Probe Mini Conn (Pointed-Tip, Type K, -418 to 1652F), McMaster-Carr, Robbinsville, NJ) for internal temperature measurements. Data from the thermocouple were recorded using a datalogger (HH306/306A, Datalogger Thermometer, Omega, Stamford, CT). Tubes were incubated in a fluidized sand bath (IFB-51 Industrial Fluidized Bath, Techne Inc., Burlington, NJ) to attain desired temperatures with rapid heat transfer rates.

Figure 4.1. Batch tubular reactor and reactor fitted with thermocouple.

4.2.2 Methods

Hot Water Pretreatment

Three temperature conditions (160, 180 and 200°C) and four reaction times (0, 10, 20 and 30 min) were evaluated using hot water pretreatment (Figure 4.2). Pretreatments were conducted at 15% solids content (d.b.) with 6.75 g dry solids added per tube. Tubes were capped after addition of biomass and distilled water. The tube reactor fitted with a thermocouple was filled with distilled water only. All tubes were immersed in a fluidized sand bath set 20°C higher than the desired temperature. Higher temperatures were used to achieve more rapid heat up times within each tube. Once the desired temperature was attained (time taken to reach temperature considered as 0 min) within the tubes, pretreatments were conducted for 10, 20 or 30 min. For 0 min, tubes were removed as soon as desired internal temperatures were achieved. Temperature profiles for 160, 180 and 200°C for a reaction time of 10 min are shown in Figure 4.3. Similar temperature profiles were obtained at other retention times.

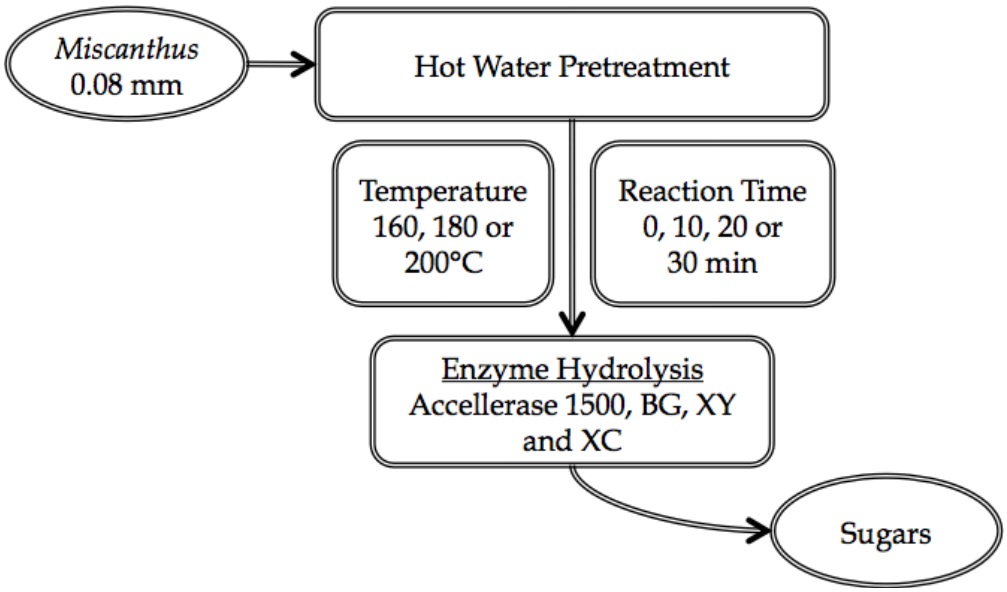

Figure 4.2. Experimental design.

Pretreatments at 0 min were conducted to evaluate effect of heat up time on biomass conversion. Once complete, reactions were stopped by immersing tubes in cold water (4°C). Pretreated solids were washed and moisture contents determined. Washed solids were stored at 4°C until further use.

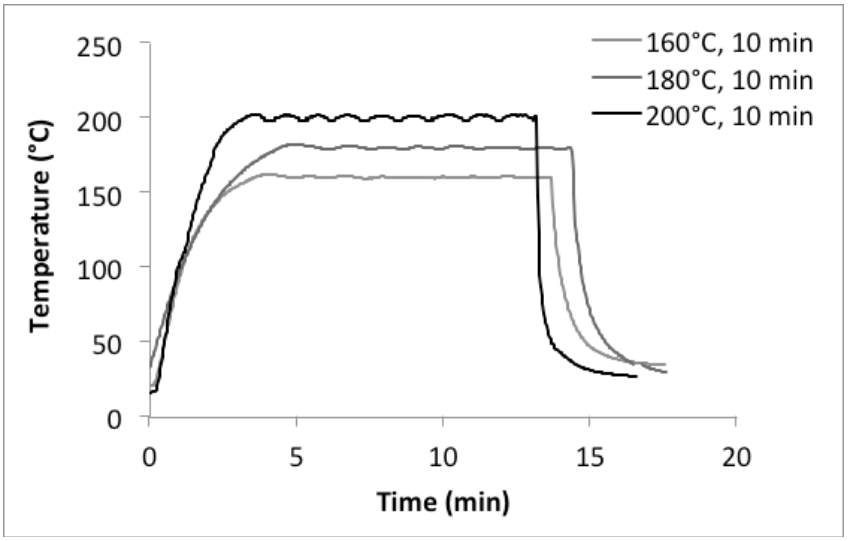

Figure 4.3. Temperature profiles during hot water pretreatment.

Enzyme Hydrolysis

Enzyme hydrolysis was conducted using a similar procedure described in NREL LAP-009 (Brown and Torget 1996). Hydrolysis was conducted at 10% solids content (d.b.) in 50 mL centrifuge tubes (Corning Inc., NY). Washed pretreated solids with known moisture content were added to preweighed tubes. Citrate buffer (1 M) was added to a final concentration of 0.05 M. Sodium azide was added to a final concentration of 0.005% to prevent microbial contamination. Based on manufacturers highest enzyme dosage recommendations, Accellerase 1500, XY, XC and BG were added at 0.25, 0.05, 0.125 and 0.09 mL/g biomass, respectively. Highest dosages were selected to eliminate enzyme activity limitations in hydrolysis experiments. Distilled water was added to bring the volume of reaction to 10% solids content. Substrate blanks were prepared for each sample similar to reaction flasks with no enzyme addition. An enzyme blank also was prepared consisting of all reaction constituents except substrate. Hydrolysis was performed on all reaction, substrate blank and enzyme blank tubes in a water bath (Gyromax 939XL, Amerex Instruments, Inc., Lafayette, CA) set at 50°C and 75 rpm. Aliquot samples (0.5 mL) were taken at 3, 12, 24, 48 and 72 hr for glucose and xylose determinations. Each sample was centrifuged at 11,230 x g (Model 5415 D, Brinkmann-Eppendorf, Hamburg, Germany) and supernatant analyzed using HPLC.

Simultaneous Saccharification and Fermentation

The pretreatment condition with highest total percent conversion to sugars was selected for simultaneous saccharification and fermentation experiments. Fermentation experiments were conducted at the National Center for Agricultural Utilization Research (NCAUR) (USDA, Peoria, IL). Moisture contents of washed pretreated solids were determined by measuring weight loss after drying at 105°C for 24 hr (Ehrman 1994). Total glucan and xylan were measured using a two stage acid hydrolysis protocol (Ruiz and Ehrman 1996). Washed pretreated solids (1 g d.b.) were added to 25 mL bottles (Corning

Inc., NY) and autoclaved for 15 min. Upon cooling, 4 mL sterile water, 0.5 mL 1 M citric acid (pH 4.5) and 1 mL 10% yeast peptone solution were added to all bottles. Filter sterilized enzymes added were Optiflow RC2 cellulase (15 FPU/g glucan), Novo 188 cellobiase (40U/g glucan) and Multifect pectinase (50 U/g glucan). Enzyme blanks were prepared by adding enzymes to 10 mL sterile water, 0.5 mL 1 M citric acid and 1 mL 10x yeast extract-peptone stock (100 g/L yeast extract and 200 g/L peptone).. *Saccharomyces cerevisiae* strain D5A was used for fermentations. For inoculum preparation, *S. cerevisiae* was transferred from -80°C to YP2D (10 g/L yeast extract, 20 g/L protease peptone, 40 g/L dextrose) plates and incubated at 32°C. A single colony was transferred from plates to 10 mL YP2D media and incubated overnight at 35°C. The culture was used to inoculate a seed flask containing 25 mL YP2D media and incubated at 35°C. Cells were concentrated to an optical density (OD) of 50 at 600 nm using a phosphorus saline buffer solution (8.5 g/L sodium chloride, 0.3g/L anhydrous potassium dihydrogen phosphate, 0.6 g/L anhydrous sodium monohydrate phosphate, 0.4 g/L peptone). Yeast inoculum was added at a final O.D.600 of 0.5 equivalent to 0.5 mL/bottle. Bottles were incubated at 35°C, 100 rpm for 72 hr. Final concentrations for monosaccharides and ethanol were measured using HPLC equipped with an organic acid column (Aminex HPX-87H, Bio-Rad, Hercules, CA).

HPLC Analyses

Glucose, xylose and ethanol were measured using HPLC. Samples (20 L) were injected onto an ion exclusion column (Aminex HPX-87H, Bio-Rad, Hercules, CA) maintained at 50°C and eluted at 0.6 ml/min with 5 mM sulfuric acid. Concentrations were measured using a refractive index detector (model 2414, Waters Corporation, Milford, MA). Data were processed using HPLC software (Waters).

