ABSTRACT

YIN, YUHAO. Consolidating Bioprocessing of Alkaline Pretreated Perennial Grasses using *Clostridium thermocellum*. (Under direction of Dr. Mari. S. Chinn).

Clostridum thermocellum, an anaerobic, thermophilic bacterium, has the capability of of growing on cellulosic substrate while producing acids and solvents. In liquid, semi-solid and solid-state fermentation conditions, this microbial catalyst has been studied for production of biochemicals of interest at varying levels, including formate, acetate, lactate, ethanol or other specific chemical substances. Lignocellulosic biomass can provide a renewable source of carbon for processing operations that require both complex and fermentable sugars. Switchgrass and miscanthus are high dry matter perennial grasses that have become herbaceous plants of interest as dedicated energy crops for bioenergy and biochemical conversion processes. However, low conversion rates and reduced efficiencies partially because of the recalcitrant structure of lignocellulosic biomass have presented limitations to using perennial grasses and other lignocellulosic crops on a wide-spread industrial scale. Chemical degradation of lignocellulosic biomass through pretreatment processes have been studied as methods to remove fractions of limiting plant fibers to improve overall processing operations, including enzymatic and microbial. Microorganisms with cellulolytic and solventogenic fermentation capabilities have also been examined as catalysts for consolidated bioprocessing operations to minimize the number of unit operations necessary to complete a conversion process from raw biomass feedstock to bio-based fuel and chemical. With those methods and the need to expose cellulose and/or hemicellulose sugars to microorganisms, combing specific pretreatments technologies with efficient microbial digestion has potential to improve production rates and reduce the system operations.

In Chapter 2, a two-stage alkaline-oxidant pretreatment approach was studied to determine its effects on changes in switchgrass and miscanthus composition. Three factors including alkaline loading level, alkaline loading time and oxidant (constant 3%

hydrogen peroxide (H_2O_2)) loading time were investigated for two different types of alkalines (sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$). NaOH showed strong lignin and hemicellulose removal ability ranging from 0.05 to 0.2 g/g initial dry matter while $Ca(OH)_2$ effects on lignin and hemicellulose were limited as a result of its poor solubility at high temperatures for both grasses. Among the three factors studied, increases in NaOH loading level and oxidant time resulted in significant differences in cellulose, hemicellulose and lignin reduction. In order to quantify the effectiveness of the pretreatment conditions on preserving cellulose while reducing hemicellulose and lignin content, a new efficiency term was coined and calculated for both switchgrass and miscanthus, Y_{Swi} and Y_{Mis}, respectively. Results of this new parameter indicate a pretreatment strategy which decreased alkaline loading while extending oxidant exposure time has a higher lignin and hemicellulose reduction combined with less loss of cellulose. Four Optimized regression models with corresponding R^2 values for cellulose reduction, hemicellulose reduction, lignin reduction and pretreatment efficiency (value Y) were established as prediction equations of composition reduction under the range of conditions studied. Established equations precisely predicted changes of the four objectives and R^2 values showed the fitness of optimized models to original data.

In Chapter 3, *C. thermocellum* 27405 was cultivated in a semi-solid consolidated bioprocessing fermentation using raw biomass and four differently pretreated substrates of switchgrass and miscanthus as carbon sources. Pretreated biomass, using either a single-stage alkaline pretreatment or a combined two-stage alkaline-oxidant pretreatment, supported improved growth, end-product formation, and solvent to acid ratios by *C. thermocellum.* Pretreated biomass with more exposed cellulose fractions and reduced lignin content enhanced substrate availability and rates of consumption by the cell. The combined presence of free electrons, drops in pH from acid production, hydrogen production from increased sugar transport and increase dehydrogenase enzyme activities contributed to the greater performance of *C. thermocellum* in metabolizing both switchgrass and miscanthus and shift in metabolism to support more ethanol production.

Consolidated Bioprocessing of Alkaline Pretreated Perennial Grasses Using *Clostridium Thermocellum*

by
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APPROVED BY:

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DEDICATION

To my beloved family

To my friends

To my advisor and mentor Dr. Mari. S. Chinn

BIOGRAPHY

Yuhao Yin was born on July 22nd, 1991 China. He received his bachlor's degree in Biological Engineering in June 2014 from NanJing Forestry University, NanJing, P.R. China and he was enrolled as a graduate student by Biological and Agricultural Department of North Carolina State University on the same year. In May 2015, he joined in Dr. Chinn's research group and launched his project about biomass pretreatment technology and anaerobic fermentation. Under support and tuition of Dr. Chinn, he is expected to receive his master degree in December 2017, and look for a potential position in relative fields after graduation.

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CHAPTER 1. LITERATURE REVIEW

1.1 Lignocellulosic Biomass

Herbaceous and woody biomass are considered to be promising carbon-sufficient feedstocks for renewable energy and bio-based products that have benefits in greenhouse gas reduction and reduced reliance on fossil fuels (Field et al., 2008, Kerckhoffs & Renquist., 2013). Lignocellulosic biomass such as agricultural residues (corn stover, sugarcane bagasse), forest products (hardwood, softwood) and dedicated energy crops (switchgrass, miscanthus) can provide substantial sources of carbon for energy and chemical production with relatively minimal competition with food supply and animal feed crops (Kumar et al., 2009, Limayem & Ricke., 2012). Cellulose, hemicellulose and lignin are three main components existing in an intricate structure of lignocellulosic biomass, which make up approximately 90% of the dry weight of most plant materials (Yan et al., 2009).

Cellulose is a linear polysaccharide polymer of cellobiose ranging from 10000~15000 units linked by β-1, 4 glycosidic linkages and its hydrolysis products (e.g. glucose) can be fermented to useful biochemical and biofuels (Moxlety et al., 2008, Zheng et al., 2014). It has crystalline regions with thermal stabilities over 300 ℃ and amorphous regions that are less ordered and repetitive in bonding patterns (Shen & Gu., 2009, Ouajai & Shanks., 2005).Fewer intrachain hydrogen bonds between individual cellulose molecules often results in amorphous regions whereas higher numbers of hydrogen bonds typically occur to form crystalline regions. Crystalline cellulose for most herbaceous plants makes up approximately 90-100g/100g cellulose weight in herbaceous plant based fibers and 60-70g/100g cellulose weight in wood based fibers (Thygesen et al., 2005). Crystallinity

index is a key property used to estimate the relative amount of crystalline material in cellulose, and can be measured by techniques including solid-state 13C NMR and X-ray diffraction (Park et al., 2010, Segal et al., 1959). Degradation of cellulose to simple sugars using enzymatic methods is highly dependent on the material's crystallinity index, where a higher index is an indication that the cellulose present will be more resistant to enzymatic hydrolysis and higher hydrolysis rates often resulting in conversion of amorphous regions in native cellulose first (Park et al., 2010, Hall et al., 2010).

Hemicellulose is the other major carbon source in lignocellulosic material and the associated xylans are the main components of secondary cell walls (Girio et al., 2010). Composition of hemicellulose varies between different plants, commonly including xylose, mannose, galactose and arabinose (Aristidou & Penttilla et al., 2000). Unlike the long chains of cellobiose units found in cellulose, hemicellulose has a branched, short-chain structure with relatively low molecular weight merely containing 100-150 units of saccharide monomers , which affects the stability of the linkages and typically makes it easier and faster to break own under high thermal conditions (200-260 °C) compared to cellulose (Mohan et al., 2006). Glycosidic linkages between monomeric sugars in hemicellulose can also be broken down using chemicals, acid or alkaline, and enzymatically with xylanases. Pentose and hexose sugars derived from hemicelluose have been fermented to ethanol or other bio-products, however those types of fermentations are not as prevalent and well-developed as glucose-based fermentations (Lee & McCaskey., 1983, Sella & Trajano., 2014).

Lignin primarily works as a structural molecule to support the sturdiness of the plant cell wall. It is a three-dimensional polyphenol connected by ester bonds between monomeric phenolic *p*-coumaryl (H), coniferyl (G) and sinapyl alcohol (S) units (Sette et al., 2011). Majority of cellulose and hemicellulose components in lignocellulosic biomass are

surrounded by lignin. In the prescence of lignin, the accessibility of the cellulose and hemicellulose to free, extracellular enzymes and exo-cellular microbial enzymes is reduced and often the enzymatic interactions and activities are not efficient (Moxley et al., 2012, Rahikainen et al., 2011). Additionally, most commercial cellulases have a tendency to bind to the surface of lignin polymers, which lowers interactions between enzymes and cellulose and the overall efficiency of biomass hydrolysis (Tu et al., 2007, Lu et al., 2002). For purposes of releasing fermentable sugars from lignocellulosic biomass for subsequent fermentation, degradation or removal of lignin plays a significant role in process development and evaluation.

1.2 Consolidated Bioprocessing.

The conversion of lignocellulosic biomass into valuable products through biochemical means typically involves three steps: 1) a pretreatment process to reduce lignin; 2) hydrolysis of cellulose and/or hemicellulose into glucose, other hexoses and pentoses using enzymes; and 3) fermentation of these sugars into useful products using microbial catalyst. Simultaneous saccharification and fermentation (SSF), which was an optimization of traditional separate enzymatic hydrolysis and fermentation steps, was initially promoted in the 1980's as a simplified method to increase overall ethanol production from cellulose substrates in a single unit operation (Gauss et al., 1976). In addition to the reduced processing steps, SSF was also considered to minimize end-product inhibition of enzymes and reduce risks of contamination (Lee et al., 1983, Olofsson et al., 2008). However, enzymes used in SSF processes often have different conditions (e.g temperature, pH) for optimal activity than the microorganism doing the fermentation and it can be difficult to recover the enzymes used once the simultaneous process is complete (Camargo et al., 2014). To address some of these challenges in lignocellulosic SSF, bacteria such as, *Clostridium cellulolyticum*, *Clostridium*

thermocellum, *Flammulina velutipe,* that are capable of producing active enzyme systems to effectively degrade polysaccharides in lignocellulose and ferment resulting sugars to useful solvents and acids in a single reaction vessel have been studied. This type of fermentation process is known as consolidated bioprocessing (CBP) and has gained attention from researchers (Lynd et al., 1991, Van Zyl et al., 2007, Zhang et al., 2005). Populus, Avicel and paper pulp have been used as potenital substrates in CBP studies for processing biofuels and/or bio-products (Dumitrache et al., 2016, Lin et al., 2015).

In general, CBP provides an alternative approach to generate and effectively use cellulolytic enzymes in biomass conversion processes for acid and solvent production with up to ten-fold lower costs since the operations (hydrolysis and fermentation) take place simultaneously in a single vessel. (Lynd et al., 2005). When considering effectivemicrobial catalysts, most bacteria applied in CBP are classified as thermophilic microorganisms, and with higher operation temperatures used, such a CBP system can often minimize chances for contamination as well as reduce need for chilling units and associated costs during the process. Wild-type and even genetically modified strains still lack tolerance to some of the metabolic end-products formed (acetate, formate, lactate) and genetic stability during long-term fermentations compared to conventional SSF methods which directly employ enzymes to proceed hydrolysis (Lynd et al., 2002, Parisutham et al., 2014). Aside from some microbial sensitivities, the structure of the lignocellulosic substrates of interest as carbon sources, especially recalcitrance portions of lignocellulosic biomass is considered another significant factor affecting direct contact between the microorganisms and complex carbohydrates (e.g. cellulose, hemicellulose) in biomass, growth rates and metabolite production

Researchers have claimed that the complexity of lignocellulosic biomass composition and structure is the major factor affecting substrate hydrolysis and product formation rates,

especially crystallinity index of cellulose complex and lignin protection layer of cells (Cheng et al., 2014). One report demonstrated that structure of pretreated corn stover were modified by Ammonia Fiber Expansion (AFEX) and strain *Clostridium phytofermentans* (ATCC 700394) produced higher ethanol concentration up to 7.0g/L after 248h, compared to 1.8g/L ethanol when raw corn stover was applied as the substrate. (Jin et al., 2012). Additionally, Zhu & Cheng (2016) applied *Clostridium thermocellum* to generate biohydrogen grown on sugarcane bagasse (SCB) pretreated with ammonia-hydrogen peroxide as substrate. The final gas concentrations increased ~3.38 times compared with raw material and finally reached to 25 mmol/L after a 6-day cultivation.

1.3 Pretreatment Methods

Enzymatic hydrolysis of raw lignocellulosic materials commonly has limited effectiveness as a result of the lignin structure, lignin's cross-linkages with hemicellulose and recalcitrance associated with cellulose crystallinity (Hu & Ragauskas., 2012). In order to improve porosity of lignocellulose structure, increase accessibility of cellulosic components to useful enzymes and thus improve overall sugar yields as well as reduce necessary enzyme concentrations a pretreatment step prior to hydrolysis through physical and/or chemical methods (Chandra et al., 2007). Pretreatment methods can be classified into three types based on distinctive mechanisms: 1) mechanical pretreatment, 2) chemical pretreatment and 3) a combination of mechanical and chemical pretretaments. The primary outcomes desired by pretreatment unit operations are to deconstruct or break down recalcitrant lignin and hemicellulose, expose polymerized cellulose surface area to help catalytic (enzymatic and/or microbial) access to inner cell wall structural polymers and achieve higher fermentable sugar yields after hydrolysis (Agbor et al., 2011, Saha et al., 2005, Kumar et al., 2009).

1.3.1 Mechanical Pretreatments

Mechanical pretreatment methods studied have typically involved particle size reduction to 0.2 to 2 mm through grinding or mill operations. Selection of particle sizes is typically dependent on energy consumption during a developing pretreatment process and adopted unti operations as well as the biomass characteristics, including structure, composition and moisture content (Kratky & Jirout., 2011, Cadoche & Lopez., 1989). The practicially of particle sizes studied should always be considered and many lab-scale studies may not take into account the feasibility of achieving sizes examined at scale. Lin et al and colleagues (2010) reported that ball milling not only reduced biomass size to 0.5 mm but also loosened fibers in corn stover and the smaller particle size made the material more accessible to enzymes , increasing enzymatic hydrolysis by 110%. However, particle size reduction alone has limited impact on the overall processing of lignocellulosic biomass conversion to fermentable sugars and chemicals since lignin and hemicellulose fibers are simply cleaved and polymers are not degraded or solubilized. Additionally, high energy consumption is required when conducting mechanical method and this approach is not considered practical for mechanical pretreatment at a large scale (Kumar et al., 2009).

1.3.2 Liquid hot water (LHW) pretreatment

Liquid hot water (LHW) pretreatment is usually conducted under high temperatures ranging from 100 to 200 ℃ in order to cleave ester and ether bonds among polysaccharides, ferulic acid and lignin, solubilize hemicellulose fractions, and preserve larger fractions of cellulose in biomass solids (Mosier et al., 2005, Zhuang et al., 2012). Wan et al (2011) reported that 80% of xylan was removed from soybean straw solids through liquid hot water pretreatment at 210℃ for 10 mins (Wan et al., 2011). Another study indicated that 62% of hemicellulose was solubilized after LHW pretreatment in hybrid poplar at 200℃ for 10 mins (Kim et al., 2009). While the LHW treatments were

successful in breaking down the hemicellulose components and seemingly minimizing cellulose losses, the fermentable sugars derived from hemicellulose have the potential to be degraded into 5-hydroxy- methylfurfural (HMF) and furfural with further degradation to formic and levulinic acid at these high temperatures which can inhibit microbial growth in subsequent processes (Ingram et al., 2009, Yu et al., 2013) Additionally, residual insoluble lignin deposited on the biomass surfaces can still a negative effect on enzymatic hydrolysis, especiallyu considering interactions of cellulase binding sites with lignin (Wang et al., 2015., Rahikainen et al., 2015).

1.3.3 Acid Pretreatment

Dilute acid pretreatment, commonly completed using less than 5% sulphuric, hydrochloric and phosphoric acid, is able to hydrolyze hemicellulose and cellulose into glucose, xylose and arabinose by cleaving intrachain linkages in polysaccharide molecules (Lavarack et al., 2002, Lee et al., 1999). Even though most of the lignin content is insoluble to dilute acid, acidic chemicals can penetrate protective outer lignin layers and directly impact cellulose and hemicellulose fractions (Lenihan et al., 2010). Acid has been applied to various species of woods (poplar, silver maple), perennial grasses (switchgrass, miscanthus) and corn resides (cobs and stover) with varied levels of structural modification under different substrate loadings, chemical concentrations, times and temperatures (Table 1.1) (Hsu et al., 2010, Kootstra et al., 2009, McMillan et al., 1999, Mitchell et al., 2014, Scordia et al., 2013). Sulfuric acid is most commonly applied in dilute acid pretreatment methods with concentrations typically below 2%. At temperatures over 110 ℃, unstable amorphous cellulose, partial hemicellulose and acid-soluble lignin fractions have been reported as effectively removed after 15 to 30 minutes of dilute sulfuric acid pretreament below 2%. Solid loading ratios vary in different reports and this parameter has been shown to be an insignificant factor

impacting pretreatment efficiency (Martinez-Patino et al., 2015). One of the most important aspects of dilute-acid pretreatment is related to changes in the lignocellulosic structure, where reports have indicated altered porosity and surface area resulting from acid corrosion. Although concentrated fermentable hydrolysates can be achieved by acid pretreatment, disadvantages of this method cannot be neglected. First of all, β-1,4-glycosidic bonds connecting glucose in cellulose are cleaved by acid and it simultaneously leads to a loss of cellulose with removal of hemicellulose (Dussan et al., 204). Additionally, acid hydrolysis of lignocellulosic material has critical requirements for reactor vessel designs, especially on prevention of acid corrosion and affordability of high solid loadings in industrial scale production (Zhu et al., 2005).

1.3.4 Alkaline Pretreatment

Alkaline pretreatment is another method which employs chemical to breakdown hemicellulose into xylose by saponifying intermolecular ester bonds and decrease crystallinity of cellulose by swelling and disrupting crosslinks between molecules (Bali et al., 2015, Sun & Cheng., 2002, Tarkow & FEIST., 1969). Delignification also occurs with alkaline treatments where esters and C-C bonds between phenyl-propane units in lignin structures are cleaved (Nenkova et al., 2008). Alkaline chemicals which can be utilized in biomass pretreatment include sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$), ammonia and potassium hydroxide (KOH). These chemcials have variable effects on biomass structure and composition, depending on the type of lignocellulose and the specific treatment conditions, including substrate loading, chemical concentration, time and temperatures (Table 1.2) (Gupta & Lee, 2010, Zhao et al., 2008, Kaar & Hotlzapple, 2000, Sharma et al., 2013).

1.3.5 Alkaline Pretreatment Combined with Oxidation.

Chemicals with strong oxidizing properties, such as hydrogen peroxide or ozone, trigger delignification reactions and solubilize lignin or related phenolics into an array of low molecular weight, water-soluble oxidation products (Gould, 1985a, Gould, 1985b, Wu et al., 2013.). Oxidative chemicals also preserve most of the carbohydrates present while significant delignifcation occurs (Sannigrahi et al., 2012). Since oxidative reactions on lignin have strict limitations on pH range, the integrate of alkaline pretreatment with oxidative chemicals is a mutually beneficial strategy to support an alkali environment for lignin oxidation with the alkaline effect on delignification (Cheng et al., 2008). Researchers have shown that an overall enzymatic glucan hydrolysis yield of 85.2 g glucan hydrolyzed/100g raw glucan can be achieved with an alkaline peroxide oxidation (pressurized oxygen) pretreatment on switchgrass (0.3g/g $Ca(OH)_2$/g raw biomass, 120°C, 6.89 bar O_2, and 240 min) (Falls et al., 2011). Another report demonstrated for a a two-stage alkaline/oxidative pretreatment application on wheat straw 56% of hemicellulose in the raw material was solubilized after a 24-hour 1% w/w alkaline soaking, along with a second oxidative step (0.3% H_2O_2 for 24 h). The latter step was effective at breaking down remnant lignin in alkaline pretreated biomass residues to minor polluting compounds (Curreli et al., 1997).

