ABSTRACT

JUNG, WOOCHUL. Characterization of Lignin Change due to Chemical Pretreatment of Herbaceous Biomass (Under the direction of Dr. Ratna R Sharma-Shivappa).

Switchgrass and *Miscanthus* x *gigantues*, which are significant bioresoureces suitable for conversion to biofuels, were pretreated with sodium hydroxide (NaOH) at various concentrations (0.5, 1.0, and 1.5% (w/v)) and pretreatment times (15, 30, and 60 min) to investigate how NaOH affects their lignin chemistry and how those changes impact enzymatic conversion efficiency. In switchgrass, higher NaOH concentrations removed xylan by up to 28.3% while there was no significant ($p > 0.05$) glucan loss due to NaOH pretreatment. NaOH resulted in significant ($p < 0.05$) delignification ranging from 44.0 to 84.6% depending on pretreatment intensity. Nitrobenzene oxidation (NBO) used to investigate changes in lignin chemistry of pretreated switchgrass by analyzing syringyl (S) to guaiacyl (G) ratio (S/G ratio) and *p*-hydroxypenyl (H) to guaiacyl ratio (H/G ratio) suggested that S/G ratio (= $0.62_{\text{raw switchgrass}}$) did not change with 15 min pretreatment, but it increased to 0.75 and 0.72, respectively, with 30 and 60 min pretreatments using 0.5% NaOH. H/G ratio (= $0.48_{\text{raw switchgrass}}$) decreased significantly ($p < 0.05$) to 0.14 at higher NaOH concentration regardless of pretreatment time. Miscanthus showed similar trends as switchgrass and delignification ranging from 42.3 to 84.6% was observed depending on pretreatment severity. S/G ratio of miscanthus (= $0.64_{\text{raw miscanthus}}$) increased to 0.77 and H/G ratio (=$0.48_{\text{raw miscanthus}}$) decreased to 0.28 at 0.5% NaOH pretreatment. While there were no significant changes in S/G ratio at 1.0 and 1.5% NaOH compared to 0.5% NaOH. H/G ratio decreased up to 0.13 with increase in NaOH concentration regardless of pretreatment times. Overall, H lignin was the most susceptible to NaOH pretreatment compared to S and G lignin. It can be inferred that H lignin can have more condensed linkage but may be formulated with smaller polymers due to

instability of free radicals for lignification via oxidative coupling. It might be inferred that NaOH can solubilize the lignin fraction containing H units relatively easily than S and G units. Although investigating lignin chemistry changes in switchgrass and miscanthus helped to partially understand which H lignin were more susceptible to alkali pretreatment, overall production of fermentable sugars did not change with change in pretreatment severity and enzymatic hydrolysis results could not be correlated to changes in lignin chemistry. Hence subsequent studies were performed to understand enzyme-lignin interaction as impacted by alkali and acid pretreatment.

Lignin isolates obtained from alkali (0.5, 1.0, and 1.5% NaOH at 121^{o}C for 30 min) and acid (1, 2, and 3% H_2SO_4 at 121^{o}C for 60 min) pretreated switchgrass and miscanthus were used to determine how changes in lignin chemistry impact cellulolytic enzymes, especially in terms of enzyme binding on lignin isolate. Also, hydrolysis of cellobiose and Avicel in suspension with and without lignin isolates was conducted with Cellic® CTec2 (5 and 10 FPU/g carbohydrate) to study how presence of lignin impacts binding of cellulolytic enzymes to the lignin and eventually influence hydrolytic sugar production. Three carbohydrate-lignin loadings (0.5:0.25, 0.5:0.5. and 0.5:1.0% (w/v)) were also used. The results of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that β-glucosidase (BG) had a strong tendency to bind to all lignin isolates from pretreated switchgrass and miscanthus. Some cellobiohydrolases (CBH), endo-1,4-β-glucanases (EG), and xylanase in CTec2 were also partially bound to lignin isolates regardless of pretreatment chemical and concentration. Though enzyme binding on lignin isolates was observed, hydrolysis in the presence of these isolates did not significantly ($p > 0.05$) impact glucose production from cellobiose and Avicel. In conclusion, in this study with biomass derived lignin isolates and model carbohydrate compounds, enzyme activity was not limited but the lignin-carbohydrate matrix in lignocellulosic biomass is different from and/or model compounds due to which the results can vary. Hence further study is needed to determine how hydrolytic efficiency changes when lignin and carbohydrates are present in a complex matrix.

Characterization of Lignin Change due to Chemical Pretreatment of Herbaceous Biomass

by
Woochul Jung

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Biological and Agricultural Engineering

Raleigh, North Carolina

2016

APPROVED BY:

Dr. Ratna R Sharma-Shivappa
Committee Chair

Dr. Praveen Kolar

Dr. Jay Jiayang Cheng

Dr. Rongda Qu

Dr. Sunkyu Park

DEDICATION

To my family

BIOGRAPHY

Woochul Jung was born in Seoul, South Korea on October 16, 1981. He received his bachelor degree in Environmental Engineering from University of Seoul, South Korea in 2008. After his undergraduate, he moved to Davis, California, USA to pursue his master's program in Civil & Environmental Engineering at University of California at Davis and received his Master degree in 2010. In August 2011, he came to the North Carolina State University to pursue the Degree of Doctor of Philosophy in the Department of Biological & Agricultural Engineering under the direction of Dr. Ratna R Sharma-Shivappa.

ACKNOWLEDGMENTS

First and foremost, I would like to give my deepest thanks to my advisor, Dr. Ratna R Sharma-Shivappa for her invaluable support and guidance during my PhD study. I would not have been able to accomplish my PhD works without her guidance and support. She has been my great mentor in my life as well as academic advisor. Her words were great encouragement to me and I will keep in mind her words for my next journey. I would like to give my sincere appreciation to my committee members, Dr. Praveen Kolar, Dr. Jay J. Cheng, Dr. Rongda Qu and Dr. Sunkyu Park for their invaluable guidance and support. I also express my sincere gratitude to Dr. Dhanalekshmi Savitri and Dr. Debbie Clare for their valuable collaboration. I gratefully acknowledge my dear colleagues: Dr. Jiele Xu, Dr. Ziyu Wang, Dr. Anushadevi Panneerselvam, Dr. Sneha Athalye, Lalitendu Das, Yane Ansanay, Rajat Sharma, Dr. Randall Etheridge, Shiqi Feng, Veronica Mbaneme and Rachel Silvka for their assistance to my research and friendship that gave me invaluable and cherishable moments during my PhD program.

Lastly, I am greatly indebted to my parents, sister and Sunghye Cho for their unconditional love, endless support and encouragement throughout my life. Thank you so much for everything.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2

CHAPTER 3

CHAPTER 1

INTRODUCTION

1.1 Significance of lignocellulosic biomass

Lignocellulosic biomass is one of the highly potential bioresources that can be converted into liquid fuel like bioethanol. It is an abundant, domestic, and sustainable resource. Many kinds of lignocellulosic feedstock like switchgrass, miscanthus, alfalfa, canarygrass, and sorghum have been investigated for conversion to cellulosic ethanol and other biofuels. These lignocelluloses have high carbohydrate proportions that can serve as an energy resource; for example, cellulose and hemicellulose can be converted into liquid energy and lignin can serve as a heat energy resource by burning. Bioenergy from lignocellulosic biomass can contribute to reduction of our energy dependence on fossil fuels for meeting the world's transportation needs. Also, utilization of lignocellulosic biomass can help reduce the emission of carbon dioxide from fossil fuels which eventually affects the global climate (Tuskan and Walsh 2001)

1.2 Structure of lignocellulosic biomass

Lignocellulosic biomass has three major components; 1) cellulose, 2) hemicellulose, and 3) lignin. Cellulose, a polysaccharide, is the main component of lignocellulosic biomass and a long chain polymer with β-(1,4)-glycosidic bonds (Kumar et al. 2009). It can be converted into glucose, a six carbon monomeric sugar, by enzymatic hydrolysis and glucose can be fermented to bioethanol. Hemicellulose is a polysaccharide which has relatively shorter polymer chains with β-(1,4)-glycosidic and β-(1,3)-glycosidic bonds. Hemicellulose mainly has five carbon molecules that can be converted to pentoses like xylose and arabinose via enzymatic hydrolysis. These components can also be transformed into bioethanol by fermentation.

The third component, lignin is a phenolic polymer which consists of three main aromatic monomeric compounds; 1) guaiacyl propanol (G-lignin), 2) syringyl propanol (S-lignin), and 3) p-hydroxyphenyl propanol (H-lignin) (Kumar et al. 2009; Taherzadeh and Karimi 2008; Zhou et al. 2011). It is the most recalcitrant component that prevents release of sugars from lignocellulosic biomass and resists microbial activity. Therefore, in terms of sugar production, lignin removal is very important to produce monomeric sugars from lignocellulosic biomass.

1.3 Switchgrass and *Miscanthus x gigantues* as biofeedstock

Among the lignocellulosic feedstocks, switchgrass and miscanthus are potential energy crops which are high yielding and fast growing (Han et al. 2011) perennial grasses (C4 species) in the United of States. Switchgrass as an energy crop is estimated to produce 540% more energy than the energy consumed (Fu et al. 2011). Switchgrass is generally composed of 32-45% cellulose, 16-31% hemicellulose, 21-24% lignin, extractives and ash (Cheng and Timilsina 2011; Falls and Holtzapple 2011; Wang et al. 2012a; Xu et al. 2011c; Xu et al. 2012). *Miscanthus giganteus* grows over 3-4 m tall per year with low moisture and soil nutrient requirements compared to feedstocks like corn and poplar (Bell 2011; El Hage et al. 2010). Miscanthus is generally composed of 40-42% cellulose, 18-25% hemicellulose, 18-28% lignin, extractives and ash (Bell 2011; Cheng and Timilsina 2011). Overall, the proportion of cellulose and hemicellulose is relatively high in these feedstock, making them valuable resources for bioethanol production.

1.4 Role of pretreatment during bioconversion of lignocellulosic biomass

A pretreatment step is required during the biochemical conversion of lignocellulosic biomass to allow cellulolytic enzymes better access to cellulose and enhances its conversion to glucose. It can also allow hemicellulose to solubilize into xylose or arabinose by breaking down or loosening the lignin structure. Therefore, a significant effort in research for maximizing ethanol production from lignocelluosic biomass has been performed on reducing the lignin content through an optimum pretreatment for various feedstocks. (Taherzadeh and Karimi 2008).

1.4.1 Mechanism of action of pretreatment on lignin breakdown including chemical and physical interactions.

Lignin is cross-ester-linked with cell wall polysaccharides, especially arabinoxylan. Carboxyl functional groups of *p*-hydroxycinnamic acid units (e.g. ferulic acid and *p*-coumaric acid) from lignin make the ester bonds with hydroxyl functional groups from the cell wall polysaccharides. Also, *p*-comuaric is cross-ester-linked to the γ-position of syringyl monolignol units and ferulic. (Yamamura et al. 2013)

Alkali and dilute acid pretreatments are mainly used to improve fermentable sugar productions during an enzymatic hydrolysis. Alkali agents (e.g. sodium hydroxide, lime, and ammonia) can cleavage ester linkage between carboxyl functional groups from lignin and hydroxyl groups from arabionxylan (Haque et al. 2013; Xu et al. 2011b). They can allow lignin polymers to be easily separated from the cell wall polysaccharides like cellulose and hemicellulose. Therefore, alkali agents have a significant effect on lignin degradation during pretreatment. Additionally, alkali agents a swelling effect on the cell walls thereby improving hydrolytic enzyme access to the polysaccharides.

Lignin degradation efficiency of dilute acid (e.g. dilute sulfuric acid) as a pretreatment agent is less than alkali (Li et al. 2016; Li et al. 2014). However, dilute acid can change lignin distribution by the following mechanisms (Yasuda et al. 2001);

1) Depolymerization of hemicellulose
2) Condensation between aromatic and benzylic carbons occur
3) Cleavage of β-syringyl ether linkage
4) Rearrangement of β-aryl ether units

Dilute acid causes the lignin to become more condensed by reacting between aromatic nuclei and benzylic carbons through the acid hydrolysis. Also, pseudo-lignin can be produced in during dilute acid pretreatment at higher pretreatment severity. (Matsushita et al. 2004; Sannigrahi et al. 2011; Yasuda et al. 2001).

1.4.2 Impact of pretreatment on lignin chemistry and/or distribution

Although there are some studies on estimating changes in lignin chemistry due different pretreatment methods, little is known about changes in lignin chemistry and/or distribution (or ratio) due to different pretreatment intensities. Belmokhtar et al (2013) studied the changes in lignin distribution after dilute sulfuric acid and aqueous ammonia pretreatments using

Miscantus x *giganteus*. Results showed that S/G ratios after both pretreatments increased in comparison to the S/G ratio of untreated miscanthus. The ester-linked phenolic acids, which are *p*-coumaric acid and ferulic acid, were also estimated after the two pretreatments. Ammonium pretreated miscanthus had significantly reduced ester-linked phenolic acid units than the unpretreated miscanthus. The concentration of *p*-coumaric acid after dilute acid pretreatment did not differ significantly from unpretreated miscanthus. The concentration of ferulic acid decreased after acid pretreatment however extent of reduction was less than with alkali pretreatment.

1.5 Effects of lignin chemistry on enzymatic digestibility

Many researches approved that the lignin content of lignocellulosic biomass prevents release of sugars during the pretreatment and the enzymatic hydrolysis. As reported by Taherzadeh et al.(2008)typically, softwoods are mainly composed of guaiacyl propanol while hardwoods have both guaiacyl propanol and syringyl alcohol. A comparison of the structure of syringyl alcohol and guaiacyl propanol indicates that syringyl alcohol is linear and has a lower degree of polymerization than guaiacyl propanol (Li et al. 2010). Li et al. (2010) suggested that guaiacyl propanol is the key deterrent because it has higher insolubility than syringyl alcohol. Thus more guaiacyl propanol in lignocelluosic biomass makes it more difficult to swell fiber and allow enzyme to access cellulose and hemicellulose compared to biomass with more syringyl alcohol (Fu et al. 2011). To sum up, guaiacyl may be the main component in lignin to impede sugar production.

Studer et al. (2011) investigated the effects of lignin content and Syringyl/Guaiacyl ratio (S/G ratio) in *Populus* on sugar production with combined hot-water pretreatment and enzymatic hydrolysis. They found that total sugars production had strongly negative correlation with the lignin content when the S/G ratio was lower than 2.0. On the other hand, the lignin content had a less negative effect on total sugar release when the S/G ratio was higher than 2.0. Also, both lignin content and S/G ratio affected the amount of glucose released. However, the amount of xylose released was only effected by S/G ratio.

Overall, cell wall degradability and thus enzymatic digestibility of bioresource that have more syringyl lignin is higher than those with more guaiacyl lignin. Higher degradability of the cell wall enables enzyme to access the carbohydrate fraction more easily for converting the lignocellulosic biomass into fermentable monomeric sugar. In conclusion, the selection of a lignocellulosic biomass that has relatively more syringyl lignin might be more economical and effective for fermentable sugar production.

1.6 Impact of lignin modification on hydrolytic sugar production

It is well know that reduced lignin content via biochemical pretreatment can improve fermentable sugar production via enzymatic hydrolysis. Reduced lignin content means less lignin inhibition through physical and structural protection from the enzyme attack.

Studies on genetic manipulation of lignin content or S/G ratio can provide a platform to determine the impacts of changes in lignin composition on hydrolysis sugar production. Li et al (2010) investigated transgenic *Arabidopsis thaliana* by overexpressing or suppressing 5-hydroxylase (F5H) gene which is an enzyme related to converting ferulate into syringyl monolignol. F5H gene was driven by cinnamate-4-hydroxylase (C4H) promoter. They produced transgenic S and G –rich Arabidopsis by overexpressing F5H gene and by suppressing F5H gene, respectively. Liquid hot water pretreatment (at 200 ^{o}C) and subsequent enzymatic hydrolysis were performed to produce fermentable sugars from S-rich, G-rich, and wild type *Arabidopsis*. Results showed that S-rich *Arabidopsis* had about 90% glucose yield while the G-rich and the wild type *Arabidopsis* had about 45% glucose yield.

Huntley et al (2003) also used the overexpression of F5H gene driven by C4H promoter to manipulate poplar for improving pulping performance. Results showed that S/G ratio increased from 1.9 in wild type poplar to 2.41~ 14.17 in 8 transgenic poplar lines. The transgenic poplar lines significantly enhanced chemical pulping efficiency by decreasing 23 kappa units. Value of > 20 ISO brightness from transgenic poplar also increased based on comparison with wild type poplar.

There were several studied related to transgenic switchgrass by suppressing the expression of 4-coumate-CoA ligase (4CL) (Wang et al. 2012a; Wang et al. 2012b; Xu et al. 2011a; Zhou et al. 2012). Wang et al (2012a) and (2012b) used alkali pretreatment with sodium hydroxide and lime, respectively, to facilitate fermentable sugar production during enzymatic hydrolysis. Based on these studies, samples with lignin suppressed by 4CL had relatively lower acid insoluble lignin and higher acid soluble lignin than lignin from control, non-transgenic switchgrass. Also, the results showed that S/G ratio in transgenic switchgrass was 12 to 165 % higher than raw switchgrass. The transgenic switchgrass had 1) 16 and 18% higher of glucan and xylan conversion efficiency than the raw swtichgrass after the sodium hydroxide pretreatment (1.0% NaOH (w/v), 30 min at 121 ^{o}C), and 2) 12% and 10% higher of glucan and xylan conversion efficiency after the lime pretreatment (0.1g/g of raw biomass and 12 hour at 50 ^{o}C). Zhou et al (2012) and Xu et al (2011a) used the dilute sulfuric acid pretreatment. It also showed that S/G ratio in the transgenic switchgrass increased and transgenic switchgrass led to enhance overall carbohydrate conversion. These studies also found that there were the positive correlation between the ratio of acid soluble lignin to acid insoluble lignin (ASL/AIL ratio) and S/G ration.

The main reason to suppress 4CL is to reduce lignin content from biofeedstock while the primary reason to overexpress F5H is to change lignin composition. In the studies about 4CL, the lignin reduction might be primary reason to improve enzymatic digestibility because enzyme can access to easily structural carbohydrates than non-transgenic feedstock because of less lignin content. Also, lignin composition changes (S/G ratio changes) via suppressing 4CL might also contribute enzymes to easily access the structural carbohydrates because it allow lignin to be easily degraded via pretreatment.

Transgenic lignin with plenty of syringyl units has more linear structure. Guaiacyl units derived from coniferyl alcohol has one methoxy group and one hydroxyl group at the 3 and 4 positions, respectively, so that free 5 position can be participated in branching reactions. Therefore, guaiacyl units can induce lignin to facilitate branching so that it can lead to form as the complicated structure. However, syringyl units derived from sinapyl alcohol has 2 methoxy group at the 3 and 5 positions and one hydroxyl group at the 4 position. Mostly, only 4 position with the hydroxyl group among 1 to 6 positions from the syringyl unit is related to the reactions with lignin monomers or polymers (e.g. β-*O*-4 linkage like β-aryl ether or α-keto-β-aryl ether). As a result, the transgenic lignin with plenty of syringyl units can allow the lignin chains to be predominantly linear. It can lead to relatively easily degrade the lignin via the chemical agents via the pretreatment step so that more fermentable monomeric sugars can be produced via the enzymatic hydrolysis by weakening the lignin inhibition during the pretreatment step.

1.7 Lignin-derived inhibition: how lignin inhibits cellulolytic enzymes

Based on previous studies, three mechanisms of lignin-derived inhibition are proposed; 1) physical and structural protection of cell wall polysaccharides from enzyme access, 2) non-productive enzyme (e.g. cellulase) adsorption on lignin, and 3) soluble lignin-derived inhibition (Berlin et al. 2006a; Kim 2012a; Lee et al. 2013; Rahikainen et al. 2011; Rahikainen et al. 2013a; Tejirian and Xu 2011; Ximenes et al. 2010; Ximenes et al. 2011)

First, lignin is cross-ester-linked with the cell wall polysaccharides like hemicellulose. Carboxyl functional groups of *p*-hydroxycinnamic acid units (e.g. ferulic acid and *p*-coumaric acid) from lignin make the ester bonds with hydroxyl functional groups from hemicellulose (Yamamura et al. 2013). This physical and structural feature can prevent enzymes from accessing the cell wall polysaccharides such that fermentable sugar production by enzyme hydrolysis is inhibited.

In another mechanism, cellulase may interact with lignin as well as cell wall polysaccharides. This results in non-productive cellulase adsorption on lignin. There are two main forces by which cellulases interact with lignin; electrostatic interactions and hydrophobic interaction. Rahikainen et al (2011; 2013a) investigated non-productive cellulase binding on lignin and reported that less cellulases were bound to lignin at higher pH. Lignin has more negative charges at higher pH conditions because the phenolic carboxyl groups from lignin can be dissociated and it contributes to the negative charge. Isoelectric points (pI) of the cellulases, *Melanocarpus albomyces* (Cel45A, endoglucanase), and mutated Cel45A with carbohydrate binding modules (CBMs) used in their study ranged from 3.4 to 4.4. At higher pH, cellulases had negative charges. Therefore, the repulsive interactions between negatively charged lignin and cellulase resulted in less enzyme being adsorbed to lignin at higher pH.

Hydrophobic interaction also has a significant role in non-productive cellulase adsorption on lignin. Rahikainen et al (2011; 2013a) showed that Cel45A without CBMs had less adsorption effect on lignin than the mutated Cel45A without CMB. CBMs was genetically modified by containing aromatic amino acids which have hydrophobic regions. Surface of lignin also has hydrophobic region induced from aromatic polymers. Therefore, CBMs from cellulases can be interacted with surface of lignin through the hydrophobic interaction.

Intensified cellulase adsorption via electrostatic and hydrophobic interactions might lead to irreversible unfolding the cellulases and change in their conformational structures such that the cellulases might be denatured.

Finally, phenolic oligomers or polymers released as soluble lignin during pretreatment can be inhibitory to the conversion of cell wall polysaccharides to monomeric sugar. Kim et al (2011), Ximenes et al (2011), and Lee et al (2013) studied the inhibitory effect of soluble lignin on cellulase activity. The results showed that phenolic oligomers like tannic acid and oligomeric proanthocyanidin have more significant inhibition effect on the cellulase activities than phenolic monomers like vanillin or coniferyl aldehyde. Also, the formation of ester bonds between the carboxyl functional groups from soluble lignin-derived and the hydroxyl functional groups from cellulose and hemicellulose can inhibit monomeric sugar production by calluses.

Kawamoto et al (1997) reported that tannins, which is lignin-derived oligomers, interacted with cellulase and decreased their activities due to denaturation. Hydrogen bonds may be a significant interaction mechanism between the lignin and the cellulases besides hydrophobic and electrostatic interactions.

Tejirian and Xu (2011) studied how monomeric and oligomeric phenolic compounds inhibit enzyme activity. The results were consistent with those of Ximenes et al (2011), and Lee et al (2013), indicating that ligomeric phenolic compounds have more inhibition effect on enzymatic celluloysis than simple phenolics. However, Tejirian and Xu (2011) also concluded that monomeric phenolic compounds could also inhibit the enzyme hydrolytic performance. They tested the enzymatic hydrolysis of the dilute acid pretreated corn stover with mixing the three monomeric phenolic compounds individually; syringaldehyde, 4-hydroxy-3-methoxy-α-methylbenzyl alcohol, and vanillin. The results showed that syringaldehyde and 4-hydroxy-3-methoxy-α-methylbenzyl alcohol led to 70% decrease in extent of hydrolysis at high phenolic monomer concentration (> 10mM). These compound however did not affect enzymatic hydrolysis performance at 1 mM. Vanillin resulted in the gelation of the hydrolysis suspension at 500 mM so that the enzymatic hydrolysis did not work. Even though oligomeric phenolic lignin-derived compounds have more inhibition effect on enzymatic hydrolytic performance than the monomeric phenolic compounds, high concentration of phenolic monomers in the hydrolyzate also inhibits enzyme activity.

1.8 Research objectives

Lignin effects for fermentable sugar production from lignocelluosic biomass can be summarized by following statements;

1) Less lignin content leads to generation of more sugars (Nlewem and Thrash, 2010)
2) Higher ratio of acid soluble lignin (ASL) to acid insoluble lignin (AIL) leads to generation of more sugars (Zhou et al., 2012 and Wang et al., 2012)
3) Syringyl propanol (S) and guaiacyl propanol (G) units have greater impact on sugar production than p-hydroxyphenyl propanol (H) unit (Studer et al., 2011)
4) Lignocellulosic biomass with more S monlignol produces more sugars than those with more G monolignol (Li et al., 2010 and Studer et al., 2011)

Even though these scientific findings are valuable information to research fermentable sugar production from lignocellulosic biomass, there is limited knowledge on the mechanism of how lignin chemistry in herbaceous feedstocks change during pretreatment and how the change in lignin chemistry impacts subsequent sugar production. Therefore, this research investigated:

1) The effect of sodium hydroxide pretreatment on lignin reduction in switchgrass and miscanthus at varying chemical concentration (0.5, 1.0, and 1.5% (w/v)) and pretreatment times (15, 30, and 60 min) at 121 ^{o}C/15 psi
2) How sodium hydroxide pretreatment influences lignin chemistry by changing S, G, H and their ratios in switchgrass and miscanthus

3) The effect of changes in lignin chemistry of switchgrass and miscanthus after sodium hydroxide pretreatment on hydrolytic sugar production

Additionally, lignin was isolated from switchgrass and miscanthus pretreated with sodium hydroxide and dilute sulfuric acid at various concentrations to investigate:

4) Which cellulolytic enzymes (Cellic® CTec2) interact with lignin isolates.

5) If lignin isolates from different pretreatment conditions influence enzyme binding.

6) How cellulase bound to lignin isolate can affect production of monomeric sugars

Lignin isolates were incubated with Cellic® CTec2 enzyme cocktail and enzymatic hydrolysis of mixtures of carbohydrate model compounds (cellobiose and Avicel) and lignin isolates was also performed.

REFERENCES

Bell AT (2011) Effects of reaction conditions on the acid-catalyzed hydrolysis of miscanthus dissolved in an ionic liquid. Abstr Pap Am Chem Soc 242.

Belmokhtar N, Habrant A, Lopes Ferreira N, Chabbert B (2013) Changes in Phenolics Distribution After Chemical Pretreatment and Enzymatic Conversion of Miscanthus x giganteus Internode 6:506-518. doi: 10.1007/s12155-012-9275-2.

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198-209. doi: 10.1016/j.jbiotec.2006.02.021.

Cheng JJ, Timilsina GR (2011) Status and barriers of advanced biofuel technologies: A review. Renewable Energy 36:3541-3549. doi: 10.1016/j.renene.2011.04.031.

El Hage R, Chrusciel L, Desharnais L, Brosse N (2010) Effect of autohydrolysis of Miscanthus x giganteus on lignin structure and organosolv delignification. Bioresour Technol 101:9321-9329. doi: 10.1016/j.biortech.2010.06.143.

Falls M, Holtzapple MT (2011) Oxidative Lime Pretreatment of Alamo Switchgrass. Appl Biochem Biotechnol 165:506-522. doi: 10.1007/s12010-011-9271-6.

Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Jr., Chen F, Foston M, Ragauskas A, Bouton J, Dixon RA, Wang Z (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci U S A 108:3803-3808. doi: 10.1073/pnas.1100310108.

Han M, Choi G, Kim Y, Koo B (2011) Bioethanol Production by Miscanthus as a Lignocellulosic Biomass: Focus on High Efficiency Conversion to Glucose and Ethanol. BioResources 6:1939-1953.

Haque MA, Barman DN, Kang TH, Kim MK, Kim J, Kim H, Yun HD (2013) Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of Miscanthus sinensis at boiling temperature with low residence time 114:294-305. doi: 10.1016/j.biosystemseng.2013.01.006.

Huntley S, Ellis D, Gilbert M, Chapple C, Mansfield S (2003) Significant increases in pulping efficiency in C4H-F5H-transformed poplars: Improved chemical savings and reduced environmental toxins. J Agric Food Chem 51:6178-6183. doi: 10.1021/jf034320o.

Kawamoto H, Mizutani K, Nakatsubo F (1997) Binding nature and denaturation of protein during interaction with galloylglucose. Phytochemistry 46:473-478. doi: 10.1016/S0031-9422(97)00330-0.

Kim TH (2012) Comparison of inhibition effects of various isolated lignins on enzymatic hydrolysis of cellulose 29:82-88. doi: 10.1007/s11814-011-0150-1.

Kim Y, Ximenes E, Mosier NS, Ladisch MR (2011) Soluble inhibitors/deactivators of cellulase enzymes from lignocellulosic biomass. Enzyme Microb Technol 48:408-415. doi: 10.1016/j.enzmictec.2011.01.007.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind Eng Chem Res 48:3713-3729. doi: 10.1021/ie801542g.

Lee H, Sanyoto B, Choi J, Ha J, Suh DJ, Lee K (2013) Effects of lignin on the ionic-liquid assisted catalytic hydrolysis of cellulose: chemical inhibition by lignin 20:2349-2358. doi: 10.1007/s10570-013-9989-0.

Li K, Wan J, Wang X, Wang J, Zhang J (2016) Comparison of dilute acid and alkali pretreatments in production of fermentable sugars from bamboo: Effect of Tween 80. Ind Crop Prod 83:414-422. doi: 10.1016/j.indcrop.2016.01.003.

Li X, Ximenes E, Kim Y, Slininger M, Meilan R, Ladisch M, Chapple C (2010) Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. Biotechnol Biofuels 3:27. doi: 10.1186/1754-6834-3-27.

Li Z, Jiang Z, Fei B, Cai Z, Pan X (2014) Comparison of bamboo green, timber and yellow in sulfite, sulfuric acid and sodium hydroxide pretreatments for enzymatic saccharification. Bioresour Technol 151:91-99. doi: 10.1016/j.biortech.2013.10.060.

Matsushita Y, Kakehi A, Miyawaki S, Yasuda S (2004) Formation and chemical structures of acid-soluble lignin II: reaction of aromatic nuclei model compounds with xylan in the presence of a counterpart for condensation, and behavior of lignin model compounds with guaiacyl and syringyl nuclei in 72% sulfuric acid. J Wood Sci 50:136-141. doi: 10.1007/s10086-003-0543-9.

Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K (2011) Inhibition of Enzymatic Hydrolysis by Residual Lignins From Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface. Biotechnol Bioeng 108:2823-2834. doi: 10.1002/bit.23242.

Rahikainen JL, Evans JD, Mikander S, Kalliola A, Puranen T, Tamminen T, Marjamaa K, Kruus K (2013) Cellulase-lignin interactions-The role of carbohydrate-binding module and pH in non-productive binding. Enzyme Microb Technol 53:315-321. doi: 10.1016/j.enzmictec.2013.07.003.

Sannigrahi P, Kim DH, Jung S, Ragauskas A (2011) Pseudo-lignin and pretreatment chemistry 4:1306-1310. doi: 10.1039/c0ee00378f.

Studer MH, DeMartini JD, Davis MF, Sykes RW, Davison B, Keller M, Tuskan GA, Wyman CE (2011) Lignin content in natural Populus variants affects sugar release. Proc Natl Acad Sci U S A 108:6300-6305. doi: 10.1073/pnas.1009252108.

Taherzadeh MJ, Karimi K (2008) Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: A review. Int J Mol Sci 9:1621-1651. doi: 10.3390/ijms9091621.

Tejirian A, Xu F (2011) Inhibition of enzymatic cellulolysis by phenolic compounds. Enzyme Microb Technol 48:239-247. doi: 10.1016/j.enzmictec.2010.11.004.

Tuskan G, Walsh M (2001) Short-rotation woody crop systems, atmospheric carbon dioxide and carbon management: A US case study. For Chron 77:259-264.

Wang Z, Li R, Xu J, Marita JM, Hatfield RD, Qu R, Cheng JJ (2012a) Sodium hydroxide pretreatment of genetically modified switchgrass for improved enzymatic release of sugars. Bioresour Technol 110:364-370. doi: 10.1016/j.biortech.2012.01.097.

Wang Z, Xu J, Pandey P, Cheng JJ, Li R, Qu R (2012b) Improvement of Sugar Production from Transgenic Switchgrass with Low-Temperature Alkali Pretreatment. Energy Fuels 26:3054-3061. doi: 10.1021/ef3004575.

Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2011) Deactivation of cellulases by phenols. Enzyme Microb Technol 48:54-60. doi: 10.1016/j.enzmictec.2010.09.006.

Ximenes E, Kim Y, Mosier N, Dien B, Ladisch M (2010) Inhibition of cellulases by phenols. Enzyme Microb Technol 46:170-176. doi: 10.1016/j.enzmictec.2009.11.001.

Xu B, Escamilla-Trevino LL, Sathitsuksanoh N, Shen Z, Shen H, Zhang YP, Dixon RA, Zhao B (2011a) Silencing of 4-coumarate:coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. New Phytol 192:611-625. doi: 10.1111/j.1469-8137.2011.03830.x.

Xu J, Zhang X, Cheng JJ (2012) Pretreatment of corn stover for sugar production with switchgrass-derived black liquor. Bioresour Technol 111:255-260. doi: 10.1016/j.biortech.2012.02.006.

Xu J, Chen Y, Cheng JJ, Sharma-Shivappa RR, Burns JC (2011b) Delignification of Switchgrass Cultivars for Bioethanol Production. BioResources 6:707-720.