Data Analysis

The experimental design for the study was a completely randomized 3x4 full factorial. Each pretreatment and enzyme hydrolysis combination was conducted in triplicates. Glucose and xylose determinations using HPLC were used to generate profiles. Glucose and xylose hydrolysis rates were determined from the slope of the linear portion (0 to12 hr) of glucose and xylose profiles. Final glucose and xylose concentrations were compared to theoretical glucose and xylose contents in *Miscanthus* to determine conversion yields:

$$Glucose_{theoretical} = \frac{W \times Glucan}{0.9} \tag{4.1}$$

$$Xylose_{theoretical} = \frac{W \times Xylan}{0.88} \tag{4.2}$$

$$Glucan\ Conversion(\%) = \frac{Glucose_{72} \times Volume}{Glucose_{theoretical}} \tag{4.3}$$

$$Xylan\ Conversion(\%) = \frac{Xylose_{72} \times Volume}{Xylose_{theoretical}} \tag{4.4}$$

$$Total\ Conversion(\%) = \frac{(Glucose + Xylose)_{72} \times Volume}{(Glucose + Xylose)_{theoretical}} \tag{4.5}$$

where;

Glucan	glucan content in *Miscanthus* (41.6%)
Xylan	xylan content in *Miscanthus* (20.6%)
W	sample weight (d.b.) added to pretreatment tubes
0.9	conversion factor of glucose to equivalent glucan
0.88	conversion factor of xylose to equivalent xylan
$Glucose_{72}$	glucose concentration (% w/v) at 72 hr
$Xylose_{72}$	xylose concentration (% w/v) at 72 hr
Volume	volume of enzymatic hydrolysis reaction (mL)

Analysis of variance (ANOVA) and Fishers least significant difference test were used

to compare sugar concentration, hydrolysis rate and conversion. Ethanol yields (% and mg/g washed pretreated biomass) were determined by comparing ethanol concentrations (72 hr) to theoretical ethanol derived from compositional analysis data of washed pretreated solids:

$$Ethanol_{theoretical} = \frac{W_p \times Glucan_p}{V_p \times 0.511} \tag{4.6}$$

$$Ethanol\ Yield(\%) = \frac{Ethanol_{72} - Ethanol_{EB}}{Ethanol_{theoretical}} \tag{4.7}$$

where;

p	denotes washed pretreated solids
W_p	weight (g d.b.) of washed pretreated solids added to fermentation
$Glucan_p$	glucan content (%) in washed solids
V_p	volume of fermentation (mL)
0.511	monosaccharide to ethanol conversion factor
$Ethanol_{72}$	ethanol concentrations (% w/v) at 72 hr
$Ethanol_{EB}$	ethanol concentration (% w/v) in enzyme blanks at 72 hr

Since *Saccharomyces cerevisiae D5A* cannot ferment five carbon sugars, ethanol yields were based only on glucan content in pretreated solids. Fermentations were conducted in duplicates. Mean monosaccharide concentrations, ethanol concentrations and ethanol yields were determined.

4.3 Results and Discussion

4.3.1 Sugar Yields from Enzymatic Hydrolysis at Different Pretreatment Conditions

Hot water pretreatment of *Miscanthus* was optimized for reaction time and temperature based upon measured glucose and xylose yields following a 72 hr enzymatic hydrolysis. Pretreated solids were washed prior to enzymatic hydrolysis to avoid interference from soluble enzyme inhibitors. Glucose, xylose and total sugar (glucose and xylose) yield efficiencies (% maximum) were based upon the carbohydrates present in the initial *Miscanthus* sample prior to pretreatment.

Results from Pretreating at 160°C

Increasing reaction time resulted in higher final sugar concentrations, sugar hydrolysis rates and better conversions (Figures 4.4 and 4.7). Maximum glucose (2.53% w/v) and xylose (1.18% w/v) concentrations for the pretreatment liquor were observed at the longest reaction time (30 min) (Table 4.2). No differences were observed in enzymatic rates for glucose and xylose production (Table 4.2) nor glucan, xylan, and total conversions (Table 4.3) between 20 and 30 min reaction times. Highest glucan (35.8%), xylan (32.8%) and total sugars (34.8%) conversions were achieved at 20 and 30 min.

At temperatures above 160°C, hemicelluloses were solubilized first, followed by lignin. Hot water disrupted hydrogen bonds among cellulose microfibers and swelled the cellulose structure (Dien et al. 2005). Pretreatment conditions at 160°C were not harsh enough to increase access of polysaccharides for enzymatic hydrolysis. Other investigators reported similar results, with optimal temperatures for hot water pretreatment above 180°C using various feedstocks (Table 4.1).

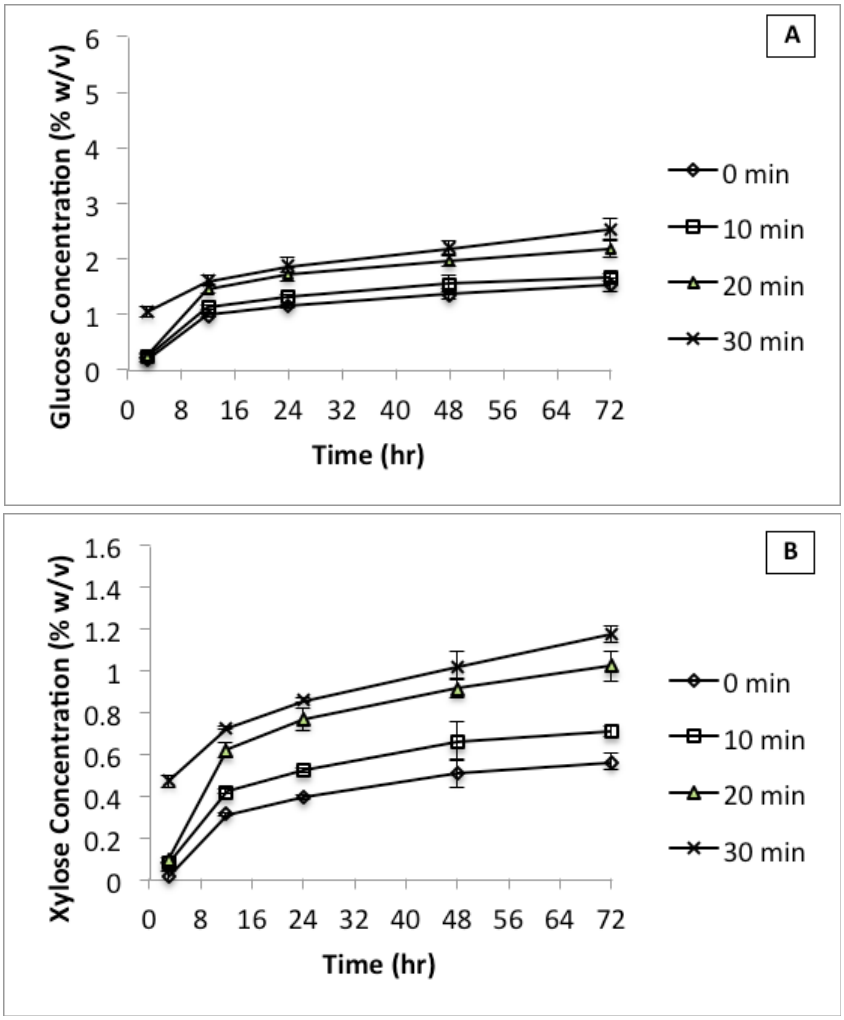

Figure 4.4. A. Glucose and B. xylose profiles during enzymatic hydrolysis of *Miscanthus* pretreated at 160°C.

Results from Pretreating at 180°C

Final glucose concentration and hydrolysis rate increased with pretreatment reaction temperature (Figure 4.5). Final glucose concentrations were higher for 20 and 30 min (4.08 and 4.16% w/v) compared to 0 and 10 min (1.53 and 3.15% w/v, respectively) reaction times (Table 4.2). Glucose hydrolysis rates increased with increasing reaction time and were highest at 30 min (0.225% w/v/hr). A different trend was observed for final xylose concentrations and xylose hydrolysis rates with increased retention time (Figure 4.5). Final xylose concentrations increased from 0 (0.63% w/v) to 10 (1.35% w/v) min but decreased

at 20 (1.08% w/v) and 30 min (0.65% w/v, respectively) (Table 4.2). Xylose hydrolysis rates peaked at 10 min (0.065% w/v/hr) and decreased when retention time was increased.

Glucan conversion increased with increase in time, but xylan conversion increased from 0 to 10 min and decreased at higher retention time (Figure 4.7). Glucan conversion was highest at 20 (57.3%) or 30 (57.7%) min; xylan conversion was highest at 10 (53.4%) min (Table 4.3). Since xylan conversion decreased after 10 min, total conversion was highest for 10 (48.7%) and 20 (52.5%) min (Table 4.3). As retention time increased, hemicellulose fractionation into pretreatment liquor increased (Mosier et al. 2005). Due to recovery of hemicellulose in pretreatment liquor, amount of xylan present in washed pretreated solids decreased resulting in lower final xylose conversions from enzyme hydrolysis.

Results from Pretreating at 200°C

The maximum final glucose concentration and hydrolysis rate were obtained at 10 (6.87% w/v and 0.348% w/v/hr, respectively) and 20 (6.85% w/v and 0.356% w/v/hr, respectively) min; whereas, xylose concentration and hydrolysis rate were highest at 0 min (1.10% w/v and 0.060% w/v/hr, respectively) (Table 4.2). Glucose concentration and hydrolysis rate peaked between 10 to 20 min but decreased when time was increased to 30 min (Figure 4.6). Xylose concentration and hydrolysis rate were highest at the shortest reaction time, and increasing time resulted in lower xylose concentrations and hydrolysis rates (Table 4.2). Glucan conversion was highest at 10 min (76.7%); xylan conversion was highest at 0 min (36.4%) (Table 4.3). Overall, total conversion was highest at 10 min (61.8%).