1.4 *Clostridium Thermocellum*

C. *thermocellum*, a well-studied consolidated bioprocessing microbial catalyst, is a gram-positive, thermophilic, anaerobic bacterium that has cellulolytic, acidogenic and solventogenic capabilities. The primary fermentation products include ethanol, lactate, acetate, formate and levulinic acid (Raman et al., 2011, Zhang & Lynd, 2005). Additionally, carbon dioxide (CO_2) and hydrogen (H_2) are released, making *C.thermocellum* a possible candidate for large-scale gas production (Levin et al., 2006).

C. thermocelllum posseses an exocellular complex cellulolytic enzyme aggregation of at least 14 units, called the cellulosome, that actively binds to cellulose surfaces and solubilizes the polymer into smaller saccharide chains, primarily cellobiose and glucose (Wang et al., 1993, Coughlan et al., 1985). The cellulosome of *C. thermocellum* has a special cellulose binding domain (CBD) which is integrated into the cellulosome with a multitude of enzyme subunits and functions as a point of attachment for the cellulosome to cellulose polymers. The presence of the CBD and the integrity of the cellulosome complex while attached to the cell are significant to the effective digestion rates of cellulose by *C. thermocellum* (Berdichevsky et al., 1999, Ong et al., 1989). Initially, an amorphour outer layer surrounding *C.thermocellum* generates CBD to anchor cellulosomes on cellulosic substrates(Bayer & Lamed., 1986, Bayer et al., 1985, Nolte & Mayer., 1989, Lemaire et al., 1998). After binding to cellulose, exo-cellular, cellulolytic enzyme complexes including endoglucanases, exoglucanases and β-glucosidases actively cleave internal sites in cellulose chains and gradually hydrolyze those shorter sugar fragments into cellobiose or glucose depending on the distinctive enzymatic functions (Lynd et al., 2002). Since the cellulosome is intimately attached to the cell surface as well as the substrate itself,, isolation of cellulolytic enzymes with similar functional activities from *C. theromocellum* cultures is difficult. When the bacteria approach death, cellulosomes shed from the cell surface and remaining enzymes attached on the cellulose decay in activity when hydrolyzing substrate chains (Bayer & Lamed., 1986, Nolte & Mayer., 1989).

As a thermophilic, anaerobic strain the optimal growth temperature of 60℃ helps preclude the growth of many types of microorganisms (contaminants) in fermentation systems, and allows *C.thermocellum* cultures to be maintained even when solid substrates are not well sterilized (Demain et al., 2005). Researchers have studied the performance of *C. thermocellum* on cellobiose and microcrystalline cellulose (e.g. Avicel) as well as solid

lignocellulosic biomass materials including paper pulps, pretreated corn stover, yellow poplar, delignified wood fibers, switchgrass and alkali extracted sorghum stover (Rani et al., 1998, Moreau et al., 2015, Shao et al., 2011, Wei et al., 2014, Levin et al., 2006, Raman et al., 2009**).** Some of the different *C. thermocellum* strains, fermentation systems studied, substrates used and related end-product concentrations formed are highlighted in Table 1.3**.** Wild-type strains of *C.thermocellum* have been reported to produce 0.08-0.29 g ethanol per g glucose equivalents fermented when growing on cellobiose as a substrate. The low ethanol tolerance of this bacteria relative to yeast is one of the major limiting factors for its industrial exploitation and research efforts have been made to increase this organism's ethanol production capabilities (Freier et al., 1988, Sai Ram & Seenayya, 1989, Mori, 1990, Sai Ram et al., 1991, Herrero & Gomez, 1980, Tailliez et al., 1989a,b).

Cellulose metabolic pathways in *C.thermocellum* are shown in Figure 1.1, where cellobiose is the preferred sugar from hydrolysis of solid substrates and ethanol, acetate, formic acid and lactic acid are the four primary hydrolyzed products (Chinn & Mbaneme et al., 2015). Cellobiose degraded from cellulosic substrates is metabolized into glucose-1-phosphate and glucose by cellobiose phosphorylase and then directed to pyruvate through the glycolysis pathway since high fluxes were examined in a ^{14}C-glucose tracer experiment (Ng & Zeikus., 1982, Zhou et al., 2013). Pyruvate is then converted to acetyl-CoA as a key intermediate metabolite, along with release of CO_2 or formate depending on electron availability in cultures (Rydzak et al., 2011). As another reduced yield from pyruvate, lactic acid yield is controlled by lactate dehydrogenase intracellular enzyme (LDH). Acetyl-CoA is reduced to acetaldehyde and ultimately to ethanol with two reduced nicotinamide cofactors (NADH and NADPH) as electron donors under catalyze of a bifunctional aldehyde/alcohol dehydrogenase enzyme, AdhE (Lo et al., 2015). Acetyl-CoA as an intermediate can also be reduced to acetate, with the formation of ATP supporting cellular energetics and maintenance .

The overall goal of this project was to explore the benefits of growing *C. thermocellum* on alkaline and alkaline-oxidant pretreated switchgrass and miscanthus in semi-solid consolidated bioprocessing fermentation conditions. Objectives were to:

1. Examine single-stage alkaline and two-stage alkaline-oxidant chemical pretreatment methods as pre-processing steps to remove lignin and hemicellulose from perennial grasses while maintaining cellulosic fractions for subsequent processing. Alkaline chemicals (sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$)), hydrogen peroxide as an oxidant, chemical concentrations and stage times were studied for overall pretreatment effects on switchgrass and miscanthus composition.

2. Evaluate *Clostridum thermocellum* performance during growth on raw and pretreated switchgrass and miscanthus substrates through measurement of end-product yields and rates of product formation.

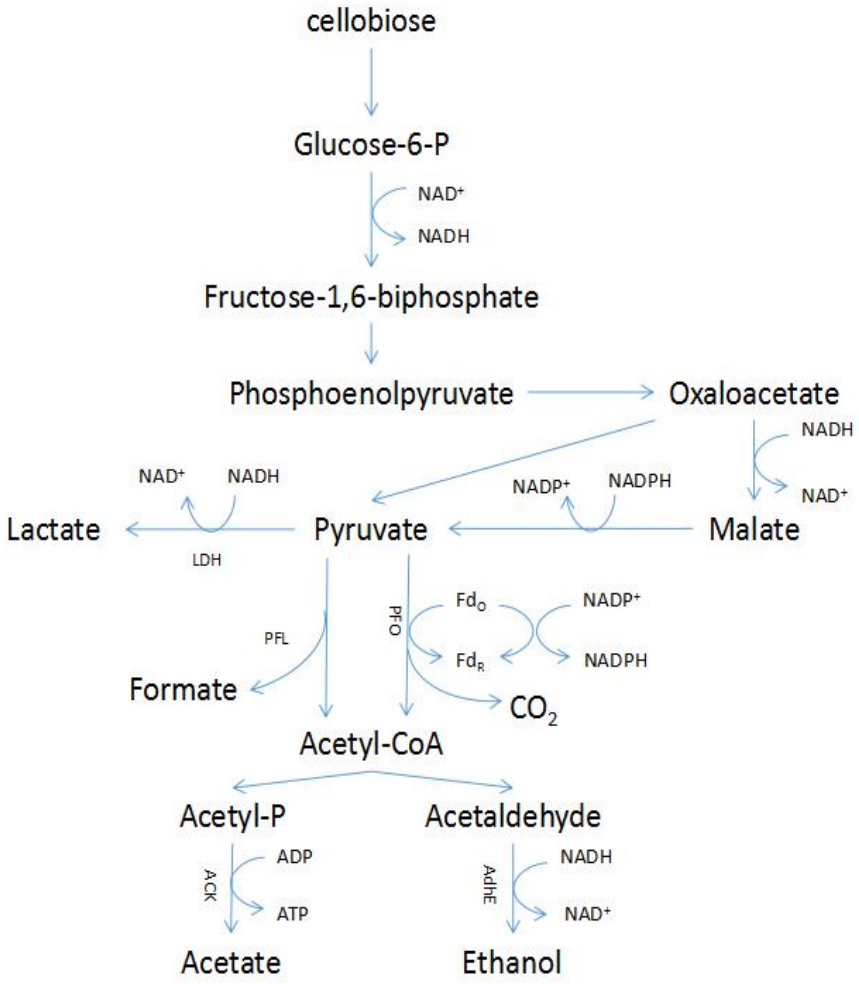

Figure 1.1 The metabolic pathway of Clostridium thermmocellum for production formation. LDH,lactate dehydrogenase (fructose-1,6-bisphosphate activated); PFO, pyruvate:fd oxidoreductase; PFL, pyruvate:formate lyase; ACK, Acetate kinase; AdhE, bifunctional enzyme composed of an aldehyde dehydrogenase and an alcohol dehydrogenase. Figure adapted from: Rydzak, T., Levin, D. B., Cicek, N., & Sparling, R. (2011). End-product induced metabolic shifts in Clostridium thermocellum ATCC 27405. Applied microbiology and biotechnology, 92(1), 199. And adapted from Tian, L., Papanek, B., Olson, D. G., Rydzak, T., Holwerda, E. K., Zheng, T., ... & Hettich, R. L. (2016). Simultaneous achievement of high ethanol yield and titer in Clostridium thermocellum. Biotechnology for biofuels, 9(1), 116.

Table 1.1 Acid Pretreatment Applications on Ligoncellulosic Biomass

Substrates	Solid to Liquid	Acid Concentration(w/w)	Heating Time (mins)	Temperature	Highlights on Structure Changes			REFERENCESs
					Cellulose	Hemicellulose	lignin	
Bagasse	1:20	2% sulfuric acid	15	111	crystal structure preserved;	Destruction enhanced	—	Chen et al., 2010
Loblolly Pine	1:8	0.5% sulfuric acid	10	180	Less ordered cellulose crystalline	—	Degree of condensation increased;	Sannigrahi et al.,2008
Bamboo	1:15	1% sulfuric acid	30	150	Amorphous part removed; Some of crystalline components broken	Surface area and porosity increased	—	Chen et al., 2014
Rice Straw	1:10	1% sulfuric acid	15	180	Surface area and pore volume increased;	Surrounding xylan decomposed	acid-soluble lignin removed	Hsu et al., 2010
Rice Straw	1:6.7	2% sulfuric acid	4	150	crystalline increased	—	exfoliated, fractured	Yu et al., 2009

Table 1.2 Alkaline Pretreatment Applications on Lignocellulosic Biomass

Substrates	Solid to Liquid	Acid Concentration(w/w)	Heating Time (mins)	Temperature	Highlights on Structure Changes			REFERENCESs
					Cellulose	Hemicellulose	Lignin	
Populus	1:10	2% Sodium Hydroxide	60	120	Degree of polymerization decreased	Gradually removed as time extend	Gradually removed as time extend	Bali et al., 2015
Moso Bamboo	1:20	8% Sodium Hydroxide	60	45	Some distortion of cellulose crystals.	Part dissolved	Cracks and collapses	Ling et al.,2008
Bamboo Stem	1:20	20% Sodium Hydroxide	240	60	Polymorphous lattice enhanced	Part deconstructed	wrinkled surface	Chen et al., 2016
Rye Straw	1:20	2% Aqueous Ammonia	60	480	Grafting of acrylamide onto cellulose	Acetyl and uronic ester groups break down	—	Domanski et al., 2016
Sorghum	1:14	0.35% Sodium Hydroxide	55	720	Both amorphous and crystalline bands decrease	fraction solublilzed	part removed	Sambusiti et al., 2013

Table 1.3 *Clostridium thermocellum* Performance on Different Substrates

Strain	Fermention Type	Substrate	Ethanol (mM)	Acetate (mM)	Total Products (mM)	REFERENCES
27405	Hemi-Solid	Delignified Wood Fiber	83.33	116.54	338.72	Levin et al., 2006
	Liquid	Cellobiose	97.48	137.44	370.71	Levin et al., 2006
	Liquid	Cellobiose	38.09	57.62	122.14	Islam et al., 2006
	Hemi-solid	Pretreated Switchgrass	8.30	12.93	—	Raman et al., 2009
	Liquid	^{15}N cellulose	26.32	23.47	—	Raman et al., 2009
	Hemi-solid	Pretreated *Populus*	6.52	13.56	—	Wilson et al., 2013
	Hemi-solid	Pretreated Switchgrass	4.35	8.47	—	Wilson et al., 2013
	Solid	paper pulp sludge	—	—	98.60	Chinn et al., 2007
	Liquid	α-cellulose	12.60	15.25	—	Islam et al., 2009
DSM 1313	Hemi-Solid	Transgenic Switchgrass	—	—	87.3	Yee et al., 2014
	Liquid	cellobiose	304.3	118.64	—	Thompson et al., 2017
JW 20	Solid	paper pulp sludge	—	—	157.4	Chinn et al., 2006
	Hemi-solid	paper pulp sludge	—	—	79.9	Chinn et al., 2006
AG 553	Liquid	Cellobiose	520.9	11.3	—	Tian et al., 2016
AG553 mutant	Liquid	Avicel	73.4	—	—	Papanek et al., 2015
M 1570	Hemi-Solid	Transgenic Switchgrass	—	—	95.8	Yee et al., 2014
DSM 2360	Hemi-Solid	Pretreated Rice Straw	21.08	11.19	—	Lü et al., 2017

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CHAPTER 2. Two-Stage Pretreatment of Switchgrass and Micanthus

2.1 Introduction

Biomass, in the form of woody and herbaceous plants and plant residues, has stored carbon energy that can be converted into fuels, chemicals or bio-materials. Biomass materials have been researched more heavily since the 1970s as an important renewable energy feedstock to supplement traditional energy sources, such as petrol, coal and natural gas, and to help mitigate current global environmental issues with carbon emission and air pollution. (Antizar & Turrion, 2008, Cao et al., 2015, Creutzig et al., 2015, Demura & Ye, 2010). Different from first-generation biofuel production using corn-derived starch or soybean oil as substrates, non-edible lignocellulosic biomass arguably has fewer negative impacts on food supplies and animal feed, with useful carbohydrates stored in structural polymers, especially cellulose and hemicellulose (Cotana et al., 2016, Shield & Boopathy., 2011). Biomass materials are expected to satisfy at least one third of the projected global energy demands by 2050 (Dornburg et al., 2010, Guo et al., 2015).

Switchgrass and miscanthus are perennial C4 grasses that are considered high dry matter lignocellulosic dedicated bioenergy crops and often studied as competitive candidates for biofuel and bio-based chemical production (McLaughlin & Kszos, 2005, Clifton-Brown et al., 2017, Scarlat et al., 2015). With relatively low soil fertility requirements and need for irrigation, these two herbaceous crop species have been harvested on marginal lands with reported yields in the U.S. up to 15 wet metric t/ha/yr and 40 wet metric t/ha/yr for switchgrass and miscanthus, respectively (Lee et al., 2015, Parrish & Fike et al., 2005, Wright & Turhollow, 2010, Qureshi et al., 2010). When considering biological approaches to converting these feedstocks to useful bio-products, both grasses, however, require a pretreatment step prior to hydrolysis for fermentable sugars. Pretreatment

methods can expose cellulose fibrils that are protected by the matrix of lignin and hemcellulose in plant cells to enzyme and microbial catalysts. (Sørensen et al., 2008, Nlewem & Thrash, 2010, Pallapolu et al., 2011).

A variety of pretreatment methods have been studied to disrupt the lignin and hemicellulose in the plant cell wall and minimize barriers to cellulose recalcitrance with aims to improve enzymatic hydrolysis or microbial digestibility of biomass substrates (Agbor et al., 2011, Keller et al., 2003). Chemical pretreatment methods, including acid and alkaline, have been broadly studied to evaluate delignfication of lignocellulosic biomass since they have strong effects on the linkages between polymerized saccharides as well as lignin and/or hemicellulose removal (Purto et al., 2016, Yelle et al., 2013). Compared to acid hydrolysis the mechanisms of alkaline pretreatment of biomass target chemical bonds in lignin and hemicellulose fractions, reducing losses of cellulose and resulting in more oligosaccharides available for conversion into fermentable sugars in subsequent steps. In addition, alkali reagents present fewer challenges with reactor vessel corrosion and lower by-product formation rates which can have inhibitory impacts on enzyme efficiency and/or microbial growth (Rajan & Carrier, 2014, Redding et al., 2011). Different kinds of alkaline pretreatment methods, such as sodium hydroxide (NaOH), calcium hydroxide ($Ca(OH)_2$) and ammonia fiber expansion (AFEX), have been demonstrated under various concentrations and other environmental conditions such as substrate loading, time, temperature and particle size (Xu et al., 2012, Bals et al., 2014, Xu et al., 2010, Yang et al., 2015). Many reports indicate that NaOH has superior capabilities in lignin and cellulose removal efficiency in combination with low alkaline concentration requirementd at ambient temperature compared to other alkali pretreatment methods. To date, NaOH has been studied on corn stover, rice straw, wheat straw, sorghum, hybrid napier grass and woody biomass (softwoods and hardwoods) and relative optimal pretreatment parameters for these substrates were reported (McIntosh & Vancov., 2011, Kim & Han., 2012., Hong et al., 2015.,Yu et al., 2014., Mohaptra et al.,

2016). $Ca(OH)_2$ is another alkaline candidate which has been successfully applied in pretreatment of corn stover, sugracane bagasse, switchgrass and rice hulls because of its cheaper price compared to NaOH (Rabelo et al., 2009, Kim & Holtzapple., 2005, Saha & Cotta., 2008., Falls & Holtzapple., 2011). However, reports on switchgrass and miscanthus pretreatments using NaOH or $Ca(OH)_2$ are limited, especially on evaluation of chemical loading concentration and pretreatment time. Additionally, most studies have primarily focused on lignocellulosic enzymatic hydrolysis efficiency and sugar release amounts after pretreatment rather than the comparisons of biomass composition before and after pretreatment.

Besides a single alkaline pretreatment on lignocellulostic substrates, hydrogen peroxide (H_2O_2) has been considered as part of a multiple reagent pretreatment to further the extent of deligninfication by triggering oxidative reactions on lignin fractions of the biomass (Enriquez et al., 2016). Banerjee et al. (2012) indicated glucose and xylose yields were 75% and 71% of the theoretical maximum for corn stover under a single stage alkaline, hydrogen peroxide (AHP) (0.125 g H_2O_2 g/g biomass, constant pH at 11.5 adjusted by NaOH) pretreatment for 48 hours at ambient temperature. Additionally, Yu and colleagues concluded that a separate two-stage aqueous ammonia pretreatment with hydrogen peroxide significantly enhanced removal of lignin up to 89.5% in miscanthus compared to the integration of alkaline and oxidative chemicals into a single mixture (Yu et al., 2014). Research on different parameters for a two-step sodium hydroxide-hydrogen peroxide pretreatment are limited and there is opportunity for further research to determine significant factors including, material loading ratios, temperature, concentrations of alkaline and H_2O_2, and duration for each step while applied to different lignocellulosic substrates,.

The objective of this research effort was to investigate compositional changes of switchgrass and miscanthus when exposed to a two-stage alkaline-oxidative chemical

pretreatment approach. Alkaline type and concentration and time were evaluated as part of stage 1 followed by time of exposure to hydrogen peroxide as part of stage 2.

