

Effect of Enzyme Interaction with Lignin Isolated from Pretreated *Miscanthus × giganteus* on Cellulolytic Efficiency

Authors:

Woochul Jung, Ratna Sharma-Shivappa, Praveen Kolar

Date Submitted: 2019-12-10

Keywords: pretreatment, inhibition, lignin, hydrolysis, miscanthus

Abstract:

The effect of binding between the lignin isolates from an alkali (NaOH) and an acid (H₂SO₄) pretreated *Miscanthus* and cellulolytic enzymes in Cellic® CTec2 was investigated. Additionally, cellobiose and Avicel were enzymatically hydrolyzed with and without lignin isolates to study how enzyme binding onto lignin affects its conversion to glucose. Three carbohydrate:lignin loadings (0.5:0.25, 0.5:0.5, and 0.5:1.0% (w/v)) were employed. The results indicated that β -glucosidase (BG) had a strong tendency to bind to all lignin isolates. The 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.

Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):

LAPSE:2019.1346

Citation (this specific file, latest version):

LAPSE:2019.1346-1

Citation (this specific file, this version):

LAPSE:2019.1346-1v1

DOI of Published Version: <https://doi.org/10.3390/pr7100755>

License: Creative Commons Attribution 4.0 International (CC BY 4.0)

Article

Effect of Enzyme Interaction with Lignin Isolated from Pretreated *Miscanthus × giganteus* on Cellulolytic Efficiency

Woochul Jung, Ratna Sharma-Shivappa * and Praveen Kolar *

Biological and Agricultural Engineering, Campus Box 7625, North Carolina State University, Raleigh, NC 27695-7625, USA; wjung3@ncsu.edu

* Correspondence: rsharm2@ncsu.edu (R.S.-S.); pkolar@ncsu.edu (P.K.);

Tel.: +1-919-515-6755 (R.S.-S.); +1-919-513-9797 (P.K.)

Received: 19 September 2019; Accepted: 10 October 2019; Published: 16 October 2019



Abstract: The effect of binding between the lignin isolates from an alkali (NaOH)– and an acid (H₂SO₄)–pretreated *Miscanthus* and cellulolytic enzymes in Cellic[®] CTec2 was investigated. Additionally, cellobiose and Avicel were enzymatically hydrolyzed with and without lignin isolates to study how enzyme binding onto lignin affects its conversion to glucose. Three carbohydrate–lignin loadings (0.5:0.25, 0.5:0.5, and 0.5:1.0% (w/v)) were employed. The results indicated that β-glucosidase (BG) had a strong tendency to bind to all lignin isolates. The 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.

Keywords: miscanthus; hydrolysis; lignin; inhibition; pretreatment

1. Introduction

There is a significant interest in renewable energy production from lignocellulosic biomasses such as *Miscanthus* sp., due to their fast growth rates and high productivity, even with minimal agronomic input [1–4]. Similarly to other biomasses, *Miscanthus* is made of cellulose, hemicellulose, and lignin chemically bonded together [5,6]. Cellulose and hemicellulose are structural carbohydrates that provide sugar for subsequent biofuel and chemical products such as bioethanol and biobutanol [7]. The remaining component, lignin, as a non-carbohydrate aromatic polymer, mainly consists of three phenolic compounds: guaiacyl (G), syringyl (S), and p-hydroxyphenyl (H) propanol [8]. It forms a complex with cellulose and hemicellulose, and this conformational feature in lignocellulosic feedstocks confers their structural stability [9]. From a biorefinery perspective, this very structural rigidity makes lignin a key obstacle to fermentable sugar productions from cellulose and hemicellulose by physically, chemically, and structurally protecting them from hydrolytic enzyme access [10,11]. Specifically, lignin-induced inhibition to enzymatic hydrolysis of structural carbohydrates is due to hydrophobic interaction [12,13], hydrogen bonding [14], and electrostatic repulsion during hydrolysis [15]. When contacted with hydrolytic enzymes, lignin, due to its hydrophobic nature, tends to adsorb the enzymes in a non-productive way, resulting in reduced sugar conversion efficiencies [16]. However, the extent of adsorption has been reported to be dependent on the lignin source. In one of their studies, Pareek et al. [17] systematically investigated six lignins derived from various biomasses and observed that the source of lignin played a significant role in the enzyme adsorption. Additionally, during the