Table 4.2. Effect of pretreatment temperature and time on final sugar concentrations (% w/v) and hydrolysis rates (% w/v/hr) following enzymatic hydrolysis

Temperature °C	**Time** (min)	**Final Glucose Concentration**[1] (% w/v)	**Final Xylose Concentration**[2] (% w/v)	**Glucose Hydrolysis Rate**[3] (% w/v/hr)	**Xylose Hydrolysis Rate**[4] (% w/v/hr)
160	0	1.52 ± 0.125 C	0.56 ± 0.041 D	0.082 ± 0.002 C	0.028 ± 0.001 C
	10	1.65 ± 0.060 C	0.71 ± 0.020 C	0.094 ± 0.002 B	0.036 ± 0.001 B
	20	2.17 ± 0.096 B	1.02 ± 0.050 B	0.123 ± 0.006 A	0.053 ± 0.003 A
	30	2.53 ± 0.193 A	1.18 ± 0.076 A	0.117 ± 0.007 A	0.053 ± 0.003 A
180	0	1.53 ± 0.037 C	0.63 ± 0.010 C	0.077 ± 0.002 D	0.028 ± 0.001 D
	10	3.15 ± 0.255 B	1.35 ± 0.029 A	0.150 ± 0.007 C	0.065 ± 0.001 A
	20	4.08 ± 0.285 A	1.08 ± 0.032 B	0.197 ± 0.007 B	0.055 ± 0.002 B
	30	4.16 ± 0.222 A	0.65 ± 0.015 C	0.225 ± 0.028 A	0.033 ± 0.002 C
200	0	2.57 ± 0.098 C	1.10 ± 0.026 A	0.150 ± 0.001 C	0.060 ± 0.003 A
	10	6.87 ± 0.064 A	0.30 ± 0.070 B	0.348 ± 0.007 A	0.015 ± 0.003 B
	20	6.85 ± 0.038 A	0.21 ± 0.014 C	0.356 ± 0.003 A	0.010 ± 0.001 BC
	30	6.34 ± 0.183 B	0.18 ± 0.010 C	0.321 ± 0.010 B	0.009 ± 0.001 C

Mean ± Standard Deviation

[1]Mean glucose concentrations followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[2]Mean xylose concentrations followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[3]Mean glucose hydrolysis rates followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

[4]Mean xylose hydrolysis rates followed by the same letter in a column within a pretreatment are not different ($P < 0.05$)

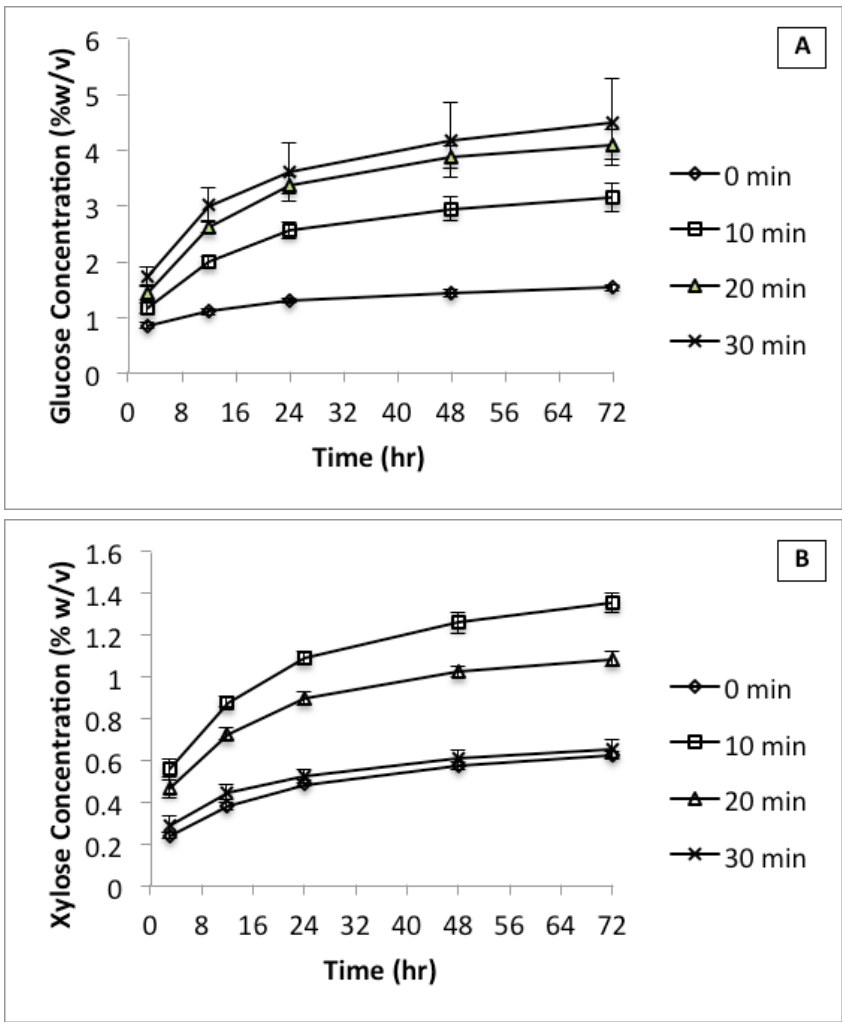

Figure 4.5. A. Glucose and B. xylose profiles during enzymatic hydrolysis of *Miscanthus* pretreated at 180°C.

Table 4.3. Effect of temperature and time on conversion (%) following enzymatic hydrolysis

Temperature	**Time**	**Glucan Conversion**[1]	**Mean Glucan Conversion**[2]	**Xylan Conversion**[3]	**Mean Xylan Conversion**[4]	**Total Conversion**[5]	**Mean Total Conversion**[6]
°C	(min)	(%)	(%)	(%)	(%)	(%)	(%)
160	0	24.4 ± 2.00 B	30.2 ± 4.90 C	17.7 ± 1.26 C	26.7 ± 6.77 A	22.2 ± 1.74 C	29.1 ± 5.48 C
	10	27.7 ± 1.01 B		23.7 ± 0.73 B		26.4 ± 0.91 B	
	20	33.0 ± 1.48 A		32.6 ± 1.80 A		32.9 ± 1.58 A	
	30	35.8 ± 2.72 A		32.8 ± 2.12 A		34.8 ± 2.52 A	
180	0	25.2 ± 0.51 C	46.8 ± 14.1 B	20.4 ± 0.39 C	33.0 ± 15.6 A	23.6 ± 0.41 C	42.3 ± 11.9 B
	10	47.0 ± 3.90 B		53.4 ± 5.41 A		48.7 ± 4.21 AB	
	20	57.3 ± 4.13 A		40.2 ± 4.57 B		52.5 ± 3.08 A	
	30	57.7 ± 3.07 A		17.8 ± 0.42 C		44.3 ± 1.91 B	
200	0	38.7 ± 1.46 D	64.3 ± 15.8 A	36.4 ± 1.33 A	14.7 ± 13.6 B	38.0 ± 1.42 D	51.1 ± 9.13 A
	10	76.7 ± 0.61 A		11.8 ± 4.69 B		61.8 ± 2.48 A	
	20	73.8 ± 0.45 B		5.60 ± 0.59 C		54.4 ± 1.00 B	
	30	68.2 ± 1.97 C		5.00 ± 0.16 C		50.3 ± 1.46 C	

Mean ± Standard Deviation

[1]Mean glucan conversion, within temperature, followed by the same letter are not significantly different ($P < 0.05$)

[2]Mean glucan conversion, across temperature, followed by the same letter are not significantly different ($P < 0.05$)

[3]Mean xylan conversion, within temperature, followed by the same letter are not significantly different ($P<0.05$)

[4]Mean xylan conversion, across temperature, followed by the same letter are not significantly different ($P<0.05$)

[5]Mean total conversion, within temperature, followed by the same letter are not significantly different ($P<0.05$)

[6]Mean total conversion, across temperature, followed by the same letter are not significantly different ($P<0.05$)

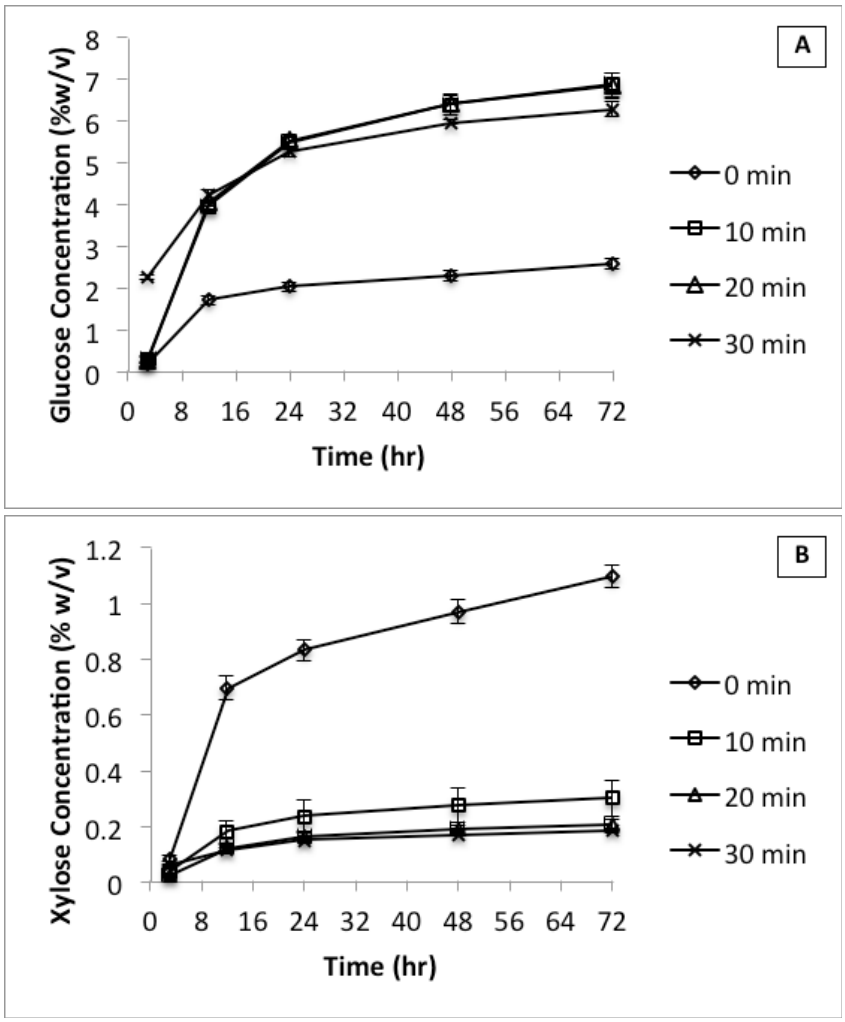

Figure 4.6. A. Glucose and B. xylose profiles during enzymatic hydrolysis of *Miscanthus* pretreated at 200°C.