Xu J, Wang Z, Sharma-Shivappa RR, Cheng JJ (2011c) Enzymatic Hydrolysis of Switchgrass and Coastal Bermuda Grass Pretreated using Different Chemical Methods. BioResources 6:2990-3003.

Yamamura M, Noda S, Hattori T, Shino A, Kikuchi J, Takabe K, Tagane S, Gau M, Uwatoko N, Mii M, Suzuki S, Shibata D, Umezawa T (2013) Characterization of lignocellulose of Erianthus arundinaceus in relation to enzymatic saccharification efficiency 30:25-35. doi: 10.5511/plantbiotechnology.12.1127a.

Yasuda S, Fukushima K, Kakehi A (2001) Formation and chemical structures of acid-soluble lignin I: sulfuric acid treatment time and acid-soluble lignin content of hardwood. J Wood Sci 47:69-72. doi: 10.1007/BF00776648.

Zhou C, Li Q, Chiang VL, Lucia LA, Griffis DP (2011) Chemical and Spatial Differentiation of Syringyl and Guaiacyl Lignins in Poplar Wood via Time-of-Flight Secondary Ion Mass Spectrometry. Anal Chem 83:7020-7026. doi: 10.1021/ac200903y.

Zhou X, Xu J, Wang Z, Cheng JJ, Li R, Qu R (2012) Dilute sulfuric acid pretreatment of transgenic switchgrass for sugar production. Bioresour Technol 104:823-827. doi: 10.1016/j.biortech.2011.11.051.

CHAPTER 2

CHANGES IN LIGNIN CHEMISTRY OF SWITCHGRASS DUE TO DELIGNIFICATION BY SODIUM HYDROXIDE PRETREATMENT

ABSTRACT

Switchgrass was pretreated with sodium hydroxide (NaOH) at various concentrations and pretreatment times to investigate how delignification caused by NaOH affects its lignin chemistry. NaOH resulted in significant delignification ranging from 44.0 to 84.6% depending on pretreatment intensity. While there was no significant glucan loss due to NaOH pretreatment, higher NaOH concentrations removed xylan by up to 28.3%. Nitrobenzene oxidation (NBO), which was used to study changes in lignin chemistry, indicated that at higher NaOH concentrations, amount of 4-hydroxygenzaldehyde (Hy) degraded from *p*-hydroxyphenyl propanol (H) lignin units was significantly reduced ($p < 0.05$). Amounts of syringic (SA) and vanillic (VA) acids, generated from syringyl (S) and guaiacyl (G) degradation, were however more at higher NaOH concentration. S/G ratio ($= 0.62_{\text{raw switchgrass}}$) did not significantly ($p > 0.05$) change with 15 min pretreatment, but it increased to 0.75 and 0.72, respectively with 30 and 60 min pretreatments ($p < 0.05$). Increase in NaOH concentration did not significantly ($p > 0.05$) change S/G ratio but H/G ratio ($= 0.48_{\text{raw switchgrass}}$) decreased significantly to 0.14 regardless of pretreatment times. Overall, H unit monolignol was found to be more susceptible to NaOH than S and G unit monolignols. Though changes in lignin chemistry due to NaOH concentration were observed, their impact on cellulolytic enzyme action during hydrolysis could not be fully understood. Further studies on

lignin isolation may help to determine how these changes in lignin chemistry by NaOH impact cellulolytic enzymes.

Keywords: lignin chemistry, syringyl, guaiacyl, *p*-hydroxyphenyl, lignin monomer ratio, nitrobenzene oxidation

1. INTRODUCTION

Lignocellulosic biomass, a resource believed to be sustainable and widely available, is mainly composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose as structural (polymeric) carbohydrates, are key substrates for production of bioethanol through enzymatic conversion to fermentable sugars like glucose and xylose. Lignin is a biosysnthesized phenolic compound formed by oxidative coupling of *p*-hydroxycinnamyl alcohols and related compounds like guaiacyl (G), syringyl (S), and *p*-phydroxyphenyl (H) propanol monolignols (Figure 2.1). G, S, and H monolignols are cross-linked by a variety of bonds (Figure 2.1). β-O-4 linked aryl ether bonds form non-condensed linkages which are relatively easier to cleave compared to condensed linkage like carbon-carbon bonds (Kishimoto et al. 2010; Liu et al. 2013; Liu et al. 2014; Ohra-aho et al. 2013; Santos et al. 2013). While softwood lignin is mainly comprised of G monolignol and hardwood contains G and S monolignols, lignin in herbaceous grasses is made of G, S, and H. Lignin fills the space between structural carbohydrates and provides structural stability to lignocellulosic biomass (Boerjan et al. 2003; Umezawa 2010). However, its complex structure makes lignin the most recalcitrant substance preventing conversion of carbohydrates to monomers by blocking cellulolytic enzyme excess.

Due to lignin's inhibitory effect on enzymatic hydrolysis of structural carbohydrates, pretreatment techniques have been developed to open up the carbohydrate-lignin matrix. Many researchers have investigated alkali pretreatment of lignocellulosic biomass and found lignin reduction to be a key factor in improving sugar production through enhanced enzyme accessibility (Haque et al. 2013; Liu et al. 2014; Wang et al. 2012b; Xu et al. 2011b). However, there is little knowledge on how pretreatment intensity affects lignin chemistry of herbaceous grasses and whether those changes can influence subsequent fermentable sugar production via enzymatic hydrolysis. Understanding the impact of these changes in lignin monomer ratios on cellulose enzyme performance can help in developing the tools to improve the conversion process.

Switchgrass (*Pancium virgatum*), a warm-season perennial grass, has been identified as a significant bioresource for 2nd generation bioethanol production because of its high carbohydrate content, rapid growth, excellent biomass yield with relatively less water and nitrogen inputs. It is also well adapted to various soil and climate conditions (Fu et al. 2011; Han et al. 2011; Jensen et al. 2007; Keshwani and Cheng 2009; Sharma et al. 2013).In this study, switchgrass was selected to investigate changes in lignin chemistry caused by sodium hydroxide (NaOH) pretreatment and how they impact enzyme hydrolysis. Effect of pretreatment at various NaOH concentrations and pretreatment times on chemical composition and lignin chemistry of pretreated switchgrass compared to raw biomass was quantified. Enzymatic hydrolysis was performed to determine if changes in lignin chemistry of switchgrass, as affected by pretreatment intensity, have an impact on fermentable sugar production

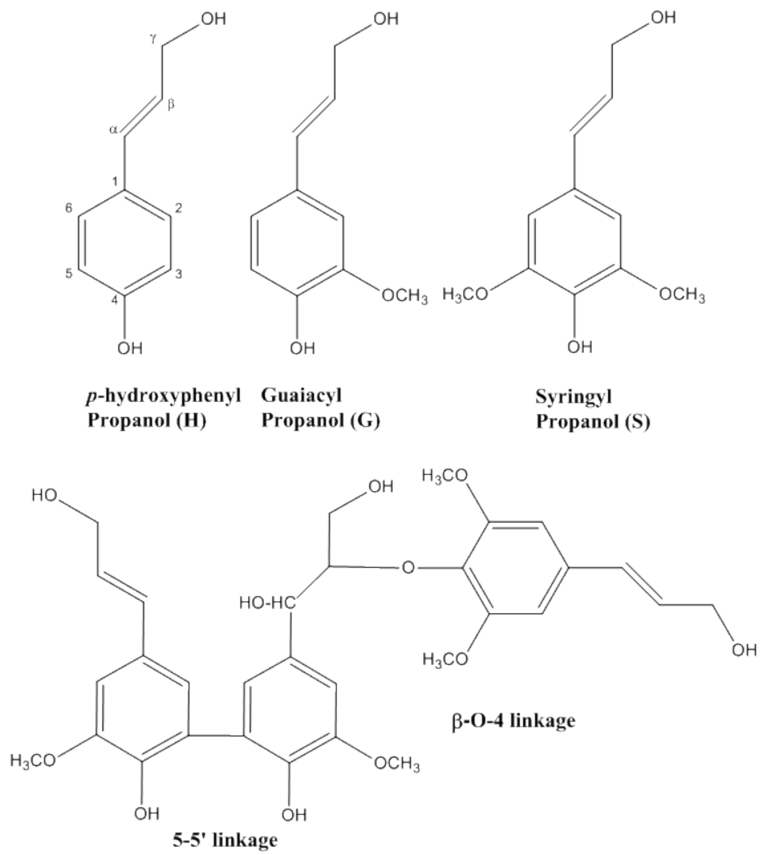


Figure 2.1. Schematic showing the structure of three main monolignols and β-O-4 linkage representing non-condensed bonds and 5-5' bonds representing condensed structure.

2. MATERIAL & METHODS

2.1 Sample preparation

Alamo switchgrass, in its fourth year at a field at Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina, was harvested and field-chopped in December 2011 for use in this study. The switchgrass was ground and passed through a 2 mm sieve by a Thomas Wiley Laboratory Mill (Model No. 4, Philadelphia, PA, USA). Acetone reflux extraction was performed to remove extractives by soxhlet extraction in cellulose thimbles for 24 hours. Extractive free samples were used for NaOH pretreatment to avoid potential interference during subsequent nitrobenzene oxidation (NBO) for determination of lignin chemistry changes.

2.2 Sodium hydroxide pretreatment

Switchgrass was pretreated in an autoclave (Model 3021, Amsco, Mentor, OH, USA) at 121 ^{o}C with a combination of three NaOH concentrations (0.5, 1.0 and 1.5%, w/v) and three pretreatment times (15, 30 and 60 min) using a full factorial experimental design. Five grams of biomass was mixed with 50 ml NaOH solution (10% w/v solid loading, dry basis) in 125 ml serum bottles which were crimp sealed for pretreatment. Washing with 500 ml of deionized (DI) water was performed to remove residual NaOH and neutralize the pretreated biomass and a vacuum filter assembly was used to recovery the solids. Approximately 1 g of the wet pretreated biomass was dried in a 105 ^{o}C convection oven to estimate moisture content and solid recovery. Also, about 4 g of the wet pretreated biomass was dried in a vacuum oven (Model 281A, Fisher Scientific, USA) at 40 ^{o}C for composition analysis and nitrobenzene oxidation for the pretreated biomass. The remaining pretreated biomass, sealed in a plastic bag, was stored at 4 ^{o}C for subsequent enzymatic hydrolysis.

2.3 Enzymatic hydrolysis

Pretreated switchgrass at 8% solid loading (dry basis) was suspended in 10 ml volume made up by 0.05M sodium citrate buffer (pH 4.8), 40 µg/ml of tetracycline, Cellic® CTec2 and HTec2 enzyme cocktails (Novozymes North America, INC, Franklinton, NC, USA) in a 50 ml centrifuge tube. Enzymatic hydrolysis was performed in a water bath shaker (Model: 89032-226, VWR international, PA) at 50 ^{o}C, 150 rpm for 72 hours. Excess enzyme loading of CTec2 (0.625 g of enzyme/g of pretreated biomass, density = 1.21 g/ml) (equivalent to 70 FPU/g dry biomass) and 0.125 g HTec2/g of pretreated biomass (density = 1.16 g/ml) was used during hydrolysis to prevent any enzyme limitation and maximize monomeric fermentable sugar production. Cellulase activity of CTec2 estimated by National Renewable Energy Laboratory's (NREL)'s Analytical Procedure (LAP) (Adney and Baker, 2008) was 110 FPU/ml. The hydrolysate was centrifuged (model 5810R, Eppendorf, Hauppauge, NY) at 4000 rpm and 4 C^{o} for 10 min and filtered supernatants were stored at -80 ^{o}C for monomeric sugars (glucose, xylose, arabinose, and galactose) analysis. Samples from the pretreatment condition identified as the most promising were also hydrolyzed at CTec2 loading of 5, 10, 15, 20, 40 and 70 FPU/ml supplemented with 12.5 g HTec2/g of pretreated biomass to further optimize hydrolysis conditions, respectively.

2.4 Analytical methods

Total solids, solid recovery, structural carbohydrates, lignin content including acid insoluble (AIL) and acid soluble (ASL) lignin, and extractives were measured based on NREL's Laboratory Analytical Protocol (Slutier et al, 2005, 2008a, 2008b and 2008c).

Monomeric sugars (e.g., glucose, xylose, arabinose, and galactose) produced from untreated and pretreated biomass via two-stage sulfuric acid hydrolysis for composition analysis and those via enzymatic hydrolysis were measured by ion-exchange chromatography (IC) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA). Amount of monomeric sugar produced from pretreated biomass during enzymatic hydrolysis (mg monomeric sugar/g pretreated biomass) was multiplied with solid recovery (%, g pretreated biomass/g untreated biomass) to determine the amount of untreated biomass needed for producing the sugars (mg monomeric sugar/g untreated biomass). Estimation of sugar polymer in untreated and pretreated biomass (e.g. glucan, xylan, arabinan, and galactan, respectively) was done by dividing the monomeric sugar numbers with 1.1. Delignification, which is percentage of lignin removed from raw biomass during pretreatment, was followed by equation 1,

$$Delignification\ (\%) = \left(\frac{LU - LP \times \frac{SR}{100}}{LU}\right) \times 100 \qquad (1)$$

where, LU: lignin content in untreatedbiomass (g lignin/g untreated biomass), LP: lignin content in pretreated biomass (g lignin/g pretreated biomass)

Lignin chemistry of untreated and pretreated biomass was analyzed by modified nitrobenzene oxidation (Kajita et al. 1996; Lima et al. 2008). Guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) propanol units, which are the main phenolic compounds making up lignin, can be oxidized into three aldehyde-lignin units (syringaldehyde (Sy), vanillin (V), 4-hyroxybenzaldehyde (Hy)), respectively, with further oxidation to acid-lignin units (syringic acid (SA), vcanillic acid (A), 4-hydroxybenzoic acid (HA)) during NBO reaction (SCHULTZ and TEMPLETON 1986) (Figure 2.2). After NBO of switchgrass samples, these phenolic compounds were extracted with methylene chloride (CH_2Cl_2) using a glass separation funnel. Shimadzu HPLC (LC-20AT, Japan) equipped with ZORBAX SB-C3 reverse phase analytical column at room temperature was used to detect the six degraded lignin monomers using a UV detector (SPD-20A, Japan). Standards solutions of Sy, SA, V, VA, Hy, and HA at concentrations of 0.0078 to 0.25 g/L were used to identify elution times of 5.3, 6.1, 6.8, 7.0, 8.5, and 9.4 min, respectively, and quantify these compounds in the switchgrass derived samples. Twenty µl of the sample solution was injected and the data acquisition time was 25 min with an eluant (a mixture of acetonitrile (CH_3CN) and water containing 10 mM formic acid (HCOOH) flow rate of 1.5 ml/min. HA was not detected in the NBO samples from untreated and pretreated switchgrass (Figure 2.3). S/G and H/G molar ratios for samples from various pretreatments were calculated by equation 2 and 3 below,

$$S/G\ molar\ ratio = \frac{Syringaldehyde\ (Sy) + Syringic\ Acid\ (SA),(\mu\mathrm{mol/g\ lignin})}{Vanillin\ (V) + Vanillin\ Acid\ (VA),(\mu\mathrm{mol/g\ lignin})} \qquad (2)$$

$$H/G\ molar\ ratio = \frac{p{-}Hydroxybenzaldehyde\ (Hy),(\mu\mathrm{mol/g\ lignin})}{Vanillin\ (V) + Vanillin\ Acid\ (VA),(\mu\mathrm{mol/g\ lignin})} \qquad (3)$$

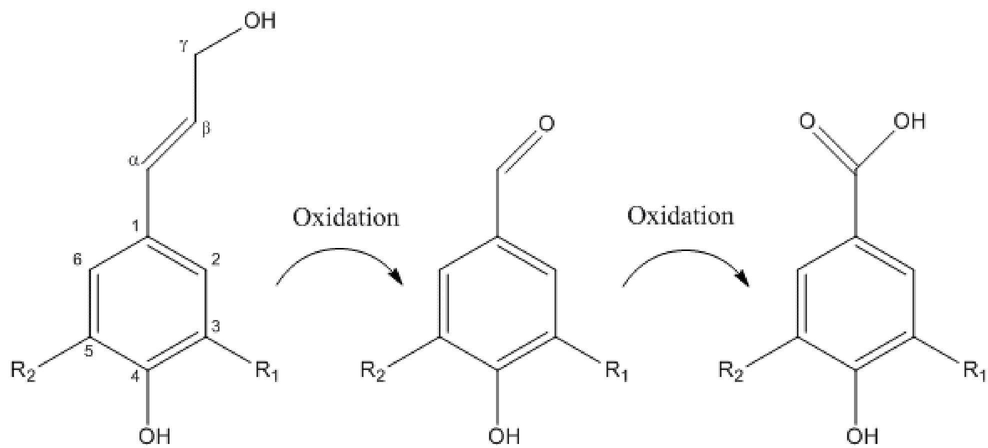


Figure 2.2. Progressive oxidation of lignin into degradation compounds during nitrobenzene oxidation reaction. (R_1, R_2 = H: *p*-hydroxylphenyl propanol → 4-hydroxybenzaldehyde → 4-hydroxybenzoic acid, R_1 = OMe, R_2=H: guaiacyl propanol → vanillin → vanillic acid, R_1, R_2 = syringyl propanol → syringaldehyde → sryingic acid)

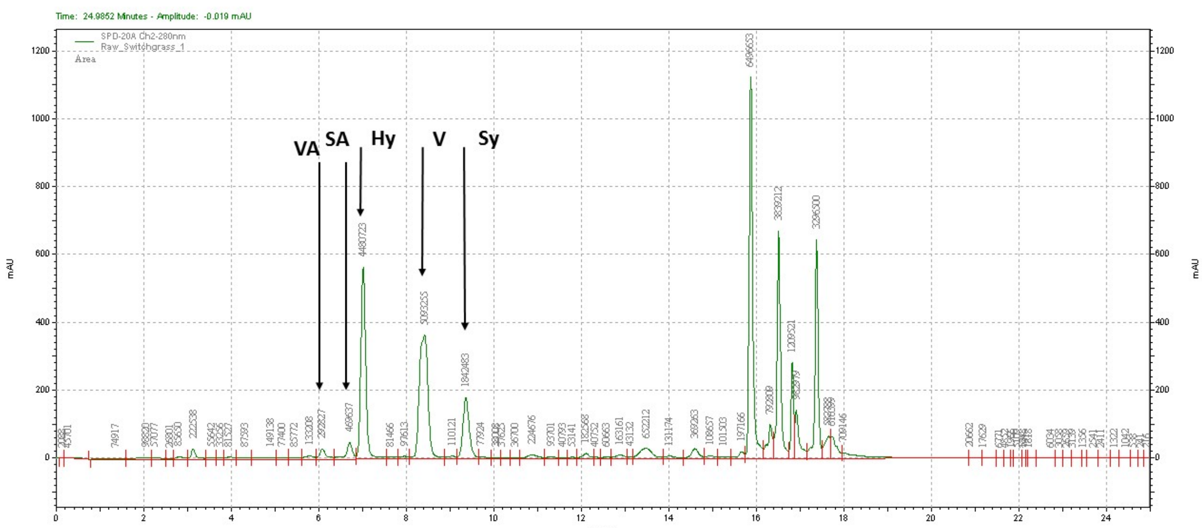


Figure 2.3. HPLC chromatograph showing lignin degradation compounds generated during NBO

2.5 Statistical analysis

All treatments were performed in triplicate. Generalized linear model (GLM) procedure with Tukey adjustment at 95% confidence level in SAS 9.3 (Cary, NC, USA) was used to statistically analyze experimental data. The main effects were NaOH concentrations (0.5, 1.0, and 1.5%) and pretreatment times (15, 30, and 60 min) and dependent variables were monomeric sugar production (i.e. glucose, xylose), lignin content including AIL and ASL, and amount of lignin degradation compounds (i.e. Sy, SA, V, VA, Hy, S/G and H/G) generated.

3. RESULTS & DISCUSSION

3.1 Characterization of Switchgrass

Composition analysis of untreated switchgrass was performed to provide a comparison with changes due to NaOH pretreatment at various conditions (Table 2.1). Glucan and xylan as the major polysaccharides, were 36.7 and 21.5 % (dry basis), respectively. Switchgrass also contained arabinan and galactan in small proportions. Lignin, including acid insoluble lignin (AIL) and acid soluble lignin (ASL), constituted 24.3% of the untreated switchgrass. Extractives and ash content were 3.2 and 2.8%, respectively.

Table 2.1. Composition of Alamo Switchgrass

Material	Alamo Switchgrass (% dry basis)
Glucan	36.7 ± 0.37
Xylan	21.5 ± 0.31
Arabinan	2.7 ± 0.01
Galactan	0.8 ± 0.02
Lignin	
Acid Soluble Lignin (ASL)	2.6 ± 0.02
Acid Insoluble Lignin (AIL)	21.7 ± 0.27
Extractives	3.2 ± 0.48
Ash	2.8 ± 0.05

3.2 Chemical composition of sodium hydroxide pretreated switchgrass

After NaOH pretreatment of switchgrass at various conditions, residual solids were separated to estimate solid recovery and chemical composition (Table 2.2). Overall, solid recovery decreased with increase in pretreatment intensity and 60 min pretreatment resulted in significantly lower ($p < 0.05$) solid recovery than 15 min pretreatment. Recovered solids ranged from 61.6 – 82.3% (g pretreated biomass/g untreated biomass) from the highest to the lowest pretreatment severity

Composition analysis of pretreated switchgrass included glucan, xylan and lignin (AIL + ASL). While no significant ($p > 0.05$) loss in glucan was observed, xylan loss occurred with increase in pretreatment intensity. Though pretreatment time did not have a significant ($p > 0.05$) effect on xylan degradation and there was no significant ($p > 0.05$) xylan degradation with 0.5% NaOH pretreatment. 1.0 and 1.5% NaOH pretreatments led to significant ($p < 0.05$) degradation in xylan. An estimation of changes in lignin content after NaOH pretreatment showed that higher concentration and longer pretreatment times led to increased delignification (Table 2.2) with delignification ranging from 44.0% (at 0.5% with 15 min) to 84.6% (at 1.5% NaOH with 30 min). Lignin has cross-ester-linkages with structural carbohydrates, especially hemicelluloses such as arabinoxylan and it is reported that NaOH comprehensively disrupts these linkages (Belmokhtar et al. 2013; Haque et al. 2013; Yamamura et al. 2013) resulting in removal of lignin and xylan from the biomass which further contributes to lower solid recovery.

Table 2.2. Solid recovery, composition of pretreated switchgrass and delignification after NaOH pretreatment at varying conditions

	Time (min)	NaOH Concentration (%)		
		0.5	1.0	1.5
Solid Recovery (wt %)	15	82.3 ± 1.3	69.8 ± 2.2	65.9 ± 1.1
	30	80.9 ± 1.3	68.3 ± 1.0	62.8 ± 0.6
	60	79.0 ± 3.1	66.0 ± 2.1	61.6 ± 0.5
Glucan (wt %)[1)]	15	39.6 ± 3.6	36.7 ± 1.5	36.7 ± 0.6
	30	39.3 ± 1.5	37.7 ± 1.5	35.4 ± 2.8
	60	38.1 ± 3.0	36.5 ± 3.6	34.5 ± 2.2
Xylan (wt %)[1)]	15	22.6 ± 2.0	18.7 ± 0.7	16.0 ± 0.3
	30	22.0 ± 1.0	18.7 ± 0.9	16.3 ± 1.3
	60	20.6 ± 1.3	17.7 ± 1.9	15.3 ± 1.1
Lignin (wt %)[1)]	15	13.7 ± 0.6	7.1 ± 0.9	4.3 ± 0.3
	30	11.4 ± 0.7	6.6 ± 0.8	3.7 ± 0.5
	60	10.8 ± 0.3	4.8 ± 0.2	3.8 ± 0.2
Delignification (wt %)	15	44.0 ± 2.3	71.0 ± 3.7	82.5 ± 1.4
	30	53.5 ± 2.7	72.9 ± 3.2	84.6 ± 1.9
	60	55.9 ± 1.3	80.2 ± 0.8	84.3 ± 0.9

[1)] (g/g dry untreated biomass)

3.3 Lignin chemistry changes in switchgrass due to sodium hydroxide pretreatment

Five lignin degradation compounds (Sy and SA from S-lignin unit, V and VA from G-lignin unit, Hy from H-lignin unit) from untreated and pretreated switchgrass were detected and quantified (Figure 2.4) and corresponding total yields calculated (Table 2.3). Sy, V and Hy were the predominantly observed compounds while the acid groups (SA, VA) produced due to further oxidized of the aldehyde groups were in relatively less amounts. Total yield of these five phenolic compounds from lignin in untreated sample was 26.1% and that from pretreated samples ranged from 17.8 to 39.9% (Table 2.3). Though reaction conditions for NBO were kept consistent for each batch, variable yields were observed. The variability in yield may to some extent be attributed to the differences in oxidation levels as switchgrass samples pretreated at each pretreatment time were analyzed in separate batches. Amount of Hy released from pretreated biomass was significantly reduced ($p < 0.05$) compared to untreated biomass and higher NaOH concentration resulted in lower production of Hy (Figure 2.4). Conversely, SA and HA generated from pretreated biomass were significantly more ($p < 0.05$) than untreated biomass and higher NaOH concentration increased the amounts of the two acid units produced. Increase in SA and HA indicates that changes in lignin structure or chemistry of switchgrass subjected to more server NaOH pretreatments make its monolignols more susceptible to oxidation.

The results of this study showed that NaOH significantly changes lignin chemistry, especially, causing reduction in H lignin units. The S:G:H ratio of untreated switchgrass was 29:48:23 as estimated from fractions of polyphenolic compounds extracted via NBO. Pretreatment with NaOH resulted in higher proportion of S and G units and lower proportion of H units in lignin compared to untreated switchgrass. Changes were also observed in S/G and H/G ratios which for untreated switchgrass were 0.62 and 0.48, respectively. The S/G ratio for samples pretreated for 15 min was not significantly ($p > 0.05$) different from untreated switchgrass but it increased significantly ($p < 0.05$) up to 0.75 and 0.72 for 30 and 60 min pretreatments, respectively, when 0.5% NaOH was applied. 30 and 60 min pretreatments had no significant ($p > 0.05$) difference of S/G ratio change with each other. On the other hand, H/G ratio significantly ($p < 0.05$) dropped to 0.37, 0.29 and 0.28 with 15, 30 and 60 min pretreatments, respectively, when 0.5% NaOH was applied. It also decreased significantly ($p < 0.05$) at all times when NaOH concentration increased.

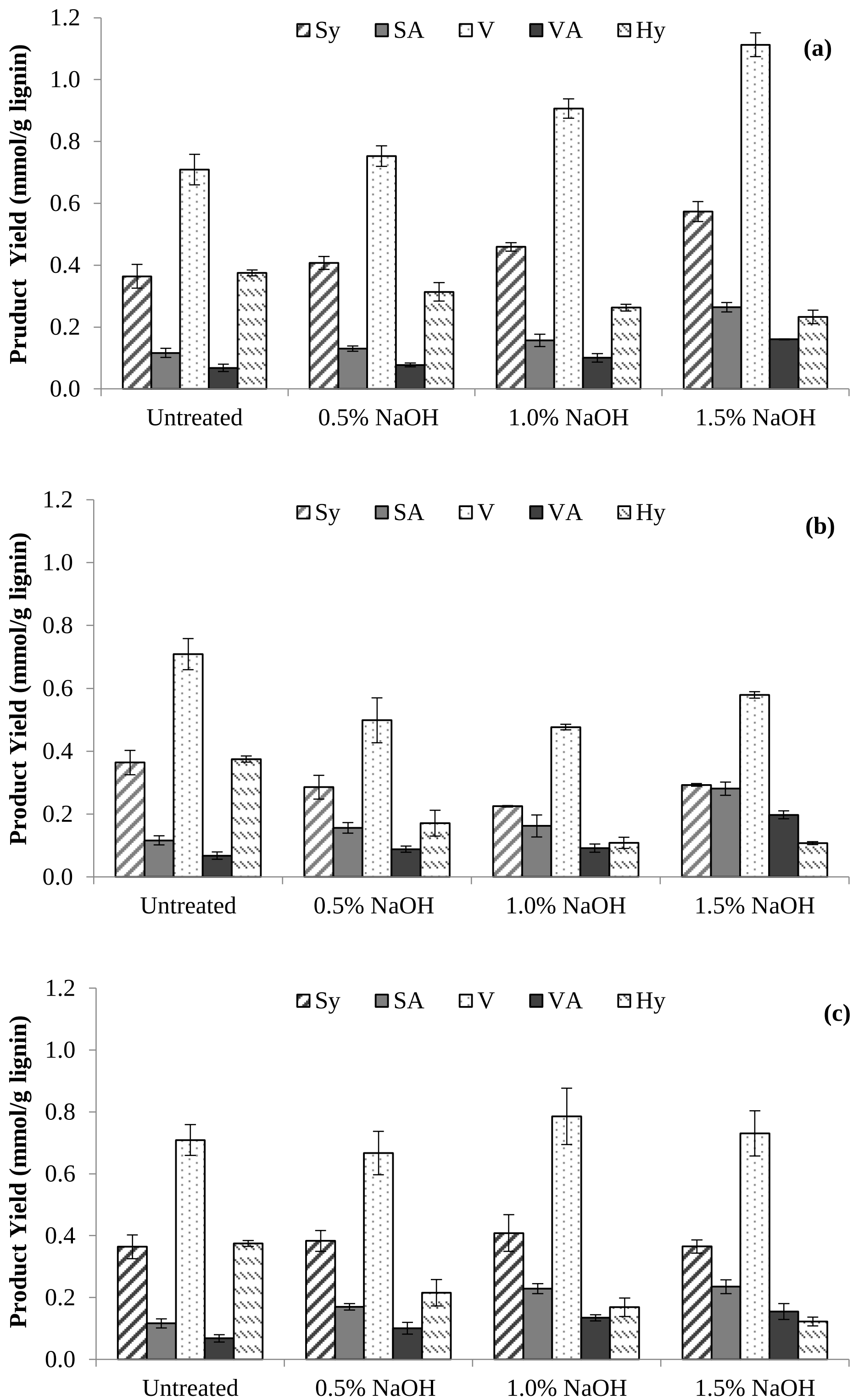
H units have more reactive sites than G and S units because they do not have a methoxy functional group at the aryl side while G and S units have one methoxy group at the 3 position and two at 3 and 5 positions, respectively. Russell et al. (2006) found that additional methoxy groups at the aryl side can aid lignification via oxidative coupling by stabilizing the free radicals. On the other hand, non-methoxylated lignin monomers might be able to terminate polymerization leading to production of smaller lignin polymers. Ziebell et al. (2010) investigated transgenic alfalfa (*Medicago sativa*) found that lignin with predominately H units in the transgenes led to decrease in molecular weight of the polymer as compared to control in which S and G units were prevalent. Since H units were observed to be the most susceptible to NaOH pretreatment in our study, delignification of switchgrass may be maximized even with less NaOH input during chemical pretreatment by genetically producing high H lignin units.

Table 2.3. Changes in ratio of S/G, H/G and S:G:H after NaOH pretreatment of switchgrass and total yield (%) by nitrobenzene oxidation

Time, min	Conc., %	S/G ratio	H/G ratio	S:G:H	Total yield [1], %
Untreated Sample		0.62 ± 0.02	0.48 ± 0.03	29:48:23	26.1 ± 2.1
15	0.5	0.64 ± 0.01	0.37 ± 0.03	32:50:18	27.5 ± 0.7
	1.0	0.61 ± 0.02	0.26 ± 0.01	33:53:14	31.6 ± 0.3
	1.5	0.65 ± 0.01	0.18 ± 0.02	35:55:10	39.9 ± 1.8
30	0.5	0.75 ± 0.02	0.29 ± 0.03	37:49:14	20.4 ± 2.9
	1.0	0.66 ± 0.03	0.19 ± 0.03	36:54:10	17.8 ± 0.8
	1.5	0.73 ± 0.03	0.14 ± 0.01	39:54:7	26.2 ± 0.8
60	0.5	0.72 ± 0.03	0.28 ± 0.02	36:50:14	25.7 ± 2.8
	1.0	0.69 ± 0.01	0.18 ± 0.01	37:53:10	30.1 ± 3.1
	1.5	0.68 ± 0.05	0.14 ± 0.01	37:55:8	29.1 ± 1.6

[1] Total yield (%) = sum of weight of total phenolic compounds produced by NBO (g) / weight of extractive-free dried lignin (g) × 100.

Figure 2.4. Production of degradation compounds through nitrobenzene oxidation of switchgrass samples (mmol/g lignin)


(a)
(b)
(c)
Sy
SA
V
VA
Hy
Pruduct Yield (mmol/g lignin)
Product Yield (mmol/g lignin)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Untreated
0.5% NaOH
1.0% NaOH
1.5% NaOH

3.4 Enzymatic hydrolysis with NaOH pretreated switchgrass

Enzymatic hydrolysis was performed to produce fermentable sugars from NaOH pretreated samples (Figure 2.5) and determine if change in lignin chemistry relative to proportion(s) S, G, and H impacted carbohydrate conversion efficiency. Hydrolysis of untreated switchgrass produced 191.8 mg glucose/g biomass and 19.0 mg xylose/g biomass, which was equivalent to 47.6 and 8.0% of glucan and xylan conversion, respectively. NaOH pretreated samples produced significantly ($p < 0.05$) higher glucose and xylose. Maximum glucose and xylose yields were 341.7 mg $_{glucose}$/g $_{untreated\ biomass}$ with 0.5% NaOH for 60 min and 149.4 mg $_{xylose}$/g $_{untreated\ biomass}$ with 1.0% NaOH for 60 min, respectively. Overall glucan conversion from all pretreated samples was 75.2 to 84.7% and xylan conversion was 53.7% and 63.3%. Though NaOH pretreated samples hydrolyzed by excess enzymes produced significantly higher glucose and xylose than untreated switchgrass, there was no significant difference ($p > 0.05$) in sugars produced from the various pretreatment conditions and 100% carbohydrate conversion was not achieved. Hemicelluloses' shielding effect and increased cellulose crystallinity due to alkali pretreatment might cause incomplete glucan conversion (Barman et al. 2014; Li et al. 2013; Ohgren et al. 2007; Sun et al. 2011; Yoshida et al. 2008). Also, partial xylan loss due to NaOH pretreatment also resulted in less than complete xylose recovery.