3.2 Materials and Methods.

3.2.1 Biomass and Chemicals

Switchgrass and miscanthus samples were both harvested from the Williamsdale Bioenergy Field Laboratory near Wallace, NC (34.765 N, 78.100 W) in December, 2013. Samples were dried at 45 ℃ in a drying oven until moisture stabilization and then ground in a hammer mill to pass through a 3/8" and then a 3/16" screen. Glucose, xylose, arabinose, sodium hydroxide, calcium hydroxide were obtained from Sigma-Aldrich (St. Louis, MO). 3% w/w H_2O_2 was obtained from VWR (Radur, PA).

3.2.2 Experiment Design

This two-stage pretreatment study was carried out in two experimental sets to minimize the number of treatment combinations and achieve the optimization of pretreatment conditions studied more efficiently for switchgrass and miscanthus. In the first experimental set, the experimental design was a randomized complete block design (RCBD) in 2 blocks (by alkaline type) with a full 2 x 2 x 2 factorial treatment structure for both switchgrass and miscanthus biomass materials. For each alkaline block (NaOH, CaOH), the factors were chemical loading ratio (X_1), alkaline pretreatment time (X_2) and 3% H_2O_2 pretreatment time (X_3). The levels for these factors were X_1: 0.05 g alkaline/g dry initial matter and 0.2 g alkaline/g initial dry matter; X_2: 0.5h and 1.5h; and X_3:0.5h and 1.5h. The main and interaction effects of each factor on mass fraction of cellulose, hemicellulose and lignin removed or lost were evaluated by performing RCBD ANOVA in SAS 9.4 (SAS institute Inc., Cary, NC). Assessments of statistical significance for treatment comparisons were made at an α level of 0.05. Analysis results from the first experimental set were used to determine the next set of 8 treatment combinations

completed in the second experimental set for both switchgrass and miscanthus biomass. If at least two first- order factors were found statistically significant for response variables in the ANOVA results from the first experimental set, those factors were carried forward to the second experimental set and used to determine treatment levels (Table 2.1). Limited statistical differences observed in $Ca(OH)_2$ treatments from set I resulted in completing experiments for just NaOH as part of experimental set II. All NaOH pretreatment data from experimental sets I & II were finally combined to determine a multiple regression model for all first-order, interaction and quadratic terms in SAS 9.4 (SAS institute Inc., Cary, NC). Assessments of statistical significance for each variable estimate were made at an α level of 0.05. Pairwise *t*-test comparison tests between treatment combinations were also made at an *p*-level of 0.05

Different from creating a simple and straightforward model that covers all variables and variable interactions in a regression equation, optimization of regression models has gained attention to limit the number of selected factors (Mitchell & Beauchamp., 1988). In addition to the impacts of main first-order variables, effects of interaction and quadratic terms also have non-negligible influences on final model accuracy. As a result regression model analysis in this study applied a fast false selection rate (FSR) method to filter optimized models through a backward selection for both switchgrass and miscanthus (Crew et al., 2011). Three primary principles were followed to pick final optimized equations: 1) the variable with the highest p-value was priorized to be excluded from model; 2) a main effect variable was never thrown out before any interaction containing it or a square of it, which is known as “enforcing strong hierarchy”; and 3) the model with lowest bayesian information criterion (BIC) were selected for final optimized equation (Weakliem., 1999). R^2 values of each equation were calculated based on method described by Edward et al., (2008).

Table 2.1 Pretreatment Factors and Levels Studied for each Alkaline Type (NaOH and $Ca(OH)_2$) using Switchgrass and Miscanthus in Experimental Sets I & II

Experiment Set	Alkaline Type	Treatment No.	Alkaline Loading (g/g initial dry matter, X_1)	Alkaline Pretreatment Time (h, X_2)	H_2O_2 Pretreatment Time (h, X_3)
1	NaOH	1	0.05	0.5	0.5
1	NaOH	2	0.05	0.5	1.5
1	NaOH	3	0.05	1.5	0.5
1	NaOH	4	0.05	1.5	1.5
1	NaOH	5	0.2	0.5	0.5
1	NaOH	6	0.2	0.5	1.5
1	NaOH	7	0.2	1.5	0.5
1	NaOH	8	0.2	1.5	1.5
1	$Ca(OH)_2$	1	0.05	0.5	0.5
1	$Ca(OH)_2$	2	0.05	0.5	1.5
1	$Ca(OH)_2$	3	0.05	1.5	0.5
1	$Ca(OH)_2$	4	0.05	1.5	1.5
1	$Ca(OH)_2$	5	0.2	0.5	0.5
1	$Ca(OH)_2$	6	0.2	0.5	1.5
1	$Ca(OH)_2$	7	0.2	1.5	0.5
1	$Ca(OH)_2$	8	0.2	1.5	1.5
2	NaOH	1	0.1	0.5	0.1
2	NaOH	2	0.1	0.5	1
2	NaOH	3	0.1	0.5	1.5
2	NaOH	4	0.15	0.5	0.5
2	NaOH	5	0.15	0.5	1
2	NaOH	6	0.15	0.5	1.5
2	NaOH	7	0.25	0.5	0.5
2	NaOH	8	0.25	0.5	1

2.2.3 Two-Stage Pretreatments

2.3.3.1 NaOH and $Ca(OH)_2$ Stage (Stage 1)

Switchgrass and miscanthus materials were dried in a 105°C oven for 24 hours before the pretreatment process to determine biomass moisture contents and normalize sample

allocations based on material dry weight (MC wet-basis-switchgrass:14.18% and MC wet-basis- miscanthus: 9.77%). A total of oven dried 2.5g biomass sample and 25 mL of NaOH or $Ca(OH)_2$ stock solution were placed in a 125 mL serum bottle to achieve a solid to liquid ratio of 0.1 g/mL (10% w/v). Two NaOH or $Ca(OH)_2$ stock solutions were prepared 1) 1.25 g NaOH or $Ca(OH)_2$ in 250 mls (12.5 mM of NaOH and 6.76 mM of $Ca(OH)_2$ or 0.5% w/w) and 2) 5 g NaOH or $Ca(OH)_2$ in 250 mls (50 mM of NaOH and 27.03 mM of $Ca(OH)_2$ or 2% w/w) to achieve 0.05g NaOH or $Ca(OH)_2$ /g intial dry biomass and 0.2g NaOH or $Ca(OH)_2$ /g initial dry biomass, respectively, with the addition of 25 mls in the pretreatment bottles. All serum bottles were sealed with rubber stoppers and crimped with aluminum seals before being placed in an autoclave (121℃, 19 psi) to achieve an elevated processing temperature. After the specified heating time (0.5h or 1.5h), bottles were removed from the autoclave and placed in an ice bath to cool. 75 mL of deionized water was added to each bottle and 2 mL aliquots were drawn from each replicate and stored at -80 ℃ for acetate and formic acid concentration analysis. Pretreated biomass replicates were washed with volumes of deionized water (300 mL per wash, ~10 washes) until pH of the outflow water stabilized at 7. The solid fractions were dried at 105℃ in a drying oven overnight and weighed to determine solid losses from this step. Material collected from each treatment combination and replicate was stored in a sealed plastic bag for H_2O_2 pretreatment on second day.

2.3.3.2 Hydrogen Peroxide (H_2O_2) Stage (Stage 2)

A total of 0.8g oven dried pretreated biomass from Stage 1 and 8 mL of 3% H_2O_2 solution were placed in a 125 mL serum bottle to achieve a solid to liquid ratio of 0.1 g/mL (10% w/v). All serum bottles were sealed with rubber stoppers and crimped with aluminum seals before being placed in an autoclave (121℃, 19 psi) to achieve the elevated processing temperature. After the specified treatment combination heating reaction time (0.5h or 1.5h), bottles were removed from the autoclave and cooled in an ice bath. Once cool, 32 mL of deionized water was added to each bottle and 2 mL aliquots were drawn

from each replicate bottle and stored at -80 ℃ for acetate and formic acid concentration analysis. Pretreated biomass replicates were washed with volumes (50 ml per wash, ~10 washes) of deionized water until pH of the outflow water stabilized at 7. The solid fractions were dried in a drying oven at 105℃ for 24 hours and weighed to determine solid losses in this step. Material collected was stored in a sealed plastic bag for composition analysis.

2.2.4 Composition Analysis

Modified Laboratory Analytical Procedures (LAP NREL/TP-51-42618, NREL/TP-510−42619 & NREL/TP-51-42620) established by National Renewable Energy Laboratory (NREL) combined with method improvement recommendations on "Sugar Recovery Standard" adopted from Whitfield et al. (2016) were used to determine total solids, ash, structural carbohydrates (cellulose, hemicellulose) and lignin of raw and pretreated grass materials (Sluiter et al., 2008(a,b), Ruiz et al., 2011, Whitfield et al., 2016). Similar to other perennial grass pretreatment experiments that report composition analysis methods, soxhlet extractions were not completed as part of the biomass material preparation for composition analysis in this study (Xu et al., 2010). Validity of this approach was checked using both raw and pretreated material prior to completion of all sample analysis. It was found that composition analysis of material with and without soxhlet extraction did not show any statistical differences in switchgrass and miscanthus used in this study (Maximum percent difference of water extractives and ethanol extractives:2.72%, 2.11%). Carbohydrate composition in biomass was determined by measuring hexoses (glucose) and pentoses (xylan and arabinose) by HPLC. Concentrations of sugar recovery standard solutions were 1 mg/mL for glucose and xylose and 0.5 mg/mL for arabinose.

2.2.5 HPLC Analysis

Acetate and formic acid concentrations were measured from sample extracts from each

pretreatment stage. Cellobiose, glucose, xylose, arabinose, galactose, acetate, formic acid, levulinic acid, 5-hydroxymethylfurfural, 2-furaldehyde were measured in samples collected from the composition analysis method. Samples were centrifuged (10 min, 13,000 *x g*) and supernatants filtered through 0.2 μm nylon filters (Whatman, Maidstone, UK). A 10 μL aliquot of each sample was analyzed using a Phenomenex (Torrance, CA) Rezex ROA column (300 mm *7.8 mm) at 50 ℃ in 50 min runs, with 0.6 mL/min 5 mM sulfuric acid in HPLC water (Sigma-Aldrich) as the eluent, and quantified by refractive index detection (RID).

2.2.6 Calculations

2.2.6.1.Pretreatment Efficiency—Quantification of Cellulose Preservation with Improved Removal of hemicellulose and lignin

For a better understanding of pretreatment efficiency, minimal cellulose loss with maximum degradation of lignin and hemicellulose, a quantifiable term (Y) was created that represents the difference between cellulose and sum of hemicellulose and lignin of pretreated biomass (switchgrass, miscanthus). The calculations involved in determining Y require the mass fractions of cellulose, hemicellulose and lignin remaining after pretreatment and are described below:

$$\mathrm{F}_{(cell)} = \frac{W_i \times P_{i(cell)} - W_f \times P_{f(cell)}}{W_i} \quad (1)$$

$$\mathrm{F}_{(hemi)} = \frac{W_i \times P_{i(hemi)} - W_f \times P_{f(hemi)}}{W_i} \quad (2)$$

$$\mathrm{F}_{(lignin)} = \frac{W_i \times P_{i(lignin)} - W_f \times P_{f(lignin)}}{W_i} \quad (3)$$

$$Y = F_{(hemi)} + F_{(lignin)} - F_{(cell)} \quad (4)$$

Where $F_{(cell)}$, $F_{(hemi)}$ and $F_{(lignin)}$ individually represent cellulose, hemicellulose and lignin mass losses over initial dry matter of the given component ($W_{i,}$ grams); $P_{i(cell)}$, $P_{i(hemi)}$ and $P_{i(lignin)}$ are fractions (decimal) of cellulose, hemicellulose and lignin in dried raw biomass and $P_{f(cell)}$, $P_{f(hemi)}$ and $P_{f(lignin)}$ are corresponding fractions (decimal) of cellulose, hemicellulose and lignin in final dried pretreated biomass.

2.2.6.2. Cellulose and hemicellulose fractions

Cellulose and hemicellulose fractions in raw and pretreated biomass were determined using a recommended method for improved quantification accuracy proposed by Whitfield et al. (2016). The calculation (below) accounts for the immediate degradation products (5-HMF, 2-furaldehyde) of glucose and adjusts for related degradation in the sugar recovery standards (SRS) (Whitfield et al., 2016).

$$R_{Hexose} = \frac{[Hexose]_f}{[Glucose]_i} \quad (5)$$

$$R_{pentose} = \frac{[Pentose]_f}{[Xylose]_i + [Arabinose]_i} \quad (6)$$

$$w_{Cellulose} = \frac{[Hexose]_{f,\,adj}}{m_{sample}} \left(\frac{162.14}{180.16}\right) \times 84mL \quad (7)$$

$$w_{Hemicellulose} = \frac{[Pentose]_{f,\,adj}}{m_{sample}} \left(\frac{132.11}{150.13}\right) \times 84mL \quad (8)$$

Where R_{Hexose} and $R_{pentose}$ respectively represent SRS yield by dividing the monosaccharide equivalents in the autoclaved SRS (indicated by f) by the concentrations

at which the SRS solution was prepared (indicated by i) (that is, the concentration prior to acid addition); $W_{cellulose}$ and $W_{hemicellulose}$ are percentages of cellulose and hemicellulose in biomass; $[Hexose]_{f,adj}$ and $[Pentose]_{f,adj}$ are final, SRS-adjusted monosaccharide equivalents; m_{sample} is the mass of sample used in the analysis.

2.2.6.3 Centered Variables and General Regression model for two-stage NaOH pretreatments

As results of experimental sets I and II were collected, all factor variables alkaline loading (X_1), stage 1 residence time (X_2) and stage 2 residence time (X_3) were centered before establishing a general regression model to reduce nonessential collinearity using the mean-centering method (Dalal & Zicker., 2011). Corresponding new variables were named as X_{1c}, X_{2c} and X_{3c}. The resulting centering equations are given below. Final four response variables (cellulose, hemicellulose, lignin and Y) are represented by T_i and were fitted to full factorial empirical equations (quadratic polynomial regression models) to identify key parameters for both switchgrass and miscanthus pretreated with NaOH:

$X_{1c}=X_1-0.140625;$

$X_{2c}=X_2-0.75;$

$X_{3c}=X_3-0.96875;$

$$T_i=\beta_0+\beta_1X_{1c}+\beta_2X_{2c}+\beta_{3c}X_{3c}+\beta_4X_{1c}X_{2c}+\beta_5X_{1c}X_{3c}+\beta_6X_{2c}X_{3c}+\beta_7X_{1c}X_{1c}+\beta_8X_{3c}X_{3c}$$

The response variable T_i represents loss of cellulose, hemicellulose and lignin and pretreatment efficiency (Y). The variables X_{1c}, X_{2c} and X_{3c} are centered values of NaOH loadings, residence time of stage 1 and residence time of stage 2 respectively. The predicted response for each variable was determined by the intercept (β_0), linear (β_1, β_2, β_3), interaction (β_4, β_5, β_6) and quadratic (β_7, β_8) parameter estimates. A parameter estimate was determined to be significant if its p-value was less than α of 0.05.

2.3 Results

2.3.1 Composition Changes of Pretreated Switchgrass and Statistical Analysis

Composition changes of pretreated switchgrass using NaOH under the different pretreatment conditions completed is presented in Table 2.2 and Table 2.3 shows the significance of NaOH pretreatment parameter estimates for main, interaction and quadratic variables on fraction losses of cellulose and hemicellulose, as well as loss or removal of lignin during each process. For NaOH on switchgrass the three first-order effects (X_1-alkaline loading), X_2-stage 1 time and X_3-stage 2 time) and one interaction term (X_2*X_3) were significant in hemicellulose digestability. Among all variables, NaOH loadings (X_1) was most influential (regression coefficient estimate=1.3693). The hemicellulose fraction loss ranged from 0.46 when all three variables were at the lowest levels for each pretreatment factor combination (X_1=0.05 g/g initial dry matter, X_2=0.5h, X_3=0.5h) to a maximum of 0.80 when NaOH loading (X_1) was 0.2g/g initial dry matter and residence time of both stages were 1.5h. Lignin reduction was affected by two first-order effects (X_1-NaOH loading and X3-stage 2 time), one second-order effect (X_1^2) and one interaction effect (X_1*X_2). Lignin losses reached up to 85.1% (X_1=0.2 g/g initial dry matter, X_2=1.5h, X_3=1.5h) of initial lignin content for pretreatments with 0.2 g NaOH/g initial dry matter (X_1), 1.5h stage 1 (X_2) and 1.5h stage 2 (X_3). This level of removal was 68.2% higher compared to treatment conditions at the lowest levels for alkaline loading X_1 (0.05g/g initial dry matter) and shortest stage residence times (X_2=0.5h, X_3=0.5). Cellulose fraction loss was also significantly impacted by first order variables (X_1, X_2 and X_3). The greatest cellulose loss was also achieved under the same conditions where the highest hemicellulose and lignin fraction losses occurred. *T*-test comparisons for cellulose fraction losses within NaOH loading level of 0.05 g/g dry matter showed no significant differences (p-value>0.05) which indicates that the cellulose present was still stable under these conditions and changes in the residence time of each stage had little influence on altering the cellulose content.

Composition changes of pretreated switchgrass using $Ca(OH)_2$ under the different pretreatment conditions completed is presented in Table 2.4 and its relative ANOVA analysis is provided in Table 2.5. Analysis of this pretreatment data indicated that $Ca(OH)_2$ loading had little effect on changes in cellulose, hemicellulose and lignin content (p-value>0.05) and only residence time in stage 2 (X_3) showed significant differences for each component (p-value<0.001). For treatments completed at 0.05g/g initial dry matter $Ca(OH)_2$ loading (X_1), across residence times of stage one (X_2), cellulose, hemicellulose and lignin losses increased by 20.2%, 91.9% and 23.8%, respectively as stage 3 residence time (X_3) extended from 0.5 to 1.5 h (p-value<0.05).

It was also found that differences between switchgrass cellulose and hemicellulose fraction losses at the lowest loading of 0.05g/g initial dry matter for $Ca(OH)_2$ (X_1) after 0.5h time in both stage 1 and stage 2 were only 5.9% higher when compared to losses in water-treated controls (same biomass loading, no chemical). This indicates that the addition of $Ca(OH)_2$ was limited in its effects on hemicellulose and cellulose composition changes. However, when comparing lignin losses between the $Ca(OH)_2$ and water only treatments, considerable lignin degradation was observed, 0.37 and 0.20 fractional losses, respectively. Much of this difference is related to the influences of H_2O_2 from stage 2 rather than the $Ca(OH)_2$ in stage 1.

Further examination of the two alkaline reagents, NaOH and $Ca(OH)_2$, on losses of hemicellulose and lignin fractions at similar treatment combinations of loading ratio (X_1) and stage residence times (X_2 and X_3) showed greater loss values in the NaOH pretreatments than the $Ca(OH)_2$ pretreatments. This observation is a strong indication that NaOH had a superior effect on removal of lignin and hemicellulose in switchgrass biomass (p-value<0.05). The highest differences in hemicellulose and lignin losses between the two alkaline reagents (NaOH and $Ca(OH)_2$) occurred when the alkaline loading ratio of 0.2 g/g initial dry matter with stage I and stage II residence times of 0.5h,

were used.