Table 4.4. Conversions (%) including sugars in pretreatment liquor

Temperature °C	**Time** (min)	**Glucan Conversion**[1] (%)	**Xylan Conversion**[2] (%)	**Total Conversion**[3] (%)
160	0	26.8 ± 1.91 D	19.8 ± 1.20 D	24.4 ± 1.67 D
	10	31.0 ± 1.02 C	27.9 ± 0.90 C	30.0 ± 0.93 C
	20	36.5 ± 1.40 B	39.8 ± 1.94 B	37.6 ± 1.57 B
	30	41.1 ± 3.04 A	48.2 ± 2.18 A	43.5 ± 2.65 A
180	0	28.2 ± 0.17 C	23.6 ± 0.78 C	26.6 ± 0.25 B
	10	50.1 ± 3.78 B	66.9 ± 7.04 A	55.7 ± 4.84 A
	20	60.0 ± 3.93 A	57.6 ± 7.34 A	59.1 ± 3.09 A
	30	63.2 ± 3.12 A	43.9 ± 2.98 B	56.7 ± 2.85 A
200	0	42.0 ± 1.40 D	46.2 ± 1.85 A	43.4 ± 1.50 D
	10	78.1 ± 0.52 A	51.3 ± 9.28 A	61.8 ± 2.48 A
	20	75.2 ± 0.45 B	30.2 ± 3.29 B	60.1 ± 1.39 B
	30	70.0 ± 1.87 C	29.8 ± 0.15 B	56.5 ± 1.31 C

Mean ± Standard Deviation

[1]Mean glucan conversion, within temperature, followed by the same letter are not significantly different ($P<0.05$)

[2]Mean xylan conversion, within temperature, followed by the same letter are not significantly different ($P<0.05$)

[3]Mean total conversion, within temperature, followed by the same letter are not significantly different ($P<0.05$)

Comparisons among Temperature Conditions

Increasing pretreatment times benefited cellulose hydrolysis as measured by end glucose concentrations, hydrolysis rates and conversion efficiencies. For xylose concentration, xylose hydrolysis rate and xylan conversion, increases were observed with increasing temperatures but decreased at 200°C for 10 and 20 min and 180°C for 30 min (Table 4.3).

Mean glucan conversion was highest at 200°C (64.3%) followed by 180°C (46.8%) and 160°C (30.2%) (Table 4.3). Mean xylan conversion rates were higher from 160 and 180°C (26.7 and 33.0%, respectively) than 200°C (14.7%). Mean total conversions increased with increasing temperature and were highest at 200°C (51.1%).

The best pretreatment condition (with highest total conversion) was observed at 200°C for 10 min (61.8%). Biomass solubilization has been observed to increase with increase in temperature, resulting in glucan enrichment in pretreated solids (Mosier et al. 2005). Pretreatment at 200°C for 10 min increased the glucan content in *Miscanthus* from 41.6 to 52.3%; whereas, xylan content decreased from 20.6 to 3.1% (Table 4.5). Total sugars (monosaccharides and oligosaccharides) were determined in the pretreatment liquor at best conditions using a dilute acid hydrolysis procedure (Ruiz and Ehrman 1996b). Pretreatment resulted in 0.49% w/v glucose and 1.82% w/v xylose concentrations in pretreatment liquor, which were 5.90 ± 0.13% and 43.4 ± 4.84% of theoretical glucose and xylose in raw *Miscanthus*, respectively.

Hot water optimization studies conducted by Mosier et al. (2005) on corn stover resulted in similar results. Mosier et al. (2005) reported hot water pretreatment at 190°C for 15 min resulted in a pretreated slurry which when enzyme hydrolyzed release 32 g/L glucose and 18 g/L xylose. Further fermentation of enzyme hydrolyzed solids using recombinant yeast 424A (LNH-ST) resulted in 88% ethanol yields. Glucose and xylose concentrations from *Miscanthus* were 69 and 3 g/L, respectively. Higher glucose

concentrations could be due to higher solids content (10%) in enzyme hydrolysis compared to conditions used by Mosier et al. (2005) (1% solids). Ethanol yield was 70% when hydrolysate was fermented using non recombinant yeast compared to 88% by Mosier et al. (2005) using a recombinant yeast. Lower ethanol yields were due to lower xylose concentrations following enzyme hydrolysis as well as use of a non recombinant yeast strain.

Table 4.5. Pretreated solids and fermentation data

Process	**Substrate/Product**	**Concentration**
Pretreatment (Washed Solids Composition, mg/g washed biomass)	Glucose	580.8 ± 27.07
	Xylose	35.4 ± 0.80
	Arabinose	2.33 ± 0.35
	Galactose	0.76 ± 1.07
	Acetate	26.2 ± 4.72
	Total Monosaccharide	644.87 mg/g
Simultaneous Saccharification and Fermentation (Final Concentrations, 72 hr, %w/v)	Glucose	0.050 ± 0.004
	Xylose	0.093 ± 0.001
	Arabinose	0.251 ± 0.005
	Xylitol	0.143 ± 0.001
	Glycerol	0.082 ± 0.001
	Acetate	0.093 ± 0.006
	Ethanol	2.040 ± 0.130
	Ethanol Yield	70.0 ± 5.71 %
		217 ± 16.9 mg/g pretreated biomass

Removal of hemicellulose has been shown to increase cellulose-cellulase interactions and thus improved biomass digestibility (Jeoh et al. 2007). Similar results were observed for hot water pretreatment (200°C, 10 min) of switchgrass (Shi et al. 2011; Table 4.1). They reported a 70% glucose yield after enzyme hydrolysis with >90% xylan removal during pretreatment. However, Shi et al. (2011) found no correlation between xylan removal and increased biomass digestibility across pretreatments (hot water, dilute acid, AFEX, lime, sulfur dioxide and soaking in aqueous ammonia), possibly due to changes in other substrate properties besides xylan removal.

4.3.2 Simultaneous Saccharification and Fermentation

Washed pretreated solids recovered from *Miscanthus* pretreated at 200°C for 10 min were simultaneously saccharified and fermented for 72 hr. Compositional analysis of pretreated solids was conducted to determine enzyme dosages and for ethanol yield calculations (Table 4.5). Final glucose, xylose, arabinose, xylitol, glycerol, acetate and ethanol concentration were measured using HPLC (Table 4.5). An ethanol yield of 70% of theoretical was achieved. The absence of residual glucose is evidence that the washed solids are suitable for fermentation. Mass balance for pretreatment and fermentation are depicted in Figure 4.8. Using an experimental fermentation efficiency of 70% of theoretical, 1 kg of *Miscanthus* can generate 0.13 kg of ethanol. Low concentrations of inhibitory compounds such as hydroxymethyl furfural (0.32 mM) and furfural (0.42 mM) were observed, which was expected because the furans would have been washed away with other soluble material following pretreatment.

Higher ethanol yields may be attainable by optimization of enzyme mixtures and dosages, or other hot water pretreatment modifications. Investigators have reported fermentation inhibition at higher temperatures (220°C) besides shorter reaction times (2 min) and lower solids loading rates (3%) (Laser et al. 2001). Perez et al. (2008) observed that using a two stage hot water pretreatment aimed at maximizing hemicellulose in the first, and glucose recovery in the second, resulted in 80% xylose and 91% glucose recovery from wheat straw. Yu et al. (2010) also reported similar results with 96.6% total sugar recovery achieved using a two stage hot water pretreatment process. However, this process would incur added capital and energy costs, which were not discussed in the paper. Sreenath et al. (1999) reported higher hemicellulose solubilization when 0.07% sulfuric acid was added to hot water pretreatments but decreased subsequent sugar hydrolysis from pretreated solids by 60%.

4.4 Conclusions

The best results for hot water pretreatment of *Miscanthus* were 200°C for 10 min based upon total sugar recoveries following both pretreatment and enzymatic hydrolysis of pretreated and washed solids. Pretreatment (at 200°C for 10 min) yielded 6% of the glucan as glucose and 43% xylose (of theoretical) in pretreatment liquor. Enzymatic hydrolysis of washed pretreated solids resulted in 77, 12 and 62% glucan, xylan and total conversion, respectively, based on starting raw *Miscanthus* solids. Best conditions generated from pretreatment were evaluated further for fermentability. Simultaneous saccharification and fermentation resulted in 70% ethanol yield based on glucan content in washed pretreated solids.