Though excess enzyme loading helped identify the most promising pretreatment (1.0% NaOH for 30 min) based on conversion efficiency and process functionality, it is important to optimize enzyme loading to improve economic feasibility of the process. Hence, enzymatic saccharification at various CTec2 and HTec2 loadings (Mixing ratio of CTec2 and HTec2 =

5:1 based on g $_{enzyme}$/g $_{pretreated\ biomass}$) equivalent to 5, 10, 15, 20, 40, and 70 FPU/g pretreated biomass (Figure 2.6) was evaluated. Overall, glucan and xylan conversion increased with increase in enzyme loading. Though glucan conversion ranged from 72.8 to 81.0% and xylan conversion ranged from 53.8 to 66.8%, the feasible enzyme dosage statistically showed no significant ($p > 0.05$) difference in enzyme digestibility compared to the excess enzymes dosage. Since there can be many factors that influence fermentable sugar production via enzymatic hydrolysis of NaOH pretreated switchgrass, a study focusing on factors related to lignin only and eliminating other factors is needed to investigate the impact of lignin chemistry changes on enzymatic digestibility (i.e. enzymatic hydrolysis with lignin isolated from NaOH pretreated switchgrass) and establish and correlations that may exist.

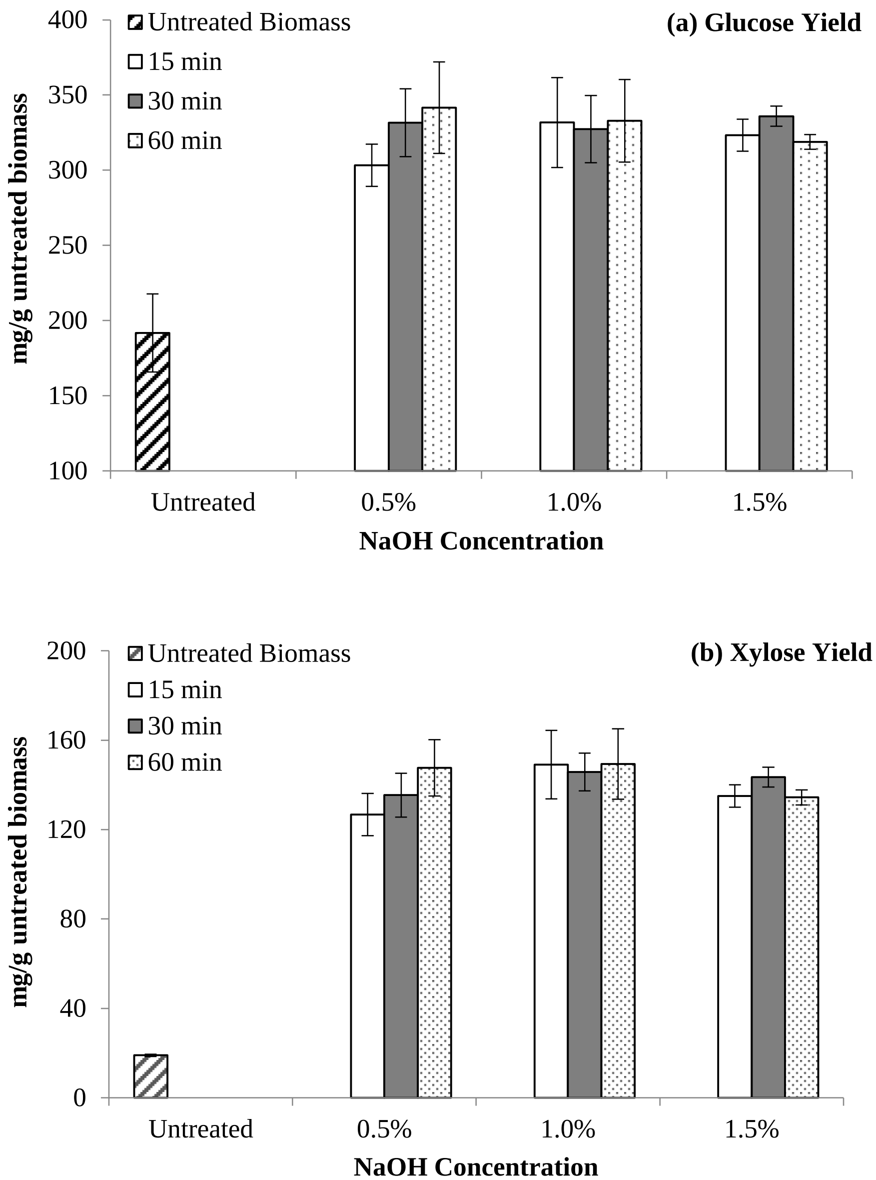


Figure 2.5. (a) Glucose and (b) xylose yield after enzymatic hydrolysis of NaOH pretreated switchgrass

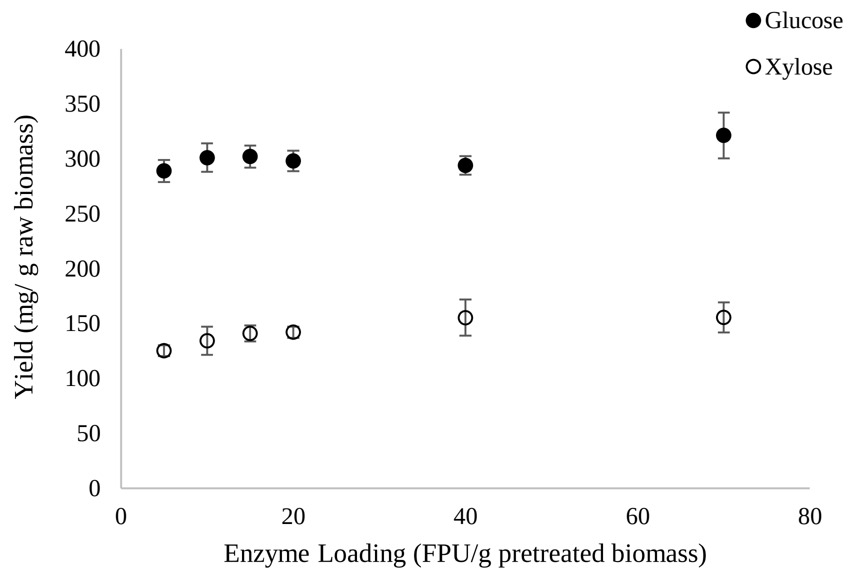


Figure 2.6. Glucose and xylose yield after enzymatic hydrolysis of switchgrass treated at 1.0% NaOH for 30 min with varying doses of CTec2 and HTec2.

4. CONCLUSION

Sodium hydroxide pretreatment of switchgrass at various conditions resulted in significant delignification and changes in lignin chemistry, especially decrease in H/G ratio in pretreated switchgrass. H monolignol appeared to be a critical factor in changing lignin chemistry of switchgrass due to its higher susceptibility to NaOH than S and G monolignol units. Although significant delignification by NaOH pretreatment was found to improve fermentable sugar production via enzymatic hydrolysis, it is important to understand what limitations exist even at excess enzyme loading. Further study employing lignin rich residue, isolated from NaOH pretreated switchgrass, may provide a better understanding of binding between lignin and cellulolytic enzymes during cellulose conversion via enzymatic hydrolysis.

REFERENCES

Adney, B. and Baker, J. (2008). Measurement of cellulase activities. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Barman DN, Haque MA, Kang TH, Kim GH, Kim TY, Kim MK, Yun HD (2014) Effect of mild alkali pretreatment on structural changes of reed (Phragmites communis Trinius) straw. Environ Technol 35:232-241. doi: 10.1080/09593330.2013.824009.

Belmokhtar N, Habrant A, Lopes Ferreira N, Chabbert B (2013) Changes in Phenolics Distribution After Chemical Pretreatment and Enzymatic Conversion of Miscanthus x giganteus Internode 6:506-518. doi: 10.1007/s12155-012-9275-2.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519-546. doi: 10.1146/annurev.arplant.54.031902.134938.

Fu C, Mielenz JR, Xiao X, Ge Y, Hamilton CY, Rodriguez M, Jr., Chen F, Foston M, Ragauskas A, Bouton J, Dixon RA, Wang Z (2011) Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. Proc Natl Acad Sci U S A 108:3803-3808. doi: 10.1073/pnas.1100310108.

Guo F, Shi W, Sun W, Li X, Wang F, Zhao J, Qu Y (2014) Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. Biotechnol Biofuels 7:38. doi: 10.1186/1754-6834-7-38.

Han M, Choi G, Kim Y, Koo B (2011) Bioethanol Production by Miscanthus as a Lignocellulosic Biomass: Focus on High Efficiency Conversion to Glucose and Ethanol. BioResources 6:1939-1953.

Haque MA, Barman DN, Kang TH, Kim MK, Kim J, Kim H, Yun HD (2013) Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of Miscanthus sinensis at boiling temperature with low residence time 114:294-305. doi: 10.1016/j.biosystemseng.2013.01.006.

Jensen K, Clark CD, Ellis P, English B, Menard J, Walsh M, Ugarte DdlT (2007) Farmer willingness to grow switchgrass for energy production. Biomass Bioenerg 31:773-781. doi: 10.1016/j.biombioe.2007.04.002.

Keshwani DR, Cheng JJ (2009) Switchgrass for bioethanol and other value-added applications: A review. Bioresour Technol 100:1515-1523. doi: 10.1016/j.biortech.2008.09.035.

Kishimoto T, Chiba W, Saito K, Fukushima K, Uraki Y, Ubukata M (2010) Influence of Syringyl to Guaiacyl Ratio on the Structure of Natural and Synthetic Lignins. J Agric Food Chem 58:895-901. doi: 10.1021/jf9035172.

Li H, Li C, Sang T, Xu J (2013) Pretreatment on Miscanthus lutarioriparious by liquid hot water for efficient ethanol production 6:76. doi: 10.1186/1754-6834-6-76.

Liu Z, Padmanabhan S, Cheng K, Xie H, Gokhale A, Afzal W, Na H, Pauly M, Bell AT, Prausnitz JM (2014) Two-Step Delignification of Miscanthus To Enhance Enzymatic Hydrolysis: Aqueous Ammonia Followed by Sodium Hydroxide and Oxidants. Energy Fuels 28:542-548. doi: 10.1021/ef401317z.

Liu Z, Padmanabhan S, Cheng K, Schwyter P, Pauly M, Bell AT, Prausnitz JM (2013) Aqueous-ammonia delignification of miscanthus followed by enzymatic hydrolysis to sugars. Bioresour Technol 135:23-29. doi: 10.1016/j.biortech.2012.10.133.

Ohgren K, Bura R, Saddler J, Zacchi G (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour Technol 98:2503-2510. doi: 10.1016/j.biortech.2006.09.003.

Ohra-aho T, Gomes FJB, Colodette JL, Tamminen T (2013) S/G ratio and lignin structure among Eucalyptus hybrids determined by Py-GC/MS and nitrobenzene oxidation. J Anal Appl Pyrolysis 101:166-171. doi: 10.1016/j.jaap.2013.01.015.

Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids 3:29-60.

Russell WR, Burkitt MJ, Scobbie L, Chesson A (2006) EPR investigation into the effects of substrate structure on peroxidase-catalyzed phenylpropanold oxidation 7:268-273. doi: 10.1021/bm050636o.

Santos RB, Hart PW, Jameel H, Chang H (2013) Wood Based Lignin Reactions Important to the Biorefinery and Pulp and Paper Industries. BioResources 8:1456-1477.

Schultz T, Templeton M (1986) Proposed Mechanism for the Nitrobenzene Oxidation of Lignin. Holzforschung 40:93-97. doi: 10.1515/hfsg.1986.40.2.93.

Sharma R, Palled V, Sharma-Shivappa RR, Osborne J (2013) Potential of Potassium Hydroxide Pretreatment of Switchgrass for Fermentable Sugar Production. Appl Biochem Biotechnol 169:761-772. doi: 10.1007/s12010-012-0009-x.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). Determination of ash in biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008a). Determination of Extractives in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Hames, B., Hyman. D., Payen, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Wolfe, J. (2008b). Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008c). Determination of Structural Carbohydrates and Lignin in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO

Sun R, Song X, Sun R, Jiang J (2011) Effect of Lignin Content on Enzymatic Hydrolysis of Furfural Residues. BioResources 6:317-328.

Umezawa T (2010) The cinnamate/monolignol pathway. Phytochem Rev 9:1-17. doi: 10.1007/s11101-009-9155-3.

Wang Z, Xu J, Pandey P, Cheng JJ, Li R, Qu R (2012) Improvement of Sugar Production from Transgenic Switchgrass with Low-Temperature Alkali Pretreatment. Energy Fuels 26:3054-3061. doi: 10.1021/ef3004575.

Xu J, Chen Y, Cheng JJ, Sharma-Shivappa RR, Burns JC (2011) Delignification of Switchgrass Cultivars for Bioethanol Production. BioResources 6:707-720.

Yamamura M, Noda S, Hattori T, Shino A, Kikuchi J, Takabe K, Tagane S, Gau M, Uwatoko N, Mii M, Suzuki S, Shibata D, Umezawa T (2013) Characterization of lignocellulose of Erianthus arundinaceus in relation to enzymatic saccharification efficiency 30:25-35. doi: 10.5511/plantbiotechnology.12.1127a.

Yoshida M, Liu Y, Uchida S, Kawarada K, Ukagami Y, Ichinose H, Kaneko S, Fukuda K (2008) Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of Miscanthus sinensis to monosaccharides. Biosci Biotechnol Biochem 72:805-810. doi: 10.1271/bbb.70689.

Ziebell A, Gracom K, Katahira R, Chen F, Pu Y, Ragauskas A, Dixon RA, Davis M (2010) Increase in 4-Coumaryl Alcohol Units during Lignification in Alfalfa (Medicago sativa) Alters the Extractability and Molecular Weight of Lignin. J Biol Chem 285:38961-38968. doi: 10.1074/jbc.M110.137315.

CHAPTER 3

EFFECT OF DELIGNIFICATION VIA SODIUM HYDROXIDE PRETREATMENT ON LIGNIN CHEMISTRY OF *Miscanthus* x *gigantues*

ABSTRACT

Miscanthus x *gigantues* was pretreated in an autoclave with various sodium hydroxide (NaOH) concentrations (0.5, 1.0, and 2.0%, w/v) and pretreatment time (15, 30, and 60 min) combinations. The influence of delignification, caused by NaOH, on lignin chemistry of *Miscanthus* x *gigantues* was investigated. NaOH had a significant effect on lignin removal with delignification ranging from 42.3 to 84.6% depending on severity of pretreatment. NaOH can comprehensively disrupt cross-ester-linkages between lignin and hemicellulose leading to release lignin from biomass solid during pretreatment. Partial hemicellulose loss (4.6 to 31.3%) was observed at more severe NaOH pretreatments. Nitrobenzene oxidation (NBO) was used to investigate changes in lignin chemistry of pretreated *M. gigantues* by analyzing syringyl to guaiacyl ratio (S/G ratio) and *p*-hydroxyphenyl to guaiacyl ratio (H/G ratio). S/G ratio of pretreated miscanthus increased from $0.64_{\text{unpretreated sample}}$ to 0.77 and H/G ratio ($=0.48_{\text{unpretreated sample}}$) decreased to 0.28 at 0.5% NaOH pretreatment. As a result of pretreatment with 1.0 and 1.5% (w/v) NaOH, S/G ratio did not undergo significant changes while H/G ratio decreased up to 0.13 regardless of pretreatment times. H unit of lignin was found to be the most susceptible to NaOH pretreatment. A non-methoxylated monomer, H unit monolignol can be easily degraded by NaOH as compared to S and G unit monolignols because it forms smaller polymers during lignification due to instability of reactive free radicals on aryl side. NaOH

concentration was a critical factor in changing lignin chemistry of pretreated switchgrass but this change did not seem to impact fermentable sugar production. A better understanding of the correlation between pretreatment severity, lignin chemistry change and enzymatic hydrolysis efficiency subsequent to pretreatment of herbaceous grasses warrants further investigations.

Keywords: lignin chemistry, syringyl, guaiacyl, *p*-hydroxyphenyl, lignin monomer ratio, nitrobenzene oxidation

1. INTRODUCTION

Lignocelluloses such as agricultural and forestry residues, and perennial grasses are high potential bio-resources suitable for conversion to biofuels like bioethanol. They can provide alternative energy to reduce our energy dependence on fossil fuels while being abundant, domestic, and sustainable in the United States (Wyman 2007). *Miscanthus*, a C4 perennial grass, has been identified as one of the significant lignocellulosic energy crops because it contains high structural carbohydrates and rapidly grows over 3 m tall with relatively less water and nitrogen inputs (Carroll and Somerville 2009; Heaton et al. 2008; Somerville et al. 2010).

Generally, lignocellulosic biomass is composed of three main structural components: cellulose, hemicellulose and lignin. Cellulose and hemicellulose are structural carbohydrates suitable for conversion to bioethanol while lignin is a complex phenol distributed within cell wall polysaccharides. Lignin mainly consists of three phenolic compounds: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) propanol units. These units are cross-linked by a variety of bonds such as ether bonds (e.g. β-O-4 and 4-O-5) and carbon-carbon bonds (e.g. β-β and 5-5) among intermonomers (Kishimoto et al. 2010; Ohra-aho et al. 2013). In grasses, lignin is made of G, S, and H units whereas hardwood lignin is comprised of G and S units, and softwood lignin mainly contains G units. The β-O-4 linkages which are predominant in grass lignin result in a non-condensed structure (Liu et al. 2013) .

Although lignin is key for the structural stability of plants, its presence in lignocellulsoic biomass obstructs conversion of plant polysaccharides into fermentable monomeric sugar. Carboxyl functional groups of *p*-hydroxycinnamic acid units (e.g. ferulic acid and *p*-coumaric acid) from lignin link through ester bonds with hydroxyl functional groups from hemicellulose. (Boerjan et al. 2003; Yamamura et al. 2013) and prevent hydrolytic enzymes from accessing cell wall polysaccharides thereby inhibiting sugar production. Thus, in order to efficiently generate fermentable sugars like glucose and xylose from lignocelluloses, it is necessary to breakdown recalcitrant lignin using a pretreatment step.

Alkali pretreatment, especially with sodium hydroxide (NaOH), has a significant effect on lignin removal from lignocellulosic biomass leading to increased accessibility of structural carbohydrates by cellulolytic enzymes and finally enhanced enzyme saccharfication efficiency (Wang et al. 2012b). Xu et al. (2011b) reported a maximum delignification of 73.6% in ‘Performer’ switchgrass after pretreatment with 3% NaOH at 121 ^{o}C for 90 min. The results showed that increase in intensity of NaOH pretreatment leads to higher lignin reduction but can also cause greater loss of total biomass. Haque et al. (2013) studied NaOH pretreatment of *Miscanthus sinensis* containing 42.2% cellulose, 28.5 % hemicellose and 18.4 % lignin with various NaOH concentrations (0.75 to 2.5%, w/v) at 105 ^{o}C for 10 min. Higher NaOH concentration led to lower hemicellulose (24.5% to 11.0%) as well as lignin (13.8% to 4.2%) content.

Although many researchers have investigated lignin degradation via NaOH pretreatment process (Haque et al. 2013; Xu et al. 2011b), the mechanism of how lignin chemistry in herbaceous lignocellulosic biomass changes during NaOH pretreatment and how the change impacts subsequent sugar production is little known. Hence, this study was undertaken to evaluate (1) the effect of NaOH pretreatment on lignin reduction in *M. gigantues* at varying chemical concentrations and pretreatment times, (2) how NaOH pretreatment influences lignin chemistry by changing S, G, H and their ratios, and (3) the effect of changes in lignin chemistry of pretreated *M. giganteus* on hydrolytic sugar production.

2. MATERIAL & METHODS

2.1 Sample preparation

Miscanthus x *giganteus* was harvested from Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina in December 2011. It was field-chopped and dried at 45^{o}C for 5 days prior to being delivered to the Bioproducts Development Lab in the Department of Biological & Agricultural Engineering at NCSU. Size reduction of the material was achieved by grinding and passing through 2 mm sieve in a Thomas Wiley Laboratory Mill (Model No. 4, Philadelphia, PA, USA). Extractives, (e.g. waxes and chlorophyll) that can potentially interfere with lignin analysis, were removed from the feedstock by soxhlet extraction in cellulose thimbles with acetone reflux for 24 h described previously (Sluiter et al., 2008a). The extractive-free sample was dried at 40^{o}C in a vacuum oven for 24 h and stored in sealed plastic bags at room temperature.

2.2 Sodium hydroxide pretreatment

The impact of NaOH pretreatment on change in lignin chemistry was investigated for pretreatments at three NaOH concentrations (0.5, 1.0 and 1.5%, w/v) and three pretreatment times (15, 30, and 60 min) in a full factorial experiment. Five grams of biomass and 50 ml NaOH solution were mixed in 125 ml glass serum bottles and crimp sealed for pretreatment at

121 ^{o}C / 15 psi in the autoclave (Model 3021, Amsco, Mentor, OH, USA) for the desired pretreatment time. Pretreated biomass (solid) was recovered by filtration through a polypropylene Buchner funnel and vacuum filter. The pretreated solid was washed with 500 ml of deionized (DI) water (100 ml DI water/g untreated biomass) to remove residual NaOH and neutralize the biomass. Wet pretreated biomass was sealed in a pre-weighed plastic bag and stored at 4 ^{o}C for subsequent enzymatic hydrolysis. About 1 g of the wet pretreated biomass was used to estimate solid recovery by drying at 105 ^{o}C for 24 h (or until constant weight was reached). Another aliquot of the wet pretreated biomass was dried in a 40 ^{o}C vacuum oven to determine composition of pretreated biomass and perform nitrobenzene oxidation (NBO) for lignin chemistry analysis of untreated and pretreated biomass.

2.3 Enzymatic Hydrolysis

Alkali pretreated biomass at 8% solid loading (w/v) in 10 ml volume was hydrolyzed with Cellic® CTec2 (blend of aggressive cellulase, high level of β-glucosidases and hemicellulase) and HTec2 (endoxylanse with cellulase background) provided by Novozymes North America, Inc. (Franklinton, NC, USA). The densities of CTec2 and HTec2 were measured to be 1.21 and 1.16 g/ml, respectively. The cellulase activity of CTec2 (= 110 FPU/ml) was determined on the basis of the National Renewable Energy Laboratory (NREL)'s Laboratory Analytical Procedures (LAP) (Adney and Baker, 2008). Excessive CTec2 dose equivalent to 70 FPU/g of dry biomass (0.625 g of enzyme/g of dry biomass) and HTec2 (0.125 g of enzyme/g of dry biomass) were used during hydrolysis to prevent any enzyme limitation and maximize fermentable sugar production. Tetracycline (40 μg/ml) was added during hydrolysis to prevent microbial contamination, and volume was made up with 0.05 M sodium citrate buffer (pH 4.8) in 50 ml centrifuge tubes. Hydrolysis was performed in a water bath shaker (Model: 89032-226, VWR International, PA) at 50 ^{o}C, 150 rpm for 72 hours. The supernatant obtained by centrifugation (model 5810R, Eppendorf, Hauppauge, NY) of the hydrolysate at 4000 rpm and 4^{o}C for 10 min was filtered through a 0.2 μm syringe filter. Filtered supernatants were stored at -80 ^{o}C for subsequent monomeric sugar (glucose, xylose, arabinose, and galactose) analysis. Conversion of structural carbohydrates to fermentable monomeric sugars via enzymatic hydrolysis was calculated by Equation 1. Untreated biomass with no enzyme addition and enzymes without biomass were also hydrolyzed as controls.

$$Polymeric\ sugar\ conversion\ (\%) = \frac{Monomeric\ sugar\ in\ hydrolysate\ (\frac{g\ sugar\ release}{g\ untreated\ biomass})}{Monomeric\ sugar\ in\ untreated\ biomass\ (\frac{g\ suagr}{g\ untreated\ biomass})} \times 100 \qquad (1)$$

2.4 Composition analysis for untreated/pretreated biomass

Chemical composition of untreated and pretreated *M. giganteus* including total solids, ash (for untreated only) was analyzed based on National Renewable Energy Laboratory (NREL)'s Laboratory Analytical Procedures (LAP) (Sluiter et al., 2005 and 2008b). Structural carbohydrate (i.e. glucan, xylan, arabinan and galatan) and lignin content including acid insoluble (AIL) and acid soluble (ASL) lignin was analyzed by a two-stage sulfuric acid hydrolysis (Sluiter et al., 2008c). Monomeric sugars from untreated and pretreated biomass and enzyme hydrolysate were measured by ion-exchange chromatography (IC) (Dionex ICS-5000, Dionex Corporation, Sunnyvale, CA, USA). The amounts of polymeric sugars were estimated by dividing the amount of monomeric sugars by 1.1.

2.5 Nitrobenzene oxidation method for lignin chemistry analysis

Modified nitrobenzene oxidation (NBO) was used to determine S/G and H/G ratio of untreated and pretreated *M. giganteus* (Kajita et al. 1996; Lima et al. 2008). Extractive-free dried biomass (0.2 g) ground to pass a 40 mesh screen was mixed with 0.5 ml of nitrobenzene and 7 ml of 2N NaOH (aqueous phase) in a stainless steel reaction tube (6.5 cm diameter and 26 cm length). The tube was placed in an oil bath at 170 ^{o}C for 2.5 h after which it was drawn from the oil bath with tongs and cooled immediately in ice water in a polypropylene tray. Subsequent processing of the mixture was performed in a fume hood. The mixture was transferred to a glass separation funnel and extracted 3 times with 25 ml each of methylene chloride (CH_2Cl_2). After the first extraction, there were two phases in the separation funnel. The bottom layer (organic phase) had methylene chloride with nitrobenzene and the upper layer (water phase) had the NaOH solution with the target components like aromatic aldehyde/acid (i.e. syringaldehyde/syringic acid and vanillin/vanillic acid). The bottom layer was discarded into a bottle for waste disposal and the upper layer was transferred to a 50 ml volumetric flask. 2N hydrochloric acid (HCl, aqueous phase) was added to the water phase in the flask until pH was between 2 and 3. The acidified solution was transferred to the separation funnel again and extracted with methylene chloride for a second extraction. After the second extraction, the organic phase (bottom layer) had the lignin compounds and wass collected in a 50 ml volumetric flask. Sodium sulfate (Na_2SO_4, anhydrous) was added to the flask to capture and dry any remaining water in the organic phase. Volume of the organic phase was adjusted to 100 ml with methylene chloride. The solution was filtered into 1.5 ml vials through a 0.2 μm syring filter and 20 μl of the solution injected into a high performance liquid chromatography (HPLC) for analysis of lignin chemistry. A Shimadzu HPLC (LC-20AT, Japan) equipped with a ZORBAX SB-C3 reverse phase analytical column at room temperature (nominally 23 ^{o}C) was used to detect the lignin monomers using a UV detector (SPD-20A, Japan). Flow rate of the mobile phase, which was mixture of acetonitrile (CH_3CN) and water containing 10 mM formic acid (HCOOH), was 1.5 ml/min.

Lignin degradation compounds including three aldehyde-lignin units (syringaldehyde (Sy), vanillin (V), 4-hydroxybenzaldehyde (Hy)) and two acid-lignin units (SA, VA) were detected from pretreated samples. Amount (μmol/g lignin) of S-, G-, and H-lignin was calculated based on the total amount of aldehyde and acid-lignin units (i.e. S (μmol/g lignin) =Sy + SA, G (μmol/g lignin) = V + VA, H (μmol/g lignin) = Hy + 4-hydroxybenozic acid (not detected)). Molar ratio (μmol/ μmol) of S/G and H/G ratio was calculated by equations 2 and 3 below.

$$S/G\ molar\ ratio = \frac{Syringaldehyde\ (Sy) + Syringic\ Acid\ (SA), (\mu\text{mol/g lignin})}{Vanillin\ (V) + Vanillin\ Acid\ (VA), (\mu\text{mol/g lignin})} \quad (2)$$

$$H/G\ molar\ ratio = \frac{p-Hydroxybenzaldehyde\ (Hy), (\mu\text{mol/g lignin})}{Vanillin\ (V) + Vanillin\ Acid\ (VA), (\mu\text{mol/g lignin})} \quad (3)$$

Total yield of phenolic compounds produced from lignin (ie. lignin converted to degraded aromatic compounds during NBO) in miscanthus samples was estimated by equation (4) below.

$$Yield\ (\%) = \frac{phenolic\ compounds\ (Sy, SA, V, VA, and\ Hy)\ released\ during\ NBO\ (g)}{lignin\ (g)} \times 100 \quad (4)$$

2.6 Statistical analysis

Experimental data were statistically analyzed by the generalized linear model (GLM) procedure in SAS 9.3 (Cary, NC, USA) with Tukey adjustment at 95% confidence level. All treatments were conducted in triplicate. Main effects were NaOH concentrations (0.5, 1.0, and 1.5%) and pretreatment times (15, 30, and 60 min) and response variables were monomeric sugar production (i.e. glucose, xylose, arabinose, galactose) and lignin attributes (i.e. Sy, SA, V, VA, Hy, S/G and H/G).

3. RESULTS & DISCUSSION

3.1 Characterization of *M. gigantues*

Composition analysis for raw miscanthus was performed for comparison with NaOH pretreated samples (Table 3.1). Glucan and xylan were estimated as 41.3 and 21.4 % (dry basis), respectively, and constituted 96% of the total carbohydrate content. Lignin including acid insoluble lignin (AIL) and acid soluble lignin (ASL) was 24.4% and the ratio of ASL to AIL was 0.1, while the extractives were 1.5%.

Table 3.1. Composition of *Miscanthus* x *gigantues*

Material	*Miscanthus* gigantues (% dry basis)
Glucan	41.3 ± 1.00
Xylan	21.4 ± 0.56
Arabinan	2.0 ± 0.05
Galactan	0.5 ± 0.03
Lignin	
Acid Soluble Lignin (ASL)	2.2 ± 0.02
Acid Insoluble Lignin (AIL)	22.2 ± 0.13
Extractives	1.5 ± 0.03
Ash	2.2 ± 0.06

3.2 Impact of sodium hydroxide pretreatment on cell wall polysaccharides and lignin content of *M. gigantues*

Total structural carbohydrates including glucan, xylan, arabinan, and galactan in raw miscanthus were 65.2%. Glucan did not undergo any significant loss ($p > 0.05$) and remained nearly unchanged even with the most severe NaOH pretreatment (1.5% NaOH concentration at 121 ^{o}C for 60 min), but xylan degradation increased up to 31.3% with increase in pretreatment intensity (Figure 3.1). Pretreatment time also impacted xylan degradation with 60 min NaOH pretreatment causing significantly ($p < 0.05$) higher loss than the 15 min NaOH pretreatment. Overall hemicellulose degradation including loss of xylan, arabinan, and galactan varied from 4.6 to 32.5% (Table 3.2) with pretreatments at 1.0 and 1.5% resulting in much higher losses.

Changes in lignin content and ASL/AIL ratio were investigated after NaOH pretreatments. AIL in untreated miscanthus reduced significantly ($p < 0.05$) from 22.2% to 5.4

– 15.4% under various alkali pretreatment conditions (Figure 3.2) and at higher NaOH concentration more AIL was solubilized into the prehydrolyzate. Pretreatment times of 30 and 60 min caused higher AIL reduction than 15 min with 1.0 and 1.5% NaOH. Amount of NaOH loading during pretreatment seemed to be more critical factor to reduce AIL content in miscanthus than pretreatment times. ASL content which varied from 1.27% (at 0.5% NaOH with 15 min) to 0.85% (at 1.5% NaOH with 60 min) in pretreated miscanthus showed reduction trends similar to AIL (data not shown). In general, increase in severity of NaOH pretreatment increased ASL/AIL ratio with values ranging between 0.07 and 0.17

Based on total lignin (AIL+ASL) reduction, delignification ranged from 42.3 to 84.6% depending on severity of NaOH pretreatment (Table 3.2). Delignification during NaOH pretreatment is reported to be caused by comprehensive disruption of the cross-ester-linkages between lignin and structural carbohydrates, especially hemicelluloses such as arabinoxylan. The main aromatic compounds in lignin that form linkages with hemicellulose are ferulic acid and *p*-coumaric acid which make ester bonds with carboxyl functional groups of these *p*-hydroxycinnamic acids and hydroxyl functional groups from arabinoxylan (Belmokhtar et al. 2013; Haque et al. 2013; Yamamura et al. 2013). More ester-linkages can be broken when high severity NaOH pretreatments are applied to lignocellulosic biomass by increasing alkali concentration (g NaOH/g biomass) or pretreatment times.