Table 2.2 Composition Changes (fraction lost) for Cellulose, Hemicellulose and Lignin Pretreated Switchgrass for Different Treatment Combinations. All values are expressed as averages of duplicate samples.*

NaOH Loading (X_1) (g/g initial switchgrass dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Fraction Loss			Total Biomass Loss (%)
			Cellulose	Hemicellulose	Lignin	
0.05	0.5	0.5	0.275	0.458	0.506	40.84%
0.05	0.5	1.5	0.291	0.588	0.573	49.66%
0.05	1.5	0.5	0.295	0.586	0.528	41.95%
0.05	1.5	1.5	0.296	0.575	0.535	52.65%
0.1	0.5	0.5	0.226	0.498	0.711	50.00%
0.1	0.5	1	0.278	0.647	0.738	57.16%
0.1	0.5	1.5	0.300	0.709	0.760	58.46%
0.15	0.5	0.5	0.323	0.604	0.772	58.91%
0.15	0.5	1	0.381	0.708	0.790	63.24%
0.15	0.5	1.5	0.347	0.750	0.806	62.87%
0.2	0.5	0.5	0.391	0.740	0.785	61.33%
0.2	0.5	1.5	0.435	0.766	0.805	65.15%
0.2	1.5	0.5	0.484	0.792	0.823	67.62%
0.2	1.5	1.5	0.496	0.802	0.851	69.61%
0.25	0.5	0.5	0.389	0.760	0.816	66.74%
0.25	0.5	1	0.382	0.774	0.815	65.12%

***Note: Raw switchgrass used in this study contained 34.57% of glucan, 23.53% of xylan and 28.43% of acid insoluble lignin.**

Table 2.3 Initial parameters estimates for all switchgrass pretreatment factors, interactions and quadratic terms on cellulose, hemicellulose, and lignin losses

	Cellulose		Hemicellulose		Lignin	
Terms	**Estimate**	***p*-value**	**Estimate**	***p*-value**	**Estimate**	***p*-value**
X_{1c}	0.969	<.0001*	1.370	<.0001*	1.429	<.0001*
X_{2c}	0.053	0.006*	0.060	0.013*	0.023	0.071
$X_{3}c$	0.031	0.034*	0.091	0.0003*	0.032	0.003*
$X_{1c}*X_{2c}$	0.336	0.133	-0.028	0.931	0.421	0.010*
$X_{1c}*X_{3c}$	-0.017	0.936	-0.406	0.202	-0.129	0.372
$X_{2c}*X_{3c}$	-0.029	0.314	-0.125	0.009*	-0.023	0.260
$X_{1c}*X_{1c}$	-2.494	0.200	-3.506	0.194	-9.590	<.0001*
$X_{3c}*X_{3c}$	-0.056	0.452	-0.064	0.524	-0.005	0.925

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

Table 2.4 Composition Changes (fraction lost) for Cellulose, Hemicellulose and Lignin Pretreated Switchgrass for Different Treatment Combinations Using Ca(OH)2. All values are expressed as averages of duplicate samples.*

$Ca(OH)_2$ Loading (X_1) (g/g initial switchgrass dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Fraction Loss			Total Biomass Loss (%)
			Cellulose	Hemicellulose	Lignin	
0.05	0.5	0.5	0.233	0.236	0.369	30.66%
0.05	0.5	1.5	0.280	0.453	0.457	42.62%
0.05	1.5	0.5	0.217	0.257	0.387	32.20%
0.05	1.5	1.5	0.296	0.476	0.491	41.47%
0.2	0.5	0.5	0.230	0.290	0.367	31.91%
0.2	0.5	1.5	0.282	0.463	0.475	42.24%
0.2	1.5	0.5	0.243	0.280	0.421	33.01%
0.2	1.5	1.5	0.299	0.438	0.487	42.32%

*** Raw switchgrass used in this study contained 34.57% of glucan, 23.53% of xylan and 28.43% of acid insoluble lignin.**

**** Cellulose, hemicellulose and lignin reduction of raw material in DI water at both stage for 0.5 h (control) is respectively 0.2200. 0.2203and 0.2021;**

Table 2.5. ANOVA Test of Compositional Changes (fraction lost) for Cellulose, Hemicellulose and Lignin Pretreated Switchgrass for Different Treatment Combinations Using $Ca(OH)_2$

	Cellulose	Hemicellulose	Lignin
Terms	*p*-value	*p*-value	*p*-value
X_1	0.327	0.545	0.286
X_2	0.297	0.919	0.220
X_3	<.0001*	<.0001*	<.0001*
X_1*X_2	0.279	0.342	0.745
X_1*X_3	0.532	0.221	0.692
X_2*X_3	0.212	0.850	0.544
$X_1*X_2*X_3$	0.303	0.836	0.186

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

2.3.2 Composition Changes of Pretreated Miscanthus and Statistical Analysis

The two-stage process studied showed similar effects and results using NaOH on miscanthus as those that were observed on switchgrass for composition changes (Table 2.4) and pretreatment parameter estimates for regression variables (Table 2.5). X_1

(alkaline loading) was most influential for all response variables, cellulose, hemicellulose and lignin reduction (p-value <0.001). As alkaline loading (X_1) increased from 0.05g NaOH/g to 0.2g NaOH/g of initial dried miscanthus there was a 1.6 fold increase in both cellulose and hemicellulose losses and up to a 1.8 fold increase in lignin reduction at the lowest residence time levels for the two stages (X_2-0.5h; X_3-0.5h). Stage 2 time (X_3) also significantly influenced the three response variables, which suggests that extended exposure to oxidants in the two stage pretreatment was beneficial for reduction of hemicellulose and lignin components. The increase in time for stage two (X_3) resulted in statistically differences (p-value<0.05) in hemicellulose and cellulose changes within the constant levels of alkaline loading (X_1) and stage 1 time (X_2) , while differences were statistically similar for lignin changes (p-value>0.05).

The composition changes in the material for the different treatment combinations are presented in Table 2.8 and the ANOVA results for the main and interaction effects are shown in Table 2.9. Pretreatment of miscanthus using $Ca(OH)_2$, showed similar results to switchgrass, where the time exposure to oxidants in stage 2 (X_3) was the only statistically significant factor for losses in cellulose, hemicellulose and lignin. As residence time of stage 2 increased from 0.5h to 1.5h, cellulose, hemicellulose and lignin losses increased by 22.8%, 78.4% and 29.7%, respectively, when treatments were completed across $Ca(OH)_2$ loadings (X_1) and across residence times of stage one (X_2),

Comparisons between alkaline types, NaOH and $Ca(OH)_2$, for miscanthus pretreatment effects on losses in hemicellulose and lignin showed that the changes in composition were significantly higher for NaOH than $Ca(OH)_2$ treatments (p-value<0.05). This finding is similar to the differences observed in alkaline types for switchgrass. However, under the same conditions cellulose loss when using 0.2g/g initial dry matter of $Ca(OH)_2$ as an alkaline reagent was on average less than 23.4% of the cellulose loss observed at a same level of NaOH loading. This reduced cellulose loss observed by $Ca(OH)_2$ compared to

NaOH in both miscanthus and switchgrass suggests the mechanisms of $Ca(OH)_2$ have cellulose preservation benefits, yet the lack of lignin reduction may not prove as useful. While for the NaOH reagent higher hemicellulose and lignin removal can be achieved simultaneously with a greater risk of solubilizing more cellulose during pretreatment.

Table 2.6 Composition Changes (Fraction Lost) of Cellulose, Hemicellulose and Lignin Pretreated Miscanthus for Different Treatment Combinations. All values are expressed as averages of duplicate samples.*

NaOH Loading (X_1) (g/g initial miscanthus dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Deduction Fraction			Total Biomass Loss (%)
			Cellulose	Hemicellulose	Lignin	
0.05	0.5	0.5	0.188	0.387	0.452	32.40%
0.05	0.5	1.5	0.247	0.598	0.533	40.42%
0.05	1.5	0.5	0.238	0.461	0.495	33.36%
0.05	1.5	1.5	0.289	0.629	0.542	41.98%
0.1	0.5	0.5	0.231	0.532	0.790	46.43%
0.1	0.5	1	0.206	0.659	0.789	51.37%
0.1	0.5	1.5	0.207	0.725	0.789	57.16%
0.15	0.5	0.5	0.171	0.612	0.773	55.53%
0.15	0.5	1	0.349	0.720	0.864	56.01%
0.15	0.5	1.5	0.288	0.781	0.841	60.45%
0.2	0.5	0.5	0.296	0.619	0.825	49.51%
0.2	0.5	1.5	0.383	0.764	0.871	59.50%
0.2	1.5	0.5	0.321	0.671	0.859	55.23%
0.2	1.5	1.5	0.400	0.783	0.898	62.96%
0.25	0.5	0.5	0.285	0.727	0.857	57.42%
0.25	0.5	1	0.255	0.735	0.855	56.81%

***Note: Raw miscanthus used in this study contained 43.27% of glucan, 26.43% of xylan and 23.70% of acid insoluble lignin.**

Table 2.7 Initial Parameter Estimates For All Miscanthus Pretreatment Factors, Interactions and Quadratic Terms on Cellulose, Hemicellulose, and Lignin Losses

	Cellulose		Hemicellulose		Lignin	
Terms	**Estimate**	***p*-value**	**Estimate**	***p*-value**	**Estimate**	***p*-value**
X_{1c}	0.651	<.0001*	1.148	<.0001*	1.765	<.0001*
X_{2c}	0.034	0.161	0.042	0.002*	0.029	0.187
X_{3c}	0.061	0.003*	0.158	<.0001*	0.046	0.011*
$X_{1c}*X_{2c}$	-0.187	0.517	-0.117	0.433	0.244	0.354
$X_{1c}*X_{3c}$	0.120	0.663	-0.554	0.001*	-0.166	0.508
$X_{2c}*X_{3c}$	0.014	0.706	-0.033	0.114	0.000	0.992
$X_{1c}*X_{1c}$	-4.144	0.112	-5.518	0.0003*	-16.64	<.0001*
$X_{3c}*X_{3c}$	-0.075	0.446	-0.035	0.482	-0.079	0.377

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

Table 2.8 Composition Changes (fraction lost) for Cellulose, Hemicellulose and Lignin Pretreated Miscanthus for Different Treatment Combinations Using $Ca(OH)_2$. All values are expressed as averages of duplicate samples.*

$Ca(OH)_2$ Loading (X_1) (g/g) initial miscanthus dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Deduction Fraction			Total Biomass Loss (%)
			Cellulose	Hemicellulose	Lignin	
0.05	0.5	0.5	0.225	0.328	0.184	23.28%
0.05	0.5	1.5	0.281	0.525	0.370	33.85%
0.05	1.5	0.5	0.218	0.311	0.244	19.81%
0.05	1.5	1.5	0.296	0.566	0.419	37.14%
0.2	0.5	0.5	0.226	0.336	0.317	26.25%
0.2	0.5	1.5	0.337	0.582	0.437	38.71%
0.2	1.5	0.5	0.249	0.336	0.320	27.53%
0.2	1.5	1.5	0.332	0.576	0.436	37.98%

*** Raw miscanthus used in this study contained 43.57 % of glucan, 26.43% of xylan and 23.7% of acid insoluble lignin.**

**** Cellulose, hemicellulose and lignin reduction of raw material in DI water at both stage for 0.5 h (control) is respectively 0.2083, 0.3002 and 0.1701;**

Table 2.9. ANOVA Test of Compositional Changes (fraction lost) for Cellulose, Hemicellulose and Lignin Pretreated Miscanthus for Different Treatment Combinations Using $Ca(OH)_2$

	Cellulose	Hemicellulose	Lignin
Terms	***p*-value**	***p*-value**	***p*-value**
X_1	0.109	0.072	0.072
X_2	0.797	0.778	0.132
X_3	0.002*	<.0001*	<.0001*
X_1*X_2	0.765	0.474	0.136
X_1*X_3	0.307	0.537	0.095
X_2*X_3	0.958	0.311	0.826
$X_1*X_2*X_3$	0.375	0.169	0.914

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

2.3.3 Pretreatment Effectiveness

As a means to quantify the effectiveness of the pretreatment conditions on preserving cellulose while reducing hemicellulose and lignin content, a new efficiency term was calculated for both switchgrass and miscanthus, Y_{Swi} and Y_{Mis}, respectively. This value is the difference between the fraction of cellulose lost in the biomass and the sum of the fractions of hemicellulose and lignin lost in the biomass after pretreatment. Higher values of this term are an indication that with meaningful reductions in lignin and hemicellulose a larger mass of cellulose is maintained for subsequent enzymatic or microbial conversion. Lower values would suggest that either little lignin and/or hemicellulose was removed or with reduction in the hemicellulose and lignin there were similarly greater losses in cellulose which would be less effective for biomass use as a sugar feedstock or fermentation substrate. Switchgrass and miscanthus efficiency values calculated for the different pretreatment conditions are shown in Table 2.10 and corresponding regression model parameters and estimates are presented in Table 2.11. The pretreatment factors of alkaline loading (X_1) and stage 2 time (X_3) were significant main effects on efficiency and the quadratic effect of X_1 also significantly influenced pretreatment efficiency of both switchgrass and miscanthus. For NaOH , the loading effect (X_1) on Y efficiency values

significantly increased when the loading ratio was increased from 0.05 to 0.1 g/g initial dry matter for both switchgrass (0.140 to 0.242) and miscanthus (0.128 to 0.228) (p-value<0.05). However, an increase in efficiency (Y) was not observed even X_1 was doubled from 0.1 to 0.2g NaOH/g initial dry matter (0.242 to 0.263 for switchgrass and 0.228 to 0.231 for miscanthus), which implies there are potentially no further gains in lignin degradation or hemicellulose removal without sacrificed cellulose loss with additional chemical.

The maximum Y_{Swi} value of 0.286 was observed for a NaOH loading (X_1) of 0.15 g/g initial dry matter and stage 1 (X_2) and 2 (X_3) residence times of 0.5h and 1.5h, respectively. This does not align well with highest treatment combination levels that supported the greatest losses in individual components (e.g. lignin and hemicellulose). This efficiency value takes the overall impact of the pretreatment effects on the biomass components into greater consideration such that the most beneficial biomass pretreatment is likely not linked to a single component. For miscanthus, the maximum Y_{Mis} was 0.287 and observed for a NaOH loading (X_1) of 0.25 g/g initial dry matter, stage 1 residence time (X_2) of 0.5h and stage 2 residence time (X_3) of 1h. A closer look at the efficiency values within miscanthus treatments indicate that the efficiency value for the maximum Y_{Mis} was statistically similar to the Y_{Mis} value determined at the NaOH loading (X_1) of 0.15 g/g initial dry matter, stage 1 residence time (X_2) of 0.5h and stage 2 residence time (X_3) of 1.5h (Maximum Y_{Swi} conditions, p-value>0.05). This would suggest that the lower loading level of NaOH and stage 1 residence time in combination with the 1.5 h oxidant stage 2 time would be equally beneficial and most optimal with respect to lignin and hemicellulose removal and preserved cellulose for both switchgrass and miscanthus pretreatment. These findings are inconsistent with results for pretreatment conditions with the highest removal of hemicellulose and lignin fractions for both grasses (NaOH loading of 0.2g/g initial dry matter level, stage 1 and 2 residence times of 1.5h).

In addition, Y_{swi} and Y_{mis} results using $Ca(OH)_2$ as an alkaline chemical are shown in Table 2.12 and the parameters estimates are listed in the next Table 2.13. Similar to the results from the individual components as response variables (cellulose, hemicellulose and lignin) for $Ca(OH)_2$, stage II residence time (X_3) was the only factor influencing Y_{swi} and Y_{mis} (p-value<0.05). Increasing X_3 time from 0.5h to 1.5h resulted in an increase in Y_{swi} from 0.002 to 0.019 and an increase in Y_{mis} from 0.007 to 0.112 for a $Ca(OH)_2$ loading ratio of 0.2 g/g initial dry matter (p-value<0.05), although the improved Y values are still comparatively lower to water-treated controls of switchgrass and miscanthus (0.034 and 0.029).

Table 2.10 Pretreatment Efficiency (difference in fraction losses between cellulose and sum of hemicellulose and lignin) of Pretreated Switchgrass and Miscanthus. All deduction values are expressed as averages of duplicate samples.*

NaOH Loading Concentration (X_1) (g/g initial dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	$Y_{(Swi)}$	$Y_{(Mis)}$
0.05	0.5	0.5	0.140	0.128
0.05	0.5	1.5	0.176	0.178
0.05	1.5	0.5	0.187	0.136
0.05	1.5	1.5	0.186	0.170
0.1	0.5	0.5	0.242	0.228
0.1	0.5	1	0.267	0.273
0.1	0.5	1.5	0.280	0.289
0.15	0.5	0.5	0.251	0.271
0.15	0.5	1	0.261	0.244
0.15	0.5	1.5	0.286	0.281
0.2	0.5	0.5	0.263	0.231
0.2	0.5	1.5	0.260	0.243
0.2	1.5	0.5	0.254	0.242
0.2	1.5	1.5	0.261	0.247
0.25	0.5	0.5	0.278	0.272
0.25	0.5	1	0.283	0.287

***.The Y_{Swi} and Y_{Mis} of raw material (Control) were respectively 0.034 and 0.029. Controlled raw material were pretreated with equivalent amount of DI water at both stages for 0.5h respectively.**

Table 2.11 Parameter Estimates of Pretreatment Factors, Interactions and Quadratic Terms for Faction Loss Difference Between Cellulose and Sum of Hemicellulose and Lignin in Pretreated Switchgrass and Miscanthus Using NaOH

	$Y_{(Swi)}$		$Y_{(Mis)}$	
Terms	**Estimate**	***p*-value**	**Estimate**	***p*-value**
X_{1c}	0.457	<.0001*	0.436	<.0001*
X_{2c}	0.010	0.345	0.006	0.516
X_{3c}	0.018	0.030*	0.027	0.001*
$X_{1c}*X_{2c}$	-0.133	0.287	0.121	0.269
$X_{1c}*X_{3c}$	-0.110	0.354	-0.227	0.034*
$X_{2c}*X_{3c}$	-0.023	0.168	-0.015	0.288
$X_{1c}*X_{1c}$	-3.791	0.002*	-2.944	0.004*
$X_{3c}*X_{3c}$	0.001	0.976	-0.013	0.727

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

Table 2.12 Pretreatment Efficiency (difference in fraction losses between cellulose and sum of hemicellulose and lignin) of Pretreated Switchgrass and Miscanthus Using $Ca(OH)_2$. All deduction values are expressed as averages of duplicate samples.*

$Ca(OH)_2$ Loading Concentration (X_1) (g/g initial dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	$Y_{(Swi)}$	$Y_{(Mis)}$
0.05	0.5	0.5	-0.020	0.021
0.05	0.5	1.5	0.018	0.088
0.05	1.5	0.5	-0.014	0.056
0.05	1.5	1.5	0.014	0.107
0.2	0.5	0.5	0.002	0.070
0.2	0.5	1.5	0.019	0.112
0.2	1.5	0.5	-0.020	0.056
0.2	1.5	1.5	-0.009	0.119

Table 2.13 Parameter Estimates of Pretreatment Factors, Interactions and Quadratic Terms for Pretreatment Efficiency (Faction Loss Difference Between Cellulose and Sum of Hemicellulose and Lignin) in Pretreated Switchgrass and Miscanthus Using $Ca(OH)_2$

	$Y_{(Swi)}$		$Y_{(Mis)}$	
Terms	**Estimate**	***p*-value**	**Estimate**	***p*-value**
X_{1c}	-0.032	0.517	0.115	0.057
X_{2c}	-0.017	0.065	0.003	0.648
X_{3c}	0.016	0.032*	0.059	<.0001*
$X_{1c}*X_{2c}$	-0.092	0.318	-0.145	0.137
$X_{1c}*X_{3c}$	-0.023	0.796	-0.173	0.083
$X_{2c}*X_{3c}$	-0.011	0.420	0.004	0.770

Note: Numbers with asterisk indicate that the term has a significant effect at 95 % confidence interval.