enzymatic hydrolysis, the hydroxyl groups in lignin are believed to interact with cellulases via hydrogen bonding, thereby decreasing the efficacy of the enzymes involved in hydrolysis reactions [16,18]. Furthermore, as has been suggested by several researchers, the electrostatic repulsion between enzymes and lignin under certain conditions can negatively impact enzymatic hydrolysis [19,20].

To decrease lignin-induced inhibition, a pretreatment step is typically necessary to allow enzymes to access structural carbohydrates with greater enzyme saccharification efficiencies [21,22]. 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 [23–25]. Alkali pretreatments, such as those with sodium hydroxide, facilitate improvements in sugar production by solubilizing and extracting lignin (or certain monomers, of lignin such as G and H units) and increasing the surface area available for enzyme attachment [26,27].

Several researchers have investigated a role of lignin in enzymatic hydrolysis [17,28–31]. However, as suggested by Li et al. [32], there is no consensus in the literature regarding lignin's ability to impede carbohydrate hydrolysis. In addition, Saini et al. [16] that lignin content alone is not a predictor of inhibition. The actual inhibition or lack of it may depend on the pretreatment method employed, experimental conditions during the hydrolysis, and even the chemistry of the lignin [33]. Therefore, 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 interacted with the isolated lignin. Model carbohydrate compounds (cellobiose and Avicel) in the presence and absence of lignin isolates were hydrolyzed to understand how variations in pretreatments might influence enzyme-lignin binding and subsequent cellulolytic efficiency.

2. Materials and Methods

2.1. Sample Preparation

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

2.2. Alkali and Acid Pretreatment

Sodium hydroxide (NaOH) and dilute sulfuric acid (H_2SO_4) were used to pretreat the miscanthus before enzymatic hydrolysis. The pretreatment temperature was set at 121 °C for both chemicals, but alkali pretreatment was conducted with NaOH concentrations 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 performed using 1, 2, and 3% (w/v) (designated as 1.0H, 2.0H, and 3.0H) for a pretreatment time of 60 min. Fifty grams of miscanthus was pretreated in five glass serum bottles (125 mL), each containing 10 g of miscanthus and 100 mL of NaOH (or H_2SO_4) with the desired concentration. The serum bottles were crimp sealed and autoclaved at 121 °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 chemicals. 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 4 g was dried in a 40 °C vacuum oven and used for composition analysis. Remaining pretreated miscanthus was stored at 4 °C for subsequent enzyme hydrolysis at a later time, typically within three days.

2.3. Lignin Isolation

Alkali- and acid-pretreated miscanthus were enzymatically hydrolyzed to recover lignin isolates (LI) with 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 at 1.23 and 1.16 g/mL, respectively, were used for hydrolysis. Miscanthus at a solid loading of 8% (w/v) in 0.05 M sodium citrate buffer (pH 4.8) was hydrolyzed with excessive CTec2 dose equivalent to 140 FPU/g dry pretreated biomass supplemented with HTec2 (0.25 g of enzyme/g dry pretreated biomass). Hydrolysis was performed at 50 °C, 150 rpm for 5 days in an air bath shaker (Series 25 incubator shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA). 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, USA) at 4000 rpm for 10 min and washed three times 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 was removed by a protease treatment with a commercial bacterial type (XXIV) protease (subtilisine, Sigma-Aldrich Co., St. Louis, MO, USA) [34,35]. Briefly, the lignin residue was suspended in 0.05 M phosphate buffer (pH 8.5) with 0.1 mg/50 mg lignin isolate of protease and incubated at 37 °C overnight. Further incubation at 90 °C for 2 h was performed to deactivate the proteases. The protease-treated solids were washed three times with DI water (pH 2.5, adjusted by HCl) and dried in a 40 °C vacuum oven and were used as “lignin isolates” (LIs).