Table 3.2. Lignin and hemicellulose degradation after NaOH pretreatment at varying conditions and carbohydrate conversions with enzymatic hydrolysis

	Time (min)	NaOH Concentration (%)		
		0.5	1.0	1.5
Pretreatment				
Delignification (%)				
	15	42.3 ± 2.7	66.4 ± 0.7	76.0 ± 0.5
	30	47.0 ± 1.5	76.6 ± 0.8	82.2 ± 1.8
	60	44.6 ± 1.4	80.1 ± 1.1	84.6 ± 2.6
Hemicellulose degradation[1)] (%)				
	15	4.6 ± 3.8	12.1 ± 4.8	24.8 ± 8.5
	30	5.8 ± 3.2	20.8 ± 5.7	29.8 ± 9.8
	60	8.6 ± 6.3	27.3 ± 7.0	32.5 ± 6.6
Enzymatic Hydrolysis				
Glucan Conversion (%)	15	68.1 ± 3.1	73.8 ± 1.1	80.8 ± 5.4
(53.9 % in untreated biomass)	30	73.3 ± 5.3	79.8 ± 5.8	76.2 ± 3.7
	60	70.7 ± 3.7	69.1 ± 1.0	71.9 ± 4.3
Xylan Conversion (%)	15	50.5 ± 3.9	60.9 ± 1.1	66.1 ± 6.0
(8.61 % in untreated biomass)	30	58.3 ± 3.8	69.3 ± 7.3	62.3 ± 3.6
	60	55.0 ± 4.8	57.6 ± 1.3	59.0 ± 5.1

[1)] = summation loss of xylan, arabinan, and galactan

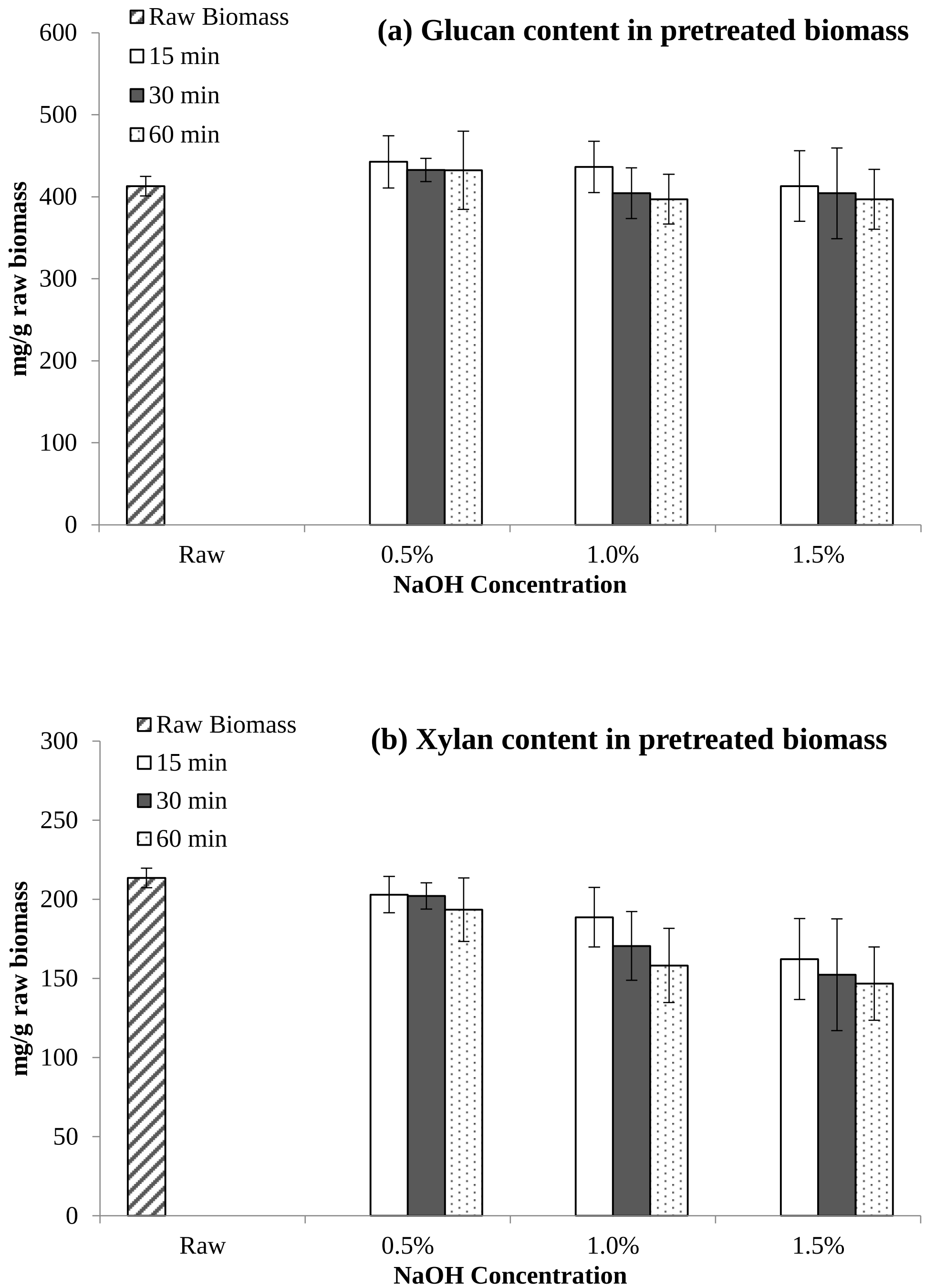


Figure 3.1. (a) Glucan and (b) xylan content in pretreated *M. gigantues* calculated per gram of untreated biomass

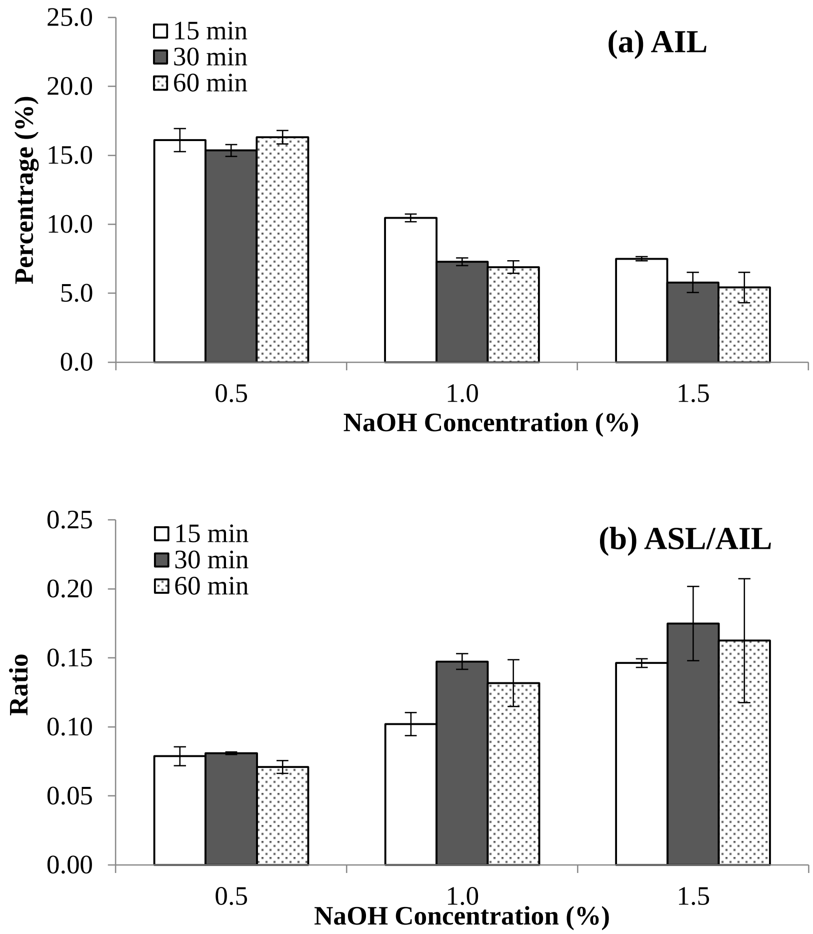


Figure 3.2. Content of (a) acid insoluble lignin content of pretreated miscanthus and changes in (b) ratio of ASL to AIL after NaOH pretreatment at various conditions

3.3 Effect of sodium hydroxide on lignin chemistry changes based on S/G and H/G ratio

Nitrobenzene oxidation (NBO) was used to investigate changes in lignin chemistry of alkali pretreated miscanthus. The main phenolic compounds making up lignin, guaiacyl (G), syringyl (S), and *p*-phydroxyphenyl (H) propanol, can be degraded into aldehyde groups like vanillin (V), syringaldehyde (Sy), and 4-hydroxybenzaldehyde (Hy) by NBO. Further oxidation by the NBO process converts aldehyde groups into acid groups like vanillic acid (VA), syringic acid (SA), and 4-hydroxybenozoic acid (HA) (SCHULTZ and TEMPLETON 1986). Amounts (μmol/g lignin) of these aldehyde and acid groups produced from lignin in untreated and NaOH pretreated samples were quantified by HPLC and corresponding total yields were calculated (Table 3.3).

It was observed that untreated miscanthus had relatively higher H-lignin content (S:G:H = 31:48:21). Amounts of Hy released from the pretreated biomass were significantly ($p < 0.05$) reduced when NaOH concentrations increased from 0.5 to 1.5% for all pretreatment times, indicating that it was potentially more susceptible to NaOH pretreatment. Conversely, overall amounts of SA and VA increased ($p < 0.05$) when NaOH concentration was up from 0.5 to 1.5%.

Total yield of phenolic compounds produced from lignin in untreated sample was 28.8%, while that from pretreated samples varied from 21.2 to 31.3% and only samples pretreated for 60 min had lower yields than untreated samples. Unless total yields are consistent, a quantitative comparison of change in lignin chemistry relative to monomeric lignin units can be challenging to estimate and prone to error. Though every effort was made to keep physical conditions of temperature and time consistent during NBO, variations in yields were observed. Hence, molar S/G and H/G ratios (μmol/ μmol) were also used to study the effect of NaOH pretreatment on lignin chemistry changes (Figure 3.3.). The S/G and H/G ratios for untreated miscanthus were 0.64 and 0.48, respectively. As a result of 15 min pretreatment with 0.5% NaOH, S/G ratio increased to 0.75 and H/G ratio decreased to 0.28. As NaOH concentration increased, there was a significant ($p < 0.05$) drop in H/G ratio to 0.13 with 1.5% NaOH pretreatment. Though S/G ratio increased when NaOH was applied, change in NaOH concentration had no significant ($p > 0.05$) impact irrespective of pretreatments. The trends for change in S/G and H/G ratio were similar for 30 and 60 min pretreatments. Overall, S/G and H/G ratios in pretreated samples ranged between 0.67 – 0.77 and 0.13 – 0.37, respectively. As a reference, maximum delignification for 0.5, 1.0 and 1.5% NaOH pretreatments was 47.0, 80.1, and 84.6%, respectively.

Alkaline hydrolysis with NaOH or ammonia is reported to have a significant effect on cleavage of β-O-4 linked aryl ether bonds among lignin units thus resulting in delignification and potentially changing the proportion of monolignols in the residual lignin (Liu et al. 2013; Liu et al. 2014; Santos et al. 2013). H lignin unit is a non-methoxylated phenolic compound that has more reactive sites to link with other lignin units via radical polymerization compared to G and S lignin units which have one and two methoxy functional groups, respectively (Ziebell et al. 2010). Russell, et al. (1996) found that the unpaired electron density in non-methoxylated phenolic compounds is greater on the carbon nuclei than the phenolic oxygen. H rich lignin has reduced β-O-4 linkages and higher C-C linkages as observed via two-dimensional NMR (Pu et al. 2009; Russell et al. 1996). Based on the reaction characteristic of H units, this might result in a condensed lignin structure (e.g. C-C linkages) which is harder to cleave than non-condensed lignin (e.g. β-O-4 linkages). However, Russell, et al. (2006) suggested that non-methoxylated phenolic compounds like H units make free radicals unstable during polymerization process and leading to production of smaller polymers. Ziebell, et al. (2010) also found that H rich lignin from transgenic alfalfa had relatively low molecular weight than control alfalfa samples having lignin with predominantly S and G units. To sum up, H rich lignin can have more condensed linkages but may be formulated with smaller polymers due to instability of free radicals for lignification via oxidative coupling. This could make them more susceptible to degradation during pretreatment of biomass

In this study, delignification increased with increase in NaOH concentration and a corresponding decrease in H/G ratios from extractable lignin portion via NBO was observed in pretreated miscanthus. It may be inferred that NaOH can solubilize the lignin fraction containing H units relatively easily because this fraction has smaller polymers than the lignin fractions with predominantly S and G units. In perspective, genetic manipulation of miscanthus so that its lignin is rich in H units may help to reduce the intensity of alkali pretreatment and ultimately contribute to reduced process cost for fermentable sugar production.

Table 3.3. Phenolic compounds production of nitrobenzene oxidation (μmol/g lignin) and total yield (%) by nitrobenzene oxidation

Time, min	Conc., %	Phenolic compounds produced by lignin oxidation, μmol/g lignin					Total yield [f], %
		Sy [a]	SA [b]	V [c]	VA [d]	Hy [e]	
Untreated Sample		424.6±31.9	115.6±6.5	785.5±49.4	59.0±7.4	375.4±6.1	28.8 ± 2.5
15	0.5	446.7±20.6	138.8±4.9	729.5±78.3	74.6±1.8	202.2±22.4	24.2 ± 3.8
	1.0	401.8±57.0	162.6±4.4	652.5±84.8	100.3±3.7	134.1±49.6	24.6 ± 2.4
	1.5	479.0±3.3	203±0.7	812.7±12.8	123.2±9.0	118.2±14.8	28.0 ± 3.0
30	0.5	502.7±63.6	165.3±29.5	817.9±31.0	90.6±13.4	336.0±30.6	31.3 ± 2.2
	1.0	561.2±50.1	241.8±14.5	964.7±76.3	145.5±3.0	267.8±34.0	28.2 ± 2.5
	1.5	627.7±56.8	259.4±18.8	1,144.6±108.5	165.6±5.1	187.5±15.4	30.0 ± 0.8
60	0.5	338.6±17.4	133.5±8.1	536.8±38.3	74.8±8.9	183.2±18.6	21.2 ± 0.7
	1.0	346.5±52.8	213.6±18.9	612.0±33.1	135.3±5.7	114.8±24.5	25.2 ± 1.3
	1.5	373.1±36.4	256.9±6.9	654.5±82.0	164.8±9.1	104.5±22.3	27.7 ± 2.4

[a] Sy=Syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde)
[b] SA=Syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid)
[c] V=Vanillin (4-hydroxy-3-methoxybenzaldehyde)
[d] VA=Vanillic acid (4-hydroxy-3-methoxybenzoic acid)
[e] Hy=*p*-Hydroxybenzaldehyde (4-hydroxybenzaldehyde)
[f] Total yield (%) = sum of weight of total phenolic compounds produced by NBO (g) / weight of extractive-free dried lignin (g) × 100.

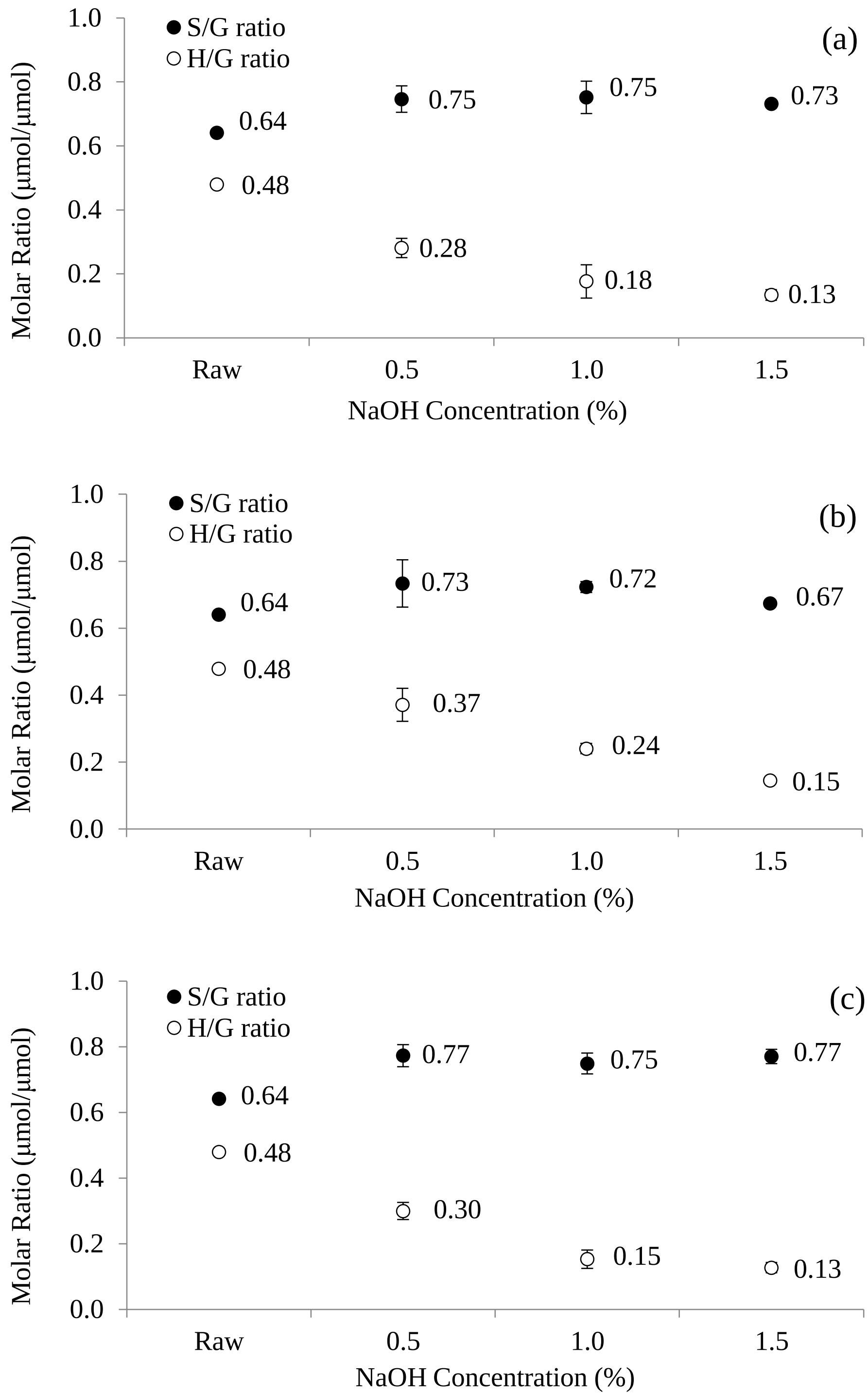


Figure 3.3. Changes in molar ratio (µmol / µmol) of S/G and H/G after NaOH pretreatment of *M. gigantues* at (a) 15 min, (b) 30 min, and (c) 60 min

3.4 Effect of sodium hydroxide on fermentable sugar production

NaOH pretreated samples were enzymatically hydrolyzed to produce monomeric sugars (Figure 3.4). Biomass treated with the least intense pretreatment (0.5% NaOH for 15 min) produced significantly ($p < 0.05$) higher glucose (> 309.06 mg $_{glucose}$/g $_{untreated\ biomass}$) and xylose ((> 118.65 mg $_{xylose}$/g $_{untreated\ biomiass}$) compared to unpretreated samples (224.2 mg $_{glucose}$/g $_{untreated\ bioimass}$ and 20.23 mg $_{xylose}$/g $_{untreated\ biomass}$). Maximum glucose and xylose were 366.80 mg $_{glucose}$/g $_{untreated\ biomass}$ from pretreatment with 1.5% NaOH for 15 min and 162.71 mg $_{xylose}$/g $_{untreated\ biomiass}$ from pretreatment with 1.0% NaOH for 30 min. The corresponding glucan and xylan conversions were 80.8% and 69.3%, respectively (Table 3.2). Compared to untreated samples, NaOH pretreatment improved efficiency of enzymatic saccharification up to 50% for glucan and 7 times for xylan.

In order to prevent enzyme limitation arising from enzyme-lignin binding and maximize sugar production from samples pretreated at various conditions, excess enzyme were used in this study. Though this aided in identifying the optimal pretreatment condition, especially time (30min), 100% glucan and xylan conversion was not achieved potentially due to 1) partial hemicellulose loss, 2) increased cellulose crystallinity (Barman et al. 2014), and 3) hemicellulose's shielding effect (Li et al. 2013; Ohgren et al. 2007; Sun et al. 2011; Yoshida et al. 2008). Since the suggested enzyme dose for economic feasibility of enzymatic hydrolysis is 5 to 15 FPU/g $_{dry\ biomass}$, subsequent hydrolysis was conducted with reduced dosage of CTec2 (0.089 g $_{enzyme}$ /g $_{dry\ biomass}$, equivalent to 10 FPU/g $_{dry\ biomass}$) and HTec2 (0.018 g $_{enzyme}$ /g $_{dry\ biomass}$) applied to biomass pretreated for 30 min (Figure 3.4). Compared to excessive enzyme loading, glucose produced with 10 FPU/g CTec2 (with HTec2) was 36 and 19% less for pretreatments with 0.5 and 1.0%, respectively, However, glucose yield from samples pretreated with 1.5% (w/v) NaOH pretreatment was statistically similar ($p > 0.05$) to that from samples hydrolyzed with excessive enzymes. Xylose yields obtained with the lower enzyme loading were also lower at 76.1, 101.1, and 104.9 $mg_{xylose}/g_{untreated\ biomass}$ for 0.5, 1.0, and 1.5% NaOH, respectively, in comparison to the yield of 136.9, 162.7, and 146.2 $mg_{xylose}/g_{untreated\ biomass}$ with excess loading. Lower enzyme doses generally showed less enzyme digestibility than excess enzyme dosage. Based on these observations, it can be inferred that presence of lignin and differences in its lignin chemistry (especially H/G ratio) have a binding effect on cellulolytic enzymes. This inhibits cellulolytic enzyme function thus producing less fermentable sugars at low enzyme loading. Since samples treated with higher NaOH concentration have lower lignin content the inhibitory effect and non-productive binding are reduced and enzymes can access cellulose and hemicellulose better.

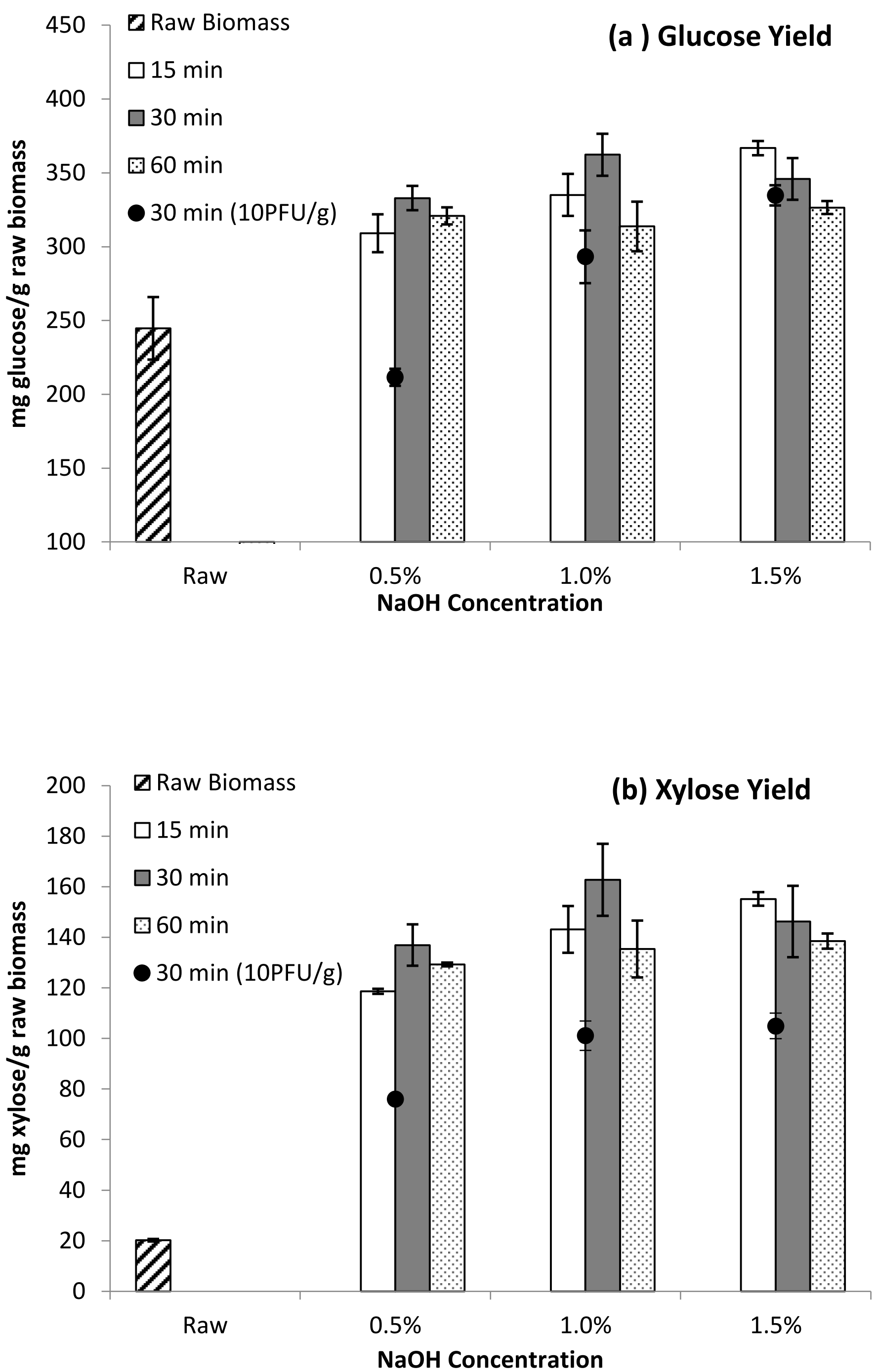


Figure 3.4. (a) Glucose yield and (b) xylose yield per gram of biomass after enzymatic hydrolysis of *M. gigantues* pretreated by NaOH at various conditions

4. CONCLUSION

Significant delignification by NaOH pretreatment can contribute to improved sugar production via enzymatic hydrolysis by removing or reducing lignin inhibition mechanisms. While results of this foundational study showed that H lignin units in miscanthus are more susceptible to alkali pretreatment, lignin isolation is needed to better understand how changes in lignin chemistry due to NaOH pretreatment influence subsequent enzymatic hydrolysis and lignin-enzyme binding. This information will help establish correlations between changes in lignin chemistry and fermentable sugar production from miscanthus thus allowing appropriate plant modifications and development of better conversion processes.

REFERENCES

Adney, B. and Baker, J. (2008). Measurement of cellulase activities. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Barman DN, Haque MA, Kang TH, Kim GH, Kim TY, Kim MK, Yun HD (2014) Effect of mild alkali pretreatment on structural changes of reed (Phragmites communis Trinius) straw. Environ Technol 35:232-241. doi: 10.1080/09593330.2013.824009.

Belmokhtar N, Habrant A, Lopes Ferreira N, Chabbert B (2013) Changes in Phenolics Distribution After Chemical Pretreatment and Enzymatic Conversion of Miscanthus x giganteus Internode 6:506-518. doi: 10.1007/s12155-012-9275-2.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519-546. doi: 10.1146/annurev.arplant.54.031902.134938.

Carroll A, Somerville C (2009) Cellulosic Biofuels. Annu Rev Plant Biol 60:165-182. doi: 10.1146/annurev.arplant.043008.092125.

Haque MA, Barman DN, Kang TH, Kim MK, Kim J, Kim H, Yun HD (2013) Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of Miscanthus sinensis at boiling temperature with low residence time 114:294-305. doi: 10.1016/j.biosystemseng.2013.01.006.

Heaton EA, Dohleman FG, Long SP (2008) Meeting US biofuel goals with less land: the potential of Miscanthus. Global Change Biol 14:2000-2014. doi: 10.1111/j.1365-2486.2008.01662.x.

Kajita S, Katayama Y, Omori S (1996) Alterations in the biosynthesis of lignin in transgenic plants with chimeric genes for 4-coumarate:coenzyme a ligase. Plant Cell Physiol 37:957-965.

Kishimoto T, Chiba W, Saito K, Fukushima K, Uraki Y, Ubukata M (2010) Influence of Syringyl to Guaiacyl Ratio on the Structure of Natural and Synthetic Lignins. J Agric Food Chem 58:895-901. doi: 10.1021/jf9035172.

Li H, Li C, Sang T, Xu J (2013) Pretreatment on Miscanthus lutarioriparious by liquid hot water for efficient ethanol production 6:76. doi: 10.1186/1754-6834-6-76.

Lima CF, Barbosa LCA, Marcelo CR, Silverio FO, Colodette JL (2008) Comparison between analytical pyrolysis and nitrobenzene oxidation for determination of syringyl/guaiacyl ratio in Eucalyptus spp. LIGNIN. BioResources 3:701-712.

Liu Z, Padmanabhan S, Cheng K, Xie H, Gokhale A, Afzal W, Na H, Pauly M, Bell AT, Prausnitz JM (2014) Two-Step Delignification of Miscanthus To Enhance Enzymatic Hydrolysis: Aqueous Ammonia Followed by Sodium Hydroxide and Oxidants. Energy Fuels 28:542-548. doi: 10.1021/ef401317z.

Liu Z, Padmanabhan S, Cheng K, Schwyter P, Pauly M, Bell AT, Prausnitz JM (2013) Aqueous-ammonia delignification of miscanthus followed by enzymatic hydrolysis to sugars. Bioresour Technol 135:23-29. doi: 10.1016/j.biortech.2012.10.133.

Ohgren K, Bura R, Saddler J, Zacchi G (2007) Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover. Bioresour Technol 98:2503-2510. doi: 10.1016/j.biortech.2006.09.003.

Ohra-aho T, Gomes FJB, Colodette JL, Tamminen T (2013) S/G ratio and lignin structure among Eucalyptus hybrids determined by Py-GC/MS and nitrobenzene oxidation. J Anal Appl Pyrolysis 101:166-171. doi: 10.1016/j.jaap.2013.01.015.

Pu Y, Chen F, Ziebell A, Davison BH, Ragauskas AJ (2009) NMR Characterization of C3H and HCT Down-Regulated Alfalfa Lignin 2:198-208. doi: 10.1007/s12155-009-9056-8.

Ralph J, Lundquist K, Brunow G, Lu F, Kim H, Schatz PF, Marita JM, Hatfield RD, Ralph SA, Christensen JH, Boerjan W (2004) Lignins: Natural polymers from oxidative coupling of 4-hydroxyphenylpropanoids 3:29-60.

Russell WR, Burkitt MJ, Scobbie L, Chesson A (2006) EPR investigation into the effects of substrate structure on peroxidase-catalyzed phenylpropanold oxidation 7:268-273. doi: 10.1021/bm050636o.

Russell WR, Forrester AR, Chesson A, Burkitt MJ (1996) Oxidative coupling during lignin polymerization is determined by unpaired electron delocalization within parent phenylpropanoid radicals. Arch Biochem Biophys 332:357-366. doi: 10.1006/abbi.1996.0353.

Santos RB, Hart PW, Jameel H, Chang H (2013) Wood Based Lignin Reactions Important to the Biorefinery and Pulp and Paper Industries. BioResources 8:1456-1477.

SCHULTZ T, TEMPLETON M (1986) Proposed Mechanism for the Nitrobenzene Oxidation of Lignin. Holzforschung 40:93-97. doi: 10.1515/hfsg.1986.40.2.93.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). Determination of ash in biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008a). Determination of Extractives in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Hames, B., Hyman. D., Payen, C., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Wolfe, J. (2008b). Determination of Total Solids in Biomass and Total Dissolved Solids in Liquid Process Samples. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Somerville C, Youngs H, Taylor C, Davis SC, Long SP (2010) Feedstocks for Lignocellulosic Biofuels. Science 329:790-792. doi: 10.1126/science.1189268.

Sun R, Song X, Sun R, Jiang J (2011) Effect of Lignin Content on Enzymatic Hydrolysis of Furfural Residues. BioResources 6:317-328.

Wang Z, Xu J, Pandey P, Cheng JJ, Li R, Qu R (2012) Improvement of Sugar Production from Transgenic Switchgrass with Low-Temperature Alkali Pretreatment. Energy Fuels 26:3054-3061. doi: 10.1021/ef3004575.

Wyman CE (2007) What is (and is not) vital to advancing cellulosic ethanol. Trends Biotechnol 25:153-157. doi: 10.1016/j.tibtech.2007.02.009.

Xu J, Chen Y, Cheng JJ, Sharma-Shivappa RR, Burns JC (2011) Delignification of Switchgrass Cultivars for Bioethanol Production. BioResources 6:707-720.

Yamamura M, Noda S, Hattori T, Shino A, Kikuchi J, Takabe K, Tagane S, Gau M, Uwatoko N, Mii M, Suzuki S, Shibata D, Umezawa T (2013) Characterization of lignocellulose of Erianthus arundinaceus in relation to enzymatic saccharification efficiency 30:25-35. doi: 10.5511/plantbiotechnology.12.1127a.

Yoshida M, Liu Y, Uchida S, Kawarada K, Ukagami Y, Ichinose H, Kaneko S, Fukuda K (2008) Effects of cellulose crystallinity, hemicellulose, and lignin on the enzymatic hydrolysis of Miscanthus sinensis to monosaccharides. Biosci Biotechnol Biochem 72:805-810. doi: 10.1271/bbb.70689.