2.3.4 Optimized Empirical Regression Model and R^2 values

Using the regression model optimization rules described in the methodology, three fitted equations for optimization of cellulose, hemicellulose and lignin and one model for parameter Y are provided in Table 2.14 for each perennial biomass using NaOH as the alkaline chemical. For any equation including X_{1c} and its quadratic term, a similar conclusion can be obtained by derivation of each response variable by X_{1c} that the NaOH loading of 0.2 g/g initial dry matter is considered an optimal set point to achieve maximum response variable values for lignin and hemicellulose and pretreatment efficiency (Y). Also, fitted equations for cellulose loss in switchgrass and miscanthus both indicate that cellulose losses were strengthened as NaOH loading increased from 0g/g to 0.24g/g initial dry matter, which supports our previous fndings regarding the risk of cellulose losses upon increased exposure to alkaline load. For most equations which contain the main effect terms of X_2 and X_3, positive effects on removal of cellulose, hemicellulose and lignin were shown when extending residence time in either stage, supporting improved reaction conditions (surface area contact and mass transfer) between alkaline-oxidant (NaOH or H_2O_2) with biomass components.

Data results on pretreated switchgrass or miscanthus using $Ca(OH)_2$ were not fitted into an optimized regression model since only one experimental set was run and statistical data analysis showed only one factor, the residence time of stage II (X_3), was significant for the different response variables.

2.3.5 Models Validation.

Additional data points were collected for pretreatment conditions used in subsequent experiments targeted for use in *C. thermocellum* fermentation. Four independent pretreatment conditions using NaOH and H_2O_2 at two levels of alkaline loading (0.1 and 0.2 g/g initial dry matter) combined with or without H_2O_2 were completed in switchgrass and miscanthus. Relative cellulose, hemicellulose and lignin reduction fractions for both grasses were compared with model prediction values and are shown in Figures 2.1 and 2.2. For cellulose reduction in switchgrass, variance between prediction values to experimental values were below 10% when the alkaline loading was at 0.2 g/g initial dry matter, while a larger errors were observed at the lower alkaline loading ratio of 0.1 g/g initial dry matter. Prediction of hemicellulose reduction fractions in switchgrass showed lower residual differences when compared to experimental replicates (maximum error: 8.5%). For Treatment I (0.1 g/g initial dry matter NaOH) there was no significant difference between the prediction values and the experimental numbers in switchgrass (p-value<0.05). For all three biomass components the differences bweteen predicted and measured experimental values were larger under treatment conditions represented by Treatment I (0.1 g/g initial dry matter), II (0.1 g/g initial dry matter plus H_2O_2) and III (0.2 g/g initial dry matter) than those differences observed for Treatment IV (0.2 g/g initial dry matter plus H_2O_2). For miscanthus, differences in residuals between measured and predicted fractional loss numbers were close to 10% for cellulose, hemicellulose and lignin in most treatment combinations, with the exception of the difference observed in cellulose reduction from pretreatment IV (~19.4% difference).

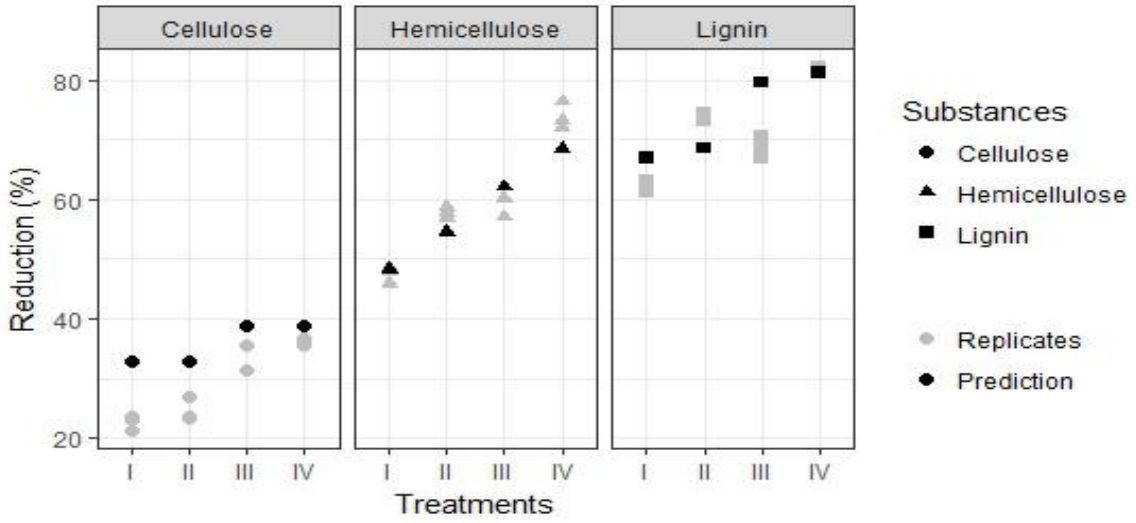

Fig 2.1 Data comparison between models prediction values to real experimental results of switchgrass pretreatment. Treatment I: 0.1 g/g NaOH dry matter, Treatment II: 0.1 g/g dry matter+3% w/w H2O2 , Treatment III: 0.2 g/g dry matter and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H2O2. Loading time for each stage was constant at 1 hour.

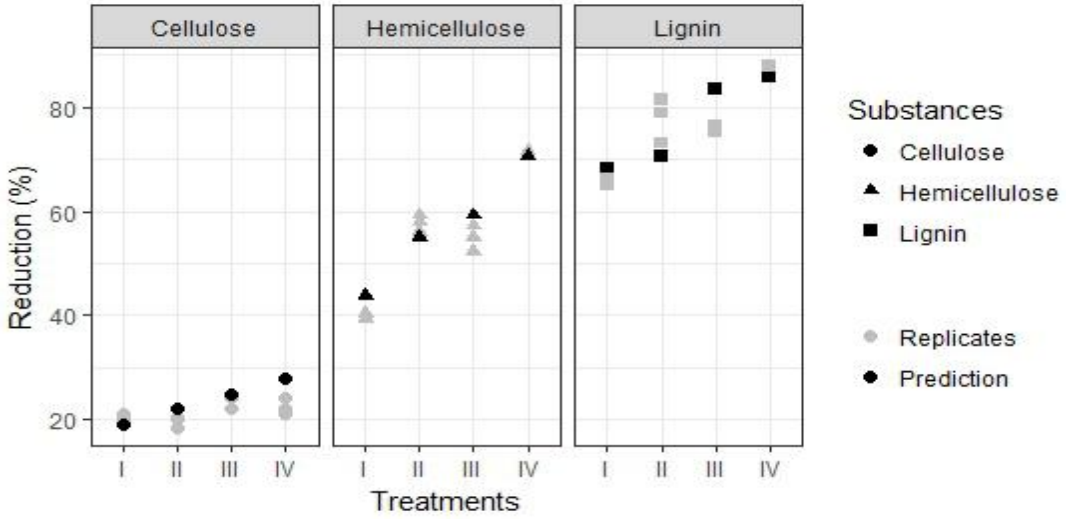

Fig 2.2 Data comparison between models prediction values to real experimental results of miscanthus pretreatment. Treatment I: 0.1 g/g NaOH dry matter, Treatment II: 0.1 g/g dry matter+3% w/w H2O2 , Treatment III: 0.2 g/g dry matter and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H2O2. Loading time for each stage was constant at 1 hour.

Table 2.14 Fitted Optimized Empirical Regression Models for Switchgrass and Miscanthus using NaOH

	Switchgrass			Miscanthus		
Response Variable	**Fitted Model Description**	**BIC**	**R^2**	**Fitted Model Description**	**BIC**	**R^2**
Cellulose	$0.3024+0.2631X_1-0.06984X_2+0.69X_1*X_2$	-95.0	0.71	$0.04858+1.5760X_1+0.03782X_2+0.05705X_3-3.3336X_1^2$	-93.7	0.65
Hemicellulose	$0.2571+1.3881X_1+0.1725X_2+0.1896X_3-0.1267X_2*X_3$	-78.3	0.83	$0.1595+3.0667X_1+0.04278X_2+0.2282X_3-5.0644X_1^2-0.5229X_1*X_3$	-121.9	0.97
Lignin	$0.3206+4.6048X_1-11.1464X_1^2+0.03218X_3$	-120.9	0.95	$0.2300+6.0294X_1+0.04428X_3-15.0346X_1^2$	-102.6	0.92
Y	$0.1282+1.4216X_1-3.5061X_1^2$	-143.7	0.73	$0.2470+1.3171X_1-3.0810X_1^2+0.028X_3$	-146.4	0.81

*** X_1 represents NaOH loading (g/g initial dry matter); X_2 represents residence time in stage 1; X_3 represents residence time in stage 2.**

2.4 Discussion

Use of perennial grasses in emerging bio-based product industries requires pretreatment methods to help alter lignocellulosic biomass structure and reduce recalcitrant components, such as hemicellulose and lignin. Composition changes observed in switchgrass and miscanthus after the two-stage pretreatment process studied suggest that the biomass materials were altered and some results were promising for further conversion to produce fermentable sugars and biochemical using enzymes and/or microbial catalysts.

In experimental set I, conditions for two alkaline reagents were evaluated for switchgrass and miscanthus pretreatment. The $Ca(OH)_2$ loading level had no significant effects on lignin or hemicellulose removal, while NaOH loading was considered the most significant factor to determine changes in cellulose, hemicellulose and lignin composition and had the highest estimated coefficients in the regression model. The solubility of $Ca(OH)_2$ may be related to the lack of differences observed in hemicellulose and lignin removal with chemical loading increases. In this study, $Ca(OH)_2$ concentration ranged from 2.5g/L (0.05g/g initial dry matter) to 10g/L (0.2g/g initial dry matter) and only a small portion of $Ca(OH)_2$ (less than 18.4%) was soluble in the liquid phase since its reported solubility is only 0.44g/L at 121°C. This lack of solubility limited contact of $Ca(OH)_2$ with hemicellulose and lignin polymers resulting in no significant differences observed for alkaline loading ratio and stage I residence time on compositional changes. These results differ from other research that have found $Ca(OH)_2$ to be beneficial to lignin removal. Fuentes et al (2011) illustrated in their study that 63.75% lignin in sugarcane bagasse was removed using 0.4 g/g (16 g/L) initial dry matter $Ca(OH)_2$ for 108h at 90°C. Notably, $Ca(OH)_2$ solubility at 90°C is 0.79 g/L and the high percentage of lignin removal reported by Fuentes et al (2011) was likely a result of their other preprocessing methods and not the presence of or concentration of $Ca(OH)_2$ as described. Hot water exposure to the

bagasse for a period of 8 hours was part of the preprocessing methods used in the study and likely in combination with the alkaline chemical had more influence on the lignin removal from bagasse than the $Ca(OH)_2$ and concentration level alone (Wang et al., 2016). Use of $Ca(OH)_2$ for alkaline pretreatments will be limited by the low solubility levels of the chemical in solvent and biomass solid loading ratios will play a significant role on the effectiveness of any given alkaline chemical loading ratio investigated and the results reported.

The significant hemicellulose losses observed in the two-stage alkaline-hydrogen peroxide process (AHP) process resulting from increases in NaOH loading and residence time of the second hydrogen peroxide stage is linked to saponification impact on intermolecular ester bonds of hemicellulose by the NaOH and removal of acetyl groups by hydrogen peroxide (Tarkow & FEIST., 1969, Alvarez-Vasco & Zhang., 2013). To break down lignin structures in plant cells, NaOH primarily cleaves esters and C-C bonds between phenyl-propane units in lignin structure (Nenkova et al., 2008) and H_2O_2 mainly reacts nucleophilically with electron deficient carbonyl and conjugated carbonyl structures (Kadla & Chang., 2001). With the extended residence time in the hydrogen peroxide stage (X_3), lignin was steadily under attack by the oxidant and losses continued to increase. The oxidation time of stage II in the switchgrass pretreatment after 0.2g NaOH /g initial dry matter treatment in stage I for 0.5h resulted in lignin losses of 80.5% up to 85.1% with the additional hour of exposure. A similar finding was determined in a two-stage alkaline-hydroxide process (AHP) where 88% of lignin in switchgrass was removed under a reported optimal 0.2g NaOH/g initial dry matter combined with 5% H_2O_2 at 85 °C for 24h at each stage, which is much longer than our residence time setting (1.5h at each stage) (Gupta & Lee., 2010). The level of lignin degradation in two-stage pretreatments has been similarly reported in integrated (single-stage) AHP processes. Xu et al. (2001) demonstrated that maximum lignin reduction in pretreated switchgrass reached up to 85.8% with a one-stage 0.2g NaOH/g initial dry matter pretreatment at

121 ℃ for 1h. While our two stage process did not show meaningful differences in lignin removal for the switchgrass study by Xu et al. (2001), the two-stage pretreatment strategy has been demonstrated as more efficient on lignin removal than a one-stage integrated method when pretreating miscanthus with NH_4OH and H_2O_2 (Yu et al., 2013). Biomass composition and structure will play a large role on the overall effectiveness of a two-stage pretreatment approach.

Cellulose has been described as less vulnerable in an alkaline environment with only its degree of polymerization susceptible to change, which improves enzymatic effectiveness (Bali et al., 2015). Cellulose losses observed in this current study ranged between 18 and 50% depending on the material and the pretreatment conditions, with greater losses in all three lignocellulosic components with increased alkaline loading level and time in each stage. Alkaline soaking in stage 1 degraded lignin and hemicellulose located in the outer portion of the lignocellulosic structure and a higher concentration of alkaline guaranteed a higher removal amount of lignin and hemicellulose (Karp et al., 2015), which simultaneously exposed more cellulose to liquor and decreased its polymerization. Hydrogen peroxide, which has been widely used in pulp and textile industries as a bleaching reagent, also likely contributed to degradation of some cellulose as a strong oxidant., (Zeronian & Inglesby., 1995). These oxidants in the second stage not only degraded residual lignin and hemicellulose but had greater access to depolymerized cellulose after biomass was pretreated at higher chemical levels (X_1>0.05 g/g dry matter) in stage I. Sun et al (2000) reported a similar result that cellulose loss in pretreated rice straw was influenced by hydrogen peroxide concentration in a two-stage pretreatment process (1% NaOH solution for 2h and varied H_2O_2 concentrations for 12h). Despite the extra oxidative effect of hydrogen peroxide on cellulose loss, conditions that maximize lignin and hemicellulose removal with efficient reduction in cellulose would need to be fully investigated for different biomass substrates of interest..

The overall goal of pretreatment on lignocellulosic biomass is to degrade obstructive lignin polymers as much as possible to improve access to cellulosic biomass components. Based on this, miscanthus had a higher maximum lignin loss than switchgrass when NaOH loading (X_1) was over 0.1 g/g initial dry matter which may be a result of the specific lignin structures in two perennial grasses. Lignin in miscanthus has a comparatively lower syringyl/guaiacy(S/G) ratio (0.7) than switchgrass and the predominant linkages in miscanthus lignin is attributed to the β-O-4 type (up to 93%), higher than 72% β-O-4 linkage in switchgrass, which is more vulnerable break down in alkali solutions than other interunits, such as phenylcoumarin, resinol, and spirodienone (Samuel et al., 2010, Villaverde et al., 2009).

Cellulose preservation is important to subsequent biochemical conversion processes, including enzymatic hydrolysis and direct microbial fermentation, since cellulose is the primary carbon polymer in biomass supporting glucose and cellobiose sugar stocks. In this case, miscanthus revealed higher cellulose preservation for each treatment combination than switchgrass. Crystalilinity of cellulose in switchgrass (Crystallinity Index=26.2) has been reported to be lower than miscanthus (Crystallinity Index=46.2) and it is less stable under alkaline pretreatment environment. This advantage of miscanthus over switchgrass was observed especially when the alkaline loading (X_1) was at the highest level (0.25 g/g initial dry matter) (Zhang et al., 2013,Li et al., 2010).

As previously mentioned, a new term to comprehensively evaluate the two-stage AHP pretreatment efficiency by systematically including variation of cellulose, hemicellulose and lignin has potential importance in defining a pretreament condition that captures value in removing as much lignin and hemicellulose while maintaining the most cellulose as possbile. The efficiency terms determined for both alkaline reagents offers a unique way to easily compare the two with respect to their overall ability to make large changes in the lignin and hemicellulose content with minimal impact on the cellulose. The

efficiency values (Y) for both switchgrass and miscanthus for $Ca(OH)_2$ were very close to 0 which in combination with the actual loss values suggests that the overall change in any given component was not substantial enough to produce an effectively pretreated biomass. On the other hand most of Y efficiency values for NaOH under all treatment combinations were more than 0.20 for switchgrass and miscanthus (p-value<0.05). This was an indication that the meaningful changes occurred in the components of the biomass. These values could be used to assess the balance in lignin and hemicellulose removal with extent of cellulose losses by a given pretreatment and a pretreatment's feasibility of use in subsequent biomass conversion processes, whether enzymatic and/or microbial.

An optimized empirical regression model presents a more realistic and simplified equation for researchers to get an estimation of variable effects on corresponding results and to narrow selection of variables among equation terms instead of accepting all terms. In the eight equations generated for cellulose, hemicellulose, lignin and efficiency (Y) for switchgrass and miscanthus, most interaction terms were excluded while the first-order effects (X_1, X_2 and X_3) and X_1 quadratic term were preserved in models. The significance of these model terms, especially the primary variable alkaline loading (X_1) on final cellulose, hemicellulose and lignin reduction ratios parallels the analysis of parameter estimations in previous studies. From this work, 0.2g/g NaOH loading was the optimal alkaline loading ratio when pretreating either switchgrass or miscanthus as determined by lignin and hemicellulose removal ratio. For other types of lignocellulosic substrates, such as sorghum bagasse, wheat straw and coastal bermuda grass, NaOH loading was addressed as the main factor that affected lignin removal or enzymatic carbohydrate hydrolysis efficiency (Jiang et al., 2016, Wang et al., 2010, Purti et al., 2017).

In addition to improved variable selection, optimized regression models can enhance accuracy of our research decisions on manipulating levels of NaOH loading or residence time at each stage. Depending on the intended purpose of a given pretreatments process,

regression models offer a comprehensive ability to predict final results when designing experiments within the range in which the model was developed. The models can also be used to adopt distinct pretreatment strategies to direct specific reductions in lignin or hemicellulose with minimal use of alkaline chemcials, which is beneficial for related economics and environmental assessment (Hu & Ragaauskas., 2012).

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CHAPTER 3 Consolidated Bioprocessing of C.thermocellum Using Raw and Pretreated Switchgrass and Miscanthus

3.1Introduction

Consolidated bioprocessing (CBP) is valued as a microbial conversion approach that integrates biomass hydrolysis to soluble sugars and fermentation of those sugars to fuels and chemicals in a single operation (Lynd et al., 1991). The microorganisms used in CBP are fundamental to the conversion efficiency and cost-effectiveness of the process. A combination of microbial catalysts that produce the enzymes for substrate, typically lignocellulosic materials, breakdown and complete conversion to desired products, such as solvents and organic acids. This process can be achieved by different microorganisms or a single organism within the same reaction vessel (Lynd et al., 2002; Xu et al., 2009, Olson et al., 2012).