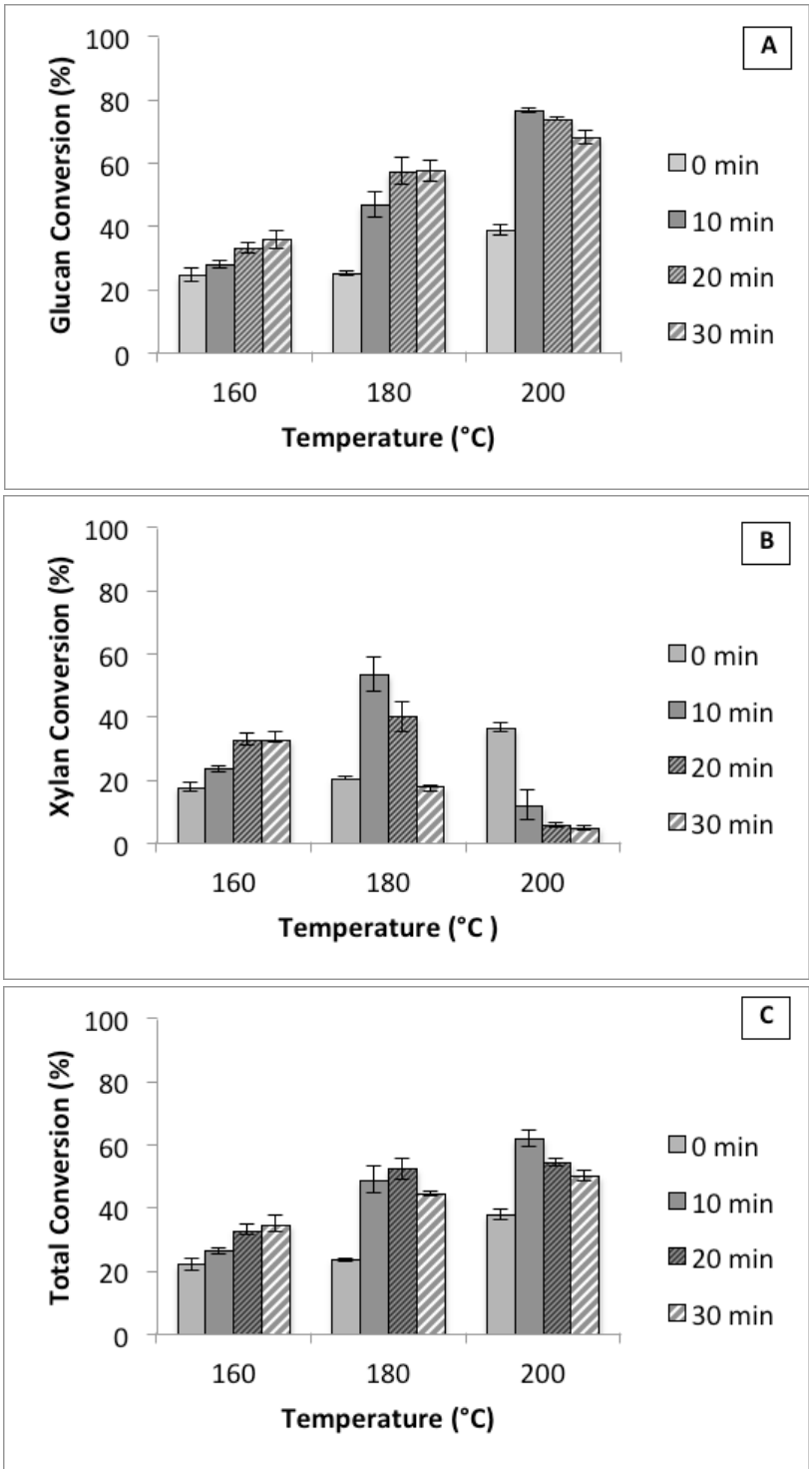

Figure 4.7. A. Glucan, B. xylan and C. total conversion (%) at different temperatures (°C) and reaction times (min).

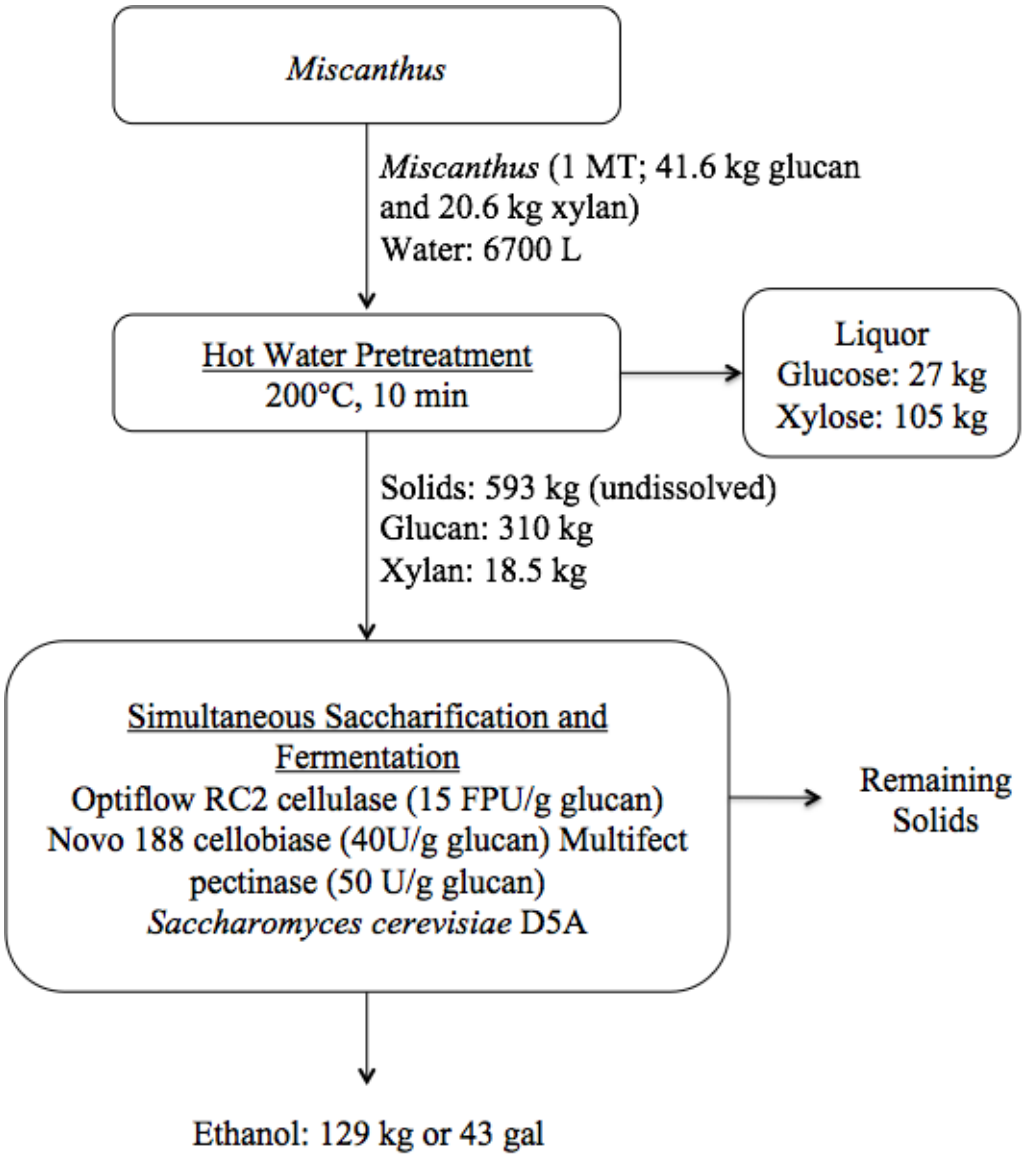

Figure 4.8. Mass balance for pretreatment and fermentation of *Miscanthus*. A 70% theoretical ethanol yield was obtained based upon glucan content of washed pretreated solids.

Chapter 5

Visualization of Physical Changes in *Miscanthus* following Hot Water Pretreatment and Enzyme Hydrolysis

5.1 Introduction

Plant cell walls are comprised of cellulose, hemicellulose, lignin and pectins (Donohoe et al. 2008). Pretreatments increase biomass digestibility by solubilization of hemicellulose (Himmel et al. 2007) and/or removal of lignin (Donohoe et al. 2008). Disruption of cell wall structures by acidic, alkaline or neutral pH pretreatments makes cellulose more accessible to enzymes (Zeng et al. 2011). An understanding of effects of pretreatments and enzymatic hydrolysis on biomass at the cellular level is needed to develop efficient pretreatment technologies. Determination of modifications at the nanoscale level alongside biochemical, chemical and genetic characterization of pretreated biomass provide information on fundamental mechanisms for biomass recalcitrance (Chundawat et al. 2011).

The objective of this study was to correlate physical changes in *Miscanthus* structure with chemical changes as a result of hot water pretreatments, using imaging techniques. Raw *Miscanthus* samples were compared to pretreated and enzyme hydrolyzed samples. Three pretreatment conditions that gave the lowest, intermediate and highest total sugar conversion (%) were selected for image analyses. Pretreated solids from each of the three conditions were hydrolyzed enzymatically and remaining solids analyzed using imaging techniques. Imaging techniques were Atomic Force Microscopy (AFM), negative staining for electron microscopy and stained thick sections for light microscopy.

5.2 Materials and Methods

5.2.1 Materials

Raw *Miscanthus*, hot water pretreated solids and enzyme hydrolyzed solids were used for image analyses. Three pretreatment conditions were selected based on total sugar conversion (Section 4.3.1). Conditions were:

1. solids from pretreatment at 160°C for 0 min, heated up to 160°C followed by quenching in a water bath (lowest total sugar conversion)

2. solids from pretreatment at 180°C for 10 min (intermediate total sugar conversion)

3. solids from pretreatment at 200°C for 10 min (highest total sugar conversion)
   Pretreated solids from the selected conditions were hydrolyzed using enzymes (Section 4.2.2.2). Solid residues from enzyme hydrolyses were collected and used for image analysis. Samples were stored at 4°C till further use.

5.2.2 Methods

Untreated *Miscanthus*, pretreated solids and enzyme hydrolyzed solids were dried overnight at 45°C in a convection oven. Once dried, samples were used for AFM, negative staining and thick section imaging techniques.

Atomic Force Microscopy (AFM)

AFM image analyses were conducted at Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois at Urbana-Champaign. Solids were bound to a glass slide by: 1) drying 0.1 mL of dilute sample (1 g dry solids in 100 mL distilled water) under an infrared lamp or 2) immobilizing dry solids onto adhesive (poly-L-lysine) coated slide. Asylum Research MFD-3D AFM was used for this study.

Samples were scanned using a BS-Tap 300AL tip (BudgetSensors, Sofia, Bulgaria) at room temperature. Contact force mode was used where the AFM tip was in contact with the sample surface. The tip was attached to the end of a cantilever; a scanner traced tip movement on the sample surface. A constant force mode was used to obtain topographical data. An optical microscope was used to view sample on the glass slide and locate areas for scanning.