Ziebell A, Gracom K, Katahira R, Chen F, Pu Y, Ragauskas A, Dixon RA, Davis M (2010) Increase in 4-Coumaryl Alcohol Units during Lignification in Alfalfa (Medicago sativa) Alters the Extractability and Molecular Weight of Lignin. J Biol Chem 285:38961-38968. doi: 10.1074/jbc.M110.137315.

CHAPTER 4

CELLULOLYTIC ENZYME BINDING ON LIGNIN ISOLATED FROM ALKALI AND ACID PRETEATED SWITCHGRASS

ABSTRACT

Binding of cellulolytic enzymes in Cellic® CTec2 onto 6 lignin isolates obtained from alkali (0.5, 1.0, and 1.5% NaOH at 121°C for 30 min) and acid (1, 2, and 3% H_2SO_4 at 121°C for 60 min) pretreated switchgrass was investigated. Hydrolysis of cellobiose and Avicel in suspension with and without (control) lignin isolates was conducted with CTec2 (5 and 10 FPU/g carbohydrate) to study whether presence of lignin and binding of cellulolytic enzymes to the isolated lignin can influence sugar production. Three carbohydrate-lignin loadings (0.5:0.25, 0.5:0.5, and 0.5:1.0 % (w/v)) were also used. Based on SDS-PAGE results, β-glucosidase (BG) was significantly bound to all lignin isolates. Some enzymes in CTec2 presumed to be cellobiohydrolases (CBH), endo-1,4-β-glucanases (EG), and xylanase, were also observed to partially bind to the lignin isolates. Identification of these enzymes could however not be accurately confirmed by SDS-PAGE due to their similar protein molecular weights. Up to 0.97 g glucose/g cellobiose was produced via hydrolysis with CTec2 (5 and 10 FPU/g carbohydrate). Similarly, up to 0.23 and 0.46 g glucose/g Avicel were produced via hydrolysis with 5 and 10 FPU/g carbohydrate, respectively. Results indicated that addition of lignin isolates during cellobiose and Avicel hydrolysis did not significantly ($p > 0.05$) reduce glucose production regardless of type and amount of lignin isolate. Hence, even though β-

glucosidase was significantly bound to lignin isolates, it could maintain their functionality as a biological catalyst in this study.

Keywords: lignin isolate, enzyme binding, cellulases, β-glucosidase, SEM, sodium hydroxide pretreatment, dilute sulfuric acid pretreatment.

1. INTRODUCTION

Lignin is an organic, non-carbohydrate, aromatic polymer whose main function is to provide physical and structural protection to cell wall polysaccharides (primarily cellulose and hemicellulose). Lignin is believed to hinder production of fermentable sugars during enzymatic hydrolysis of the polysaccharides in lignocelluloses. Its inhibitory action is mainly attributed to impediment of enzyme (cellulase) access to plant polysaccharides and non-productive enzyme adsorption on lignin instead of cellulose and hemicellulose (Berlin et al. 2006a; Kim 2012a; Rahikainen et al. 2011; Rahikainen et al. 2013a).

Interaction of cellulase and lignin is facilitated by two main forces; electrostatic and hydrophobic (Rahikainen et al. 2011; Rahikainen et al. 2013a). Electrostatic binding between cellulose and lignin due to opposing charges may be impacted by various factors. Rahikainen et al (2011; 2013a) investigated non-productive cellulase binding on lignin and the results indicated that less cellulase was bound to lignin at higher pH. They employed cellulases from *M. albomyces* (Cel45A, endoglucanase) and mutated Cel45A with carbohydrate binding modules (CBMs) whose isoelectric points (pI) ranged from 3.4 - 4.4. At higher pH, cellulase had negative charge and the phenolic carboxyl groups from lignin were also dissociated thus contributing to the negative charges on lignin. The repulsive interaction between negatively charged lignin and cellulase prevented enzyme adsorption. Hydrophobic interaction between cellulose and lignin also plays a significant role in non-productive cellulase adsorption on lignin. Rahikainen et al (2011; 2013a) showed that Cel45A without CBMs had less adsorption effect on lignin than the mutated Cel45A with CBMs. CBMs were genetically modified to contain aromatic amino acids with hydrophobic regions. Lignin also consists of aromatic polymers with hydrophobic character induced by cross-linked racemic phenolic molecules. Thus, CBMs from cellulase interact with the surface of lignin as well as crystalline cellulose through hydrophobic interaction. The intensified cellulase adsorption via electrostatic and hydrophobic interactions can result in irreversible denaturation of cellulase due to changes to their conformational structures eventually leading to reduced hydrolytic efficiency.

Haven and Jorgensen (2013) and Ko et al. (2015) investigated cellulase (Cellic® CTec2, Novozymes) binding on lignin residue from steam-pretreated wheat straw and mixed hard wood chips pretreated by liquid hot water pretreatment, respectively. They found that a significant amount of β-glucosidase was bound to the lignin residue. Ko et al (2015) also found that when higher guaicyl units are present in lignin, and majority of the enzymatic activity was lost from the supernatant. Nevertheless, Haven and Jorgensen (2013) reported that β-glucosidase can remain active even when it is intensively bound to the lignin residue. Even though it has been shown that cellulolytic enzymes have varying tendencies to be bound to lignin during hydrolysis of lignocellulosic biomass, the effect of varying pretreatments on enzyme adsorption and functionality is not fully understood.

In this study, lignin was isolated from switchgrass pretreated with sodium hydroxide (NaOH) and dilute sulfuric acid (H_2SO_4) at various concentrations to investigate (1) which cellulolytic enzymes interact with isolated lignin and (2) if lignin isolates from different pretreatment conditions influence enzyme binding. The lignin isolates were incubated with Cellic® CTec2 enzyme cocktail and enzymatic hydrolysis of mixtures of model carbohydrates and lignin isolates was also performed to study how cellulase bound to lignin isolate can affect production of monomeric sugars.

2. MATERIIALS & METHODS

2.1 Sample preparation

Alamo switchgrass was obtained from Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina. The switchgrass was ground and passed through a 2 mm sieve by a Thomas Wiley Laboratory Mill (Model No. 4, Philadelphia, PA, USA). Extractives removal was achieved by soxhlet extraction in cellulose thimbles with acetone reflux for 24 hours to prevent potential interference with lignin analysis and during processes for recovering lignin isolates. Extractive-free switchgrass was used as raw material for alkali and acid pretreatment.

2.2 Alkali and acid pretreatment

Switchgrass was pretreated using two pretreatment methods: sodium hydroxide (NaOH) and dilute sulfuric acid (H_2SO_4) pretreatments. Three chemical concentrations were used for pretreatment at 121°C for predetermined treatment times (NaOH: 0.5, 1.0 and 1.5% for 30 minutes were designated as 0.5N, 1.0N, and 1.5N, respectively; H_2SO_4: 1, 2 and 3% for 60 min designated as 1.0H, 2.0H, and 3.0H, respectively). Ten grams of extractive free biomass and 100 ml NaOH or H_2SO_4 solution of desired concentration were mixed at 10% (w/v) solid loading in 125 ml glass serum bottles which were crimp sealed prior to pretreating in the autoclave at 121°C/15 psi (Model 3021, Amsco, Mentor, OH, USA). Pretreated biomass was washed with 500 ml deionized (DI) water and recovered by vacuum filtration. In all, 50 g of switchgrass was pretreated at each condition and biomass from the 5 serum bottles was combined into one 500 ml polypropylene bottle after pretreatment to obtain a well-mixed bigger batch.

2.3 Enzyme hydrolysis and lignin isolation

Cellic® CTec2 and HTec2 enzyme cocktails (Novozymes North America, INC, Franklinton, NC, USA) were used in excess for maximum removal of structural carbohydrates from the pretreated switchgrass. Density of CTec2 and HTec2 was measured to be 1.23 and 1.16 g/ml, respectively. Cellulase activity of CTec2 was estimated as 103.5 FPU/ml according to National Renewable Energy Laboratory (NREL)'s Laboratory Analytical Procedures (LAP) (Adney and Baker, 2008).

2.3.1 Lignin isolation

Alkali and acid pretreated biomass at 8% solid loading (w/v) was hydrolyzed in 0.05M sodium citrate buffer (pH 4.8) with excessive Cellic® CTec2 dose equivalent to 140 FPU/g dry pretreated biomass and supplemented with HTec2 (0.25 g of enzyme /g dry pretreated biomass) to maximize enzyme saccharfication efficiency. Tetracycline (40 µg/ml) was added during enzymatic hydrolysis to prevent microbial contamination and hydrolysis was performed for 120 h at 50^{o}C, 150 rpm in an air bath shaker (Series 25 incubator shaker, New Brunswick Scientific Co., INC, Edison, NJ). After hydrolysis, the suspension was centrifuged (model 5810R, Eppendorf, Hauppauge, NY) and supernatant removed. The remaining solids were washed thrice with DI water adjusted to a pH of 2.5 by adding hydrochloric acid (HCl) to prevent lignin solubilization during the washing step (Rahikainen et al. 2011). A commercial bacterial protease (Type (XXIV), EC # 232-752-2, Sigma-Aldrich Co., St. Louis, MO) was used to remove residual enzymes bound to the lignin rich hydrolyzed. The protease treatment method was modified from Tamminent and Hortling (2005), Berlin (2006b) and Rahikainen et al. (2011). Briefly, washed solids were incubated overnight in 0.05 M phosphate buffer (pH 8.5) containing 0.1 mg protease/50mg lignin in an air bath shaker at 37^{o}C. After treatment, the proteases were deactivated by placing the samples at 90^{o}C in a convection oven for 2 hours. Protease treated solids were further washed with DI water adjusted to pH 2.5 with HCl three times. Finally, the solids were dried in a 40^{o}C vacuum oven and used as 'lignin isolates (LI)'.

Composition analysis including glucan, xylan, and lignin of the lignin isolates as well as raw switchgrass was performed according to NREL's LAP (Sluiter et al. 2008) prior to conducting the lignin-enzyme binding study. Scanning electron microscopy (SEM) images of raw switchgrass and lignin isolates obtained from alkali and acid pretreated switchgrass were also obtained to investigate structural changes via comparative visual analyses. A Hitachi S-3200N variable pressure scanning electron microscope (VPSEM) equipment available at North Carolina State University's Analytical Instrumental Facility (AIF) was used in this study. Dried samples were placed on a stub with carbon tape, followed by sputter coating with Gold-Palladium (Au-Pd) in high vacuum mode. Further, coated samples were placed on a chamber to be visualized through the VPSEM.

2.4 Lignin-enzyme binding study

Six types of lignin isolates (LI) were prepared from switchgrass pretreated with three NaOH and three H_2SO_4 pretreatments, to study lignin-enzyme binding. Enzyme binding was investigated in microcentrifuge tubes by mixing 1% (w/v) lignin isolate in 1.5 ml 0.05 M sodium citrate buffer (pH 4.8) with CTec2 equivalent to 5 FPU/g lignin isolate. As control, 1% (w/v) lignin isolates were suspended in 1.5 ml of 0.05 M sodium citrate buffer (pH 4.8) only. The experimental and control tubes were incubated for conventional enzymatic hydrolysis (at 50^{o}C, 150 rpm for 72 hours). After 72 hours, the supernatant and lignin isolate were separated by centrifugation at 14,000 rpm for 10 min.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize enzyme-lignin isolate binding. For supernatant analysis (liquid fraction (LF)), 0.3 ml of 5X SDS sample buffer was mixed with the supernatant (approximately 1.5 ml) and the mixture was incubated at 90^{o}C in a heat block for 10 min. Enzyme binding on the lignin isolates (solid fraction (SF)) was studied by washing the isolates with 1.5 ml of 0. 05 M sodium citrate (pH 4.8) followed by centrifugation to remove the wash liquid. The lignin isolate was resuspended in 1.5 ml of 0.05 M sodium citrate (pH 4.8) and mixed with 0.3 ml of 5X SDS sample buffer. The mixture was incubated at 90^{o}C in the heat block for 10 min and centrifuged to obtain the liquid for SDS-PAGE analysis using Mini-Protean® TGX^{TM} Precast Gels (Bio-Rad, Hercules, CA, USA) with 10 wells and Tris/glycine/SDS running buffer. Eight micro liter protein standard ladder (Precision Plus Protein Kaleidoscope Standards, Bio-Rad, Hercules, CA, USA) and 20 µl samples (from liquid and solid fractions) were loaded into the wells on the gel. The gels were run at 120 V (constant) for approximately 60 min. Coomassie blue staining solution was applied to the gel for protein staining, followed by application of a destaining solution to remove the stain from the non-protein portions of the gel. Gel

Documentation EQ System (Bio-RAD, Hercules, CA, USA) was used to take gel images and analyze enzyme binding onto lignin isolates.

2.5 Carbohydrates hydrolysis with or without lignin isolate

Cellobiose (Sigma-Aldrich) and crystalline Avicel (Sigma-Aldrich) were individually hydrolyzed with CTec2 in the presence and absence of lignin isolates to investigate how binding of cellulolytic enzymes to the lignin isolate affects production of glucose from these two carbohydrates. Generally, β-glucosidase (BG) plays a significant role in converting cellobiose into glucose while BG, cellobiohydrolases (CBH) and endo-1,4-β-glucanases (EG) are needed to convert Avicel into glucose. Therefore, BG's conversion efficiency with cellobiose may be explained when hydrolysis is performed with and without lignin isolates. Similarly, changes in CBH and EG conversion efficiency can be indirectly estimated by analyzing amount of sugar generated from Avicel in the presence and absence of lignin isolates.

In order to better understand the functionality and binding characteristics of cellulolytic enzymes, the following experimental variations were investigated during hydrolysis of cellobiose and Avicel with and without lignin isolates: a) CTec2 at 5 and 10 FPU/g carbohydrates was used to study how changes in enzyme loading affect glucose production, and b) the amounts of carbohydrate and lignin in the hydrolysate were varied to simulate various carbohydrate:lignin ratios to determine if higher lignin content affected sugar production from the two carbohydrate types (cellobiose and Avicel). The lignin isolates (LI):carbohydrate ratios tested were 0.25% (w/v, equivalent to 2.5 g/L) LI:0.5% (w/v, equivalent to 5 g/L) carbohydrate, 0.5:0.5%, and 1.0:0.5%. Hydrolysis was performed by mixing the desired amounts of cellobiose or Avicel and lignin isolate with 1.5 ml of 0.05 M sodium citrate buffer (pH 4.8) in a microcentrifuge with a CTec2 loading of 5 and 10 FPU/g carbohydrate. A number of control samples were also prepared to establish baseline values i) blank control (just 0.05 M sodium citrate buffer), ii) substrate control (cellulose and Avicel only), iii) CTec2 control (5 and 10 FPU/g carbohydrate) and iv) lignin isolate only. Glucose content of the hydrolysate was determined by centrifuging and analyzing the supernatant with a YSI 2950 Biochemistry Analyzer (Xylem Inc., Yellow Springs, USA).

3. RESULTS & DISCUSSION

3.1 Chemical compositions of raw switchgrass and lignin isolates

Composition analysis for raw switchgrass and lignin isolates obtained from switchgrass pretreated by NaOH and H_2SO_4 at the desired conditions was performed to determine residual carbohydrates (e.g. glucan and xylan) and lignin contents (Table 4.1). Raw switchgrass contained 35.1% glucan, 23.4% xylan and 24.4% total lignin (AIL + ASL). Lignin in the isolates obtained from NaOH pretreated switchgrass ranged from 36.6 to 58.8%. Higher NaOH concentration resulted in higher lignin content in the isolates. Even though excessive CTec2 and HTec2 loadings were used to maximize removal of structural carbohydrates, significant amounts of glucan and xylan remained in lignin isolates from NaOH pretreatment, especially those pretreated by 0.5% NaOH. Therefore, the lignin isolate from 0.5% NaOH pretreated switchgrass was subjected to repeat enzymatic hydrolysis at the same conditions as first enzymatic hydrolysis to remove the remaining glucan and xylan and improve lignin content. However, no significant change ($p > 0.05$) in lignin content occurred (Data not shown). It is inferred that the remaining glucan and xylan were resistant to any further cellulolytic enzyme action for conversion to soluble polysaccharides or monomeric sugars. Thus these isolates were considered suitable for use in lignin-binding studies. In comparison to NaOH, lignin isolates from H_2SO_4 pretreatment had higher lignin contents ranging from 63.1 to 70.4%. Xylan was not detected in the isolates potentially because H_2SO_4 pretreatment in known to efficiently solubilize hemicellulose like xylan (Dien et al. 2006; Yang et al. 2009)

Table 4.1. Composition of raw switchgrass and lignin isolate

Material	Type of LI	Glucan (%)	Xylan (%)	Lignin (%)
Raw		35.1 ± 0.51	23.4 ± 0.46	24.4 ± 0.26
Lignin Isolates	0.5N	35.4 ± 0.11	9.2 ± 0.56	36.3 ± 2.07
	1.0N	25.5 ± 0.09	6.9 ± 0.75	50.8 ± 0.49
	1.5N	20.4 ± 0.50	4.1 ± 0.95	58.8 ± 1.36
	1.0H	29.2 ± 0.68	ND[1]	63.1 ± 0.32
	2.0H	19.4 ± 1.92	ND	69.6 ± 2.15
	3.0H	16.9 ± 1.04	ND	70.4 ± 0.73

[1] Not detected

3.2 Visualizing the impact of pretreatment conditions on structure of switchgrass

Scanning electron microscopy (SEM) was performed to observe structural changes in lignin isolates generated from switchgrass pretreated by alkali and acid at various conditions. According to SEM analysis, no significant difference was apparent in the structure of lignin isolates due to change in concentration of NaOH or H_2SO_4. SEM images of raw switchgrass and lignin residues pretreated by 1% NaOH and 3% H_2SO_4 are presented as representatives (Figure 4.1). While raw switchgrass showed a clear and intact outer core, its surface was relatively rough (Figure 4.1A). Lignin isolated from 1% NaOH pretreated switchgrass showed significant disruption compared to raw switchgrass (Figure 4.1B) and elongated structures along with a clumped clay like shapeless substance were seen. Generally, cellulose (represented by glucan in this study) is a long chain polymer with β-(1,4)-glycosidic bonds between glucose molecules and appears as bundles of cellulose microfibrils in plants (Boudet et al. 2003; Kumar et al. 2009). Based on the SEM image (Figure 4.1B), the elongated structures may be related to glucan and the clumped substance may be related to lignin and xylan complex. Composition analysis of this particular lignin isolate showed 25.5% glucan, 6.9% xylan, and 50.8% lignin (Table 4.1)

The lignin isolate from 3% H_2SO_4 pretreatment (Figure 4.1C) predominately showed the clumped shapeless substance. It may be inferred that this substance might be lignin since the isolate contained 70.4% lignin and 16.9% glucan with no xylan being detected. Overall, both lignin residues pretreated by NaOH and H_2SO_4 had significant structural changes with relatively higher lignin content than raw switchgrass.

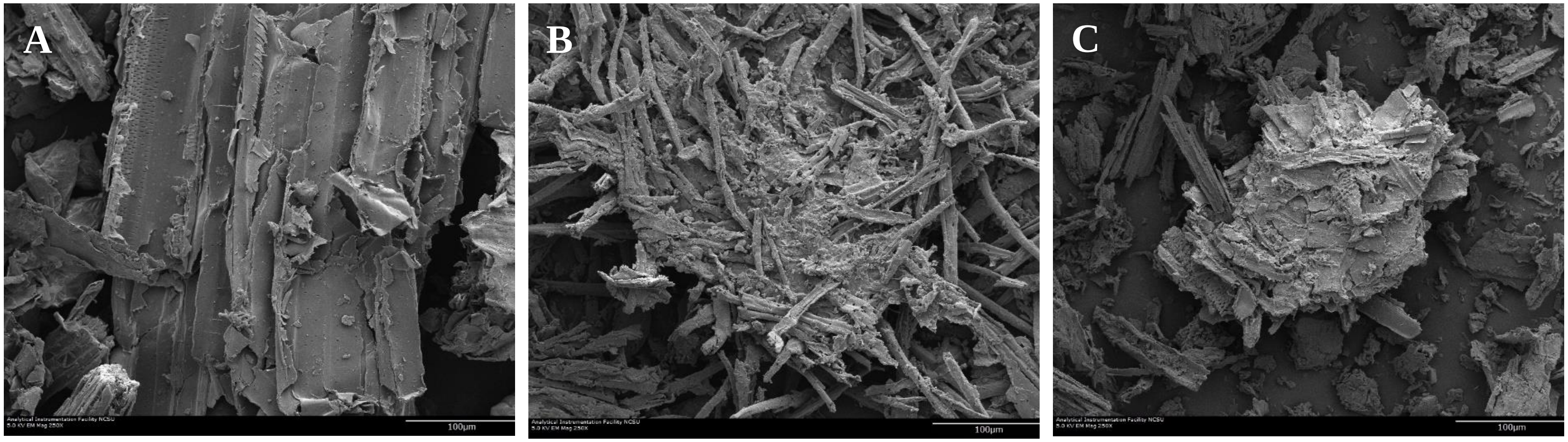


Figure 4.1. SEM Images of (A) raw switchgrass, (B) lignin isolated from switchgrass pretreated by 1% NaOH at 121 ^{o}C for 30 min, and (C) lignin isolated from switchgrass pretreated by 3% H_2SO_4 at 121^{o}C for 60 min (at 500X magnification)

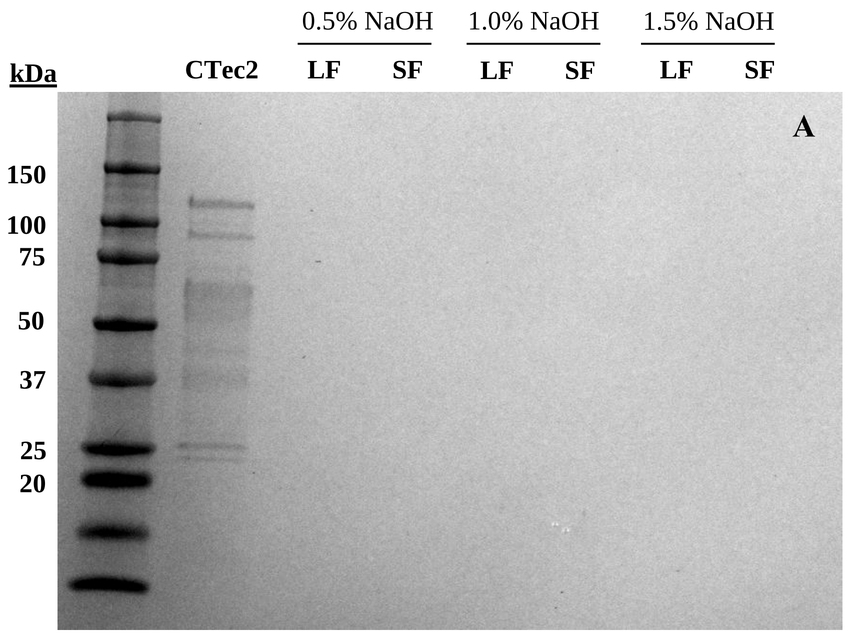
3.3 SDS-PAGE visualization of cellulolytic enzyme binding on lignin isolates

Lignin isolates were incubated with CTec2 cellulolytic enzyme cocktail to investigate how (if any) enzyme binding on lignin occurred. To interpret protein bands on the SDS-PAGE gel, information about molecular weight of proteins in needed. Cellic® CTec2 (Novozymes North America, INC, USA) used in this study is a commercial enzyme blend including 1) cellulases like cellobiohydrolases (CBH) and endo-1,4-β-glucanases (EG), 2) β-glucosidase (BG), and 3) hemicellulase like xylanase (XYN) (Novozymes, 2012). Detailed information on protein distribution in CTec2 could not be found, hence the following putative enzyme information on Cellic® CTec (a previous generation enzyme blend) was used to interpret results. CTec derived from *Trichoderma reesei* is known to contain 1) BG (EC 3.2.1.21) with additional BG, 2) at least the two main CBH (EC 3.2.1.91: Cel6A as CBHI and Cel7A as CBHII) and five kinds of EG (EC 3.2.1.4: EGI, EGII, EGIII, EGIV, and EGV), which are Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A, respectively (Rosgaard et al. 2007; Sorensen et al. 2005).

Electrophoresis of samples drawn from lignin isolates incubated in buffer without CTec2 showed that there were no proteins/enzymes in the liquid (LF, supernatant) and solid fractions (SF, lignin residue) regardless of lignin isolate type (Figure 4.2). Absence of enzymes in the solid fraction indicated that no residual cellulolytic enzymes (from hydrolysis performed to prepare the isolates) were present on the lignin isolates. On the other hand, SDS-PAGE images of samples drawn from lignin isolates incubated with CTec2 showed several protein bands (Figure 4.3). While some seemed to be individual bands, other bands could not be reliably identified from the gel due to similarity between molecular weights of enzymes. For ease of interpretation, the protein bands were divided into 4 zones on the basis of CTec2 control lane in the gel. Zone 1, covering molecular weights between 75 and 120 kDa, was identified as being related to BG which has relatively high molecular weight compared to other cellulases (Haven and Jorgensen 2013; Ko et al. 2015). Zone 2 (approximately 50 to 75 kDa) is expected to be related to CBHI (60~70 kDa), CBHII (60~70 kDa) and EGI (50~60 kDa) (Pribowo et al. 2013; Rahikainen et al. 2011). Zone 3 (approximately 30 to 50 kDa) is likely related to EGII, EGIV, XYNIII (xyn10a) and Zone 4 (approximately 20 to 30 kDa) to EGIII, EGV, XYNI (xyn11a) and XYNII (xyn11b) (Ko et al. 2015).

In Zone 1, related to BG, intense bands were seen for solid fractions of lignin isolates from both NaOH and H_2SO_4 pretreatments (Figure 4.3A and 4.3B). The liquid fraction lanes did not seem to show BG bands regardless of chemical type and concentration. This demonstrates that BG was significantly bound to all lignin isolate solids. Some cellulases related to CBHI, CBHII and EGI in Zone 2 were detected on SF, with bands appearing to be more intense for NaOH derived lignin isolates than H_2SO_4 derived isolates. Since no clear protein bands could be seen below zone 2, enzyme binding on SF of lignin isolates in zone 3 could not be determined. The presence of bands related to zone 3 in LF lanes shows that there may have been limited enzyme-lignin binding in this zone. In zone 4, two distinguishable protein bands potentially related to some EG and XYN were observed on CTec2 control lane. However, only one upper band was detected in LF lanes (Figure 4.3A) indicating that enzymes related to the lower band may be adsorbed on lignin isolates from NaOH pretreatment. In addition, higher H_2SO_4 concentration pretreatments seemed to draw more enzymes to the solid fraction.

Guo et al. (2014) also investigated the cellulase (from *Penicillium oxalicum*) adsorption on lignin from six types of lignocellulosic biomass (aspen, pine, corn, kenaf, and two *Arabidopsis* lines). These six lignins were extracted from aqueous dioxane (96%) solution. They found that lignin with low syringyl/guaiacyl (S/G) ratio had a high enzyme adsorption capacity, and also suggested that the amount of *p*-hydroxyphenyl (H) units from lignin may not affect to the enzyme capacity. In chapter 2 of this dissertation related to changes in lignin chemistry of switchgrass, higher NaOH concentration during pretreatment had the significance effect to reduce amount of H/G ratio ($p < 0.05$). However, increase in NaOH concentration did not significantly ($p > 0.05$) change S/G ratio. Even though changes in lignin chemistry of swithgrass was found by increasing NaOH concentration (0.5 to 1.5%), the difference of the enzyme binding was not detected based on SDS-PAGE (Figure 4.3B). Like Guo et al. (2014)'s inference, changes in amount of H unit might not have significant effect to the CTec2 binding capacity on the lignin isolates.



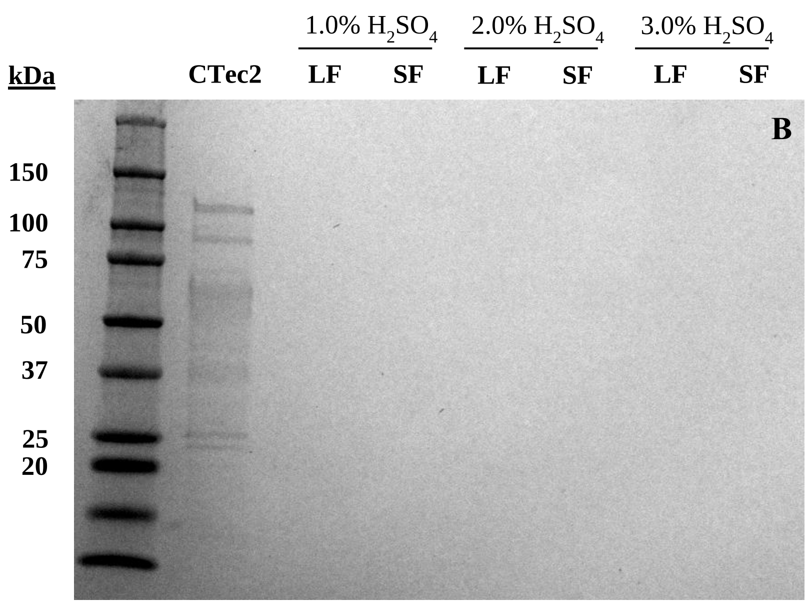


Figure 4.2. SDS-PAGE for liquid (LF) and solid fraction (SF) obtained after control hydrolysis of lignin isolated from (A) NaOH pretreated switchgrass and (B) H_2SO_4 pretreated switchgrass without CTec2

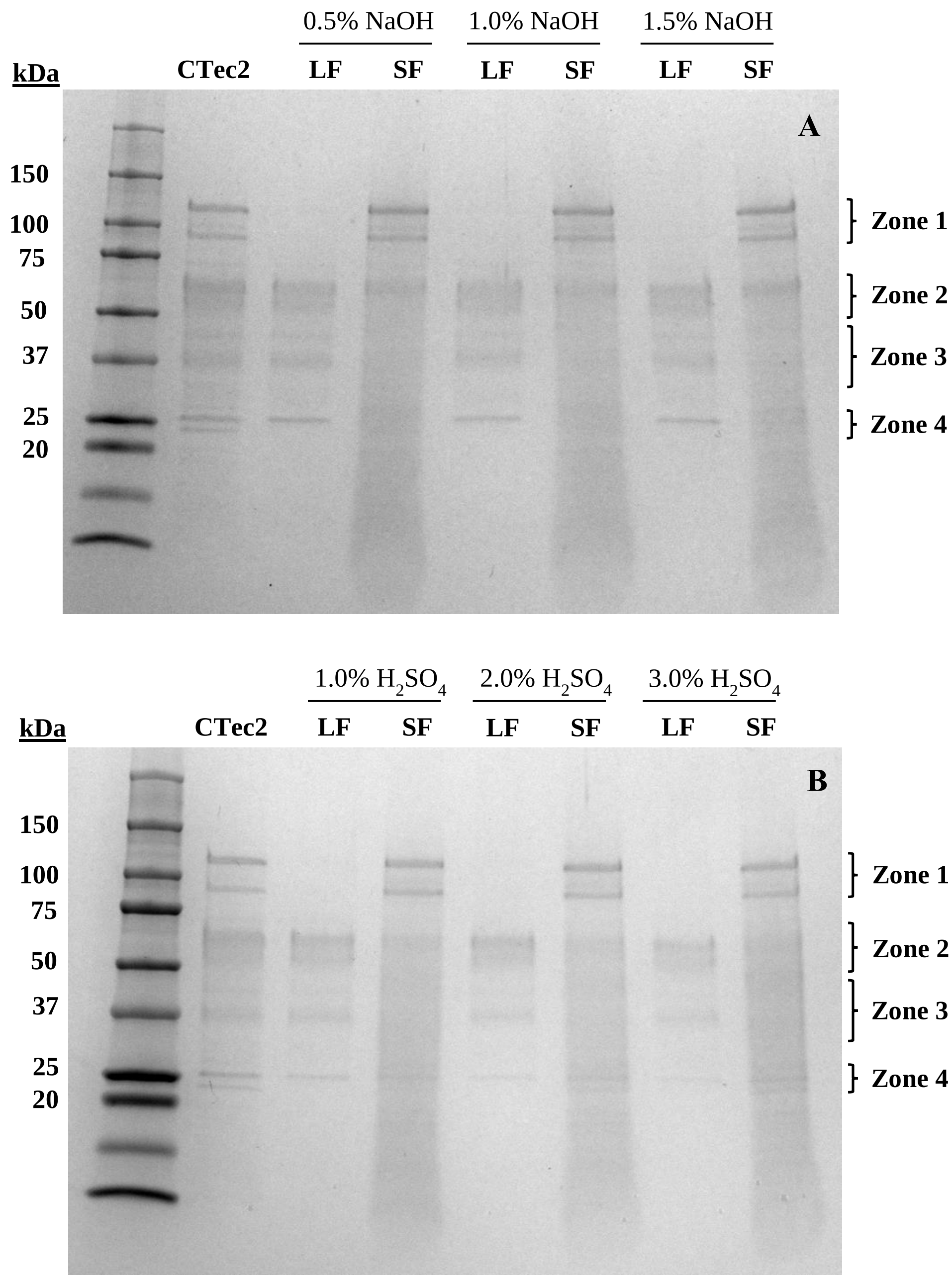


Figure 4.3. SDS-PAGE for liquid (LF) and solid fraction (SF) after hydrolysis of lignin isolated from (A) NaOH pretreated switchgrass and (B) H_2SO_4 pretreated switchgrass with CTec2 (5 FPU/g lignin isolate)

3.4 Glucose production during hydrolysis of carbohydrate model compounds

Since lignin isolates were not homogenous materials containing only lignin, the carbohydrates present in the isolates had the potential to produce glucose during enzymatic hydrolysis for lignin-enzyme binding studies. Hence, a preliminary hydrolysis of lignin isolates was performed with 5 and 10 FPU/g CTec2 to establish baseline glucose levels which were then subtracted from glucose generated during carbohydrate model compound-lignin isolate hydrolysis (Table 4.3) discussed in this section. Glucose yield from glucan remaining in lignin isolates from NaOH and H_2SO_4 pretreatments ranged from 0.01 to 0.09 g/g and 0.01 to 0.05 g/g, respectively.