Clostridium thermocellum, a rod shaped, anaerobic bacterium which converts cellulosic substances, such as Avicel, cellobiose and paper pulp sludge, into organic acids, ethanol, carbon dioxide and hydrogen, has been studied as a promising candidate for CBP to contribute to production of renewable energy and bio-based products (Ng et al., 1977, Lynd et al., 1987, Chinn et al., 2007). A unique characteristic of *C. thermocellum* is its sophisticated exocellular cellulase system called the cellulosome. The cellulosome has a cellulose binding domain that allows the cell to become intimately attached to cellulosic substrates and includes various cellulases and hemicellulases to digest polysaccharides (Lamed & Bayer., 1988, Bayer & Lamed., 1986). This, in combination with its acidogenic and solventogenic capabilities, makes *C. thermocellum* a favorable microbial catalyst in development of CBP processes. As a thermophile, *C. thermocellum* typically demonstrates optimal growth at 60 ℃ which has the added benefit of minimizing the growth of other microorganisms (contaminants) in CBP fermentation systems (Demain et

al., 2005). While majority of research has been completed on pure cellulosic materials (e.g. Avicel, microcrystalline cellulose), physiology and performance investigations have also been completed on different plant-derived cellulosic biomass materials, such as sugarcane bagasse and transgenic switchgrass (Cheng & Zhu., 2016, Yee et al., 2014, Johnson et al., 1982). Process development efforts have targeted improving ethanol tolerance and production ratios through alteration of environmental parameters, including type of substrate, substrate loading, gaseous headspace concentrations and shaking (Zhu et al., 2013, Shao et al., 2011, Biswas et al., 2015, Papanek et al., 2015, Ramachandran et al., 2011). Useful information has been gained regarding *C. thermocellum's* performance, however much still remains to be discovered to fully exploit the CBP potential of this bacterium, including strategies that target enhancing substrate use and product formation through integration or preprocessing methods of the biomass. According to Lynd et al (1989) a major obstacle impeding cellulosomes from breaking down available cellulose in lignocellulosic biomass is the surrounding lignin and hemicellulose matrices.

Switchgrass and miscanthus are perennial grasses primarily harvested in Untied States and Europe (Schmer et al., 2008, Brosse et al., 2012) that have been promoted as feedstocks to support production of bioenergy and bio-based chemicals (McLaughlin & Kszos., 2005, Clifton-brown et al., 2004). Pretreatment methods can assist with removal of lignin and relative aromatic components as well as enlarge surface area to reduce recalcitrance during enzymatic digestion (Tao et al., 2011, Li et al., 2013). Among all pretreatments methods, sodium hydroxide has shown superior removal of both hemicellulose and lignin up to 66% and 85%, respectively, while preserving cellulose fractions. This is in comparison to other alkaline reagents such as $Ca(OH)_2$ and ammonia fiber explosion that require either greater residence times and/or special reaction vessel designs (Xu & Cheng, 2011, Xu et al., 2011, Zhang et al., 2013, Fuentes et al 2011). You et al (2013) reported 79.5% of lignin in corn stover was removed when 6% w/w NaOH solution was applied at 50 °C and Silverstein et al (2007) demonstrated that 2% w/w

NaOH solution was able to achieve 65.63% delignification of cotton stalks at 121 ℃ (15 psi). Simultaneously, cellulose loss was limited to below 18% in both studies, which implies over 80% of available cellulosic carbon was preserved in the biomass evaluated. To date, the objective of most alkaline pretreatment studies has been to enhance enzymatic hydrolysis efficiency of biomass by removing as much lignin or hemicellulose as possible and reducing the degree of polymerization in crystalline cellulose. However, exploration of using alkaline pretreatment methods to enhance the performance of microbial fermentation systems, including the extent of lignin and hemicellulose removal necessary has not been well documented. The combined process of substrate pretreatment and CBP using *C. thermocellum* warrants further study and a greater understanding of how structural changes in solid substrates can improve growth, end-product yields and conversion efficiency deserves attention.

The objective of this research effort was to evaluate *C. thermocellum* performance in semi-solid CBP fermentation using switchgrass and miscanthus feedstocks pretreated under different alkaline conditions. The benefits of pretreatment and the conditions necessary to achieve improved end-product formation were central to the study.

3.2 Materials and Methods

3.2.1 Perennial Grasses and Pretreatment Chemicals

Switchgrass and miscanthus biomass samples were harvested at the Williamsdale Biofuels Field Laboratory near Wallace, NC (34.765 N, 78.100 W) in December 2013. Biomass was dried at 45 ℃ in a drying oven until moisture stabilized and then ground in a hammer mill to pass through ¾" and $^{3}/_{16}$" screens. Sodium hydroxide was obtained from Sigma-Aldrich (St. Louis, MO) and 3% w/w H_2O_2 was obtained from VWR (Radur, PA) for use in biomass substrate pretreatment preparation.

3.2.2 Experimental Design

The effects of pretreated perennial grasses on the growth performance of *C. thermocellum* 27405 in semi-solid fermentation were investigated. Several treatments of alkaline and two-stage alkaline-oxidative pretreated grasses (switchgrass and miscanthus) were studied as fermentation substrates and included 1) raw material; 2) 0.1g NaOH /g initial dry matter; 3) 0.2g NaOH/g initial dry matter; 4) 0.1g NaOH/g initial dry matter with second stage addition of 3% w/w H_2O_2; and 5) 0.2g NaOH /g initial dry matter with second stage addition of 3% w/w H_2O_2. The five substrate preparations were inoculated with *C. thermocellum* in triplicate and a single no inoculum experimental unit was used as a substrate only control. Fermentation treatments were sampled at time zero and every two days for 14 days as a repeated measure.

The response variables measured over time were the primary metabolic end products (lactate, ethanol, formic acid and acetate concentrations) produced by the organism from the switchgrass and miscanthus carbon substrates. The amount of product on day zero was subtracted from each measurement of end-product to represent the amount of product formed as a result of solid substrate metabolized. The main and interaction effects of NaOH and H_2O_2 treatment combinations on individual products (mg/g initial cellulose) and total product yields (mmol/l) were evaluated by performing ANOVA in SAS 9.4 (SAS institute Inc., Cary, NC). Maximum individual (mg/g initial cellulose) and total end-products (mM/l) were evaluated by paired t-test comparison to determine differences between sample days) Assessments of statistical significance for treatment comparisons were made at an α level of 0.05.

3.2.3 Sodium Hydroxide and Sodium Hydroxide-Hydrogen Peroxide Biomass Pretreatment

Pretreatment conditions studied were determined based on previous experimental findings on effectiveness toward lignin and hemicellulose reduction. Both switchgrass and

miscanthus materials were dried in the oven (105℃; 24 hours) prior to pretreatment to determine moisture content and normalize sample allocations based on material dry weight (MC wet-basis-switchgrass:14.18% and MC wet-basis- miscanthus: 9.77%). A total of oven dried 5g biomass sample was combined with NaOH solution (50 ml) in a 125 ml serum bottle to achieve a solid to liquid loading ratio of 10%w/v). Two NaOH stock solutions were prepared 1) 2.5 g NaOH in 250 mls (25 mM or 1% w/w) and 2) 5 g NaOH in 250 mls (50 mM or 2% w/w) to achieve 0.1g NaOH/g intial dry biomass and 0.2g NaOH/g initial dry biomass, respectively, with the addition of 50 mls in the pretreatment bottles. All serum bottles were sealed with rubber stoppers and crimped with aluminum seals before autoclaving (121℃, 19 psi) for 1 hour. Once reaction times were complete, bottles were removed and cooled to room temperature in an ice water bath. NaOH pretreated biomass was washed with multiple volumes (~300 ml per wash, ~10 washes) of deionized water until pH of the residual water stabilized at 7. The solid fractions were dried at 105℃ drying oven overnight and weighted to determine solid loss from this step. Material collected was stored in a sealed plastic bag for composition analysis and for use in the semi-solid CBP fermentation study. This stage was repeated again to prepare enough substrates for the subsequent H_2O_2 pretreatment (Stage 2).

Pretreated biomass (2 g) from Stage 1 and 20 ml of 3% H_2O_2 solution were placed in a 125 mL serum bottle to achieve a solid/liquid ratio of 0.1 g/ml. All serum bottles were sealed with rubber stoppers and crimped with aluminum seals prior to autoclaving at 121℃ (19 psi; 1 hour). Bottles were removed from the autoclave and transferred to an ice water bath for cooling to room termperature. Biomass was washed with multiple volumes (~50 ml per wash, ~10 washes) of deionized water until pH of the residual water stabilized at 7. The solid fractions were dried overnight in a drying oven (105℃) and weighed to determine solid loss from this step. Pretreated material was stored in a sealed plastic bag for composition analysis and semi-solild CBP fermentation experiments.

3.2.4 Basal Medium and Inoculum Preparation

The T4YE medium was prepared based on procedures described by Freier et al. (1988) and contained per liter (pH 6.7): $Na_2HPO_4 \cdot 12H_2O$ (4.2 g), KH_2PO_4 (1.5 g), NH_4Cl (0.5 g), $(NH_4)_2SO_4$ (0.5 g), $MgCl_2 \cdot 6H_2O$ (90 mg), $CaCl_2$ (30 mg), yeast extract (4 g), standard vitamins (10 ml), modified metals (5 ml), cysteine hydrochloride (0.5 g), 8% sodium carbonate (50 ml) prepared under a CO_2 atmosphere and 0.1% resazurin (1 ml). The standard vitamin solution contained (per 100 ml of distilled H_2O): pyradoxal 2HCl (10 mg), riboflavin (20 mg), thiamine HCl (20 mg), nicotinamide (20 mg), CaD pantothenate (20 mg), lipoic acid (10 mg), p-aminobenzoic acid (1 mg), folic acid (0.5 mg), biotin (0.5 mg), cobalamin (0.5 mg), pyridoxal HCl (10 mg) and pyridoxine (10 mg). The modified metals contained the following components (per liter): Na_4EDTA (500 mg), 200 mg $FeSO_4 \cdot 7H_2O$, 10 mg $ZnSO_4 \cdot 7H_2O$, 200 mg $MnCl_2 \cdot 4H_2O$, 20 mg H_3BO_3, 20 g $CoCl_2 \cdot 6H_2O$, 1 mg $CuCl_2 \cdot 2H_2O$, 2 mg $NiCl_2 \cdot 6H_2O$, 3 mg $Na_2MoO_4 \cdot 2H_2O$, 10 mg $Na_2WO_4 \cdot 2H_2O$, and 1 mg Na_2SeO_3. T4YE medium used for inoculum stocks and semi-solid fermentation experiments was prepared under a 20% CO_2, 80% N_2 headspace. Balch tubes (9 ml medium) and 125 ml serum bottles (45 ml medium) were prepared and used for inoculum stocks. Tubes and bottles were sealed with rubber stoppers, crimped with aluminum seals and placed in an autoclave (121℃, 19 psi, 20 mins). Cellobiose stock solutions (8% w/v) were prepared under an anaerobic environment (20% CO_2, 80% N_2) as the carbon source for inoculum cultures and autoclaved (121℃, 19 psi, 20 mins).

C. thermocellum 27405 (ATCC 27405) cells (0.5 ml; 5% inoculum) were transferred aseptically and anaerobically from frozen culture stocks (-80 ℃) into Balch tubes containing 9 ml of T4YE medium and 0.5 ml cellobiose stock (8% w/v cellobiose) as a soluble carbon source (4 g/L) for cell growth. The culture tubes were incubated in a water bath for 48 h at 60 ℃. A second transfer of 0.5 mL *C. thermocellum* cell culture (5% inoculum) to a new 9.5 mL Balch tube (4 g/l cellobiose) was performed and incubated at 60 ℃ for 24 hours. A third transfer of 0.5 mL *C. thermocellum* cell culture (5% inoculum)

to a new 9.5 mL Balch (4g/l cellobiose) tube was performed and incubated at 60 ℃ for 12 hours to achieve the desired cell densities. Once the cells reached an OD ranging from 1.5 to 2.0, the culture (2.5 ml) was transferred into a 125 mL serum bottles containing of 45 ml of T4YE medium and 2.5 ml cellobiose stocks (8% w/v cellobiose). Serum bottles were incubated in a water bath for 12 h at 60 ℃ to use as inoculum for the semi-solid fermentation study.

3.2.5 Semi-solid CBP fermentation

Pretreated and unpretreated biomass materials (0.25 g dry switchgrass, 0.25 g dry miscanthus) were weighed into 125 ml serum bottles. These materials represented the raw material and the four pretreatment levels: 1) 0.1g NaOH/g initial dry matter, 2) 0.1g NaOH/g initial dry matter with 3% w/w H_2O_2; 3) 0.2g NaOH/g initial dry matter; and 4) 0.2g NaOH/g initial dry matter with 3% w/w H_2O_2. T4YE medium (47.5 ml) was added into each bottle and sparged with a 20% CO_2, 80% N_2 gas mixture to create an anaerobic condition/headspace. Each serum bottle was sealed with rubber stoppers, crimped with aluminum seals and placed in the autoclave (121℃, 19 psi, 20 mins.) All experimental bottles were prepared in triplicate and one substrate-only control bottle was completed for each treatment level. Treatments were inoculated with *C. thermocellum* 27405 cells (2.5 ml; 1.8 OD) previously grown on cellobiose. All bottles were incubated in a water bath (60 ℃). Liquid samples (2 ml aliquots) were taken at time 0 and every 48 hours and stored at -80°C.. Gas samples (1 ml) were also saved in gas tight syringes on day 0, 6, and 14 and measured for composition by gas chromatography.

3.2.6 Gas Analysis

Headspace gas samples (1 ml) were pulled on Day 0, 6 and 14 using a gas tight syringe (5 ml). Samples were injected in to a gas chromatograph (GC-2014, Shimadzu Crop., Kyoto, Japan) within 24 hours after collection through an actuating valve for precision injection (125 µL H_2, N_2 and CO_2 concentrations were quantified using a Carbosieve S-II, 100/120

mesh stainless column. The injector and detector (TCD) temperatures were 200℃ and 250℃, respectively. Helium was used as the carrier gas (30 ml/min) and the column temperature was programmed to run isothermally at 50 ℃ for 9 min, rise to 200 at 32℃/min and hold for 3 min and then rise to 225℃ at 32℃/min and hold for 7 mins.

3.2.7 End-Products Analysis

Thawed liquid samples were centrifuged (10 min, 13,000 *x g*) and filtered through 0.2 μm nylon filters (Whatman, Maidstone, UK) into HPLC vials and sealed. A 10 μL aliquot of each sample was analyzed using a Phenomenex (Torrance, CA) Rezex ROA column (300 mm × 7.8 mm) at 50 ℃ in 50 min runs, with 0.6 mL/min 5 mM sulfuric acid in HPLC water (Sigma-Aldrich) as the eluent, and quantified by refractive index detection (RID) for cellobiose,glucose, xylose, arabinose, acetate, formic acid, lactic acid, ethanol, levulinic acid, 5-hydroxymethylfurfural, 2-furaldehyde.

3.3 Results

3.3.1 Composition of Raw and Pretreated Materials

Means of cellulose, hemicellulose and lignin fractions in raw and pretreated biomass substrates under four different alkaline pretreatment conditions are presented in Table 3.1 and Table 3.2 for switchgrass and miscanthus, respectively. The pretreatment methods had a substantial effect on the composition of the biomass, where the lignin and hemicellulose content were lowered and the cellulose content increased on a dry matter basis in comparison to the raw material for both grasses. The lignin content in the pretreated grasses ranged between 14-18% for switchgrass and 6-12% for mischanthus. The hemicellulose content was similar for both grasses ranging between 15-23%. The percent cellulose available in the pretreated switchgrass ranged between 52-59% while the cellulose in miscanthus was between 61-68%. These values were at least 18% higher in comparison to the raw material cellulose fractions. Lower lignin and hemicellulose

content in combination with higher cellulose content in the materials was observed as NaOH alkaline concentration was increased and with the addition of 3% (w/v) H_2O_2.

Table 3.1 Initial Composition of Raw and Pretreated Switchgrass

Treatment	NaOH Loading (X_1) (g/g)initial switchgrass dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Raw	0	0	0	34.6 ± 1.30	23.53 ± 0.81	28.43 ± 1.53
I	0.1	0.5	0	52.13 ± 2.51	21.27 ± 1.00	18.57 ± 0.58
II	0.1	0.5	0.5	55.43 ± 1.17	20.20 ± 1.28	15.23 ± 0.49
III	0.2	0.5	0	56.27 ± 0.85	18.17 ± 0.65	16.80 ± 0.90
IV	0.2	0.5	0.5	59.63 ± 1.53	16.33 ± 1.17	14.13 ± 0.67

Table 3.2 Initial Composition of Raw and Pretreated Miscanthus

Treatment	NaOH Loading (X_1) (g/g initial miscanthus dry matter)	Pretreatment Stage 1 Time (X_2) (h)	Pretreatment Stage 2 time (X_3) (h)	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Raw	0	0	0	43.27 ± 2.23	26.43 ± 0.59	23.70 ± 0.82
I	0.1	0.5	0	61.60 ± 2.91	23.27 ± 1.19	12.00 ± 1.04
II	0.1	0.5	0.5	66.03 ± 1.98	19.13 ± 0.70	9.03 ± 0.32
III	0.2	0.5	0	63.27 ± 1.21	20.90 ± 1.28	10.13 ± 1.70
IV	0.2	0.5	0.5	68.07± 1.59	15.37 ± 1.16	6.27 ± 0.31

3.3.2 Total End-Product Concentration Accumulations

Average values of total end-products (sum of ethanol, acetate, formate and acetate) measured every two days (Day 0~14) are presented in Figure 3.1 for raw and pretreated switchgrass (Figure 3.1A) and miscanthus (Figure 3.1B) substrates during growth of C.thermocellum. Fermentations with pretreated substrates (switchgrass or miscanthus) followed similar accumulation trends of total end products. Increases in total end products formed between day 0 and 6 were statistically significant (p-value<0.05). Cultures

reached stationary growth and production between days 6 and 12 with the highest total product accumulations of 45mM and 43 mM for switchgrass and miscanthus, respectively with the two-stage pretreated substrates. This is a considerable difference compared to the raw materials where total product accumulations were less than 10 mM overall. Interestingly, total end-product concentrations in the four pretreated switchgrass cultures increased significantly ranging from 25.9% to 36.1% between Day 12-14 (p-value<0.0002), while such increases were not observed in pretreated miscanthus cultures (p-value>0.05).

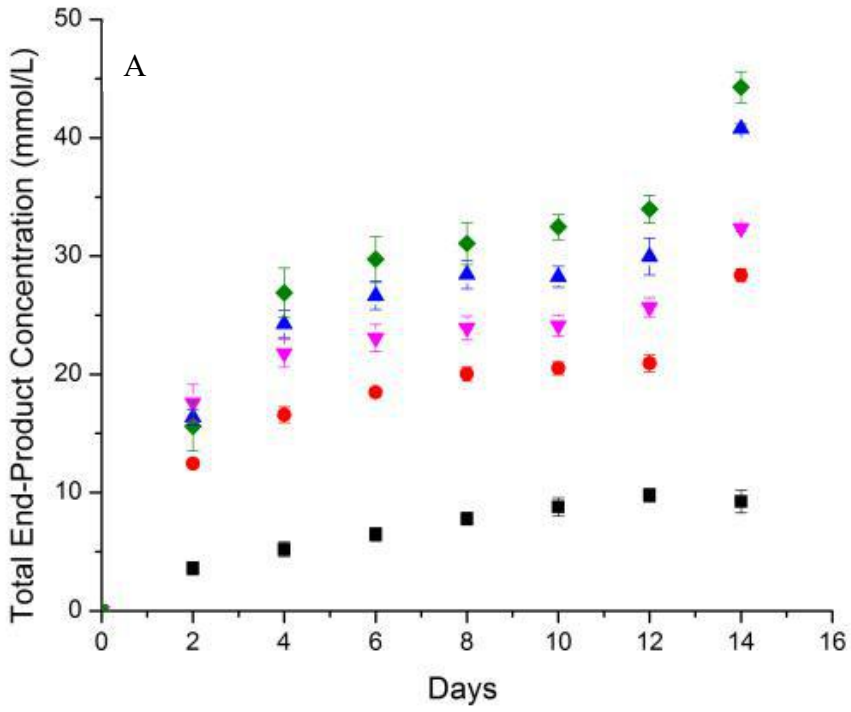

Figure 3.1 Total Metabolic End-product Concentration (mM) of Raw and Pretreated Switchgrass. Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond).