2.4. Lignin–Enzyme Binding Study

Incubation of lignin isolated from various pretreatments with the CTec2 cellulase cocktail was performed to investigate enzyme binding onto lignin isolate. First, 1% (w/v) lignin isolate was suspended in 1.5 mL of 0.05 M sodium citrate buffer (pH 4.8) with 5 FPU equivalent CTec2/g lignin isolate and incubated at 50 °C, 150 rpm for 72 h in the air bath shaker (Series 25 incubator shaker, New Brunswick Scientific Co., Inc., Edison, NJ, USA). Supernatant and hydrolyzed lignin isolate were separated by centrifugation at 14,000 rpm for 10 min. The analysis of the supernatant was performed by mixing 1.5 mL of the supernatant liquid with SDS buffer. For analysis of the solid fractions, the lignin isolates were washed using 1.5 mL of 0.05 M sodium citrate buffer, and the wash liquid was subsequently extracted via centrifugation. The process was repeated and the isolates were mixed with 0.3 mL of SDS buffer, as described in [36]. 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. Gel Documentation EQ System (Bio-RAD, Hercules, CA, USA) was used for the SDS-PAGE gel imaging and investigating 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 affected enzymatic hydrolysis efficiency, two model carbohydrate compounds, cellobiose (a disaccharide with two glucose molecules bound via β -(1,4)-glycosidic bonds, Sigma-Aldrich, St. Louis, MO, USA) and Avicel (microcrystalline cellulose, Sigma-Aldrich, St. Louis, MO, USA), were individually hydrolyzed with each of the six 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 1). The cellobiose (or Avicel) with lignin isolate suspension was hydrolyzed with CTec2 at 50 °C, 150 rpm for 72 h in an air bath shaker. The hydrolysate containing glucose produced from cellobiose (or Avicel) was centrifuged and the supernatant was recovered. Control experiments (in 1.5 mL sodium citrate buffer) were also performed, which included (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 1. Experimental design for carbohydrate model compound and lignin isolate hydrolysis.

Material	Experimental Variables for Hydrolysis
Cellobiose and Avicel	0.5% (w/v)
Six types of LI ¹	0.25, 0.5 and 1.0% (w/v)
CTec2	5 and 10 FPU/g carbohydrate

¹ Pretreated with NaOH (0.5, 1.0, and 1.5%) and H₂SO₄ (1, 2, and 3%).

2.6. Composition Analysis

Chemical compositions 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) [37,38]. 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 (glucose and xylose) for composition analysis and glucose in the hydrolysates was determined using YSI 2950 Biochemistry Analyzer (Xylem Inc., Yellow Springs, OH, USA). Monomeric sugar (i.e., glucose and xylose) data were 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 the untreated miscanthus and lignin isolates obtained from alkali- and acid-pretreated miscanthus. The analysis was performed with a 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 the 95% confidence level in SAS 9.3 (Cary, NC, USA) was used to analyze experimental data. All treatments were conducted in duplicate. The main effects were (1) six kinds of pretreatment methods (three from NaOH with 0.5, 1.0, and 1.5%, and other three from H₂SO₄ 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 and Discussion

3.1. Changes in the Chemical Composition of Alkali- and Acid-Pretreated Miscanthus

Analysis of structural carbohydrate content (e.g., glucan and xylan) indicated that the xylan in miscanthus was degraded by both NaOH and H₂SO₄ pretreatments, while glucan remained nearly unchanged (Table 2). NaOH pretreatments at 0.5, 1.0, and 1.5% degraded xylan by 7.3, 27.0, and 31.9%, respectively, while H₂SO₄ degraded much higher amounts of xylan, up to 95.7%. Delignification due to comprehensive disruption of the cross-ester linkages between lignin and hemicellulose [27,39,40] during NaOH pretreatment ranged from 33.2 to 72.8% depending on the NaOH concentration. Lignin content in NaOH-pretreated miscanthus was significantly less than in untreated miscanthus ($p < 0.05$), while that in H₂SO₄-pretreated miscanthus remained nearly unchanged.

