Changes in Lignin Chemistry of Switchgrass due to Delignification by Sodium Hydroxide Pretreatment

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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) was used to study changes in lignin chemistry, and indicated that at higher NaOH concentrations, the amount of 4-hydroxygenzaldehyde (Hy) degraded from p-hydroxyphenyl propanol (H) lignin units was significantly reduced (p < 0.05). However, amounts of syringic (SA) and vanillic (VA) acids generated from syringyl (S) and guaiacyl (G) degradation were greater at higher NaOH concentration. S/G ratio (=0.62 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 raw switchgrass) decreased significantly to 0.14 regardless of pretreatment times. Overall, the H unit 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: switchgrass; lignin chemistry; syringyl; guaiacyl; p-hydroxyphenyl; lignin monomer ratio; nitrobenzene oxidation

1. Introduction

Lignocellulosic biomass, a resource which is 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 the production of bioethanol through enzymatic conversion to fermentable sugars such as glucose and xylose. On the other hand, lignin is a biosynthesized phenolic compound formed by the oxidative coupling of p-hydroxycinnamyl alcohols and related compounds like guaiacyl (G), syringyl (S), and p-phenolylpropanol (H) monolignols cross-linked by a variety of bonds. β-O-4-linked aryl ether bonds form non-condensed linkages which are relatively easier to cleave compared to condensed linkages like carbon-carbon bonds [1–5]. 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 [6,7]. However, its complex structure makes lignin the most recalcitrant substance, preventing the conversion of carbohydrates to monomers by blocking cellulolytic enzyme excess.
Due to lignin’s inhibitory effect on the 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 [3,8–10]. However, there is little knowledge on how pretreatment intensity affects the 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 (Panicum virgatum) is a warm-season perennial grass that has been identified as a significant bioresource for second-generation bioethanol production because of its high carbohydrate content, rapid growth, and excellent biomass yield with relatively less water and nitrogen inputs. It is also well-adapted to various soil and climate conditions [11–15]. Hence, in this study, switchgrass was selected to investigate changes in lignin chemistry caused by sodium hydroxide (NaOH) pretreatment and how they impact enzyme hydrolysis. The effect of pretreatment at various NaOH concentrations and pretreatment times on the 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.

2. Materials and 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 h. 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 °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 based on previous research by our group and others [16–19]. 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 recover the solids. Approximately 1 g of the wet pretreated biomass was dried in a 105 °C convection oven to estimate moisture content and solid recovery. Additionally, about 4 g of the wet pretreated biomass was dried in a vacuum oven (Model 281A, Fisher Scientific, Waltham, MA, USA) at 40 °C for composition analysis and nitrobenzene oxidation of the pretreated biomass.

2.3. Enzymatic Hydrolysis

Pretreated switchgrass at 8% solid loading (dry basis) was suspended in 10 mL volume made up by 0.05 M sodium citrate buffer (pH 4.8), 40 µg/mL of tetracycline, CTe2 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, Randor, PA, USA) at 50 °C, 150 rpm for 72 h. Excess enzyme loading of CTe2 (0.625 g of enzyme/g of
pretreated biomass, density = 1.21 g/mL; equivalent to 70 filter paper units (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 the National Renewable Energy Laboratory’s (NREL)’s Analytical Procedure (LAP) was 110 FPU/mL [20]. The hydrolysate was centrifuged (model 5810R, Eppendorf, Hauppauge, NY, USA) at 4000 rpm and 4 °C for 10 min, and filtered supernatants were stored at −80 °C for monomeric sugars (glucose, xylose, arabinose, and galactose) analysis. Samples from the pretreatment condition identified as the most promising were also hydrolyzed at a 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 [21–24]. 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). The 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). The 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 by 1.1. Delignification percentage—the percentage of lignin removed from raw biomass during pretreatment—was determined by Equation (1):

\[
\text{Delignification (\%) = } \left(\frac{(LU - LP \times SR/100)}{LU}\right) \times 100
\]

where LU is lignin content in untreated biomass (g lignin/g untreated biomass), and LP is lignin content in pretreated biomass (g lignin/g pretreated biomass).