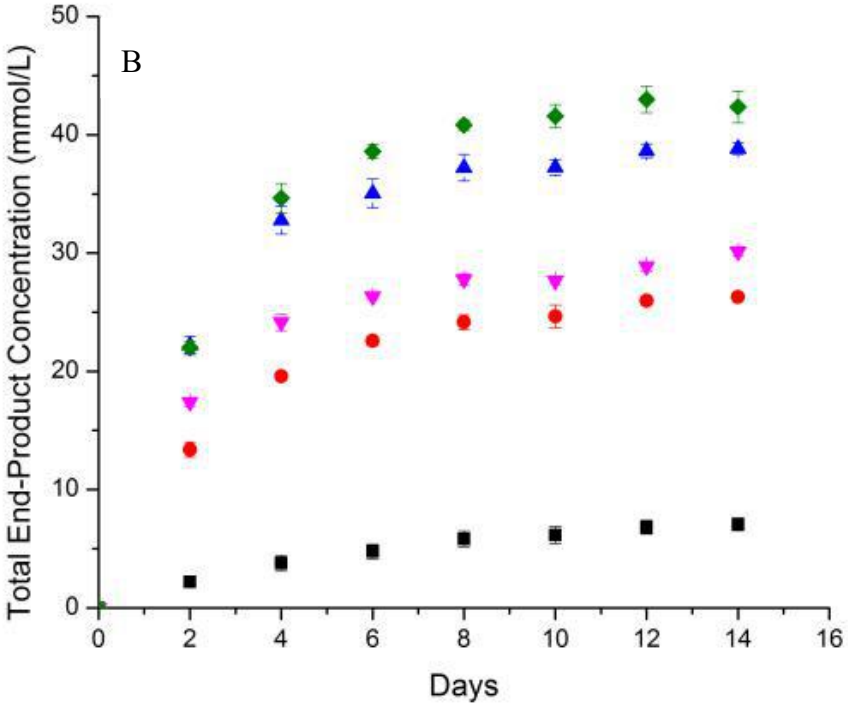

Figure 3.2 Total Metabolic End-product Concentration (mM) of Raw and Pretreated Miscanthus. Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond).

3.3.3 Individual End-product Yields Using Raw and Pretreated Switchgrass

Average product yields over time for ethanol, acetate, lactate and formate from C. thermocellum cultures grown on raw and pretreated switchgrass are presented in Figure 3.2 (A~D). The primary end products, ethanol, acetate and formate, slowed to a stationary production rate on day 6 and entered a second increased rate of production between day 10~14 when growing on pretreated material. Ethanol and formate yields in the raw substrate cultures accumulated at a significantly slower rate than in cultures with pretreated substrates. The rates of ethanol and formate formation in raw switchgrass were at most 40% and 27.3% of the rates observed for pretreated switchgrass cultures after four

days. With the lower production rates, the final yields were at least 149.44 and 20.57 mg/g initial dry cellulose matter lower than pretreated switchgrass cultivations, for ethanol and formate, respectively. Acetate was the primary end-product when cells were cultivated on raw switchgrass and had a similar accumulation trend with a slower rate and lower maximum yields compared to the other four pretreated switchgrass cultures , Cultures grown on switchgrass pretreated using two-stage pretreatment methods showed significantly higher acetate and ethanol yields than single-stage alkaline pretreated switchgrass cultures, while the highest formate yields on each day were produced in the switchgrass culture only pretreated with 0.2g/g NaOH at stage 1. Lactate accumulation (Fig 3.2.C) was fairly constant for an extended period of time, reaching maximum production after eight days. Similar to other end-products, lactate accumulation also increased at a higher rate of production between days 10~12 or day 12~14 and depending on the pretreatment method percent increase in production rate was considerable higher when compared to other products (P-value<0.05).

Maximum values of each individual end-product and ethanol:acetate:lactate ratios are displayed in Table 3.5. Compared to raw switchgrass cultures, end product yields for cultures grown on the pretreated substrates were higher by 145%, 11.2%, 70.0% and 28.1% for ethanol, acetate, formate and lactate, respectively (p-value<0.05). For switchgrass, maximum product yields occurred on day 14 of the fermentation for all treatments with the lowest yields achieved in the raw substrate and the highest yields observed in grasses pretreated with the two-stage alkaline-hydrogen peroxide process followed by the single stage alkaline only treatments. The differences in the treatments were statistically significant for all maximum product yields except for lactate; and within a given single-stage pretreated or two-stage pretreated substrate culture the product formation was greater when the switchgrass was treated with a higher alkaline loading. Ethanol production values were greater than the acetate values for cultures with pretreated substrate where the maximum ethanol to acetate ratio for the raw material treatments was

0.65:1 and the ratios for cultures with pretreated substrates were greater than 1 and ranged from 1.3:1 to 1.9 to 1.

3.3.4 Individual End-product Yield Using Raw and Pretreated Miscanthus

Average product yields over time for ethanol, acetate, lactate and formate from C. thermocellum cultures grown on raw and pretreated miscanthus are presented in Figure 3.3 (A~D). Ethanol and acetate continued to accumulate for the first 8 days of the experiment and slowed formation rates with similar production concentrations occurred between Day 8~14. The highest productivity was observed within the first 2 days for ethanol and acetate for cultures grown on miscanthus pretreated with 0.2g NaOH loading/g initial dry matter for 0.5h (stage 1) combined with 3% H_2O_2 pretreatment for 0.5h (stage 2). These cells grown on miscanthus pretreated using two-stage pretreatment methods produced ethanol at a rate of 88.4 mg/g initial cellulose/day and acetate at a rate of 64.62 mg/.g initial cellulose/day, respectively. These rates were 34.6% and 18.4% higher than ethanol and acetate production rates observed for cells grown on single-stage pretreated miscanthus. When considering ethanol and acetate formation of cultures grown on raw maiscanthusm the rates of production were at least 88.7% and 54.5% lower than the rates observed in the cultures with single-stage pretreated miscanthus. Unlike the other culture treatments, formate was not produced with significant increases after day 4 and the maximum formation rate was found on day 2 (12.9 mg/g initial cellulose/day) for cells grown on miscanthus pretreated with only 0.2g NaOH loading/g initial dry matter for 0.5h (stage 1). With the exception of cultures with miscanthus pretreated using the two-stage 0.2g NaOH loading/g initial dry matter and 3% H_2O_2, the other three treatments accumulated lactate before day 8 and showed a fluctuation in production between days 8 and 14. Ethanol, formate and acetate yields for raw miscanthus cultures were very low compared to the other 4 pretreatment cultures. The acetate end product in the raw miscanthus cultures showed a lower but similar trend in accumulation to the other treatment production curves during the 14-day experiments.

Corresponding maximum values for miscanthus cultures are provided in Table 3.6 for a better understanding of different effects from the four pretreated substrate conditions. Majority of the maximum values observed still occurred on day 14 and significant differences in maximum ethanol, acetate and formate yields were found between each culture treatment. Compared to raw miscanthus as a fermentation substrate, ethanol, acetate and formate yields increased by more than 483.6%, 43.9% and 210.7%, respectively in cultures with pretreated miscanthus (p-values<0.05). Lactate was not detectable for the raw miscanthus culture. Trends in the ethanol to acetate ratios observed were similar to those described for switchgrass where ratios ranged from 1.3:1 to nearly 2:1 for cells grown on pretreated miscanthus.

Table 3.3 Maximum individual and total end-products yields (mg/g initial cellulose) using raw and pretreated switchgrass*

Switchgrass	Max Ethanol Yield (mg/g)	Max Acetate Yield (mg/g)	Max Formate Yield (mg/g)	Max Lactate Yield (mg/g)	Max Total Yield (mmol/L)	Max Ethanol:Acetate
Raw	102.78^{d} #	213.30^{d}	29.37^{e}	9.19^{b}	9.78^{e} #	0.65
I	252.22^{c}	247.12^{b}	49.94^{d}	11.77^{ab}	28.39^{d}	1.31
II	392.64^{ab}	286.00^{a}	63.34^{a}	13.37^{a}	40.78^{b}	1.76
III	277.88^{b}	237.15^{c}	59.55^{b}	14.02^{a}	32.36^{c}	1.40
IV	413.54^{a} #	280.94^{ab}	54.07^{c}	12.74^{a}	44.27^{a}	1.89

Note: I, II, III, IV represent four pretreatment condition applied in this test and details of each condition are shown as below: I: 0.1g/g dry matter NaOH (1h); II: 0.1g/g dry matter NaOH (1h)+3% w/w H_2O_2 (1h); III: 0.2g/g dry matter NaOH (1h); IV: 0.2g/g dry matter NaOH (1h)+3% w/wH_2O_2(1h). The presence of different letters are superscripts within a column indicates that the means differed significantly among raw matter and four pretreatments(I,II,III,IV) . The letters were listed in a descending order.

***All maximum total end-products, individual products and ethanol:acetate:lactic acid ratio values were determined on Day 14 except those denoted with a #, which were observed on Day 12. The product ratios represent relationships between products derived from acetyl-CoA**

Table 3.4 Maximum individual and total end-products yields (mg/g initial cellulose) using raw and pretreated miscanthus*

Miscanthus	Max Ethanol Yield (mg/g)	Max Acetate Yield (mg/g)	Max Formate Yield (mg/g)	Max Lactate Yield (mg/g)	Max Total Yield (mmol/L)	Max Ethanol:Acetate
Raw	35.71^{e}	139.40^{d}	9.79^{d}	ND	7.06^{e} #	0.37
I	208.06^{d}	201.60^{c}	30.42^{c} #	2.59^{b} #	26.28^{d}	1.32
II	316.32^{b}	228.59^{b}	45.24^{a} #	6.05^{a} #	38.82^{b}	1.78
III	239.63^{c}	200.56^{c}	41.62^{b}	5.94^{a}	30.13^{c}	1.53
IV	360.09^{a}#	234.50^{a}	39.05^{bc}	6.06^{a}	42.99^{a} #	1.98#

Note: I, II, III, IV represent four pretreatment condition applied in this test and details of each condition are shown as below: I: 0.1g/g dry matter NaOH (1h); II: 0.1g/g dry matter NaOH (1h)+3% w/w H_2O_2 (1h); III: 0.2g/g dry matter NaOH (1h); IV: 0.2g/g dry matter NaOH (1h)+3% w/wH_2O_2(1h). The presence of different letters are superscripts within a column indicates that the means differed significantly among raw matter and four pretreatments(I,II,III,IV) . The letters were listed in a descending order.

***All maximum total end-products, individual products and ethanol:acetate ratio values were determined on Day 14 except those denoted with a #, which were observed on Day 12. The product ratios represent relationships between products derived from acetyl-CoA**

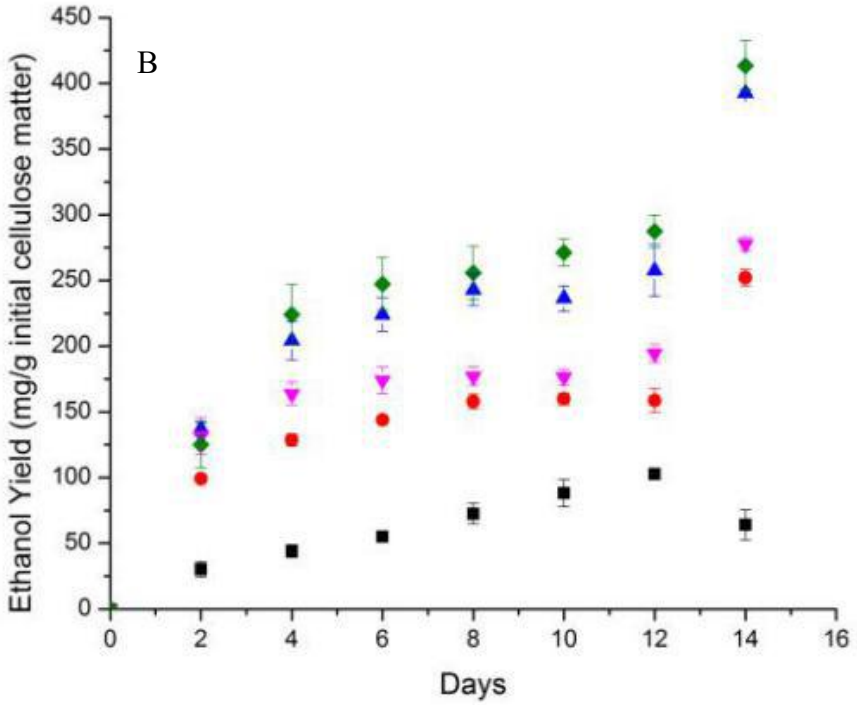

Figure 3.3 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Switchgrass. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw switchgrass ; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

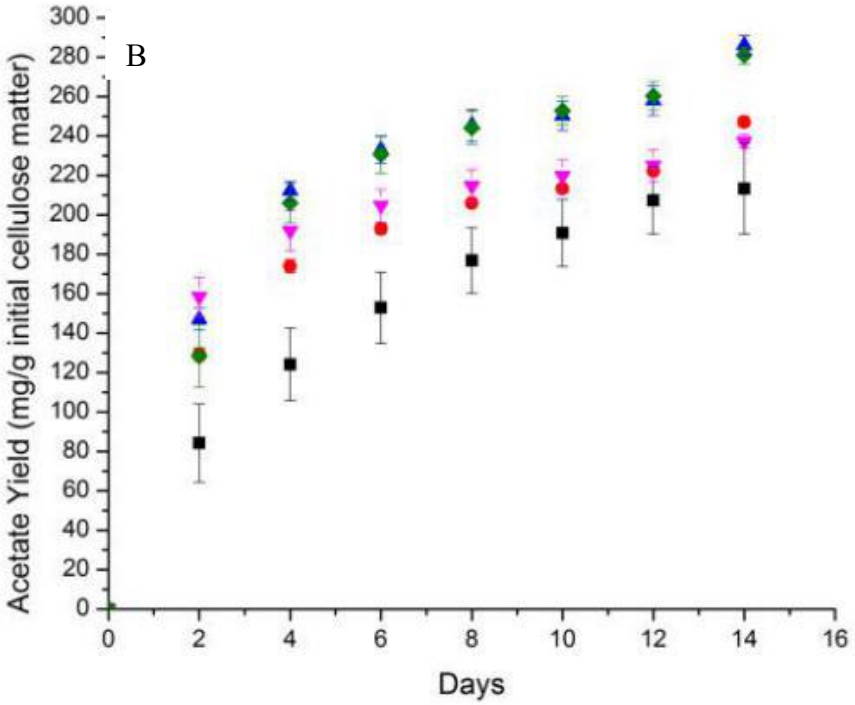

Figure 3.4 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Switchgrass. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw switchgrass ; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H2O2 (green diamond) were determined as indicated in Materials and Methods.

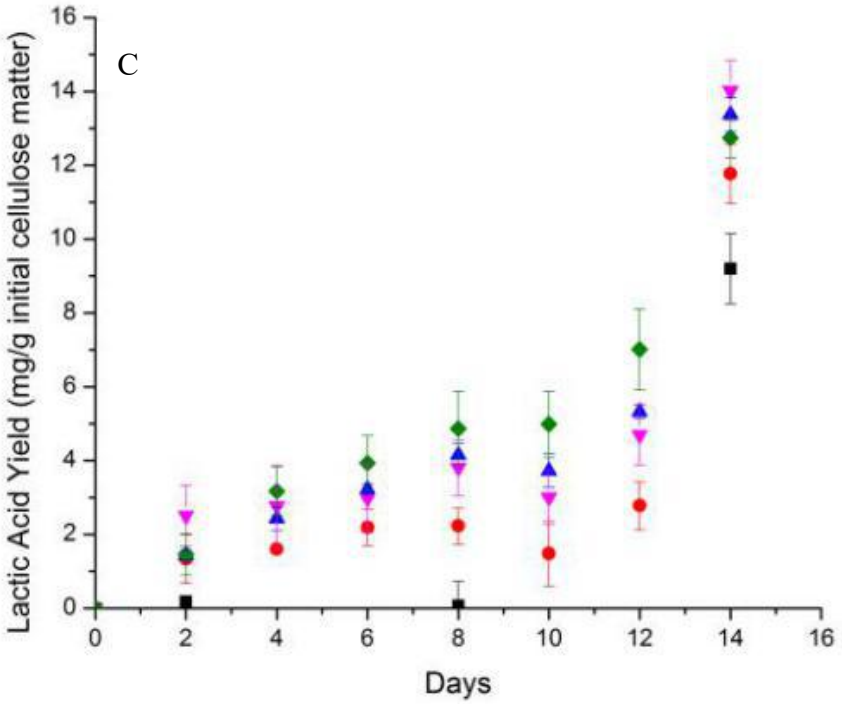

Figure 3.5 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Switchgrass. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw switchgrass ; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

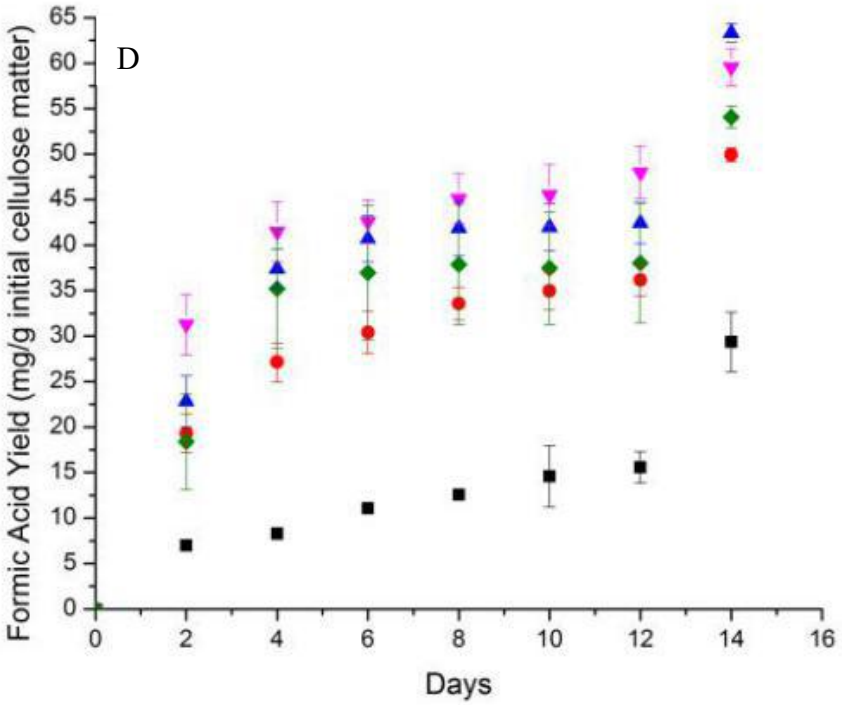

Figure 3.6 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Switchgrass. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw switchgrass ; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment treatment IV: 0.2 g/g dry matter+3% w/w H2O2 (green diamond) were determined as indicated in Materials and Methods.