Cellobiose and Avicel hydrolysis with and without lignin isolates was performed to determine if binding of enzymes on lignin isolates affected their conversion to glucose. Glucose yields from cellobiose (0.5% w/v) at CTec2 loadings of 5 and 10 FPU/g cellobiose were 0.97 g/g with no significant difference between the values (Table 4.2). Since the role of BG is to convert cellobiose into glucose, high conversion efficiency showed that BG in 5 FPU/g of CTec2 was enough to produce glucose from cellobiose. On the other hand, glucose yield from Avicel was 0.30 with a CTec2 dose of 5 FPU/g Avicel and increased significantly to 0.46 g/g when 10 FPU/g was used (Table 4.2). Avicel is crystalline cellulose with a relatively more resistant structure than amorphous cellulose in cellobiose and needs BG, CBH, and EG for efficient conversion to glucose. Thus, it can be inferred that higher CBH and EG loading might be needed to improve glucose production from Avicel.

Glucose yields from hydrolysis of cellobiose in the presence of various lignin isolates was not significantly different ($p > 0.05$) from those with cellobiose only hydrolysis. (Figure

4.4). Pretreatment chemicals and concentrations used prior to lignin isolation as well as increasing the amount of lignin residue mixed with cellobiose during hydrolysis did not significantly ($p > 0.05$) influence glucose yields. Though SDS-PAGE results pointed towards binding of BG to lignin isolates (Figure 4.3), these results indicate that BG remained catalytically active for cellobiose conversion.

Hydrolysis of Avicel in the presence of various lignin isolates at conditions similar to those for cellobiose hydrolysis showed that a CTec2 loading of 10 FPU/g generated significantly ($p < 0.05$) more glucose than 5 FPU/g (Figure 4.5). However, presence of lignin isolate as well as its type and amount did not have a significant effect on glucose yield ($p <$ 005). As with cellobiose hydrolysis, glucose production during Avicel hydrolysis did not change even though cellulase related to CBH and EG seemed to be partially bound to the lignin isolates (Figure 4.3).

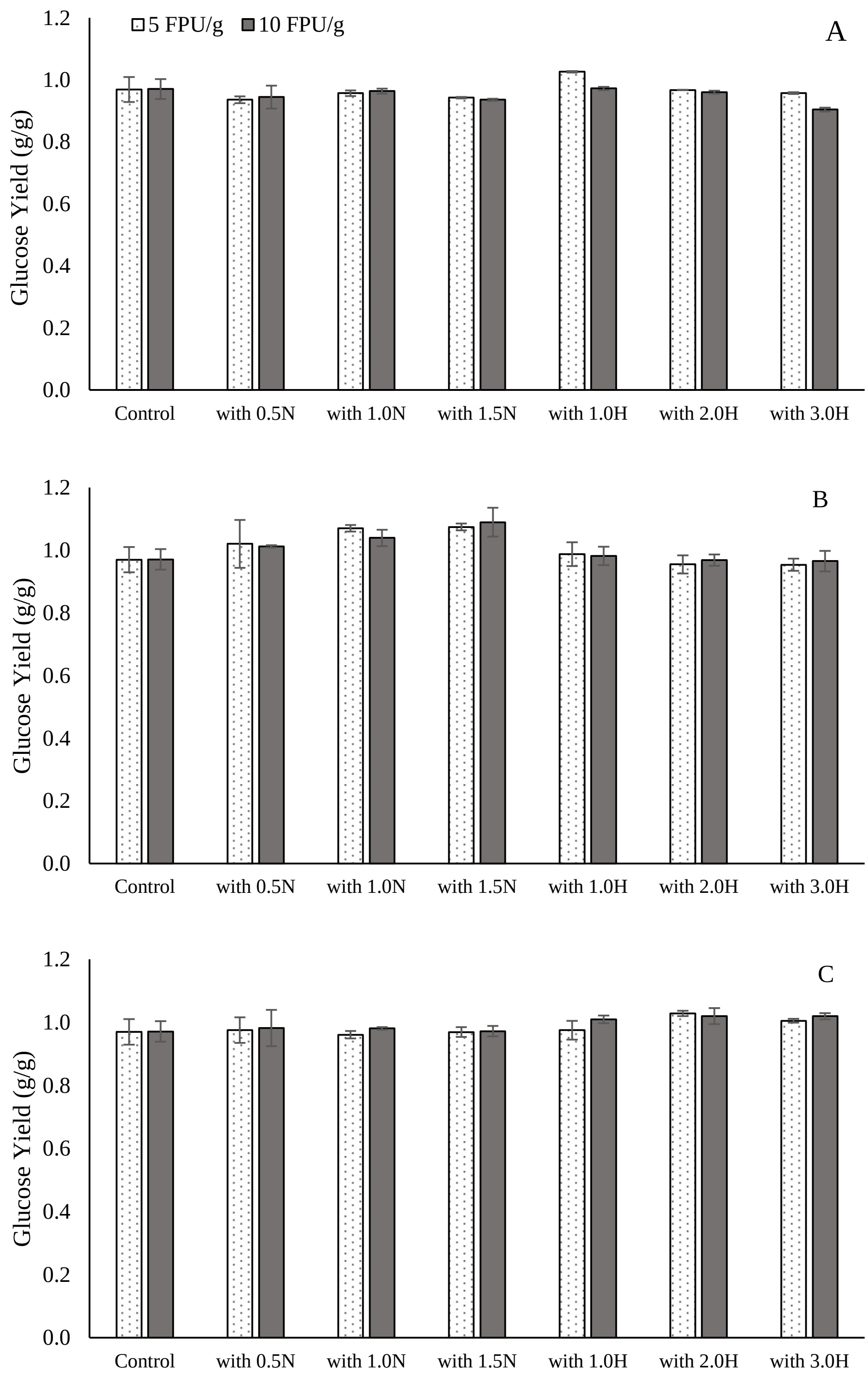
Table 4.2. Glucose production through hydrolysis of Cellobiose and Avicel. Includes data from controls.

Sample type	Glucose (g/L)	Glucose Yield (g/g $_{carbohydrate}$)
Buffer only	0	-
Carbohydrates only		
0.5% (w/v) Cellobiose	0.02 ± 0.007	0.003 ± 0.0013
0.5% (w/v) Avicel	0	0
CTec2 only		
5 FPU/g carbohydrates	0.01 ± 0.005	-
10 FPU/g carbohydrates	0.02 ± 0.007	-
Cellobiose + CTec2		
5 FPU/g carbohydrates	5.06 ± 0.210	0.97 ± 0.04
10 FPU/g carbohydrates	5.07 ± 0.170	0.97 ± 0.03
Avicel + CTec2		
5 FPU/g carbohydrates	1.62 ± 0.264	0.30 ± 0.05
10 FPU/g carbohydrates	2.53 ± 0.156	0.46 ± 0.03
Lignin isolate only		
0.5, 1.0, 1.5% NaOH	0	-
1, 2, 3 % H_2SO_4	0	-

Table 4.3. Glucose yield from glucan remaining in lignin isolates during hydrolysis

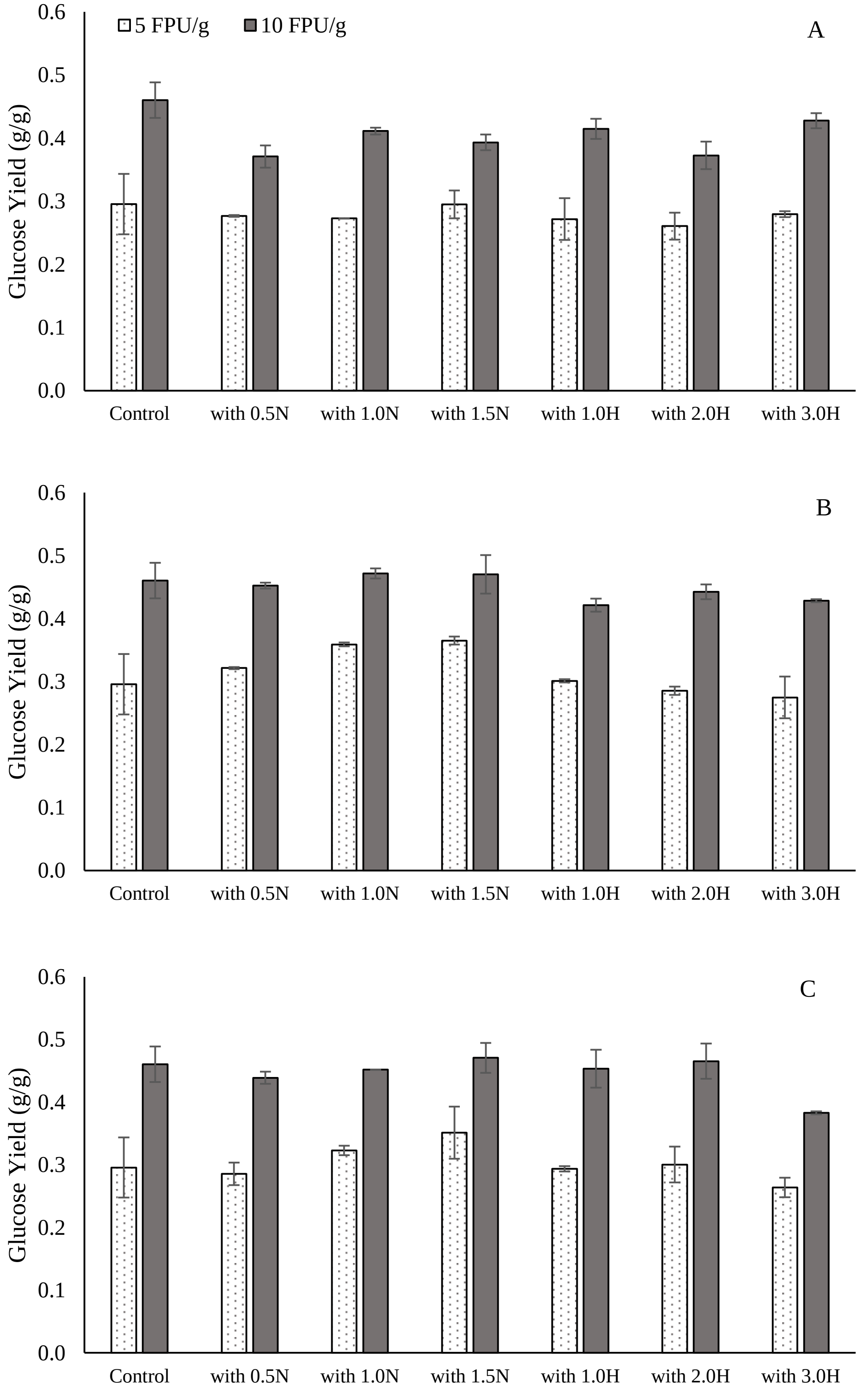
		Glucose Yield (g/g)		
Type of LI	CTec2 loading (FPU/g $_{\text{lignin isolate}}$)	0.25% LI	0.5% LI	1.0% LI
0.5N	5	0.02 ± 0.009	0.02 ± 0.022	0.01 ± 0.000
	10	0.03 ± 0.010	0.02 ± 0.004	0.02 ± -0.003
1.0N	5	0.07 ± 0.005	0.07 ± 0.001	0.06 ± 0.004
	10	0.08 ± 0.004	0.08 ± 0.011	0.09 ± 0.002
1.5N	5	0.06 ± 0.005	0.08 ± 0.007	0.08 ± 0.009
	10	0.07 ± 0.021	0.08 ± 0.006	0.09 ± 0.011
1.0H	5	0.02 ± 0.006	0.02 ± 0.002	0.01 ± 0.003
	10	0.03 ± 0.009	0.03 ± 0.017	0.02 ± 0.001
2.0H	5	0.02 ± 0.006	0.02 ± 0.002	0.02 ± 0.003
	10	0.05 ± 0.003	0.03 ± 0.003	0.02 ± 0.000
3.0H	5	0.03 ± 0.003	0.03 ± 0.011	0.02 ± 0.002
	10	0.04 ± 0.003	0.03 ± 0.007	0.04 ± 0.003

Figure 4.4. Glucose production through hydrolysis of cellobiose and lignin isolates from various pretreatments mixed as (A) 0.25% LI:0.5% cellobiose, (B) 0.5% LI:0.5% cellobiose, and (C) 1.0% LI:0.5% cellobiose



5 FPU/g
10 FPU/g
A
B
C
Glucose Yield (g/g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Control
with 0.5N
with 1.0N
with 1.5N
with 1.0H
with 2.0H
with 3.0H

Figure 4.5. Glucose production through hydrolysis of Avicel and lignin isolates from various pretreatments mixed as (A) 0.25% LI:0.5% Avicel, (B) 0.5% LI:0.5% Avicel, and (C) 1.0% LI:0.5% Avicel


5 FPU/g
10 FPU/g
A
B
C
Glucose Yield (g/g)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Control
with 0.5N
with 1.0N
with 1.5N
with 1.0H
with 2.0H
with 3.0H

4. CONCLUSION

The amount of lignin in isolates derived from NaOH and H_2SO_4 pretreated switchgrass increased with increase in chemical concentration. Though β-glucosidase was observed to be significantly bound to lignin isolates from NaOH and H_2SO_4 pretreated switchgrass, it exhibited sufficient catalytic activity during carbohydrate hydrolysis. Some cellulases related to cellobiohydrolases and endo-1,4-β-glucanases also showed a tendency to bind to lignin isolates. It appeared that lignin isolates from H_2SO_4 pretreatment allowed more enzymes related to some xylanases (XYNI, XYNII) and endo-1,4-β-glucanases (EGIII and EGV) to be bound to the isolates than NaOH. Addition of lignin isolates at various levels during hydrolysis of carbohydrates model compounds (cellobiose and Avicel) did not impact glucose production by CTec2 though more glucose was produced from Avicel hydrolyzed at higher enzyme loading. Overall, binding of enzymes in the cellulolytic enzyme cocktail on lignin isolates did not negatively impact glucose production. It might be possible that other cellulases bound to the lignin isolates can remain active like β-glucosidase. Also cellulases in the liquid fraction (not bound to the lignin isolate) may have catalytic activity which is sufficient for Avicel hydrolysis. Individual enzyme activity testing is needed to further investigate the mechanism of cellulase binding on lignin isolates and its impact on hydrolytic activity.

REFERENCES

Adney, B. and Baker, J. (2008). Measurement of cellulase activities. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006a) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198-209. doi: 10.1016/j.jbiotec.2006.02.021.

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006b) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198-209. doi: 10.1016/j.jbiotec.2006.02.021.

Boudet A, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci 8:576-581. doi: 10.1016/j.tplants.2003.10.001.

Dien BS, Jung HG, Vogel KP, Casler MD, Lamb JFS, Iten L, Mitchell RB, Sarath G (2006) Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass Bioenerg 30:880-891. doi: 10.1016/j.biombioe.2006.02.004.

Guo F, Shi W, Sun W, Li X, Wang F, Zhao J, Qu Y (2014) Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. Biotechnol Biofuels 7:38. doi: 10.1186/1754-6834-7-38.

Haven MO, Jorgensen H (2013) Adsorption of beta-glucosidases in two commercial preparations onto pretreated biomass and lignin. Biotechnol Biofuels 6:165. doi: 10.1186/1754-6834-6-165.

Kim TH (2012) Comparison of inhibition effects of various isolated lignins on enzymatic hydrolysis of cellulose 29:82-88. doi: 10.1007/s11814-011-0150-1.

Ko JK, Ximenes E, Kim Y, Ladisch MR (2015) Adsorption of Enzyme Onto Lignins of Liquid Hot Water Pretreated Hardwoods. Biotechnol Bioeng 112:447-456. doi: 10.1002/bit.25359.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind Eng Chem Res 48:3713-3729. doi: 10.1021/ie801542g.

Novozymes (2012) Application sheet, Novozymes Cellic© CTec2 and HTec2 – Enzymes for hydrolysis of lignocellulosic materials. Retrieved from http://bioenergy.novozymes.com/en/cellulosic-ethanol/CellicCTec3/Documents/AS_2010-01668-03.pdf

Pribowo AY, Hu J, Arantes V, Saddler JN (2013) The development and use of an ELISA-based method to follow the distribution of cellulase monocomponents during the hydrolysis of pretreated corn stover. Biotechnol Biofuels 6:80. doi: 10.1186/1754-6834-6-80.

Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K (2011) Inhibition of Enzymatic Hydrolysis by Residual Lignins From Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface. Biotechnol Bioeng 108:2823-2834. doi: 10.1002/bit.23242.

Rahikainen JL, Evans JD, Mikander S, Kalliola A, Puranen T, Tamminen T, Marjamaa K, Kruus K (2013) Cellulase-lignin interactions-The role of carbohydrate-binding module and pH in non-productive binding. Enzyme Microb Technol 53:315-321. doi: 10.1016/j.enzmictec.2013.07.003.

Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS (2007) Evaluation of minimal Trichoderma reesei cellulase mixtures on differently pretreated barley straw substrates. Biotechnol Prog 23:1270-1276. doi: 10.1021/bp070329p.

Slutier, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). Determination of Structural Carbohydrates and Lignin in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Sorensen H, Pedersen S, Vikso-Nielsen A, Meyer A (2005) Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in an industrial ethanol fermentation residue. Enzyme Microb Technol 36:773-784. doi: 10.1016/j.enzmictec.2005.01.007.

Yang Y, Sharma-Shivappa R, Burns JC, Cheng JJ (2009) Dilute Acid Pretreatment of Oven-dried Switchgrass Germplasms for Bioethanol Production. Energy Fuels 23:3759-3766. doi: 10.1021/ef900043z.

CHAPTER 5

EFFECT OF ENZYME INTERACTION WITH LIGNIN ISOLATED FROM PRETREATED *Miscanthus* x *gigantues* ON CELLULOLYTIC EFFICIENCY

ABSTRACT

Lignin isolates from alkali (0.5, 1.0, and 1.5% NaOH at 121°C for 30 min) and acid (1, 2, and 3% H_2SO_4 at 121°C for 60 min) pretreated miscanthus were used to identify which cellulolytic enzymes in Cellic® CTec2 have a tendency to bind to lignin. Also, cellobiose and Avicel were enzymatically hydrolyzed with and without lignin isolates to study how enzyme binding onto lignin affects their conversion to glucose. Three carbohydrate-lignin loadings (0.5:0.25, 0.5:0.5, and 0.5:1.0% (w/v)) were also used. The results of SDS-PAGE showed that β-glucosidase (BG) had a strong tendency to bind to all lignin isolates. Some cellobiohydrolases (CBH), endo-1,4-β-glucanases (EG), and xylanases in CTec2 were also observed to partially bind to the lignin isolates. Overall tendency of enzyme binding onto lignin isolate was similar regardless of pretreatment chemical concentration. Though enzyme binding onto lignin isolates was observed, hydrolysis in the presence of these isolates did not have a significant ($p > 0.05$) impact on glucose production from cellobiose and Avicel. Cellobiose to glucose conversion of 99% was achieved via hydrolysis at both 5 and 10 FPU/g carbohydrate. Hydrolysis of Avicel with 5 and 10 FPU/g CTec2 resulted in 29.3 and 47.7% conversion to glucose, respectively. While the cellulolytic enzymes used in this study seemed to retain hydrolytic activity, further study is needed to determine how hydrolytic efficiency can change when lignin and carbohydrates are present in a complex matrix.

Keywords: lignin isolate, enzyme binding, cellulases, β-glucosidase, CTec2, SEM, sodium hydroxide pretreatment, dilute sulfuric acid pretreatment.

1. INTRODUCTION

Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin. Cellulose and hemicellulose are structural carbohydrates that provide sugar resources for biofuels production like bioethanol and biobutanol. Lignin, as a non-carbohydrate aromatic polymer, mainly consists of three phenolic compounds: guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) propanol. It forms a complex with cellulose and hemicellulose and this conformational feature in lignocellulosic feedstocks confers them structural stability (Boerjan et al. 2003; Umezawa 2010). From a biorefinery view point, this feature makes lignin a key obstruction to fermentable sugar productions from cellulose and hemicellulose by physically and structurally protecting them from hydrolytic enzyme access. Therefore, a pretreatment step is necessary to allow enzymes to access structural carbohydrates with greater enzyme saccharificaton efficiencies.

Dilute sulfuric acid (H_2SO_4) pretreatment has a significant effect on sugar production through solubilization of hemicellulose and redistribution of lignin thus increasing cellulolytic enzyme accessibility. Acid also renders lignin to become more condensed. (Dien et al. 2006; Yang et al. 2009). Alkali pretreatments, such as those with sodium hydroxide (NaOH) facilitate improvements in sugar production by solubilizing lignin and increasing surface area available for enzyme attachment (Haque et al. 2013; Liu et al. 2013; Wang et al. 2012b; Xu et al. 2011b). Jung (Chapter 3, Doctoral dissertation, 2016) found that NaOH pretreatment changed lignin chemistry in pretreated miscanthus and increased S/G ratio from 0.64 in untreated biomass to 0.73 - 0.77 after pretreatment with 0.5 – 1.5% NaOH. Conversely, the H/G ratio (0.48 untreated biomass) decreased to 0.13 as NaOH concentration increased regardless of pretreatment time.

Even though acid and alkali pretreatments effect lignin chemistry and/or structure, how the alteration affects cellulolytic enzyme performance is not fully understood, especially, in terms of non-productive enzyme binding and other mechanisms of lignin-derived inhibition (Berlin et al. 2006c; Kim 2012b; Rahikainen et al. 2011; Rahikainen et al. 2013a; Rahikainen et al. 2013b)

In this study, lignin isolated from miscanthus pretreated with NaOH and H_2SO_4 at various concentrations was incubated with a cellulolytic enzyme cocktail to investigate which cellulolytic enzymes interact with isolated lignin. Model carbohydrate compounds (cellobiose and Avicel) in the presence and absence of lignin isolates were hydrolyzed to understand how variations in pretreatments influence enzyme-lignin binding and subsequent cellulolytic efficiency.

2. MATERIAL & METHODS

2.1 Sample preparation

Miscanthus x *giganteus* was harvested from Mountain Horticultural Crops Research and Extension Center, Mills River, North Carolina in December 2011. The field dried and chopped miscanthus was milled (Model No. 4, Philadelphia, PA, USA) and ground to pass a 2 mm sieve. Extractive-free miscanthus obtained by acetone extraction was dried and used in this study.

2.2 Alkali and acid pretreatment

Sodium hydroxide (NaOH) and dilute sulfuric acid (H_2SO_4) were used to pretreat miscanthus prior to enzymatic hydrolysis. The pretreatment temperature was set at 121^{o}C for both chemicals but alkali pretreatment was conducted with NaOH concentration of 0.5, 1.0, and 1.5% (w/v) (designated as 0.5N, 1.0N and 1.5N) for 30 min, while H_2SO_4 concentration was 1, 2, and 3 % (w/v) (designated as 1.0H, 2.0H and 3.0H) corresponding with a pretreatment time of 60 min. Fifty g of miscanthus was pretreated in 5 glass serum bottles (125 ml) each containing 10 g of miscanthus and 100 ml of NaOH (or H_2SO_4) with desired concentration. The serum bottles with crimp seal were autoclaved at 121^{o}C/15 psi (Model 3021, Amsco, Mentor, OH, USA) for the desired pretreatment times. Pretreated miscanthus was recovered by vacuum filtration and washed with 500 ml of deionized (DI) water to remove any residual chemical. Biomass recovered from the five serum bottles was mixed in 500 ml plastic bottles. About 1 g of the wet pretreated miscanthus was taken to estimate moisture content and solid recovery. Another 4g was dried in a 40^{o}C vacuum oven and used for composition analysis. Remaining pretreated miscanthus was stored at 4^{o}C for subsequent enzyme hydrolysis at a later time, typically within 3 days.

2.3 Lignin isolation

Alkali and acid pretreated miscanthus was enzymatically hydrolyzed to recover lignin isolates which has minimum structural carbohydrate and maximum lignin content. Cellic® CTec2 (103.5 FPU/ml as experimental value) and HTec2 enzyme cocktails (Novozymes North America, Inc, Franklinton, NC, USA) with density measured to be 1.23 and 1.16 g/ml, respectively, were used for hydrolysis. Miscanthus at a solid loading of 8% (w/v) in 0.05M sodium citrate buffer (pH 4.8) was hydrolyzed with excessive CTec2 dose equivalent to 140 FPU/g $_{\text{dry pretreated biomass}}$ supplemented with HTec2 (0.25 g of enzyme/g $_{\text{dry pretreated biomass}}$). Hydrolysis was performed at 50 ^{o}C, 150 rpm for 5 days in an air bath shaker (Series 25 incubator shaker, New Brunswick Scientific Co., INC, Edison, NJ). Tetracycline (40 µg/ml) was added during the enzymatic hydrolysis to prevent microbial contamination.

After hydrolysis, solids in the hydrolysate were separated by centrifugation (Model 5810R, Eppendorf, Hauppauge, NY) at 4000 rpm for 10 min and washed three time with DI water (pH 2.5 adjusted by HCl) to remove residual enzymes and prevent lignin solubilization. Any potential enzyme bound to the hydrolysate solid were removed by a protease treatment with a commercial bacterial type (XXIV) proteases (subtilisine, Sigma-Aldrich Co., St. Louis, MO) (Berlin et al. 2006b; Rahikainen et al. 2011). Briefly, the lignin reside was suspended in 0.05M phosphate buffer (pH 8.5) with 0.1 mg/50mg $_{\text{lignin isolate}}$ of protease and incubated at 37^{o}C overnight. Further, incubation at 90^{o}C for 2 h was performed to deactivate the proteases. The protease treated solids were washed three time with DI water (pH 2.5 adjusted by HCl) and dried in 40^{o}C vacuum oven. These dry solids were used as ‘lignin isolate (LI)’

2.4 Lignin-enzyme binding study

Incubation of lignin isolated from various pretreatments with CTec2 cellulase cocktail was performed to investigate enzyme binding onto lignin isolate. 1% (w/v) of lignin isolate was suspended in 1.5 ml of 0.05M sodium citrate buffer (pH 4.8) with 5 FPU equivalent CTec2/g $_{\text{lignin isolate}}$ and incubated at 50 ^{o}C, 150 rpm for 72 hours in the air bath shaker (Series

25 incubator shaker, New Brunswick Scientific Co., INC, Edison, NJ). Supernatant and hydrolyzed lignin isolate was separated by centrifugation at 14,000 rpm for 10 min. Lignin isolate was washed and resuspended in 0.05M sodium citrate buffer (pH 4.8) to remove free enzymes which are not bound to the isolate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize which enzymes were suspended in the supernatant and which were bound to the washed lignin isolate (Jung, Chapter 4, Doctoral Dissertation. 2016). Gel Documentation EQ System (Bio-RAD, Hercules, CA, USA) was used to take the SDS-PAGE gel image and investigate enzyme binding onto the lignin isolate.

2.5 Effect of enzyme binding on hydrolysis efficiency for cellobiose and Avicel

To study how enzyme binding on lignin isolates affects enzymatic hydrolysis efficiency, two model carbohydrate compounds, cellobiose (a disaccharide with 2 glucose molecules bound via β-(1,4)-glycosidic bonds, Sigma-Aldrich, USA) and Avicel (microcrystalline cellulose, Sigma-Aldrich, USA), were individually hydrolyzed with each of the 6 types of lignin isolates in 1.5 ml of 0.05 M sodium citrate buffer (pH 4.8). A full factorial experiment with variations in CTec2 dose and amount of lignin isolate mixed with 0.5% cellobiose or Avicel was conducted (Table 5.1). The cellobiose (or Avicel) with lignin isolate suspension was hydrolyzed with CTec2 at 50ºC, 150 rpm for 72 hours in an air bath shaker. The hydrolysate containing glucose produced from cellobiose (or Avicel) was centrifuged and supernatant was recovered. The following controls (Table 5.4) in 1.5 ml sodium citrate buffer were also hydrolyzed to calculate accurate glucose production; 1) blank control (just 0.05 M sodium citrate buffer), 2) substrate control (0.5% w/v cellulose or Avicel only), 3) CTec2 control (5 and 10 FPU/g carbohydrate), 4) lignin isolate control (0.25, 0.5, and 1.0 % w/v lignin isolate only) and 5) lignin isolate hydrolysis control (lignin isolate + CTec2).

Table 5.1. Experimental design for carbohydrate model compound and lignin isolate hydrolysis

Material	Experimental variables for hydrolysis
Cellobiose and Avicel	0.5 % (w/v)
6 types of LI[1)]	0.25, 0.5 and 1.0% (w/v)
CTec2	5 and 10 FPU/g $_{carbohydrate}$

[1)] Pretreated by NaOH (0.5, 1.0 and 1.5%) and H_2SO_4 (1, 2 and 3%)

2.6 Composition analysis

Chemical composition of untreated and pretreated miscanthus, and lignin isolates from the pretreated miscanthus were analyzed based on National Renewable Energy Laboratory (NREL)'s Laboratory Analytical Procedures (LAP) (Sluiter et al., 2008a and 2008b). A two-stage sulfuric acid hydrolysis was used to analyze structural carbohydrates (i.e. glucan and xylan) and lignin content including acid insoluble (AIL) and acid soluble (ASL) lignin. Monomeric sugars (glcucose and xylose) for composition analysis and glucose in the hydrolysates was determined using YSI 2950 Biochemistry Analyzer (Xylem Inc., Yellow Springs, USA). Monomeric sugars (i.e. glucose and xylose) data was divided by 1.1 to estimate the amount of polymeric sugars (glucan and xylan).

2.7 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to visualize structural changes between untreated miscanthus and lignin isolates obtained from alkali and acid pretreated miscanthus. Analysis was performed with Hitachi S-3200N variable pressure scanning electron microscope (VPSEM) at the Analytical Instrumental Facility (AIF) in North Carolina State University. Vacuum dried samples were placed on a stub with carbon tape, followed by sputter coating with Gold-Palladium (Au-Pd) in high vacuum mode. The coated samples were placed in a chamber to be visualized through the VPSEM.

2.8 Statistical analysis

The generalized linear model (GLM) procedure with Tukey adjustment at 95% confidence level in SAS 9.3 (Cary, NC, USA) was used to statistically analyze experimental data. All treatments were conducted in 2 replicates. Main effects were 1) 6 kinds of pretreatment methods (three from NaOH with 0.5, 1.0, and 1.5%, and other three from H_2SO_4 with 1, 2, and 3%), 2) mixing ratio of cellobiose (or Avicel) and lignin isolate, and 3) CTec2 loading (5 and 10 FPU/$g_{carbohydrate}$) while the dependent variable was glucose production from cellobiose and Avicel.

3. RESULTS & DISCUSSION

3.1 Changes in chemical composition of alkali and acid pretreated miscanthus

Analysis of structural carbohydrate content (e.g. glucan and xylan) showed that xylan in miscanthus was degraded by both NaOH and H_2SO_4 pretreatments while glucan remained nearly unchanged (Table 5.2). NaOH pretreatments at 0.5, 1.0, and 1.5% degraded xylan by 7.3, 27.0 and 31.9%, respectively while H_2SO_4 degraded much higher amount of xylan up to 95.7%. Delignification due to comprehensive disruption of the cross-ester-linkages between lignin and hemicellulose (Belmokhtar et al. 2013; Haque et al. 2013; Yamamura et al. 2013) during NaOH pretreatment ranged from 33.2 to 72.8% depending on NaOH concentration. Lignin content in NaOH pretreated miscanthus was significantly less than untreated miscanthus ($p < 0.05$) while that in H_2SO_4 pretreated miscanthus remained nearly unchanged.

Table 5.2. Composition analysis (% dry untreated biomass basis) of untreated and pretreated miscanthus

Pretreatment	Conc. (%)	SR[1] (%)	Glucan (%)	Xylan (%)	AIL (%)	ASL (%)	Lignin (%)
Untreated	-	-	41.3 ± 1.09	24.1 ± 0.07	22.2 ± 0.13	2.2 ± 0.02	24.3 ± 0.13
NaOH	0.5	76.0 ± 0.89	38.9 ± 0.17	22.3 ± 0.16	15.0 ± 1.08	1.2 ± 0.06	16.3 ± 1.14
	1.0	66.3 ± 0.69	40.5 ± 0.19	17.6 ± 0.36	6.5 ± 0.08	1.0 ± 0.02	7.5 ± 0.06
	1.5	65.1 ± 2.63	42.3 ± 0.05	16.4 ± 2.56	5.7 ± 0.38	0.9 ± 0.02	6.6 ± 0.40
	1.0	72.3 ± 0.66	41.5 ± 0.66	2.7 ± 0.14	23.0 ± 0.74	1.2 ± 0.02	24.2 ± 0.76
	2.0	67.8 ± 1.25	38.4 ± 0.03	1.4 ± 0.12	22.9 ± 0.5	1.2 ± 0.02	24.1 ± 0.54
	3.0	65.8 ± 1.16	38.7 ± 0.71	1.0 ± 0.13	22.7 ± 0.38	1.1 ± 0.01	23.8 ± 0.39

[1] Solid Recovery

3.2 Characterization of lignin isolates

Composition of lignin isolates obtained from NaOH and H_2SO_4 pretreated miscanthus is presented in Table 5.3. Higher NaOH concentration resulted in higher lignin content (42.1 – 69.5%) in the isolates while lignin content in isolates from H_2SO_4 pretreated miscanthus was similar (58.9 – 65.9%) ($p > 0.05$). Even though excessive CTec2 and HTec2 were used to remove carbohydrates, lignin isolates from NaOH and H_2SO_4 pretreated miscanthus contained up to 46.0 and 32.2% glucan and xylan, respectively. The residual glucan and xylan are inferred to be highly resilient polysaccharides resisting conversion to oligomers or monomeric sugars.