Negative Staining using Transmission Electron Microscopy

Negative staining was conducted at Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois at Urbana-Champaign. Dry solids (0.05 g) were suspended in 0.5 mL distilled water in a 1.5 mL microcentrifuge tube (Fisherbrand, Fisher Scientific, Pittsburg, PA). Equal volume (0.5 mL) of stain (2% phosphotungstic acid) was added. A drop of the mixture was placed on a plastic surface and a prepared grid was mounted on top of the drop for 8 to 20 min. Grids were tiny copper wafers with 100 to 300 bar count/inch mesh (Pelco Grids, Ted Pella Inc., Redding, CA). Grids were coated with a thin film of formvar (polyvinyl formal) plastic and coated with carbon particles to stabilize the plastic. Sample not stuck to the plastic was wicked off and the grid dried for 15 min before visualization using TEM (Hitachi H600, Hitachi, Europe) at a magnification of 20,000 X.

Thick Sections for Light Microscopy

Thick sections for light microscopy were developed at Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois at Urbana-Champaign. Samples were first embedded in epoxy before cutting thick sections for viewing under the light microscope.

Embedding

Rapid embedding procedures employing microwave energy were used to accomplish sample embedment (Giammara 1993, Login and Dvorak 1993, Miller 1982). Dry biomass samples were added to a 1.5 mL microcentrifuge tube (Fisherbrand, Fisher Scientific, Pittsburg, PA). Distilled water was added to just cover the sample followed by addition of Karnovskys fixative and incubation on ice. Primary fixation was conducted using a microwave technique, wherein uncapped samples were heated in a water bath using a microwave for 38 s followed by cooling on ice for 20 s. The process was repeated 4 times and samples were washed 3 times using cacodylate buffer. A secondary fixative, 2% aqueous osmium tetraoxide, was added to just cover the sample. Samples were cooled on ice for 20 to 30 s, followed by microwaving and chilling for 20 s. The process was repeated every 5 min for a total of 25 min. Equal volumes of an osmium tetraoxide reducer, 3% aqueous potassium cyanate, were added to the vial. Samples were incubated (without microwaving) with rotation for 15 min. Samples were rinsed with distilled water (3 times) and water was removed from the sample after final washing step. Saturated uranyl acetate was added to just cover the sample. Samples were microwaved, capped and rotated for 30 min. Uranyl acetate was removed by incubating with 10% ethanol for 8 min. Ethanol concentrations were increased (25, 50, 75, 95 and 100%) and incubated for 8 min at each concentration. Samples were incubated for 8 min using 1:1 mixture of 100% ethanol:acetonitrile. Two subsequent incubations were conducted for 8 min in 100% acetonitrile. Acetonitrile was removed from sample; a 1:1 mixture of acetonitrile:epoxy was added to the sample. Samples were vortexed, microwaved for 20 s and revortexed. Tubes were incubated in a rotator for 10 min. A mixture of 1:1 acetonitrile:epoxy was added and steps repeated. Epoxy was removed and 1:3 mixture of acetonitrile:epoxy was added to the sample. Sample was vortexed, microwaved for 20 s, revortexed and incubated in a rotator for 20 min. Incubation with 1:3 mixture was repeated. Epoxy was removed from the

sample and pure epoxy was added. Samples were vortexed, microwaved for 30 s, vortexed again and incubated in the rotator for 30 min. Sample epoxy was replaced with fresh epoxy and incubated for 1 hr. Sample was removed and placed over an absorbent tissue to drain off epoxy and to allow any dehydrants present to evaporate. Samples were transferred to the bottom of a mold well containing one drop of epoxy. Bubbles, if present, were removed using a wooden tool and the mold was filled just below the top with epoxy. The mold was placed in a histodryer at 85°C for 8 to 15 hr. Molds were cooled at room temperature or at 4°C for 20 min before removal.

Thick Sections

Once hardened (process also know as curing), molds are called blocks. Blocks were trimmed under a microscope to eliminate excess epoxy on sample and to improve visibility under a microscope. A knife was used to trim the blocks and level the surface. Glass knives were used to generate sections (0.5 μm thick). Sections were then picked up and placed onto a drop of water on a glass slide. Sections were bound to the glass slide by drying using a hot plate. Sections were stained with Toluidine Blue (0.5% toluidine blue and 1.0% sodium borate) and Basic Fuchsine (3:1 mixture of 0.65% sodium borate:1% basic fuchsine) (Hoffman et al. 1983). Slides were stained for 30 to 90 s and excess stain removed using distilled water. Slides were dried on a hot plate for 15 to 20 s. Slides were viewed under a light microscope (Olympus, Center Valley, PA) at a magnification of 400 X.

5.3 Results and Discussion

5.3.1 AFM

Miscanthus samples were used for AFM imaging were without the extensive sample preparation applied by Hanley et al. (2002), Ding and Himmel (2006) and Tetard et al. (2011). A number of technical problems were encountered using AFM to image biomass

that was pretreated or enzyme hydrolyzed. Issues identified were;

1. *Wide particle size distribution*

   Biomass samples were ground using a hammer mill equipped with a 0.08 mm sieve screen. The particle size distribution for ground biomass is shown in Figure 5.1. Distributions indicate particle sizes varying from 1.0 μm to 450 μm. When visualized under the optical microscope for positioning of the AFM tip, a large variety of particle sizes were observed. Due to the broad range or particle sizes present, selection of particles for AFM imaging was difficult.

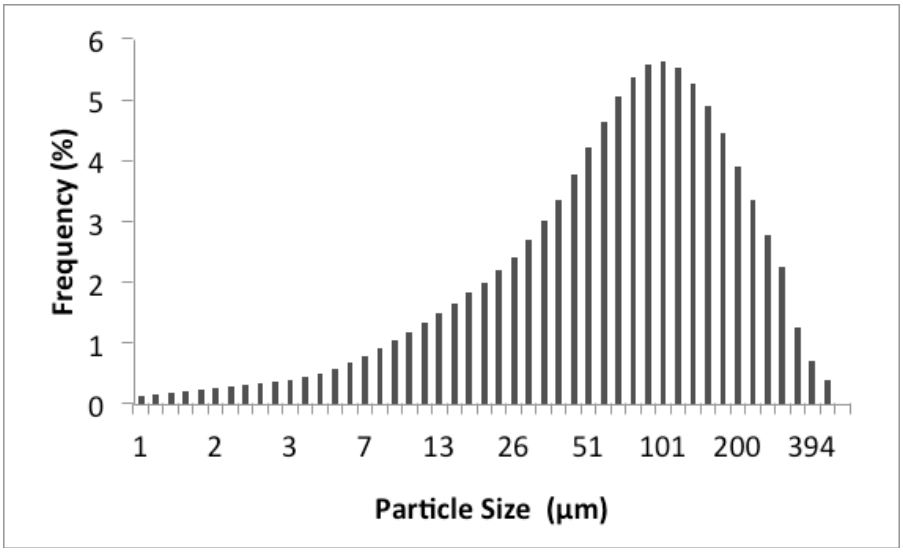

Figure 5.1. Particle size distribution for *Miscanthus* ground using 0.08 mm sieve screen.

2. *Sticky nature of sample*

   Since pretreated and enzyme hydrolyzed samples contained monomeric sugars and polysaccharides, when used in native solution form, they generated a sticky substrate for AFM analysis. This caused sample to bind to the tip surface resulting in sample displacement.

3. *Binding sample to glass slide*

   To adhere sample to glass slide, a drop of suspended solids was dried on the glass slide, or dry solids were dusted on the surface of epoxy coated glass slide. Issues with

imaging dry sample were that cellulose microfibrils tend to stand upright like hairs causing interference with tip movement on the surface (Kirby et al. 1996).

4. *Lack of information on surface components*

   Information on topography (height changes) and elasticity (phase changes) were obtained from the AFM (Ding and Himmel 2006). However, no information on chemical constituents of the surface was obtained.

AFM has been used in conjunction with other imaging techniques such as TEM, SEM, NMR, laser scanning confocal fluorescence microscopy (LSCM) and electron spectroscopy for chemical analysis (ESCA) to study the effect of AFEX pretreatment on biomass (Chundawat et al. 2011). Zuluaga et al. (2010) characterized cellulose microfibrils using AFM with SEM, TEM and FTIR techniques. Other investigators used different modes such as mode synthesizing atomic force microscopy (MSAFM), which used nonlinear mechanical coupling between the probe and sample to obtain more surface and subsurface information, although not chemical information (Tetard et al. 2010). Ding and Himmel (2006) discussed the use of functionalized AFM tips for characterization of cell wall structure. AFM, when integrated with other analytical tools such as IR, NMR, XRD or TEM, provides both chemical composition and atomic level resolution cellulose structure information (Harris et al. 2010). Some amount of sample preparation also was required prior to AFM imaging such as hand dissected sections of tissue (Ding and Himmel 2006, Chundawat et al. 2011), microtoming (Tetard et al. 2010), TEM sample preparation methods (Hanley et al. 1992), cellulose microfibril isolation procedures (Zuluaga et al. 2007) or milling and homogenization (Kirby et al. 1996).

Using sample preparation steps to purify/fractionate samples prior to AFM imaging, as well as using AFM in conjunction with other analytical methods that provide local chemical composition information, could add to AFM imaging capabilities. Pretreated or enzyme hydrolyzed biomass samples will require further treatment (e.g.

washing) prior to AFM imaging.

5.3.2 Negative Staining using Transmission Electron Microscopy

Magnifications of 20,000 times were achieved using negative staining techniques. Using this technique, exposed cellulose microfibrils were visible (Figure 5.2).

However, disadvantages with this technique were that only small particles attached to the grid were visible. Also, in most cases images were not clear and did not provide a clear representation of the sample. For better visualization, thick sections were prepared for viewing under a light microscope. Biomass samples were embedded, sectioned and stained to visualize cell types.

Figure 5.2. Negative staining image using TEM (20,000 X magnification) of *Miscanthus* pretreated at 160°C.

5.3.3 Thick Sections for Light Microscopy

Plant stem tissues are comprised of epidermis, vascular bundles and parenchyma pith (Ding and Himmel 2008). The epidermis acts as a protective layer and contains

epidermal cells, guard cells and subsidiary cells. Beneath the epidermal layer are schlerenchyma cells and 1 to 3 layers of collenchyma cells. Collenchyma cells are non lignified, elongated axially with irregular thickened walls. Parenchyma cells form the bulk of the stem and have thin, non lignified walls. Vascular bundles, xylem and phloem are surrounded by a bundle sheath (fiber).