Table 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
H ₂ SO ₄	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. AIL is acid-insoluble lignin and ASL is acid-soluble lignin.

3.2. Characterization of Lignin Isolates

The compositions of the lignin isolates obtained from NaOH- and H₂SO₄-pretreated miscanthus are presented in Table 3. Higher NaOH concentrations resulted in higher lignin content (42.1–69.5%) in the isolates, while lignin content in isolates from H₂SO₄-pretreated miscanthus remained similar (58.9–65.9%) ($p > 0.05$). Even though excessive CTec2 and HTec2 were used to remove carbohydrates, lignin isolates from NaOH- and H₂SO₄-pretreated miscanthus contained up to 46.0 and 32.2% glucan and xylan, respectively. The residual glucan and xylan were inferred to be highly resilient polysaccharides resisting conversion to oligomers or monomeric sugars.

Table 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 ND: not detected.

Morphological Changes of Lignin Isolate

Untreated miscanthus and sub-samples of lignin isolates obtained from NaOH- and H₂SO₄-pretreated miscanthus were studied by electron microscopy (SEM) to better understand morphological changes in lignin due to pretreatment (Figure S1). The 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 S1A,B). The structure of lignin isolated from NaOH (1% w/v)-pretreated miscanthus was disrupted and fragmented (Figure S1C,D). Many elongated structures and a clumped clay-like shapeless substance were observed. In comparison, SEM images of lignin isolates from H₂SO₄ (2%)-pretreated miscanthus predominantly showed a clumped substance with some elongated structures (Figure S1E,F). Based on the composition of lignin isolates (Table 3) derived from 1% NaOH (29.1% glucan, 4.4% xylan, and 59.1% lignin) and 2% H₂SO₄ (32.2% glucan and 58.9% lignin), it was inferred that the elongated structures may have been related to linear glucose chains linked by β -(1,4)-glycosidic bonds. Isolation of lignin via chemical pretreatment and enzymatic hydrolysis can disrupt miscanthus structure, as seen in Supplementary Figure S1.

3.3. Enzyme Binding Study of Lignin Isolates—SDS-PAGE Visualization

To better understand the 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. As per Reference, CTec2 derived from *Trichoderma reesei* is reported to contain (1) β -glucosidase with additional BG, (2) at least the two main key cellobiohydrolases (CBH), (Cel6A and Cel7A) and (3) five kinds of endo-glucanases (EG), namely Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A. Some xylanases (XYN) were 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 reveal 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 the type of lignin isolate (data not shown). However, when lignin isolates were incubated with CTec2, investigation of samples from the LF and SF via electrophoresis (Figure 1) showed several protein bands. In their research, Zanchetta et al. [41] studied the adsorption of cellulases on sugarcane-derived lignin and separated Ctec2 into 12 protein components based on molecular weights that ranged between 25 kDa and 150 kDa. Similarly, after identifying 10 proteins in Ctec2, Yarbrough et al. [42] divided the proteins into three groups: large molecular weight greater than 80 kDa, medium molecular weight 30–80 kDa, and lower molecular weight proteins which were less than 30 kDa. Therefore, for easy interpretation, the protein bands in our research were divided into four zones on the basis of the CTec2 control ladder in the gel. Zone 1 (around 75 to 120 kDa) was related to β -glucosidase (BG) which has a relatively high molecular weight compared to other cellulases [30,31]. Zone 2 (around 50 to 75 kDa) was identified as being related to cellobiohydrolase and endo-glucanases, Cel7A, Cel6A, and Cel7B. Zone 3 (around 30 to 50 kDa) was expected to be Cel5A Cel12A, xylanase, and (xyn10a), and Zone 4 (around 20 to 30 kDa) was related to Cel12A, Cel45A, and xylanases (xyn11a and xyn11b), as described by Ko et al. [30].