3.2.1 Morphological changes of lignin isolate

Untreated miscanthus and sub-samples of lignin isolates obtained from NaOH and H_2SO_4 pretreated miscanthus were scanned by electron microscopy (SEM) to better understand morphological changes in lignin due to pretreatment (Figure 5.1). Overall external appearance of untreated miscanthus was clear and smooth, but outer edges seemed to be uneven potentially due to mechanical milling for size reduction. Many pores could be seen on the surface (Figure 5.1A and B). The structure of lignin isolated from NaOH (1% w/v) pretreated miscanthus was disrupted and fragmented (Figure 5.1C and D). Many elongated structures and a clumped clay like shapeless substance were observed. In comparison, SEM images of lignin isolates from H_2SO_4 (2%) pretreated miscanthus, predominantly had the clumped substance with some elongated structures (Figure 5.1E and F). Based on the composition of lignin isolates (Table 5.3) derived from 1% NaOH (29.1% glucan, 4.4% xylan and 59.1% lignin) and 2% H_2SO_4 (32.2% glucan and 58.9% lignin), it was inferred that the elongated structures may be related to linear glucose chains linked by β-(1,4)-glycosidic bonds. These chains are put together as cellulose microfibrils via covalent bonds, hydrogen bonds and Van der Waals forces. Cellulose fibers can then be formed by bundling the cellulose microfibrils. In the lignocellulose matrix, cellulose fibers are embedded in a network of hemicellulose and lignin which have amorphous shape (Kumar et al. 2009); Ratanakhanolchai et al. 2013). Isolation of lignin via chemical pretreatment and enzymatic hydrolysis can disrupt miscanthus' structure as seen in Figure 5.1.

Table 5.3. Composition of lignin isolate (% dry lignin isolated basis)

Type of LI	Glucan (%)	Xylan (%)	AIL (%)	ASL (%)	Lignin (%)
0.5N	38.8 ± 0.27	7.2 ± 0.01	40.3 ± 2.19	1.8 ± 0.06	42.1 ± 2.13
1.0N	29.1 ± 0.21	4.4 ± 0.33	57.6 ± 1.40	1.5 ± 0.02	59.1 ± 1.42
1.5N	18.4 ± 0.11	2.5 ± 0.81	67.8 ± 0.52	1.7 ± 0.06	69.5 ± 0.46
1.0H	29.7 ± 5.32	ND[1]	58.8 ± 1.72	1.6 ± 0.01	60.4 ± 1.72
2.0H	32.2 ± 2.14	ND	57.4 ± 2.48	1.5 ± 0.04	58.9 ± 2.48
3.0H	29.2 ± 1.34	ND	64.3 ± 2.98	1.6 ± 0.04	65.9 ± 3.00

[1] Not detected

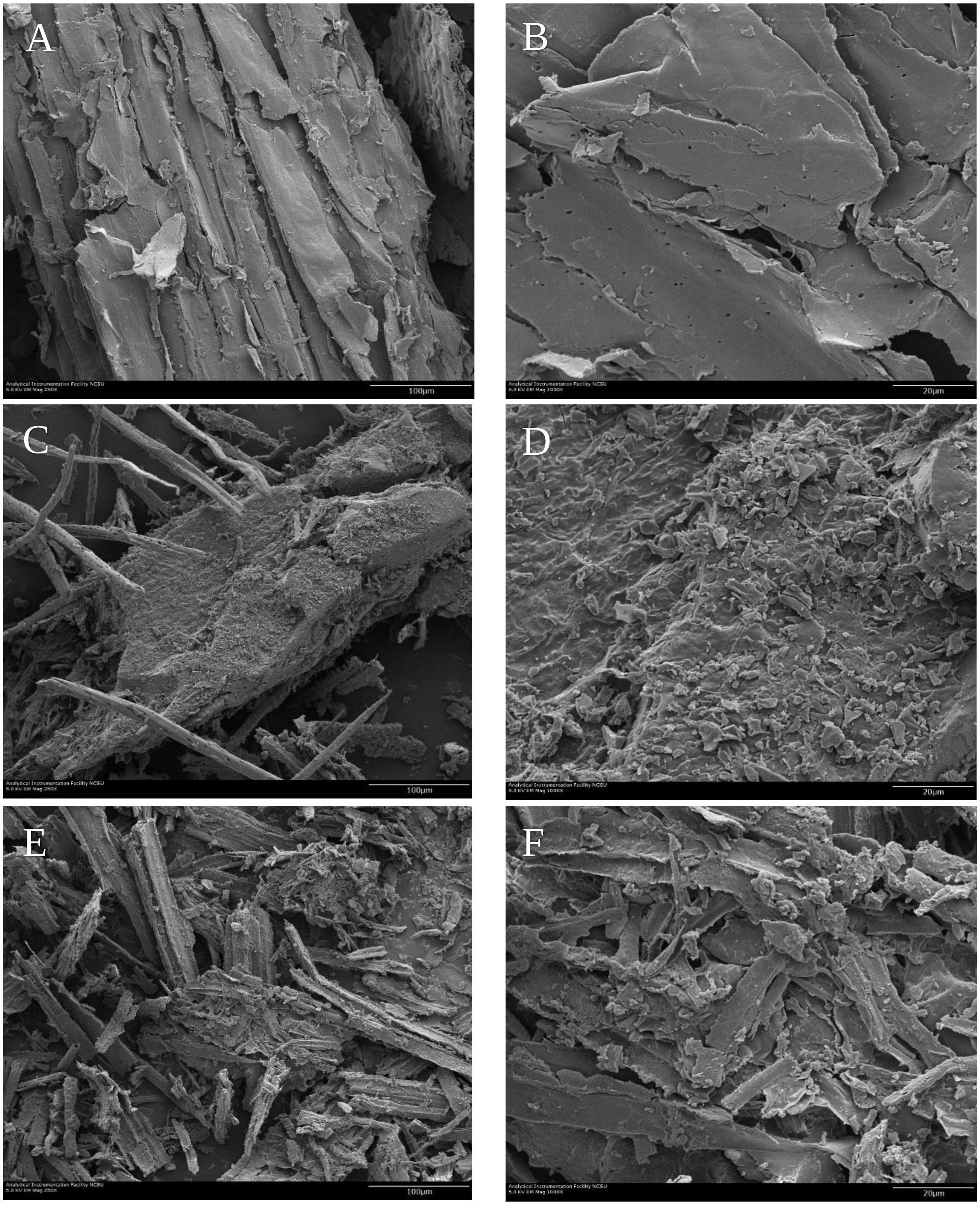


Figure 5.1. SEM images of untreated miscanthus (A and B, magnification 250X and 1000X), lignin isolated from miscanthus pretreated by 1% NaOH at 121 ^{o}C for 30 min (C and D, magnification 250X and 1000X), and lignin isolated from miscanthus pretreated by 2% H_2SO_4 at 121^{o}C for 60 min (E and F, magnification 250X and 1000X).

3.3 Enzyme binding study on lignin isolates - SDS-PAGE visualization

In order to better understand cellulolytic enzyme binding on lignin, lignin isolates from pretreated miscanthus were incubated with CTec2 cellulolytic enzyme cocktail and enzymes suspended in the liquid fraction (LF) and on the solid fraction (SF) were visualized by gel electrophoresis. Since tangible information on molecular weight of enzymes and their distribution in CTec2 could not be found, available enzyme information on Cellic® CTec (a previous generation enzyme blend) was used to interpret the gels. CTec derived from *Trichoderma reesei* is reported to contain 1) β-glucosidase (BG, EC 3.2.1.21) with additional BG, 2) at least the two main key cellobiohydrolases (CBH, EC 3.2.1.91: Cel6A as CBHI and Cel7A as CBHII) and 3) five kinds of endo-1,4-β-glucanases (EG, EC 3.2.1.4: EGI, EGII, EGIII, EGIV, and EGV), which are Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A, respectively (Rosgaard et al. 2007; Sorensen et al. 2005). Some xylanase (XYN) are also expected to be present in the cocktail.

Incubation of lignin isolates without CTec2 under conditions similar to those for incubation with CTec2 did not show any residual cellulolytic enzymes. The absence of bands on the SDS-PAGE gel indicated that there were no enzymes in the liquid and solid fractions, regardless of type of lignin isolates (data not shown). However, when lignin isolates were incubated with CTec2, investigation of samples from LF and SF via electrophoresis (Figure 5.2) showed several protein bands. Some bands were well separated showing individual proteins while others with similar molecular weights appeared as thick bands and were difficult to identify. Therefore, the protein bands were divided into 4 zones on the basis of CTec2 control ladder in the gel. Zone 1 (around 75 to 120 kDa) was related to β-glycosidase (BG) which has relatively high molecular weight compared to other cellulases (Haven and Jorgensen 2013; Ko et al. 2015). Zone 2 (around 50 to 75 kDa) was identified as being related to cellobiohydralase and endo-glucanases, CBHI (60~70 kDa), CBHII (60~70 kDa) and EGI (50~60 kDa) (Pribowo et al. 2013; Rahikainen et al. 2011). Zone 3 (around to 30 to 50 kDa) was expected to be EGII, EGIV, xylanase XYNIII (xyn10a) and Zone 4 (around 20 to 30 kDa) was related to EGIII, EGV, XYNI (xyn11a) and XYNII (xyn11b) (Ko et al. 2015).

In Zone 1, intense bands were observed in lanes with SF of lignin isolates obtained from NaOH and H_2SO_4 pretreated miscanthus regardless of isolate type (Figure 5.2A and B). On the other hand, LF lanes seemed to be clear indicating significant binding of BG to isolated lignin solids. Have and Jorgensen (2013) investigated CTec2 binding on lignin residue from steam-pretreated wheat straw and also found that a significant amount of BG was bound to the lignin residue when hydrolysis was performed at pH 4.8 in 0.05M sodium citrate buffer. Since proteins gain a net negative charge as pH increases above isoelectric point (pI) and vice versa, pH can have a significant effect on binding of BG which has a theoretical pI of 5.7-6.4 (Ko et al. 2015) and experimental reported pI of 8.0 – 8.5 (Chauve et al. 2010; Chirico and Brown 1987; Hui et al. 2001; Medve et al. 1998; Nakagame et al. 2011a; Vinzant et al. 2001). Ko et al. (2015) investigated the effect of pH (4-6) on enzyme adsorption onto lignin and showed that free CTec2 enzymes in supernatant increased with increase in pH. This may be due to repulsion between lignin and BG caused by increase in the negative charge of BG as well as that of lignin due to dissociation of the phenolic carboxyl groups (Dong et al. 1996; Lou et al. 2013; Nakagame et al. 2011b; Rahikainen et al. 2013a).

Cellulases related to CBHI, CBHII and EGI in Zone 2 showed some binding tendencies based on bands from SF both pretreatment methods. Due to appearance of unclear regions, enzyme binding on lignin isolates in zone 3 could not be determined. However, based on the presence of bands from LF in Zone 3, it might be inferred that enzymes related to zone 3 were still suspended, at least partially. In zone 4, two distinct bands potentially related to some EG and XYN were observed with CTec2 control. However, only one upper band was detected from LF (Figure 5.2A and B) suggesting partial loss due to binding on SF. Overall, the tendency of CTec2 binding onto lignin isolates was similar with NaOH and H_2SO_4 derived isolates.

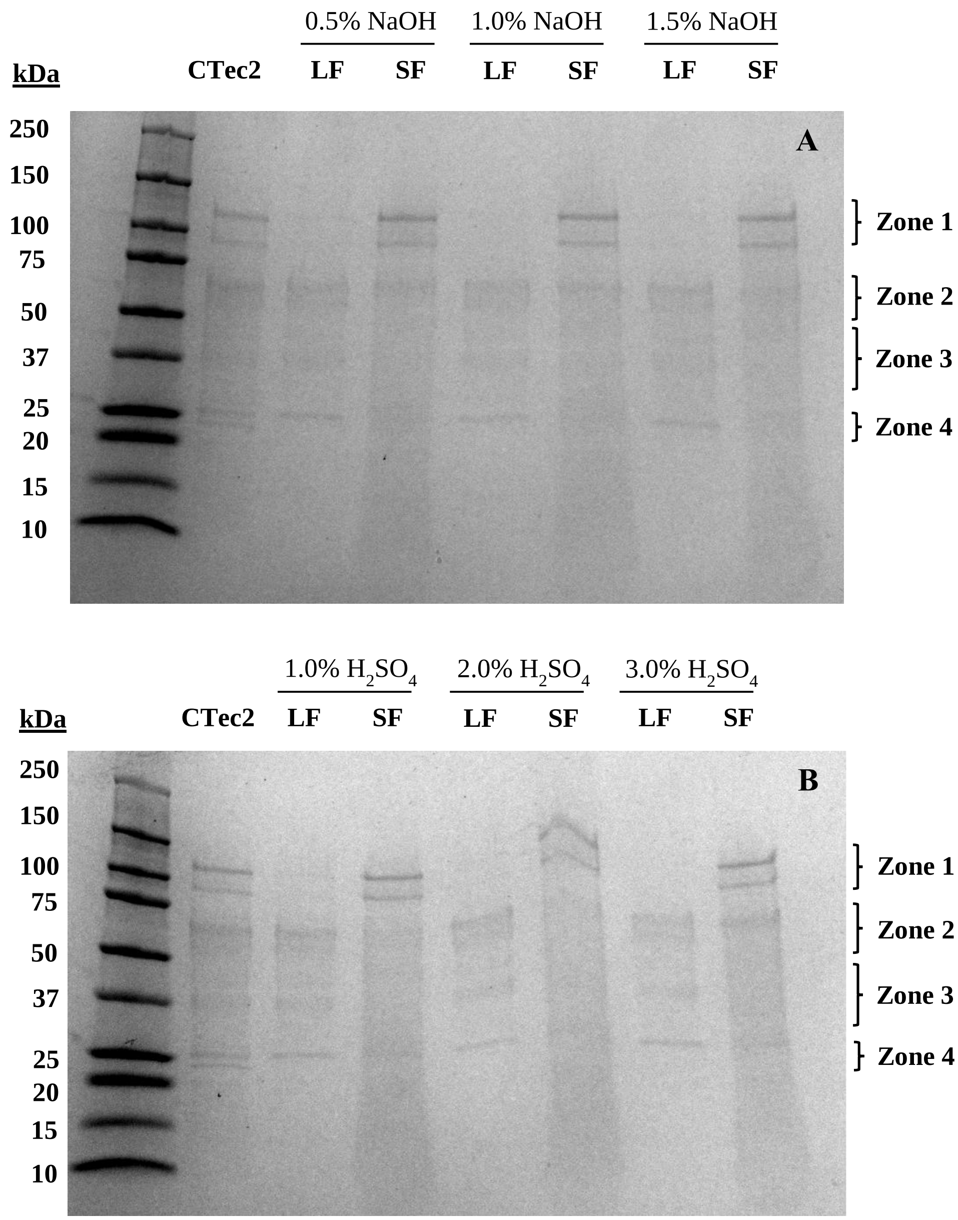


Figure 5.2. SDS-PAGE gel images for liquid (LF) and solid (SF) fraction after hydrolysis (with CTec2 at 5 FPU/g lignin isolate) of lignin isolated from (A) NaOH pretreated miscanthus and (B) H_2SO_4 pretreated miscanthus

3.4 Glucose production during hydrolysis of carbohydrate model compounds

Prior to performing enzymatic hydrolysis of carbohydrate model compounds with lignin isolates, glucose generated from control samples was estimated (Table 5.4). No glucose was detected in the buffer and hydrolysate from lignin isolate incubation without CTec2. Cellobiose released 0.003 g glucose/g cellobiose while Avicel did not generate any glucose when CTec2 was not added. In order to establish base line glucose production from residual carbohydrates in lignin isolates, the isolates were hydrolyzed with CTec2 at 5 and 10 FPU/g (Table 5.5). Overall, 3.3-10.7% of residual glucan in lignin isolates obtained from NaOH pretreatments was converted into glucose during the hydrolysis while 1.7-3.6% of glucan in lignin isolates from H_2SO_4 pretreated miscanthus was converted. The amount of glucose generated from lignin isolates and control factors was subtracted from glucose produced during carbohydrate model compound and lignin isolate hydrolysis.

Cellobiose and Avicel were hydrolyzed with and without lignin isolates to determine whether enzyme binding onto lignin isolate impacted their conversion to glucose. Cellobiose hydrolysis with CTec2 (5 and 10 FPU/g cellobiose) showed that approximately 99% of cellobiose was converted into glucose regardless of CTec2 dose (Table 5.4). However, 29.3 and 47.7% of Avicel was converted into glucose at 5 and 10 FPU/g Avicel of CTec2 loading, respectively. Avicel is crystalline cellulose and interlinking of the cellulose microfibrils via hydrogen bonds may limit hydrolysis (Klemm et al. 2005). BG, CBH, and EG are needed for Avicel conversion to glucose and results suggest that higher levels of CBH and EG may be needed to further improve glucose production from Avicel.

Hydrolysis of cellobiose in the presence of lignin isolates showed that regardless of type of lignin isolate, cellobiose conversion was not significantly different ($p > 0.05$) from when no lignin isolates were present (Figure 5.3). Also, increasing the amount of isolate present in the suspension with cellobiose had no significant ($p > 0.05$) impact on cellobiose conversion. Although BG was significantly bound to lignin isolates (Figure 5.2), it appeared to retain sufficient catalytic activity needed for effective cellobiose hydrolysis.

Avicel conversion also showed similar results as cellobiose (Figure 5.4). Though a higher CTec2 dose (10 FPU/g) produced significantly ($p < 0.05$) more glucose from Avicel than 5 FPU/g CTec2, presence of lignin isolates did not significantly ($p > 0.05$) effect Avicel conversion regardless of type and amount of lignin isolate. Partial cellulase binding related to CBH and EG as observed by SDS-PAGE did not seem to have a significant impact on Avicel conversion in this study. Amount of Lignin (0.25-1.0%, w/v) used in this study might be smaller than the actual amount of lignin from a lignocellulosic biomass during a conventional enzymatic hydrolysis process. Also, the hydrolysis reaction time (72 hour) as conventional hydrolysis period) in this study might be enough time for free CBH and EG (or these CBH and EG bound to the lignin isolates) to convert into glucose which had similar amount from Avicel hydrolysis without lignin isolates. Therefore, as a further study, Avicel hydrolysis with the supernatants (which contains free enzymes not bound to lignin isolates) from lignin-enzyme binding study can aid in better understanding whether residual activity of enzyme is sufficient to hydrolyze or whether enzymes bound to lignin isolates retain their catalytic activity.

Table 5.4. Glucose production through hydrolysis of cellobiose and Avicel. Includes data from controls

Control	Glucose (g/L)
Buffer only	0
Substrate only	
0.5% (w/v) Cellobiose	0.02 ± 0.007
0.5% (w/v) Avicel	0
CTec2 only	
5 FPU/g carbohydrates	0.01 ± 0.004
10 FPU/g carbohydrates	0.02 ± 0.002
Cellobiose + CTec2	
5 FPU/g carbohydrates	5.21 ± 0.299
10 FPU/g carbohydrates	5.14 ± 0.233
Avicel + CTec2	
5 FPU/g carbohydrates	1.61 ± 0.263
10 FPU/g carbohydrates	2.53 ± 0.156
Lignin isolates only	
0.5, 1.0, 1.5% NaOH	0
1, 2, 3 % H_2SO_4	0

Table 5.5. Glucan conversion during hydrolysis of residual glucan in lignin isolate

		Glucan Conversion (%)		
Type of LI	CTec2 loading (FPU/g $_{\text{lignin isolate}}$)	0.25% LI	0.5% LI	1.0% LI
0.5N	5	4.6 ± 0.33	4.4 ± 1.50	3.3 ± 0.57
	10	5.2 ± 0.48	5.9 ± 1.65	4.4 ± 1.36
1.0N	5	7.9 ± 1.54	8.0 ± 1.05	6.4 ± 0.90
	10	9.5 ± 1.13	9.4 ± 0.31	9.7 ± 0.97
1.5N	5	7.9 ± 0.92	8.2 ± 0.80	7.8 ± 0.56
	10	8.5 ± 0.67	10.7 ± 0.76	9.5 ± 0.34
1.0H	5	3.6 ± 0.51	2.3 ± 0.47	2.5 ± 0.55
	10	3.0 ± 0.87	2.6 ± 0.39	2.4 ± 0.23
2.0H	5	3.2 ± 0.04	1.7 ± 0.06	1.9 ± 0.23
	10	3.5 ± 1.27	2.7 ± 0.38	2.0 ± 0.01
3.0H	5	2.8 ± 0.46	2.0 ± 0.54	1.7 ± 0.21
	10	2.7 ± 1.46	2.3 ± 0.30	2.4 ± 0.16

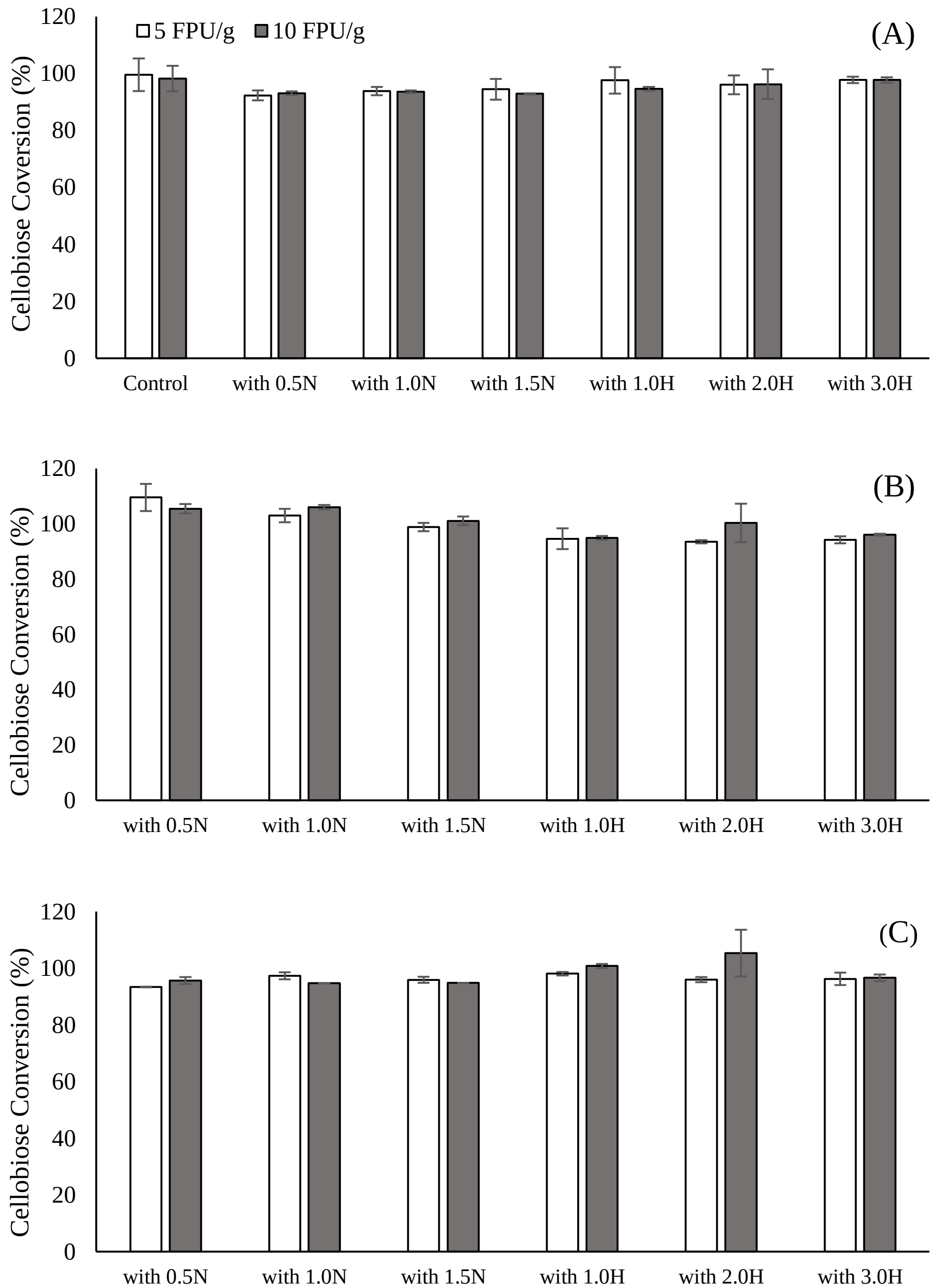


Figure 5.3. Cellobiose conversion during hydrolysis of lignin isolates from various pretreatments mixed with cellobiose as (A) 0.25% LI:0.5% cellobiose, (B) 0.5% LI:0.5% cellobiose, and (C) 1.0% LI:0.5% cellobiose. Control as cellobiose only

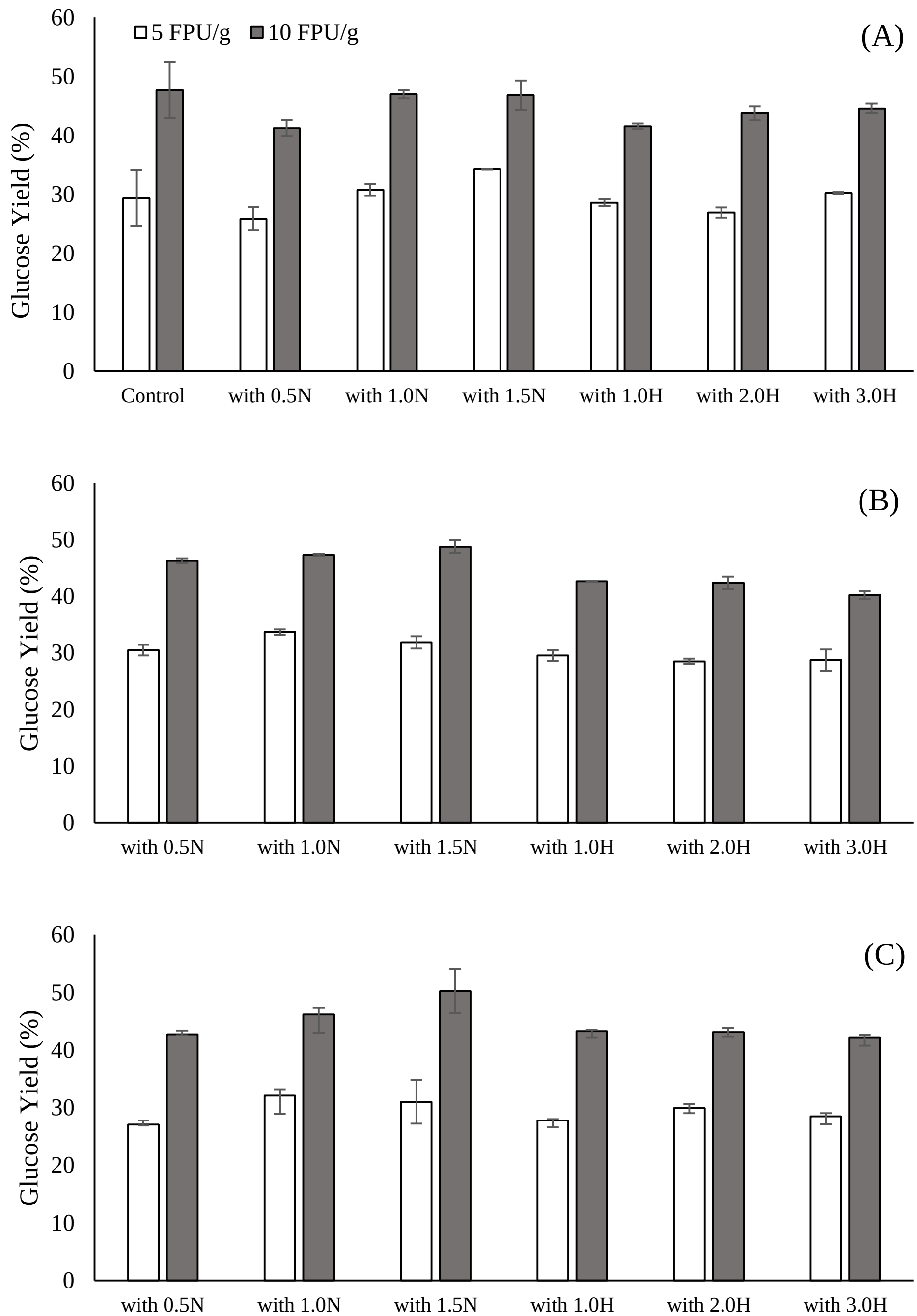


Figure 5.4. Avicel conversion during hydrolysis of lignin isolates from various pretreatments mixed with Avicel as (A) 0.25% LI:0.5% Avicel, (B) 0.5% LI:0.5% Avicel, and (C) 1.0% LI:0.5% Avicel. Control as Avicel only

4. CONCLUSION

Higher content of lignin in isolates derived from NaOH pretreated miscanthus was observed with increase in chemical concentration. On the other hand, H_2SO_4 concentration did not have an impact on lignin content in the isolates. Though morphological changes in lignin isolates due to the two chemical pretreatments were observed, tendency of enzyme binding onto lignin isolates was similar. Of all the cellulolytic enzymes likely to comprise CTec2, β-glucosidase had a significantly higher tendency to bind to lignin isolates. Some CBH, EG and XYN were observed to be partially bound to lignin isolates. Nevertheless, addition of these isolates in various amounts during hydrolysis of carbohydrate model compounds (cellobiose and Avicel) did not significantly affect their conversion to glucose indicating that the enzymes were catalytically active despite being bound to lignin. Although this study was performed with carbohydrate model compounds suspended freely with lignin isolates unlike lignocellulosic biomass in which cellulose and hemicellulose exist in a complex matrix with lignin, it provides notable information about varying tendencies of cellulolytic enzymes towards binding on lignin. Researchers have shown evidence of non-productive binding but results of this study indicate that binding alone may not reduce or limit enzymatic efficiency. Lower lignin loading (0.25-1.0%, w/v) and longer hydrolysis time (72 hour) used in this study, which were than other researches like Guo et al. (2014) (3.3% lignin and 48 hour), might provide free CBH and EG (or these enzymes bound to lignin isolates) with enough condition to produce maximum glucose within the limits of the possible from Avicel.

REFERENCES

Belmokhtar N, Habrant A, Lopes Ferreira N, Chabbert B (2013) Changes in Phenolics Distribution After Chemical Pretreatment and Enzymatic Conversion of Miscanthus x giganteus Internode 6:506-518. doi: 10.1007/s12155-012-9275-2.

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006a) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198-209. doi: 10.1016/j.jbiotec.2006.02.021.

Berlin A, Balakshin M, Gilkes N, Kadla J, Maximenko V, Kubo S, Saddler J (2006b) Inhibition of cellulase, xylanase and beta-glucosidase activities by softwood lignin preparations. J Biotechnol 125:198-209. doi: 10.1016/j.jbiotec.2006.02.021.

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519-546. doi: 10.1146/annurev.arplant.54.031902.134938.

Chauve M, Mathis H, Huc D, Casanave D, Monot F, Lopes Ferreira N (2010) Comparative kinetic analysis of two fungal beta-glucosidases. Biotechnol Biofuels 3:3. doi: 10.1186/1754-6834-3-3.

Chirico WJ, Brown RD (1987) Purification and Characterization of a Beta-Glucosidase from Trichoderma-Reesei 165:333-341. doi: 10.1111/j.1432-1033.1987.tb11446.x.

Dien BS, Jung HG, Vogel KP, Casler MD, Lamb JFS, Iten L, Mitchell RB, Sarath G (2006) Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. Biomass Bioenerg 30:880-891. doi: 10.1016/j.biombioe.2006.02.004.

Dong D, Fricke A, Moudgil B, Johnson H (1996) Electrokinetic study of kraft lignin. Tappi J 79:191-197.

Guo F, Shi W, Sun W, Li X, Wang F, Zhao J, Qu Y (2014) Differences in the adsorption of enzymes onto lignins from diverse types of lignocellulosic biomass and the underlying mechanism. Biotechnol Biofuels 7:38. doi: 10.1186/1754-6834-7-38.

Haque MA, Barman DN, Kang TH, Kim MK, Kim J, Kim H, Yun HD (2013) Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of Miscanthus sinensis at boiling temperature with low residence time 114:294-305. doi: 10.1016/j.biosystemseng.2013.01.006.

Haven MO, Jorgensen H (2013) Adsorption of beta-glucosidases in two commercial preparations onto pretreated biomass and lignin. Biotechnol Biofuels 6:165. doi: 10.1186/1754-6834-6-165.

Hui JPM, Lanthier P, White TC, McHugh SG, Yaguchi M, Roy R, Thibault P (2001) Characterization of cellobiohydrolase I (Cel7A) glycoforms from extracts of Trichoderma reesei using capillary isoelectric focusing and electrospray mass spectrometry 752:349-368. doi: 10.1016/S0378-4347(00)00373-X.