The lignin chemistry of untreated and pretreated biomass was analyzed by modified nitrobenzene oxidation [25,26]. Guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) propanol units—the main phenolic compounds making up lignin—can be oxidized into three aldehyde-lignin units (syringaldehyde (Sy), vanillin (V), and 4-hydroxybenzaldehyde (Hy)), respectively, with further oxidation to acid-lignin units (syringic acid (SA), vanillic acid (VA), and 4-hydroxybenzoic acid (HA)) during NBO reaction [27]. After the NBO of switchgrass samples, these phenolic compounds were extracted with methylene chloride (CH₂Cl₂) 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 for HA (Retention Time (RT) = 5.3 min), VA (RT = 6.1 min), SA (RT = 6.8 min), Hy (RT = 7.3 min), V (RT = 8.5 min), and Sy (RT = 9.4 min), were prepared (0.0078 to 0.25 g/L) for quantification of products obtained from switchgrass-derived samples. Typically, twenty micro-liters of the sample solution was injected, and the data acquisition time was 25 min with an eluent (a mixture of acetonitrile (CH₃CN) and water containing 10 mM formic acid (HCOOH) flow rate of 1.5 mL/min). S/G and H/G molar ratios for samples from various pretreatments were calculated by Equations (2) and (3) below:

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

\[
\text{H/G molar ratio} = \frac{\text{(p-Hydroxybenzaldehyde (Hy), (\mu mol/g lignin))}}{\text{(Vanillin (V) + Vanillin Acid (VA), (\mu mol/g lignin))}}}
\]
2.5. Statistical Analysis

All treatments were performed in triplicate. A 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 and 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 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 contents were 3.2% and 2.8%, respectively.

Table 1. The 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). Overall, solid recovery decreased with an 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 to 82.3% (g pretreated biomass/g untreated biomass) from the highest to the lowest pretreatment severity.

Table 2. Solid recovery, composition of pretreated switchgrass, and delignification after NaOH pretreatment at varying conditions.

| Parameter                        | Time (min) | NaOH Concentration (%) |
|----------------------------------|------------|------------------------|
|                                  |            | 0.5        | 1.0        | 1.5        |
| Solid Recovery (wt %) $^1$       | 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 %) $^2$               | 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 %) $^2$                | 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 |
Composition analysis of pretreated switchgrass included glucan, xylan, and lignin (AIL + ASL). The data suggested that there was no significant \( p > 0.05 \) loss in glucan due to pretreatment. However, a change in xylan was observed with an increase in pretreatment intensity. Though pretreatment time did not have a significant \( p > 0.05 \) effect on xylan reduction and there was no significant \( p > 0.05 \) loss in xylan with 0.5% NaOH pretreatment, NaOH pretreatments at 1.0 and 1.5% led to significant \( p < 0.05 \) reduction in xylan, perhaps due to either extraction or degradation under NaOH environment \[28,29\]. An estimation of changes in lignin content after NaOH pretreatment showed that higher concentration and longer pretreatment times led to increased delignification (Table 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 \[8,30,31\], resulting in the removal of lignin and xylan from the biomass, which further contributes to lower solid recovery.

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, and Hy from H-lignin unit) from untreated and pretreated switchgrass were detected and quantified, and corresponding total yields were calculated (Table 3). HA was not detected in the NBO samples from untreated and pretreated switchgrass (Figure 1). Sy, V, and Hy were the predominantly observed compounds, while the acid groups (SA, VA) produced due to further oxidation of the aldehyde groups were in relatively lower amounts.

Table 2. Cont.

| Parameter                  | Time (min) | 0.5       | 1.0       | 1.5       |
|----------------------------|------------|-----------|-----------|-----------|
| Lignin (wt %) \(^2\)       | 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 %) \(^3\)| 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 pretreated biomass g\(^{-1}\) untreated biomass); \(^2\) (g g\(^{-1}\) dry untreated biomass); \(^3\) (lignin removed from raw biomass).