A number of different cell types were observed for raw *Miscanthus* when viewed under the light microscope (Figure 5.3). Cells were spherical, oval or elongated. Most cells had thick cell walls, although some had thinner walls. After pretreatment at 160°C for 0 min (Figure 5.4 A, B), intact cell networks as well as cell debris were observed. Even after subsequent enzymatic hydrolysis, intact cell structures were present (Figure 5.4 C, D).

Hot water pretreatment at 180°C for 10 min resulted in biomass where individual cells as well as cell networks were visible (Figure 5.5 A, B). Following enzymatic hydrolysis of pretreated solids, cells were intact as observed with pretreatment at 160°C (Figure 5.5 C, D). Pretreated solids from 200°C for 10 min showed more broken cells than cellular networks (Figure 5.6 A, B). Enzymatic hydrolysis of pretreated solids showed few intact cells; most cell walls were digested or broken down (Figure 5.6 C, D). Comparing images from solids generated by the worst (160°C, 0 min), intermediate (180°C, 10 min) and best (200°C, 10 min) pretreatment conditions, at optimal pretreatment conditions most cells were broken down at the end of enzyme hydrolysis; whereas, in other cases cells were intact.

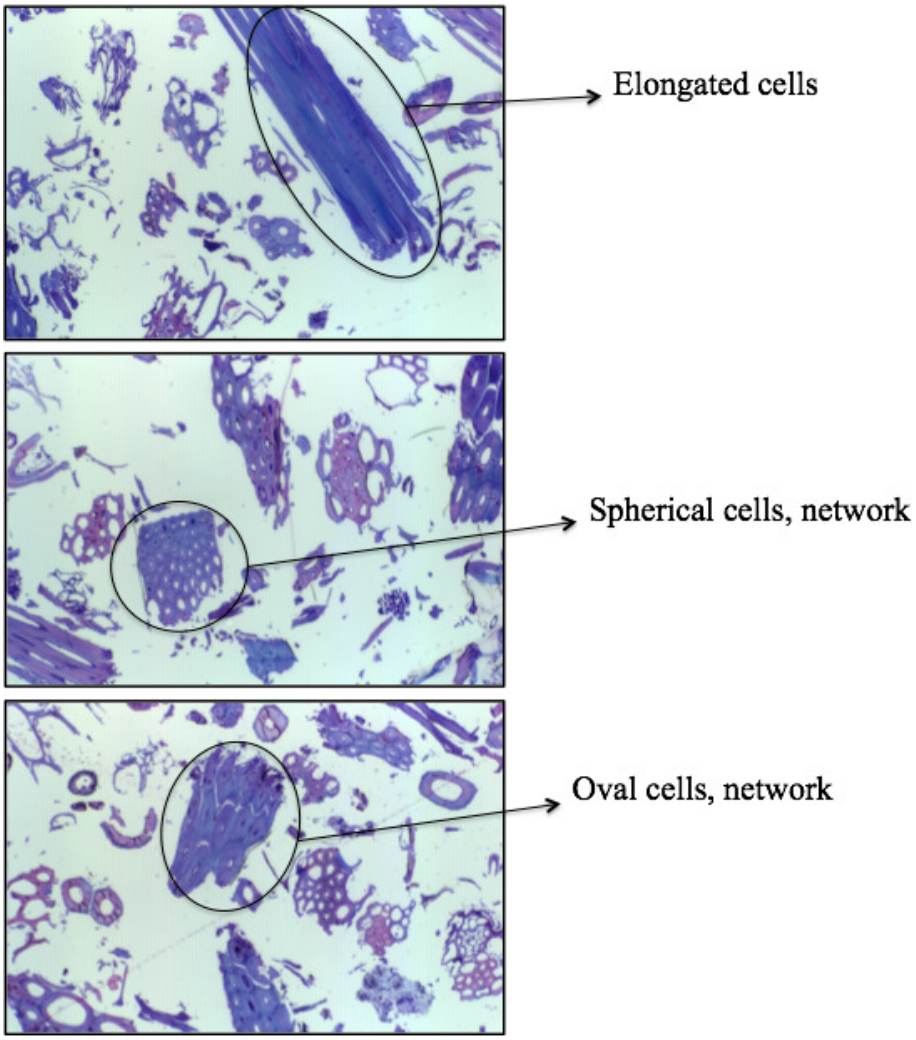

Figure 5.3. Raw *Miscanthus* using light microscopy (400 X magnification).

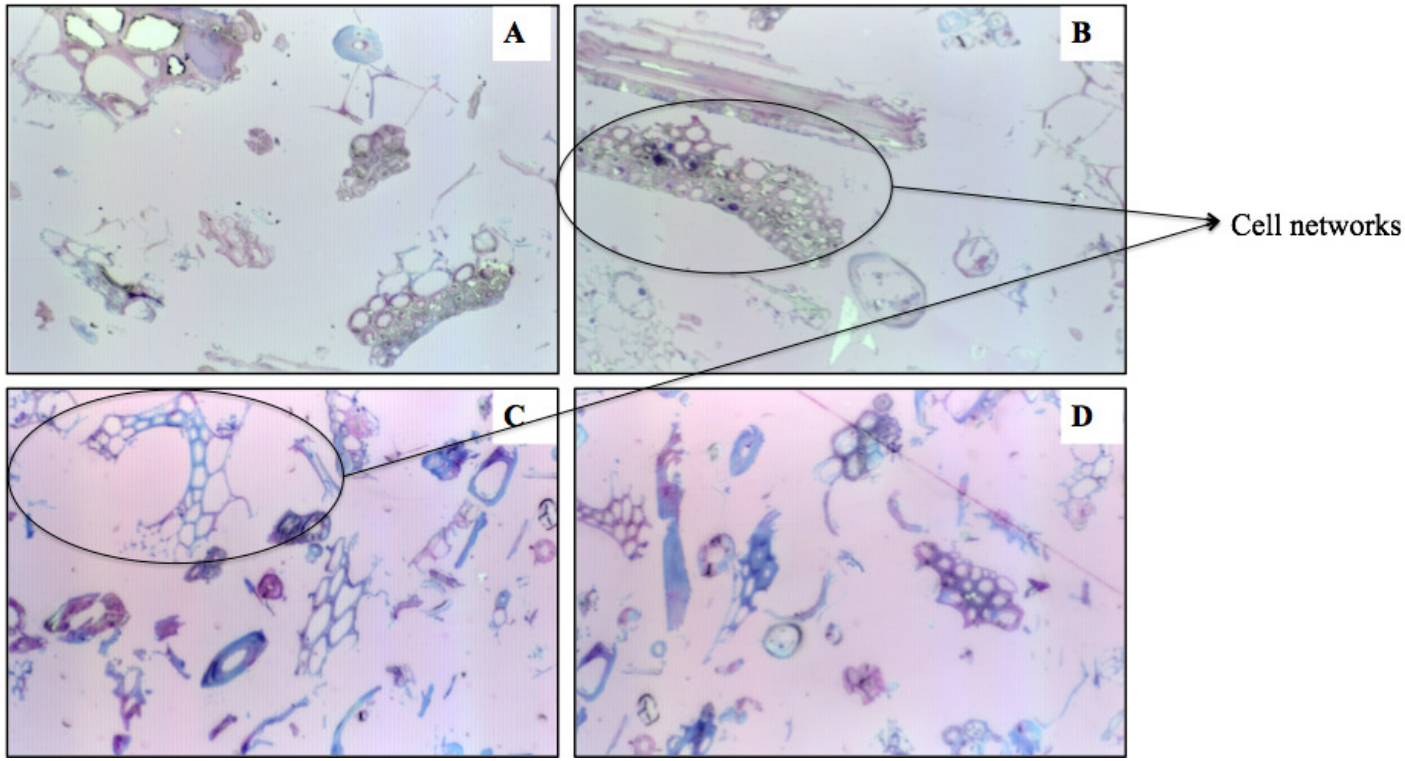

Figure 5.4. Solids pretreated at 160°C (A and B) followed by enzymatic hydrolysis (C and D), using light microscopy (400 X magnification).