In Zone 1, intense bands were observed in lanes with SF of lignin isolates obtained from NaOH- and H₂SO₄-pretreated miscanthus regardless of isolate type (Figure 1A,B). On the other hand, the LF lanes seemed to be clear, indicating significant binding of BG to isolated lignin solids. Similar results were reported by Yarbrough et al. [42], who studied the binding of Ctec2 on lignin obtained from corn stover and observed that most of the higher molecular weight proteins bound to lignin were BG. Further, Zanchetta et al. [41] also noted that BG tended to adsorb onto lignin, although the authors observed that the acid-derived sugarcane bagasse lignin exhibited higher adsorption of the enzymes relative to the lignin obtained via enzymatic hydrolysis. The authors of [31] 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 0.05 M sodium citrate buffer. Since proteins gain a net negative charge as pH increases above the isoelectric point (pI) and vice versa, pH can have a significant effect on the binding of BG, which has a theoretical pI of 5.7–6.4 [30] and experimentally reported pI of 8.0–8.5 [43–48]. The authors of [30] 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 have been due to repulsion between lignin and BG caused by an increase in the negative charge of BG as well as that of lignin due to dissociation of the phenolic carboxyl groups [20,49–51].

Cellulases related to Cel7A, Cel6A, and Cel7B in Zone 2 showed some binding tendencies based on the bands from the SF both pretreatment methods. Due to the 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 the CTec2 control. However, only one upper band was detected from the LF (Figure 1A,B) suggesting partial loss due to binding on SF. Overall, the tendency of CTec2 binding onto lignin isolates was similar for NaOH- and H₂SO₄-derived isolates.