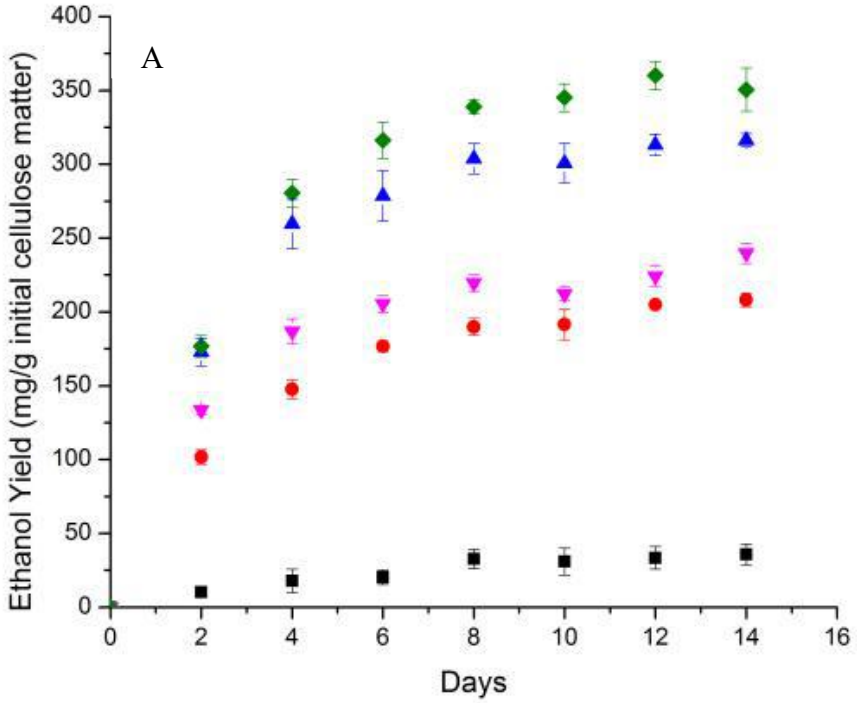

Figure 3.7 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Miscanthus. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw miscanthus; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

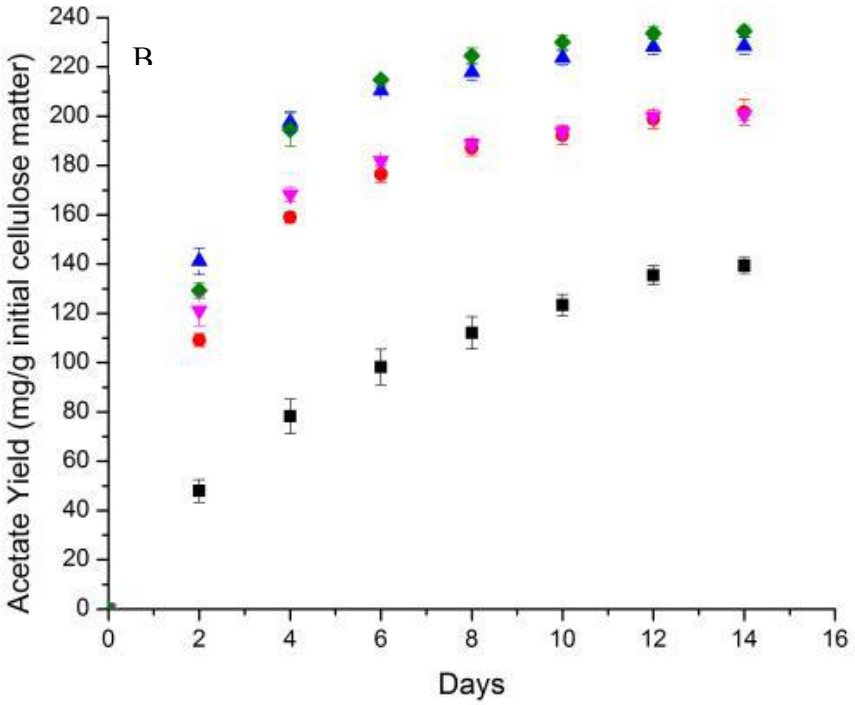

Figure 3.8 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Miscanthus. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw miscanthus; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

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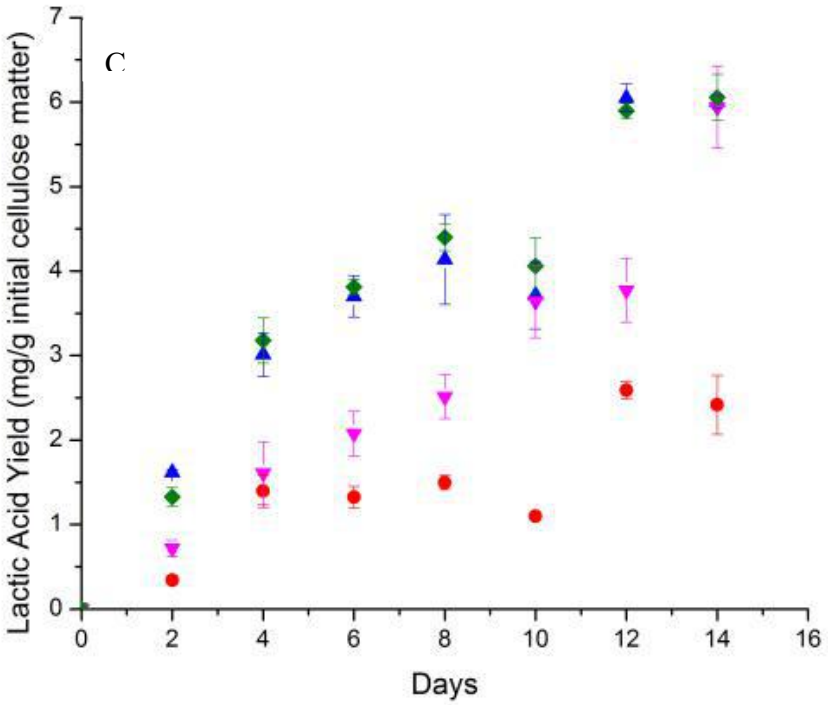

Figure 3.9 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Miscanthus. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw miscanthus; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

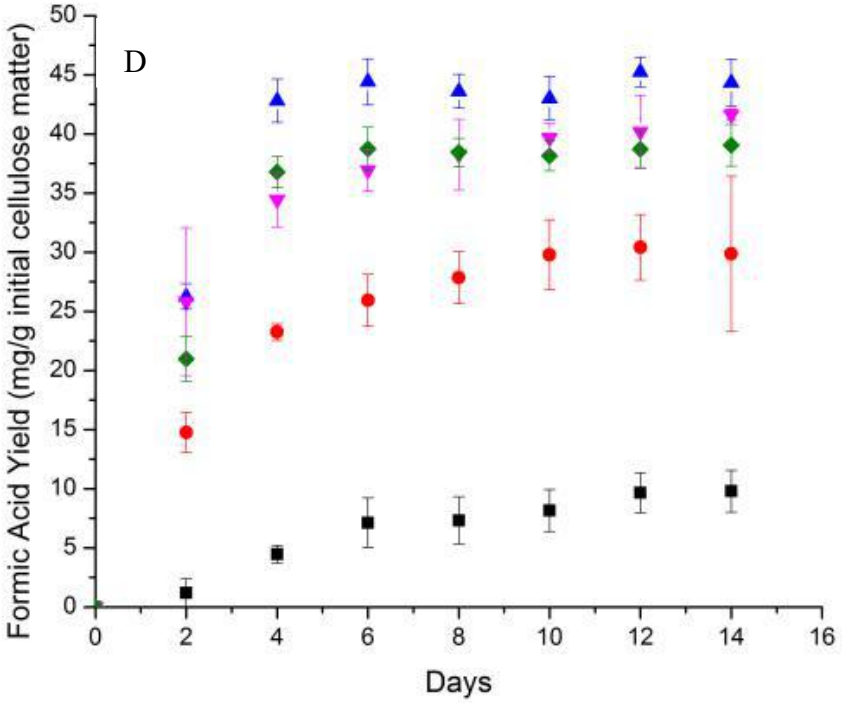

Figure 3.10 Individual Metabolic End-Product Formation (mg/g Initial Cellulose Matter) of Raw and Pretreated Miscanthus. Accumulation of Ethanol (A), Acetate (B), Lactate (C) and Formate (D) in grams per gram of initial cellulose during growth of C. thermocellum on pretreated and raw miscanthus; Raw material (black square), Treatment I: 0.1 g/g NaOH dry matter (red circle), Treatment II: 0.1 g/g dry matter+3% w/w H_2O_2 (blue upright triangle), Treatment III: 0.2 g/g dry matter (pink downward tringle) and Treatment IV: 0.2 g/g dry matter+3% w/w H_2O_2 (green diamond) were determined as indicated in Materials and Methods.

3.4 Discussion.

As a promising microbial catalyst for biochemical production in CBP systems, *C.thermocellum* has caught attention from researchers because of its high efficiency in digesting cellulosic materials and directly fermenting the sugars. Majority of current research efforts on *C. thermocellum* are focused on altering processing conditions to affect key genes and metabolic shifts between end-products during growth on pure cellulosic substrates, such as cellobiose, Avicel or microcrystalline cellulose, while

limited reports of cultivating cells on lignocellulosic biomass have been published (Weimer & Zeikus., 1977, Lynd et al., 1989, Wood & Ingram., 1992., Johnson et al., 1982a,b). In this study, two types of perennial grasses promoted as dedicated bioenergy crops, switchgrass and miscanthus, were pretreated under different alkaline and oxidant reagent conditions and used as a substrate for *C. thermocellum* growth. The primary purpose of pretreatment is to remove lignin from plant cell wall structures, alter cellulose crystallinity and increase accessibility of the inner cellulose matrix (Puri, 1984). As reported, both NaOH and H_2O_2 have strong lignin degradation ability in lignocellulosic biomass pretreatment under higher temperature conditions (Chapter 2; Monlua et al., 2012). H_2O_2 has the additional effect of decreasing degree of polymerization of cellulose and altering crystalline cellulose into a more amorphous form, which is easier to be hydrolyzed by enzymes (Li et al., 2016). Alkaline pretreatments or combined alkaline-oxidant pretreatments used in this study helped *C. thermocelllum* achieve a significant increase in individual end products formed as well as total product concentrations compared to the raw switchgrass and miscanthus substrates (P-value<0.05). Using raw and pretreated biomass as fermentation substrates showed significantly different results for ethanol to acetate ratios produced by *C. thermocellum*. In raw material cultures, ethanol yields were the lowest among the five treatments, only reaching 102.78 and 35.71 mg/g initial cellulose for switchgrass and miscanthus respectively after a 14-day fermentation. Not only were the ethanol yields lower but the accumulation of acetate and formate were lowest compared with other treatments for the raw biomass material. Composition analysis of these two raw biomass materials indicates that lignin was present in the highest percentages for the raw switchgrass and miscanthus. Additionally, lignin composition in raw switchgrass was higher than that present in the raw miscanthus we studied. This lignin prevents direct contact between the cellulose substrate and the cellulosome enzyme groups anchored on the surface of the *C. thermocellum* bacteria. Higher cellular energy is likely required to manage cellulosome activity around the outer protective layer of lignin and promote enzymatic hydrolysis.

Higher levels and rates of acetate production were observed in raw biomass cultures and growth on pretreated materials that presented higher lignin and/or hemicellulose composition. In this circumstance, *C.thermocellum* may have preferred acetate as a final end-product in the raw materials compared to the pretreated substrate cultures and in the switchgrass cultures when compared to the miscanthus cultures since it generates associated ATP energy for the cell (Rydzak et al., 2011).

Formate is one of the primary metabolites of *C. thermocellum* and it was reported as a key factor determining pH value during fermentation (Rydzak et al., 2011). In this study, observed formate yields were more than 3 times greater in cultures loaded with pretreated biomass compared to raw biomass, either in switchgrass or miscanthus. According to the metabolic pathway diagram described in Chapter 1 (Fig.1), formate mainly originates from degradation of intermediate pyruvate into Acetyl-CoA. If formate is not formed, pyruvate is reduced to Acetyl-coA and CO_2 via another pathway. Pathway pREFERENCES in reduction of pyruvate seems dependent on existence of free electrons in the medium. Previous reports have indicated that plenty of electron are generated in the process of single alkaline pretreatment or combined alkaline oxidant pretreatment since both alkali chemical and oxidant are capable of lignin side chain cleavage and consequently result in accumulation of abundant free electrons (Li et al., 2012). Considering the pretreatment methods used in this study and enhanced formate levels, electrons released and present in the pretreated biomass likely influenced the carbon flow from pyruvate into formate instead of CO_2 since the concentration of electrons was adequate enough to satisfy requirements for reducing power in the cell.

As acetate and formic acid accumulated more quickly and in larger concentrations in the pretreated switchgrass and miscanthus cultures, medium pH likely dropped. For *C. thermocellum*, in addition to acetate, ethanol is another derivative from reduction of acetyl-CoA with nicotinamide cofactor (NADH or NADPH) as electron donor (Rydzak et

al., 2011). According to recent literature, two bifunctional acetaldehyde/alcohol dehydrogenases, AdhB and AdhE were proven as key enzymes which separately control formation of ethanol at early and later growth stages in *C. thermocellum* (Lo et al., 2015., Pei et al., 2010). The optimal pH environment for AdhB and AdhE has been described as 6.6 and they are considered stable when pH varies from 5.1 to 6.6 (Pei et al., 2010). Lowered medium pH within the lower range of 5.1 to 6.6 from 6.9 at time zero as a result of the accumulation of formate and acetate in pretreated cultures seemed to enhance the activities of these two enzymes supporting the increases in ethanol formation and production rates observed on the pretreated biomass substrates.

This phenomenon of activated dehydrogenases in stable weak acidic media promoting greater ethanol accumulation rates was also evident in the raw switchgrass cultures in comparison to the raw miscanthus cultures (Table 3.5 and Table 3.6). In raw miscanthus material, the lower lignin composition and accessibility of cellulosic fractions as a result of structural differences influenced cellular energetics (e.g. ATP) required for substrate consumption and likely contributed to less acid formation in comparison to raw switchgrass. Smaller amounts of formate and acetate accumulated in the raw miscanthus cultures during the first 2-4 days of the cultures compared to the raw switchgrass cultures, resulting in a slower pH drop to a level optimal for enhanced dehydrogenase activity. This difference in metabolic end-product formation likely contributed to the smaller ethanol to acetate ratio observed in raw miscanthus cultures (0.37 mM/mM) compared to raw switchgrass cultures (0.65 mM/mM). Similarly, majority of studies on unpretreated biomass commonly report ethanol to acetate ratios less than 1.0. (Ng et al., 1981, Freier et al., 1988.,Levin et al., 2006),

In fermentation of biomass pretreated with NaOH reagent or a combination of NaOH with 3% H_2O_2, final ethanol yields were significantly enhanced by 2.5 to 4.0 times compared to raw switchgrass ethanol production, and up to 10 times in miscanthus

pretreated compared to the raw substrate culture. The composition analysis and results of our previous pretreatment study demonstrated that significant lignin and hemicellulose fraction losses were established with the two-stage alkaline loading and extended oxidant exposure (Chapter 2). The related changes in composition and biomass structure contribute to the improved substrate use and related end-product formation rates by *C. thermocellum* that are observed in the culture treatments. As a result of the pretreatments, parts of the lignin components were removed from the material, reducing biomass recalcitrance to bioconversion and enhancing cellulosic substrate accessibility. As expected the pretreated biomass provided a carbon source to the *C. thermocellum* that increased substrate availability and/or affinity and likely reduced energy requirements of the cells as the need to overcome impeding lignin polymers was lessened. More active cellulosomes (substrate availability/affinity) and lower energy requirements (ATP; associated acetate) likely lead to the metabolism shifts to ethanol observed in pretreated switchgrass and miscanthus cultures.

Still, initial lignin content was higher in the pretreated switchgrass treatments than the miscanthus treatments which comparatively accelerated the acetate formation rate in pretreated switchgrass, up to 2.9 mmol/L/day in the first 4 days. Similar to what was stated previously, this acid production also supported a weak acidic environment which proved beneficial to intracellular AdhE and AdhB enzymatic activity and solvent production in the pretreated switchgrass cultures, especially the two-stage 0.2g NaOH/g initial dry matter with 3% H_2O_2 pretreated switchgrass culture. Similar findings were reported by Avgerinos & Wang (1983). This group described cultivation of a mixed culture of *C. thermocellum* and *C. thermosaccharolyticum* on alkaline pretreated corn stover (up to 67% delignification) that resulted in a 400% increase in rate of substrate degradation compared with growth on raw corn stover. This study also showed final ethanol and acetate concentrations up to 480 mM/L and 220 mM/L respectively and an ethanol to acetate ratio of 2.17 (mM/mM) after 3 days in pretreated corn stover culture,

while ethanol and acetate were lower than 4 g/L in unpretreated Solka Floc culture.

The improved ethanol to acetate ratios observed in the pretreated biomass cultures can be attributed to the substrate availability/affinity as previously described as well as differences in hydrogen concentrations, all of which are related to reduced acetate formation. Higher levels of hydrogen accumulation were observed in the headspace of pretreated biomass cultures than in raw biomass substrate cultures (data not shown).The pretreated miscanthus and switchgrass cultures provided a lignocellulosic substrate with lower lignin and hemicellulose content and increased substrate accessibility, supporting an environment of enhanced substrate consumption. This likely contributed to the differences in levels of acetate formation and related energetics (e.g. ATP) since the pretreated cultures did not require the same cellular energy to access and hydrolyze cellulose. This ease of sugar transformation also likely contributed to the increased headspace hydrogen levels observed in the pretreated biomass cultures, with hydrogen produced during sugar transport.

In unstrirred cultures like those in this study, hydrogen produced will also begin to saturate the liquid phase and excess gas will move into the culture headspace, influencing lower production levels of acetate. Lamed et al (1988) demonstrated that additional hydrogen in headspace in unstirred fermentations increases ethanol/acetate ratios since hydrogen saturated in the liquid phase (greater presence in the gas phase) inhibits acetate production. Ethanol to acetate ratios in our study ranged from 1.3:1 to 1.9: 1 in pretreated switchgrass treatments and 1.3:1 to 2:1 in pretreated miscanthus. This difference in comparison to the less than 1 ratios observed in the raw biomass cultures can also be linked to the increased hydrogen concentrations present in pretreated cultures and the unstirred system. Similar results were revealed by Lynd et al. (2007) who reported an ethanol to acetate ratio of 1.8: 1 achieved in dilute-acid pretreated wood fermented by *C. thermocellum* after 8 days. Besides that, Saddler and Chan (1984) demonstrated an

ethanol to acetate ratio that was reversed from 0.41: 1 to 1.9: 1 when raw wheat straw substrate was replaced with alkali pretreated biomass cultivated with same *C. thermocellum* strain. The growth and end-product formation of *C. thermocellum* cultures grown on pretreated substrates can enhance solvent over acid production. The improved substrate composition and reduced structural complexity create an environment that triggers many of the factors that increase ethanol formation rates and yields, including regulated energetics and reducing power, lower pH levels and hydrogen saturation.

3.5 Conclusion

Pretreament of switchgrass and miscanthus biomass material by both single-stage alkaline pretreatment and combined two-stage alkaline-oxidant pretreatment were successful in reducing the recalcitrant effect of the lignocellulosic substrates on growth and product formation of *C. thermocellum*. Use of the pretreated substrates promoted a significant increase in all end-products formed and demonstrated improved shifts in metabolism from acids to solvents. The improved substrate affinity and accessibility of pretreated grasses improved the energetics of *C. thermocellum* as suggested by the rates and types of acids formed during the early stages of culture growth. In addition, the substrate composition and lowered complexity of the pretreated grasses influenced electron flow, hydrogen saturation and reducing power available to the cells supporting shifts in metabolism toward ethanol.

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