Figure 1. A representative HPLC chromatograph showing lignin degradation compounds generated during nitrobenzene oxidation (NBO). Hy: 4-hydroxybenzaldehyde; SA: syringic acid; Sy: syringaldehyde; V: vanillin; VA: vanillic acid.
Table 3. Changes in the ratio of S/guaiacyl (G), p-hydroxyphenyl (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 | 32:48:23 | 26.1 ± 2.1 |
| 15       | 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       | 0.75 ± 0.02 | 0.29 ± 0.03 | 37:49:14 | 20.4 ± 2.9 |
| 0.5      | 0.66 ± 0.03 | 0.19 ± 0.03 | 36:54:10 | 17.8 ± 0.8 |
| 1.0      | 0.73 ± 0.03 | 0.14 ± 0.01 | 39:54:7  | 26.2 ± 0.8 |
| 1.5      | 0.72 ± 0.03 | 0.28 ± 0.02 | 36:50:14 | 25.7 ± 2.8 |
| 60       | 0.69 ± 0.01 | 0.18 ± 0.01 | 37:53:10 | 30.1 ± 3.1 |
| 0.5      | 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.

The 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 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). Conversely, the amounts of SA and VA generated from pretreated biomass were significantly higher (\(p < 0.05\)) than untreated biomass, and higher NaOH concentration increased the amounts of the two acid units produced. Increase in SA and VA indicated that changes in lignin structure of switchgrass subjected to more severe NaOH pretreatments made its lignin units more susceptible to oxidation.

The results of this study showed that NaOH significantly changes lignin chemistry, especially causing a 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 a higher proportion of S and G units and a lower proportion of H units in lignin compared to untreated switchgrass. Changes were also observed in S/H and G/H 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. Pretreatments of 30 and 60 min had no significant (\(p > 0.05\)) difference of S/G ratio change compared to 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. found that additional methoxy groups at the aryl side can aid lignification via oxidative coupling by stabilizing the free radicals [32]. On the other hand, non-methoxylated lignin monomers might be able to terminate polymerization, leading to the production of smaller lignin polymers. Based on our results and a report by [33], it appears that during pretreatment, NaOH may have selectively cleaved bonds between cellulose, hemicellulose, and H-lignin. Subsequently, this reaction [34] may have allowed fragmented lignin to dissolve in NaOH, resulting in the decrease of H-lignin in switchgrass. Ziebell et al. investigated transgenic alfalfa (Medicago sativa) and found that lignin with predominately H units in the transgenes led to a decrease in the molecular weight of the polymer as compared to control, in which S and G units were prevalent [35]. 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.

Figure 2. Production of degradation compounds through nitrobenzene oxidation of switchgrass samples (mmol/g lignin) pretreated for (a) 15 min; (b) 30 min; and (c) 60 min.

Figure 2. Production of degradation compounds through nitrobenzene oxidation of switchgrass samples (mmol/g lignin) pretreated for (a) 15 min; (b) 30 min; and (c) 60 min.
3.4. Enzymatic Hydrolysis with NaOH-Pretreated Switchgrass

Enzymatic hydrolysis was performed to produce fermentable sugars from NaOH-pretreated samples (Figure 3) and determine if a change in lignin chemistry relative to the proportion(s) of 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 75.2–84.7%, and xylan conversion was 53.7% and 63.3%. Because of the extraction of lignin from switchgrass during pretreatment with NaOH, switchgrass fibers may have become porous, thus allowing greater enzyme access to cellulose and hemicellulose and increasing yields of glucose and xylose [28,36]. Similar results were reported by [37], who also observed increased porosity in switchgrass after pretreatment with NaOH.

Although 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. It was also observed that 100% carbohydrate conversion was not achieved. Hemicelluloses’ shielding effect and increased cellulose crystallinity due to alkali pretreatment might cause incomplete glucan conversion [2,38–40]. Additionally, partial xylan loss due to NaOH pretreatment also resulted in less-than-complete xylose recovery. However, from an economic perspective, our results suggest that even the lower concentration of NaOH (0.5%) employed in this research may be adequate for optimal pretreatment of switchgrass.

Though excess enzyme loading helped to 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 the 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 4) was evaluated.

**Figure 4.** Glucose and xylose yield after enzymatic hydrolysis of switchgrass treated at 1.0% NaOH for 30 min with varying doses of CTec2 and HTec2.

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 correlations that may exist.

4. Conclusions

Sodium hydroxide pretreatment of switchgrass at various conditions resulted in significant delignification and changes in lignin chemistry—especially a decrease in H/G ratio in pretreated switchgrass. Results indicated that H-units are more susceptible to NaOH pretreatment than S and G-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 studies 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.

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Conflicts of Interest: The authors declare no conflict of interest.

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