Kim TH (2012) Comparison of inhibition effects of various isolated lignins on enzymatic hydrolysis of cellulose. Korean J Chem Eng 29:82-88. doi: 10.1007/s11814-011-0150-1.

Klemm D, Heublein B, Fink H, Bohn A (2005) Cellulose: Fascinating biopolymer and sustainable raw material. Angew Chem -Int Edit 44:3358-3393. doi: 10.1002/anie.200460587.

Ko JK, Ximenes E, Kim Y, Ladisch MR (2015) Adsorption of Enzyme Onto Lignins of Liquid Hot Water Pretreated Hardwoods. Biotechnol Bioeng 112:447-456. doi: 10.1002/bit.25359.

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. Ind Eng Chem Res 48:3713-3729. doi: 10.1021/ie801542g.

Liu Z, Padmanabhan S, Cheng K, Schwyter P, Pauly M, Bell AT, Prausnitz JM (2013) Aqueous-ammonia delignification of miscanthus followed by enzymatic hydrolysis to sugars. Bioresour Technol 135:23-29. doi: 10.1016/j.biortech.2012.10.133.

Lou H, Zhu JY, Lan TQ, Lai H, Qiu X (2013) pH-Induced Lignin Surface Modification to Reduce Nonspecific Cellulase Binding and Enhance Enzymatic Saccharification of Lignocelluloses. ChemSusChem 6:919-927. doi: 10.1002/cssc.201200859.

Medve J, Lee D, Tjerneld F (1998) Ion-exchange chromatographic purification and quantitative analysis of Trichoderma reesei cellulases cellobiohydrolase I, II and endoglucanase II by fast protein liquid chromatography 808:153-165. doi: 10.1016/S0021-9673(98)00132-0.

Nakagame S, Chandra RP, Kadla JF, Saddler JN (2011a) The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. Bioresour Technol 102:4507-4517. doi: 10.1016/j.biortech.2010.12.082.

Nakagame S, Chandra RP, Kadla JF, Saddler JN (2011b) Enhancing the Enzymatic Hydrolysis of Lignocellulosic Biomass by Increasing the Carboxylic Acid Content of the Associated Lignin. Biotechnol Bioeng 108:538-548. doi: 10.1002/bit.22981.

Pribowo AY, Hu J, Arantes V, Saddler JN (2013) The development and use of an ELISA-based method to follow the distribution of cellulase monocomponents during the hydrolysis of pretreated corn stover. Biotechnol Biofuels 6:80. doi: 10.1186/1754-6834-6-80.

Rahikainen J, Mikander S, Marjamaa K, Tamminen T, Lappas A, Viikari L, Kruus K (2011) Inhibition of Enzymatic Hydrolysis by Residual Lignins From Softwood-Study of Enzyme Binding and Inactivation on Lignin-Rich Surface. Biotechnol Bioeng 108:2823-2834. doi: 10.1002/bit.23242.

Rahikainen JL, Evans JD, Mikander S, Kalliola A, Puranen T, Tamminen T, Marjamaa K, Kruus K (2013a) Cellulase-lignin interactions-The role of carbohydrate-binding module and pH in non-productive binding. Enzyme Microb Technol 53:315-321. doi: 10.1016/j.enzmictec.2013.07.003.

Rahikainen JL, Moilanen U, Nurmi-Rantala S, Lappas A, Koivula A, Viikari L, Kruus K (2013b) Effect of temperature on lignin-derived inhibition studied with three structurally different cellobiohydrolases. Bioresour Technol 146:118-125. doi: 10.1016/j.biortech.2013.07.069.

Rosgaard L, Pedersen S, Langston J, Akerhielm D, Cherry JR, Meyer AS (2007) Evaluation of minimal Trichoderma reesei cellulase mixtures on differently pretreated barley straw substrates. Biotechnol Prog 23:1270-1276. doi: 10.1021/bp070329p.

Slutier, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008a). Determination of Extractives in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Slutier, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008b). Determination of Structural Carbohydrates and Lignin in Biomass. NREL Biomass Analysis Technology Team Laboratory Analytical Procedure, NREL, Golden, CO.

Sorensen H, Pedersen S, Vikso-Nielsen A, Meyer A (2005) Efficiencies of designed enzyme combinations in releasing arabinose and xylose from wheat arabinoxylan in an industrial ethanol fermentation residue. Enzyme Microb Technol 36:773-784. doi: 10.1016/j.enzmictec.2005.01.007.

Umezawa T (2010) The cinnamate/monolignol pathway. Phytochem Rev 9:1-17. doi: 10.1007/s11101-009-9155-3.

Vinzant TB, Adney WS, Decker SR, Baker JO, Kinter MT, Sherman NE, Fox JW, Himmel ME (2001) Fingerprinting Trichoderma reesei hydrolases in a commerical cellulase preparation. Appl Biochem Biotechnol 91-3:99-107. doi: 10.1385/ABAB:91-93:1-9:99.

Wang Z, Xu J, Pandey P, Cheng JJ, Li R, Qu R (2012) Improvement of Sugar Production from Transgenic Switchgrass with Low-Temperature Alkali Pretreatment. Energy Fuels 26:3054-3061. doi: 10.1021/ef3004575.

Xu J, Chen Y, Cheng JJ, Sharma-Shivappa RR, Burns JC (2011) Delignification of Switchgrass Cultivars for Bioethanol Production. BioResources 6:707-720.

Yamamura M, Noda S, Hattori T, Shino A, Kikuchi J, Takabe K, Tagane S, Gau M, Uwatoko N, Mii M, Suzuki S, Shibata D, Umezawa T (2013) Characterization of lignocellulose of Erianthus arundinaceus in relation to enzymatic saccharification efficiency 30:25-35. doi: 10.5511/plantbiotechnology.12.1127a.

Yang Y, Sharma-Shivappa R, Burns JC, Cheng JJ (2009) Dilute Acid Pretreatment of Oven-dried Switchgrass Germplasms for Bioethanol Production. Energy Fuels 23:3759-3766. doi: 10.1021/ef900043z.

CHAPTER 6

CONCLUSION AND FUTURE WORK

In this study, switchgrass and *Miscanthus* x *gigantues* were used to investigate how sodium hydroxide (NaOH) pretreatment at various concentration (0.5, 1.0, and 1.5% (w/v)) and pretreatment times (15, 30, and 60 min) affects their lignin chemistry and subsequent ability of cellulolytic enzymes to hydrolyze carbohydrates present in the pretreated biomass. The major conclusions are listed below:

1. While there was no significant ($p > 0.05$) glucan loss due to NaOH pretreatment, higher sodium hydroxide concentration removed xylan from switchgrass and miscanthus by up to 28.3% and 31.3%, respectively.
2. NaOH resulted in significant ($p > 0.05$) delignification of switchgrass and miscanthus ranging from 44.0 to 84.6% and 42.3 to 84.6%, respectively, depending on severity of NaOH pretreatment.
3. Based on nitrobenzene oxidation analysis for studying changes in lignin chemistry of lignocellulosic biomass, NaOH concentration was a critical factor effecting *p*-hydroxypenyl to guaiacyl ratio (H/G ratio). The H/G ratio of raw switchgrass and miscanthus (both H/G = 0.48_{raw}) decreased significantly to 0.14-0.18 and 0.13-0.15, respectively, when treated at higher NaOH concentration regardless of pretreatment times ($p < 0.05$). On the other hand, S/G ratio (= $0.62_{\text{raw switchgrass}}$) of switchgrass did not change with 15 min pretreatment but increased ($p < 0.05$) to 0.75 and 0.72 with 0.5% NaOH with 30 and 60 min pretreatment. In miscanthus, S/G ratio (=$0.64_{\text{raw miscanthus}}$)

increased to 0.77 with 0.5% NaOH pretreatment but there was no significant change at higher NaOH concentrations. These results indicate that H unit monolignol can be more easily degraded by NaOH than S and G unit monolignols. It can be inferred that the lignin fractions with high proportion of H units have smaller polymers due to the instability of free radicals (for lignification) on aryl side from non-methoxylated phenolic compounds like H units. Smaller polymers can be easily degraded by NaOH. These findings suggest that increasing the proportion of H units in lignin via genetic manipulation of lignocellulosic biomass may enhance the functional feasibility of the carbohydrate to sugar conversion process.

4. Enzymatic hydrolysis of NaOH pretreated switchgrass and miscanthus with excess Cellic® CTec2 (0.625 g enzyme/g dry pretreated biomass, equivalent to 70 FPU/g pretreated biomass) and HTec2 (0.125 g enzyme/g dry pretreated biomass) produced significantly higher glucose and xylose. However, there was no significantly difference ($p > 0.05$) in sugars produced from biomass obtained from various pretreatment conditions. Maximum glucose and xylose yields from switchgrass and miscanthus were 341.7 $mg_{glucose}/g_{raw\ switchgrass}$ (0.5% NaOH for 60min) and 149.4 $mg_{xylose}/g_{raw\ switchgrass}$ (1.0% NaOH for 60min), and 366.8 $mg_{glucose}/g_{raw\ miscanthus}$ (1.5% NaOH for 15 min) and 162.71 $mg_{xylose}/g_{raw\ miscanthus}$ (1.0% NaOH for 30 min), respectively.

Investigating monolignol changes in switchgrass and miscanthus provided some insight into the effect of alkali pretreatment on overall lignin chemistry but the effect of these changes on cellulolytic enzyme hydrolysis and sugar production could not be clearly explained. Hence to investigate how changes in lignin chemistry impact cellulolytic enzymes, binding of cellulolytic enzymes from Cellic® CTec2 on 6 types of lignin isolates obtained from alkali (0.5, 1.0, and 1.5% NaOH at 121ºC for 30 min) and acid (1, 2, and 3% H_2SO_4 at 121ºC for 60 min) pretreated switchgrass and miscanthus was investigated. Carbohydrate model compounds (cellobiose and Avicel) were enzymatically hydrolyzed in the presence and absence of lignin rich isolates to study how enzyme binding on lignin affects their hydrolytic efficiency. Three carbohydrate-lignin loadings (0.5:0.25, 0.5:0.5, and 0.5:1.0 % (w/v)) were also used. The major conclusions from this study are listed below:

1. Based on SDS-PAGE results, β-glucosidase (BG) was found to be significantly bound to all lignin isolates. Some enzymes in CTec2, which might be under the classification of cellobiohydrolases (CBH), endo-1,4-β-glucanases (EG), and xylanase, were also observed to partially bind to the lignin isolates regardless of pretreatment chemical and concentration.
2. Even though enzyme binding to lignin isolates was observed, hydrolysis in the presence of these isolates did not have a significant ($p > 0.05$) impact on glucose production from cellobiose and Avicel. Overall, 99% of cellobiose to glucose conversion was achieved via hydrolysis with CTec2 at both 5 and 10 FPU/g carbohydrates. Approximately 29.5 and 47.0% of Avicel was converted into glucose during hydrolysis with 5 and 10 FPU/g, respectively.
3. β-glucosidase (BG) had a strong tendency to bind to all lignin isolates, but it could still play an important role as a biological catalyst for conversion of cellobiose to glucose. Also, based on the result of Avicel conversion to glucose, it can be inferred that other enzymes in CTec2 that were partially bound to lignin isolates also maintained

functionality. Further investigation is needed to determine if cellulases, in the liquid fraction from lignin-carbohydrate hydrolysis, which were not bound to lignin isolates contain catalytic activity which is sufficient for cellulose hydrolysis. This will also help in elucidating the effect of enzyme binding on carbohydrates conversion.

Some other suggestions for future work include:

1. Individual enzyme activity testing is needed to identify the cellulases specific interaction between enzymes and lignin isolates. Identity of these enzymes could not be accurately confirmed by SDS-PAGE due to their similar protein molecular weights. These test could help establish the changes in enzyme binding intensity based on type of lignin isolates.
2. Since pH of the hydrolysate can impact electrostatic charges on lignin and cellulases, various pH conditions should be investigated to understand electrostatic interactions of cellulolytic enzymes with lignin isolates.
3. Studying the hydrophobicity of lignin isolates obtained from different pretreatment conditions may also help to understand how changes in lignin chemistry affect their overall hydrophobic features and how changes in hydrophobic features affect enzyme binding on lignin isolates.

APPENDICES

Appendix A

Sample SAS code

This SAS code was used to evaluates the effects of pretreatment time, concentration (Conc) and their interaction on glucose yield (Glu), glucan conversion (GluCon), xylose yield (Xyl) and xylan conversion (XylCon) for NaOH pretreatment of switchgrass at 121^{o}C.

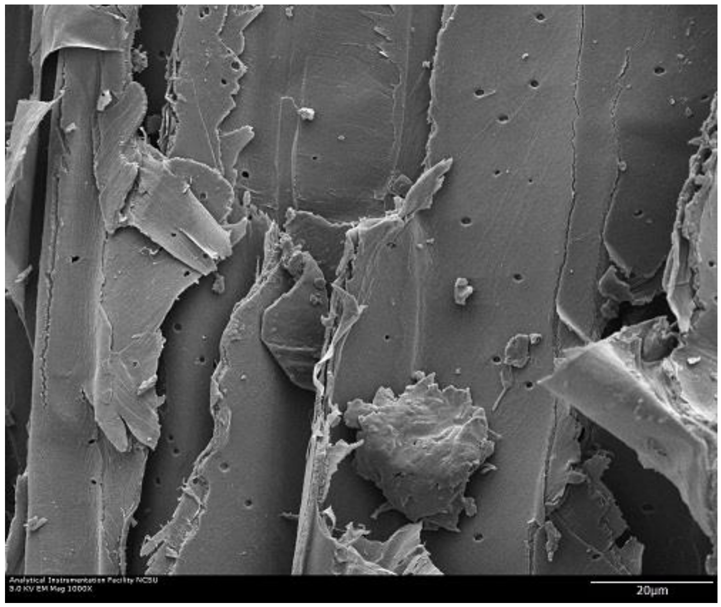
```
data Sugar;
input Time$ Conc$ Glu GluCon Xyl XylCon;
datalines;
15    0.5   288.6626725 78.72961377 124.1135666 57.86388745
15    0.5   275.4847701 75.13548379 114.5196728 53.39104856
15    0.5   262.9900398 71.72768159 106.915329  49.84577216
15    1     311.8175107 85.04484481 141.8392858 66.12792376
15    1     270.7439702 73.84248202 119.5629214 55.74229809
15    1     322.0980044 87.84873799 145.148174  67.67058458
15    1.5   302.0906965 82.39196171 123.8064408 57.72070011
15    1.5   296.1994768 80.78519542 126.6360341 59.03990538
15    1.5   283.2422798 77.25126045 117.7292957 54.88743016
30    0.5   324.8898111 88.610173   132.3534621 61.70546902
30    0.5   286.5423009 78.15130541 114.5278396 53.39485606
30    0.5   293.0787811 79.9340595  122.5192659 57.12059696
30    1     318.9871837 87.00029538 140.4921559 65.49986854
30    1     294.8477903 80.41653758 131.8733807 61.48164677
30    1     278.6425721 75.99674004 125.2321701 58.38539974
30    1.5   302.266731  82.43997322 126.3847434 58.92274929
30    1.5   301.4160394 82.20795631 130.3429984 60.76815611
30    1.5   312.4996134 85.23088093 134.4487993 62.68235135
60    0.5   326.4000579 89.02207644 141.4391431 65.94137033
60    0.5   326.7887863 89.12809789 140.1195327 65.32614518
60    0.5   278.680172  76.00699501 120.9418035 56.38515676
60    1     273.914282  74.70715018 119.3267269 55.63218017
60    1     319.8036572 87.22297968 145.0886031 67.64281158
60    1     314.0025038 85.64077799 142.9699163 66.65504322
60    1.5   284.8446133 77.68827952 118.9034431 55.43483795
60    1.5   291.2975911 79.44825924 122.8429024 57.27148188
60    1.5   293.4435487 80.03354593 124.8519966 58.20815626

;
proc print data=lignin;
run;

proc glm;
class Time Conc;
model Glu=Time|Conc;
```

```
lsmeans Time|Conc/pdiff adjust=tukey;
run;

proc glm;
class Time Conc;
model GluCon=Time|Conc;
lsmeans Time|Conc/pdiff adjust=tukey;
run;

proc glm;
class Time Conc;
model Xyl=Time|Conc;
lsmeans Time|Conc/pdiff adjust=tukey;
run;

proc glm;
class Time Conc;
model XylCon=Time|Conc;
lsmeans Time|Conc/pdiff adjust=tukey;
run;
```

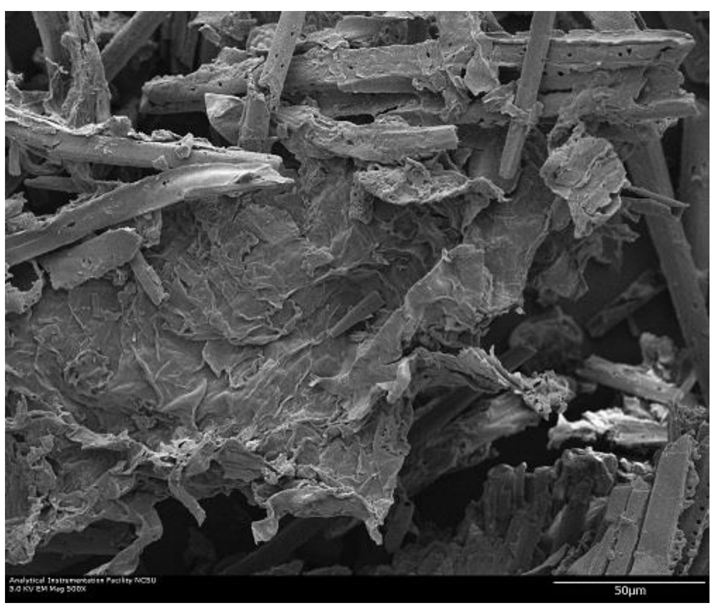
Appendix B

Additional SEM images for lignin isolates obtained from pretreated switchgrass

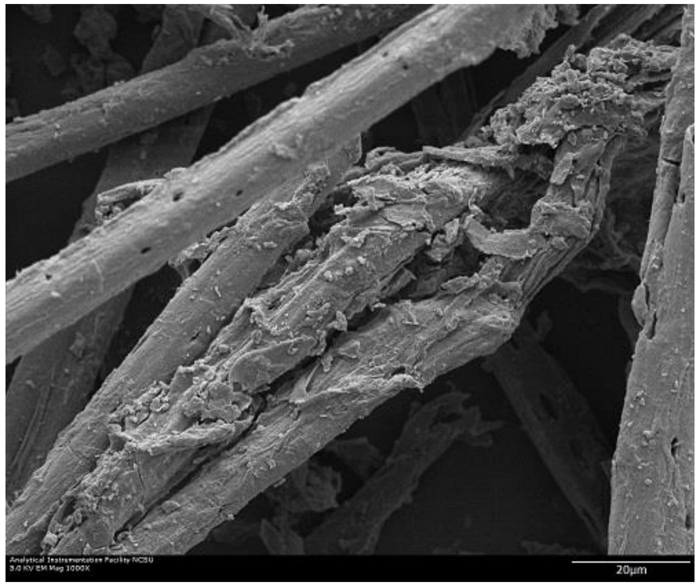
Appendix B.1 – Raw switchgrass (Magnification 1000X)



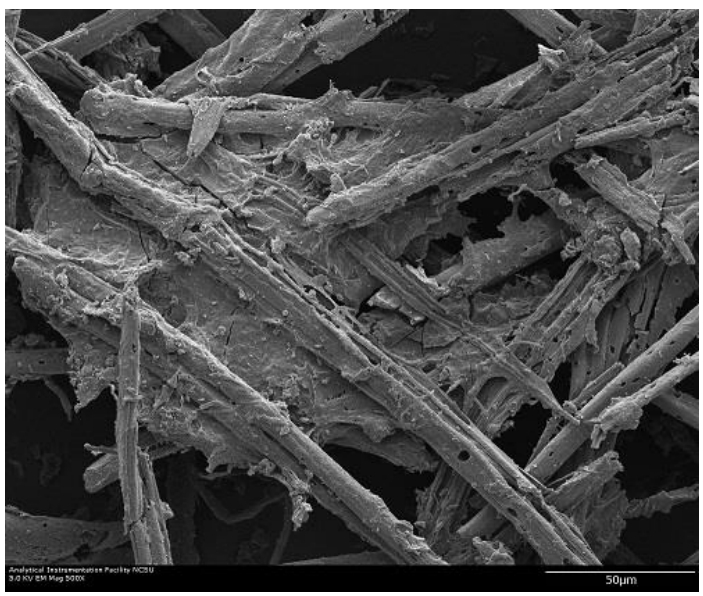
Appendix B.2 – Lignin isolate obtained from switchgrass pretreated with 0.5% NaOH at 121°C for 30 min (Magnification 500X)



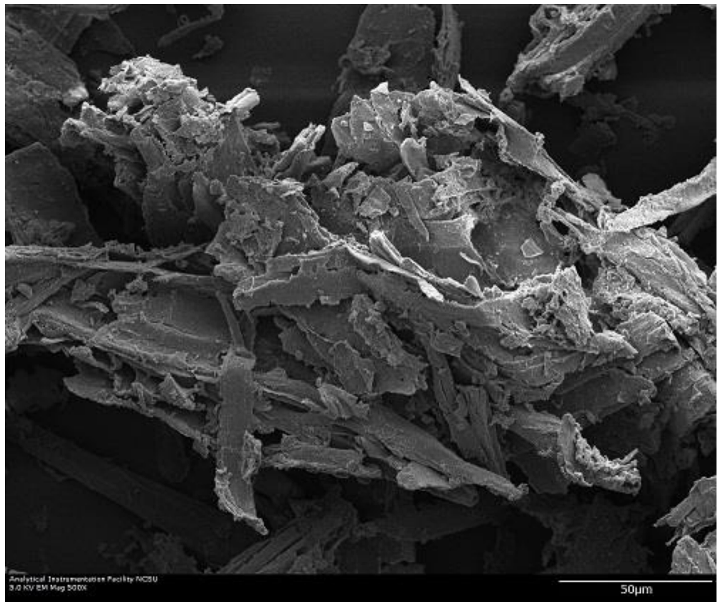
Appendix B.3 – Lignin isolate obtained from switchgrass pretreated with 1.0% NaOH at 121ºC for 30 min (Magnification 1000X)



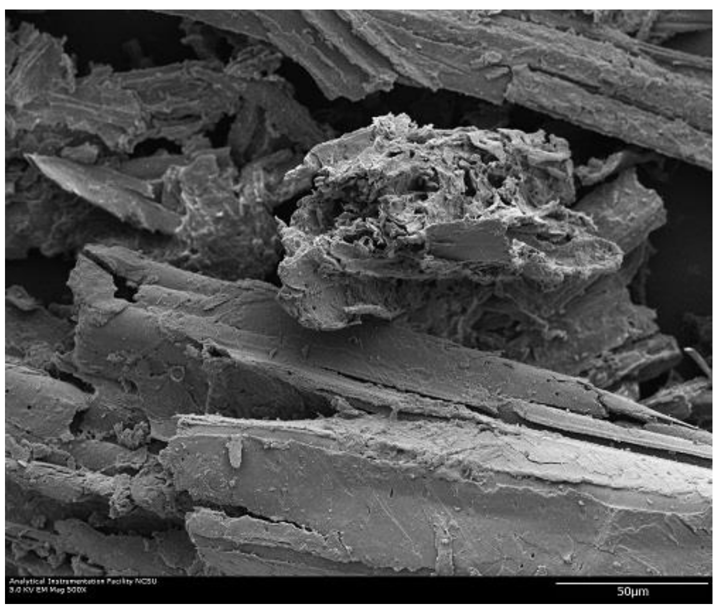
Appendix B.4 – Lignin isolate obtained from switchgrass pretreated with 1.5% NaOH at 121ºC for 30 min (Magnification 500X)



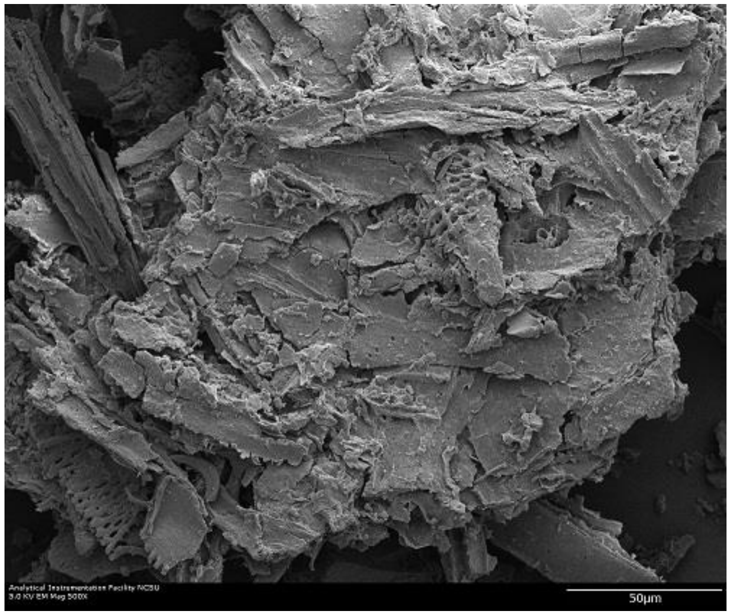
Appendix B.5 – Lignin isolate obtained from switchgrass pretreated with 1.0% H_2SO_4 at 121^{o}C for 60 min (Magnification 500X)



Appendix B.6 – Lignin isolate obtained from switchgrass pretreated with 2.0% H_2SO_4 at 121^{o}C for 60 min (Magnification 500X)



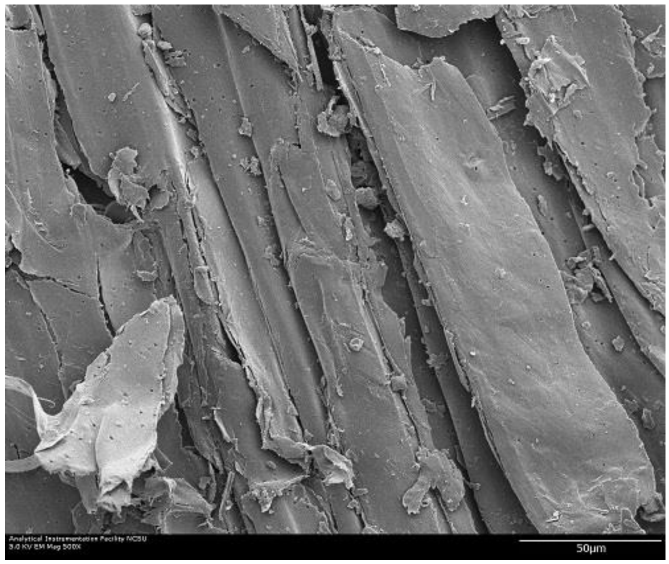
Appendix B.7 – Lignin isolate obtained from switchgrass pretreated with 3.0% H_2SO_4 at 121°C for 60 min (Magnification 500X)



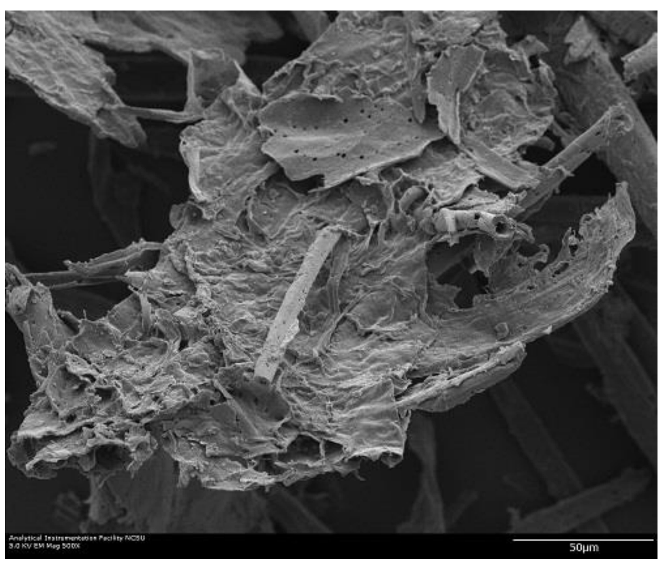
Appendix C

Additional SEM images for lignin isolates obtained from pretreated miscanthus

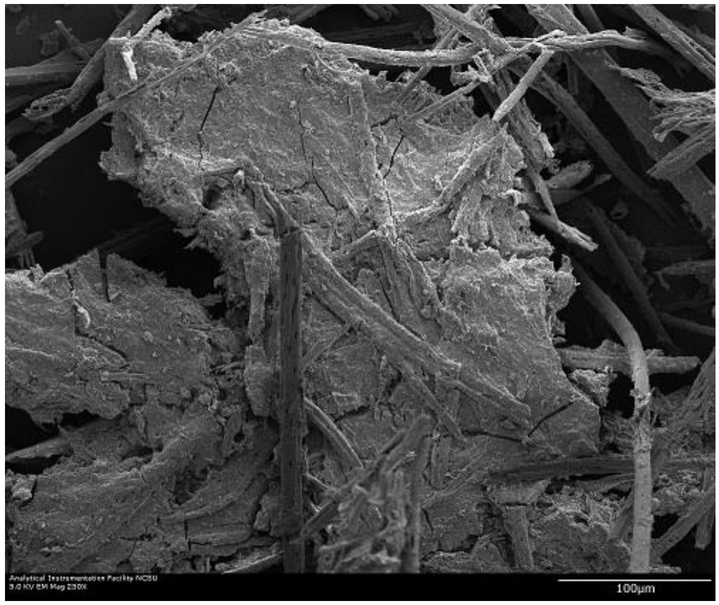
Appendix C.1 – Raw miscanthus (Magnification 500X)



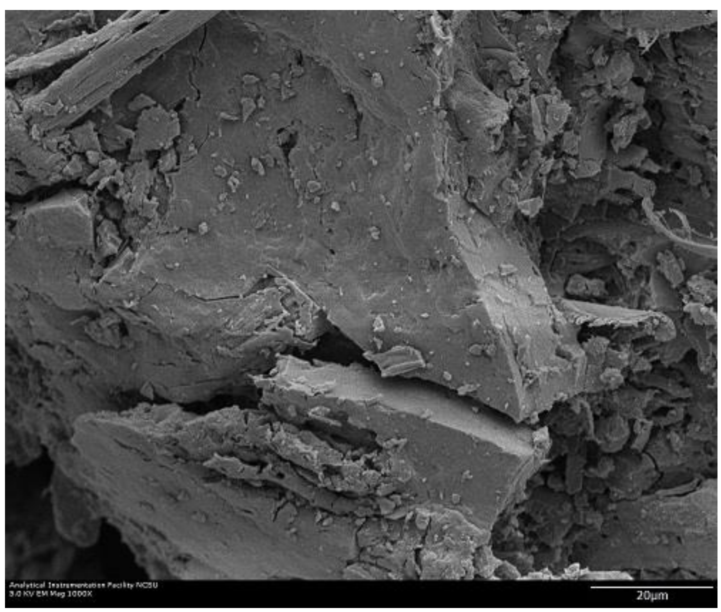
Appendix C.2 – Lignin isolate obtained from miscanthus pretreated with 0.5% NaOH at 121^{o}C for 30 min (Magnification 500X)



Appendix C.3 – Lignin isolate obtained from miscanthus pretreated with 1.0% NaOH at 121°C for 30 min (Magnification 250X)

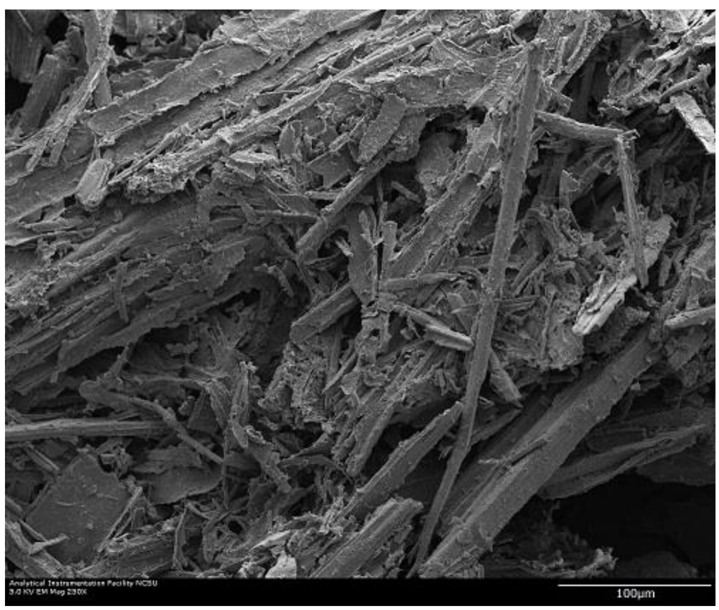


Appendix C.4 – Lignin isolate obtained from miscanthus pretreated with 1.5% NaOH at 121°C for 30 min (Magnification 1000X)



Appendix C.5 – Lignin isolate obtained from miscanthus pretreated with 1.0% H_2SO_4 at 121^{o}C for 60 min (Magnification 250X)

Appendix C.6 – Lignin isolate obtained from miscanthus pretreated with 2.0% H_2SO_4 at 121^{o}C for 60 min (Magnification 250X)



Appendix C.7 – Lignin isolate obtained from miscanthus pretreated with 3.0% H_2SO_4 at 121°C for 60 min (Magnification 500X)