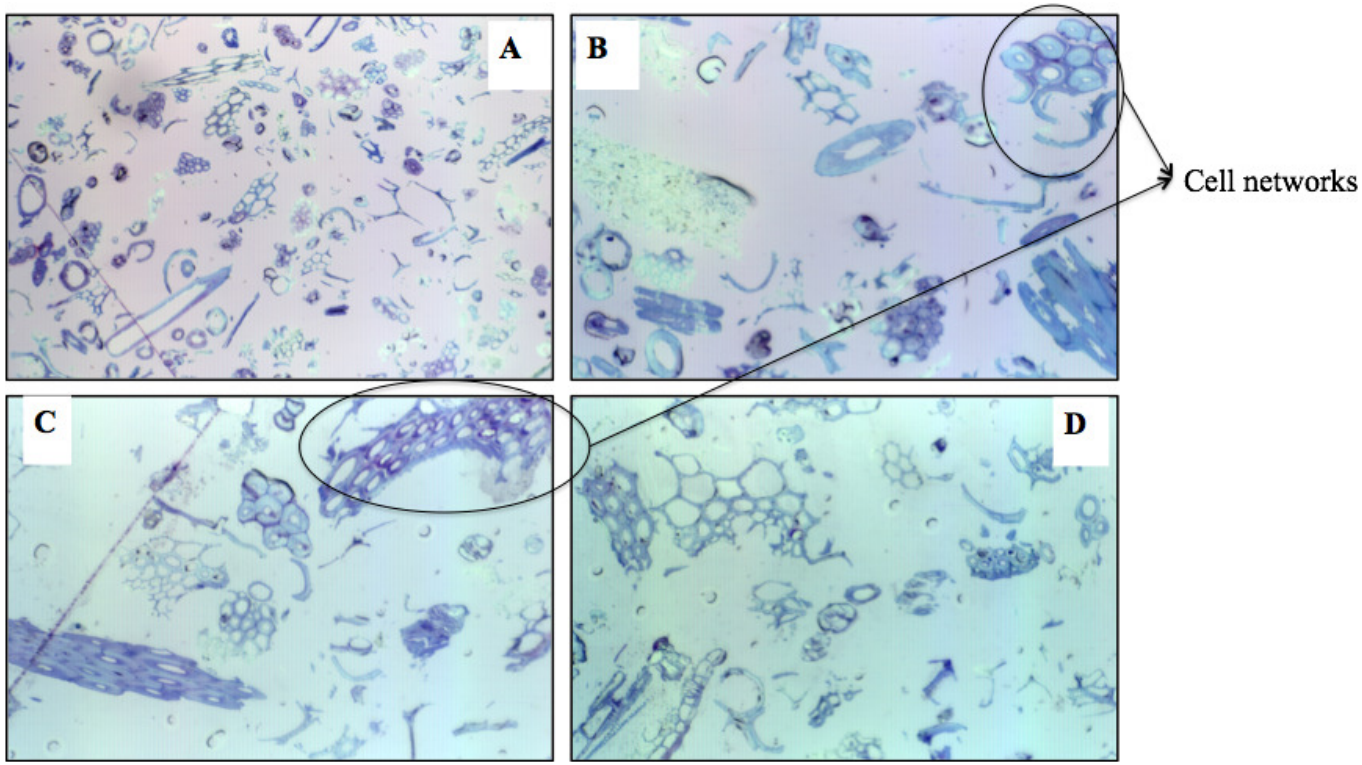

Figure 5.5. Solids pretreated at 180°C (A and B) followed by enzymatic hydrolysis (C and D), using light microscopy (400 X magnification).

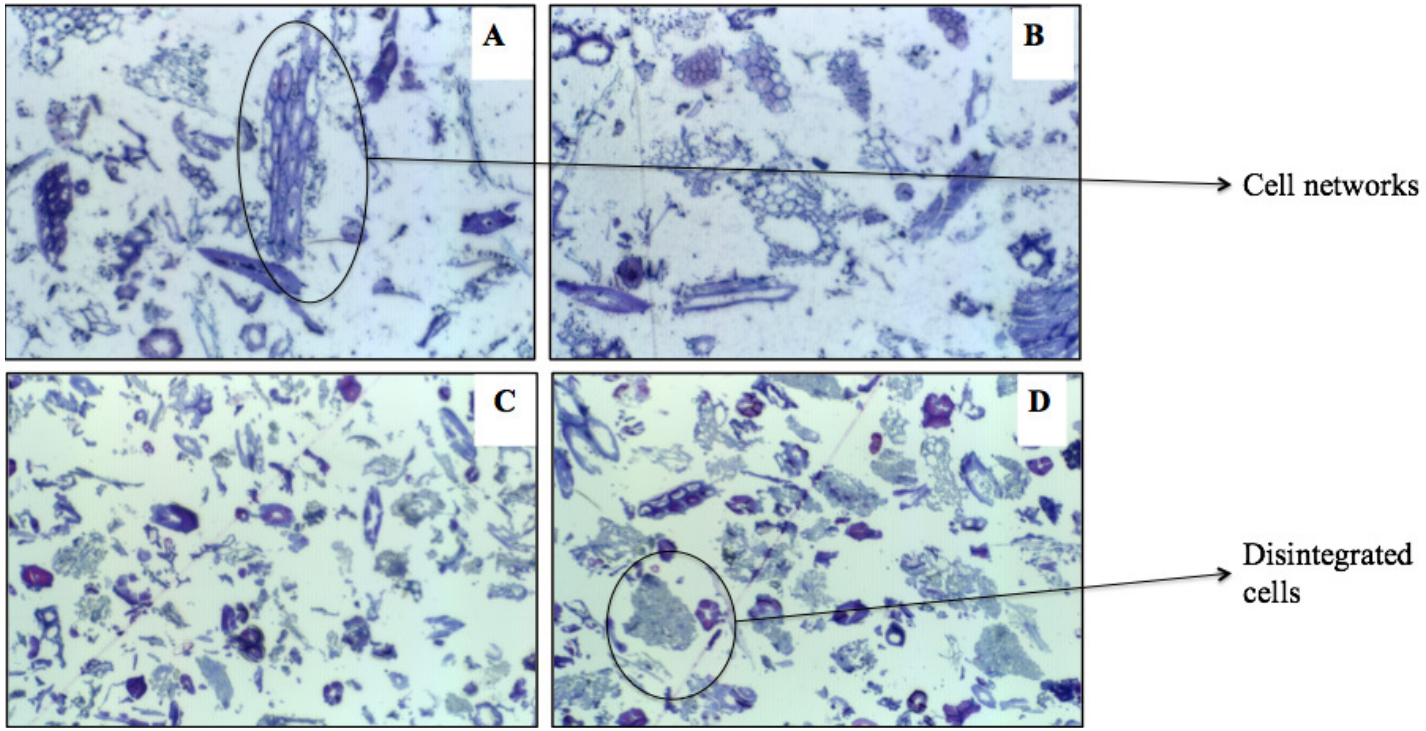

Figure 5.6. Solids pretreated at 200°C (A and B) followed by enzymatic hydrolysis (C and D), using light microscopy (400 X magnification).

Investigators were not able to detect differences in corn stover cellular structure but saw differences in corn leaves using SEM following hot water pretreatment at 190°C for 10 min (Zeng et al. 2011). Zeng et al. (2011) reported changes in parenchyma cells, with production of pores following hot water pretreatment. They observed redeposition of material (lignin or waxes) on the surface following pretreatment. They also studied the effect of enzyme hydrolysis on cell structure and reported correlations between structural changes and cellulose conversion. Enzymatic hydrolysis of pretreated pith gave highest cellulose conversion (90%) compared to leaves (70 to 80%) and rind (50 to 60%). SEM images for pretreated pith resulted in exposed secondary walls at much lower enzyme dosages (5 FPU/g glucan) than for leaves (60 FPU/g glucan) and rind (15 FPU/g glucan).

Li et al. (2010) studied the effect of hot water pretreatment on anatomical changes in *Arabidopsis* tissues. Hot water pretreatment broke pith cells and detached phloem cells from xylem cells. For pretreated and enzyme hydrolyzed samples, stem cross sections were collapsed for the mutant (high S-lignin content) compared to the wild type. Since S-lignin is more linear than G-lignin, higher amounts of S-lignin facilitated better redistribution during pretreatment and increased enzyme access to cellulose (Li et al. 2010).

Donohoe et al. (2009) reported increased cellulase penetration in thick cell walls with increased severity of dilute acid pretreatment. Dilute acid pretreatment (100°C, 20 min) resulted in <1% penetration of secondary cell wall thickness by enzymes; whereas, pretreatments conducted at 150°C for 20 min resulted in 100% penetration of even the thickest walls. Enzyme and polymer specific antibodies were used for labeling and visualization using TEM.

5.4 Conclusions

Pretreated and enzymatically hydrolyzed *Miscanthus* samples were analyzed using imaging techniques. Due to inherent stickiness of samples, varying particle sizes, low

binding affinity to glass slides and lack of local chemical compositional information, AFM analyses were not successful. Due to ineffective binding of sample onto the grid, negative staining techniques also were not used to compare samples. Samples embedded, stained and sectioned into thick sections were useful in providing a qualitative comparison of biomass. Thick sections from biomass pretreated at optimal conditions when viewed under the microscope showed disintegrated cells following enzyme hydrolysis; whereas, biomass pretreated at conditions with the lowest total sugar conversion had intact cellular networks present after enzyme hydrolysis. Thick sections were useful in viewing and qualitatively determining changes in pretreated *Miscanthus* samples.

Chapter 6

Conclusions and Recommendations for Future Work

The goal of this dissertation was to determine the effect of particle size on pretreatments and to evaluate different conditions for hot water pretreatment for sugar production using *Miscanthus*. Based on the specific objectives stated in the Introduction, the main conclusions were:

1. An increase in total polysaccharide conversion was observed when particle size was decreased for all pretreatments (hot water, dilute acid and dilute ammonium hydroxide) studied. Unpreated biomass also had increased total polysaccharide conversion with decrease in particle size, although total conversions were lower compared to chemical pretreatments.

2. Optimal conditions for hot water pretreatment of *Miscanthus* were 200C for 10 min. Enzymatic hydrolysis of washed pretreated solids resulted in 77% glucan, 12% xylan and 62% total conversion of raw *Miscanthus*. Simultaneous saccharification and fermentation resulted in 70% ethanol yield efficiency. From SSF results, 43 gal of ethanol can be produced per dry ton of *Miscanthus*.

3. Solids from pretreatment at optimal conditions were compared to those with lowest and intermediate total sugar conversions using imaging techniques. Viewing thick stained sections using light microscopy showed higher cell disintegration for solids pretreated at optimal conditions compared to other selected conditions.

Based on observations and results from this study, the following issues are recommended for further investigation:

1. Since particle size reduction increased total polysaccharide conversion, a study should be conducted to determine whether particle size reduction could decrease enzyme loadings. As particle size is decreased, the amount of energy used for grinding increases. It would be interesting to investigate whether particle size reduction has an effect on enzyme dosage, hence offsetting costs required for grinding.

2. Biomass porosity is a major factor that determines the extent of cellulose conversion. Since decreasing particle size increased biomass digestibility, an investigation on effect of size reduction on porosity would help understand methodology behind increased conversions. Methods like Simon staining could be employed to determine porosity of biomass.

3. Hot water optimization studies were conducted in tube reactors. What effect does scale up have on the optimal conditions? Tube reactor contents were static during pretreatment. For higher solids concentration, mixing might be critical for effective heat transfer.

4. Fermentation of solids pretreated using optimal conditions generated 70% ethanol yields. Identification of factors affecting ethanol yields from *Miscanthus* would be beneficial to increase ethanol yields. Modifications to hot water pretreatment, such as addition of low concentrations of chemicals could be evaluated. Also, optimizing enzyme dosages or using commercial enzyme cocktails for SSF could be evaluated.

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