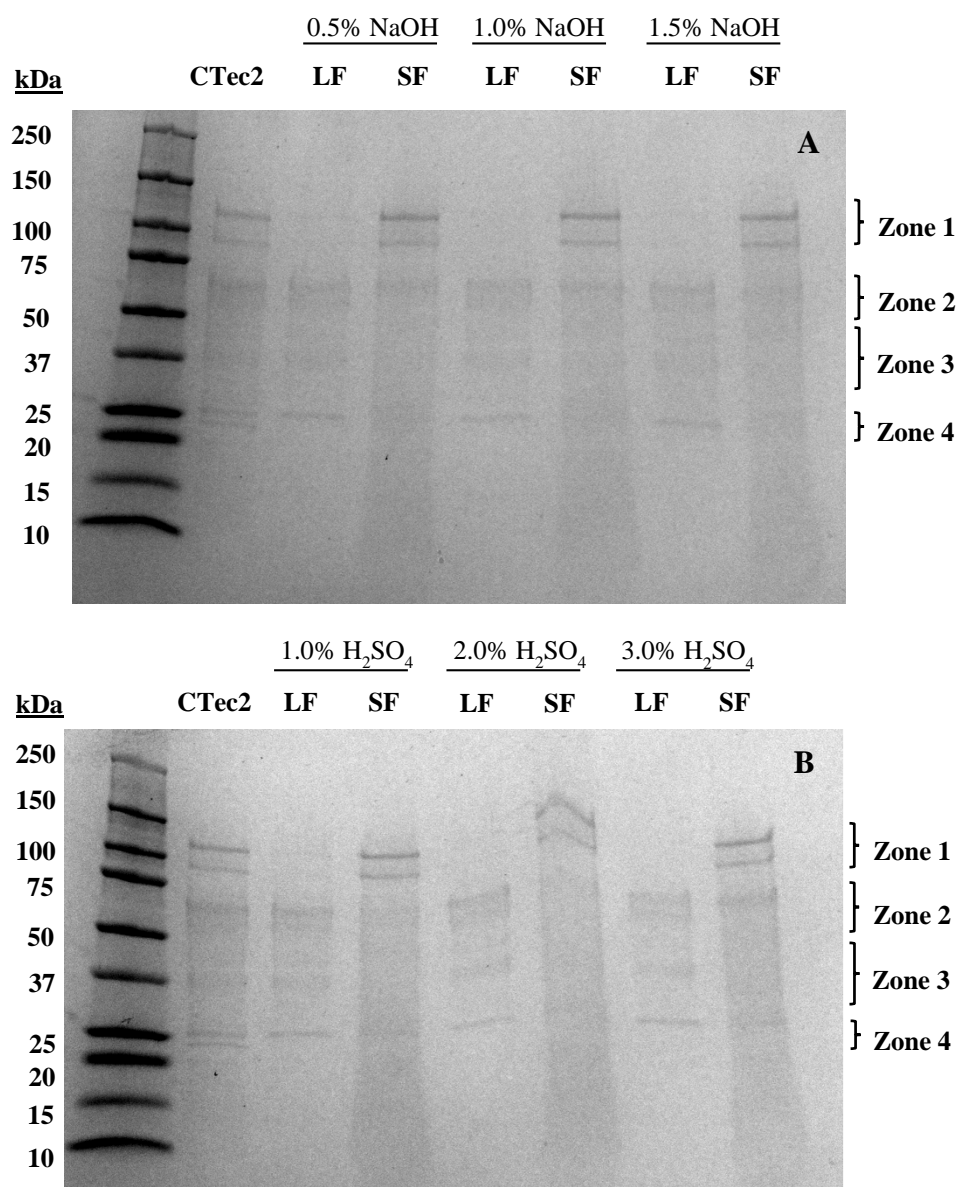


Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel images for liquid (LF) and solid (SF) fractions after hydrolysis (with CTec2 at 5 FPU/g lignin isolate) of lignin isolated from (A) NaOH-pretreated miscanthus and (B) H₂SO₄-pretreated miscanthus.

3.4. Glucose Production during Hydrolysis of Carbohydrate Model Compounds

Before performing enzymatic hydrolysis of carbohydrate model compounds with lignin isolates, the glucose generated from the control samples was estimated (Table 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 baseline glucose production from residual carbohydrates in lignin isolates, the isolates were hydrolyzed with CTec2 at 5 and 10 FPU/g (Table 5). Overall, 3.3–10.7% of the residual glucan in lignin isolates obtained from NaOH pretreatments was converted into glucose during the hydrolysis, while 1.7–3.6% of the glucan in lignin isolates from H₂SO₄-pretreated miscanthus was converted. The amount of glucose generated from lignin isolates and control factors was subtracted from glucose produced during the carbohydrate model compound and lignin isolate hydrolysis.

Table 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 + CTec	
5 FPU/g carbohydrates	5.21 ± 0.299
10 FPU/g carbohydrates	5.14 ± 0.233
Avicel + CTec	
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 ₂ SO ₄	0

Table 5. Glucan conversion during hydrolysis of residual glucan in lignin isolate.

Type of LI	CTec2 Loading (FPU/g lignin isolate)	Glucan Conversion (%)		
		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

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. 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 the interlinking of the cellulose microfibrils via hydrogen bonds may limit hydrolysis [52]. BG, CBH, and EG are needed for Avicel conversion to glucose, and the results suggested that higher levels of CBH and EG might be needed to further improve glucose production from Avicel.

The data from the hydrolysis of cellobiose in the presence of lignin isolates suggested that regardless of the type of lignin isolate, cellobiose conversion was not significantly different ($p > 0.05$) when no lignin isolates were present (Figure 2). Additionally, increasing the amount of isolate present in the suspension with cellobiose had no significant ($p > 0.05$) impact on cellobiose conversion. Although BG appeared to be bound to lignin isolates (Figure 1), it appeared to retain sufficient catalytic activity for effective cellobiose hydrolysis.

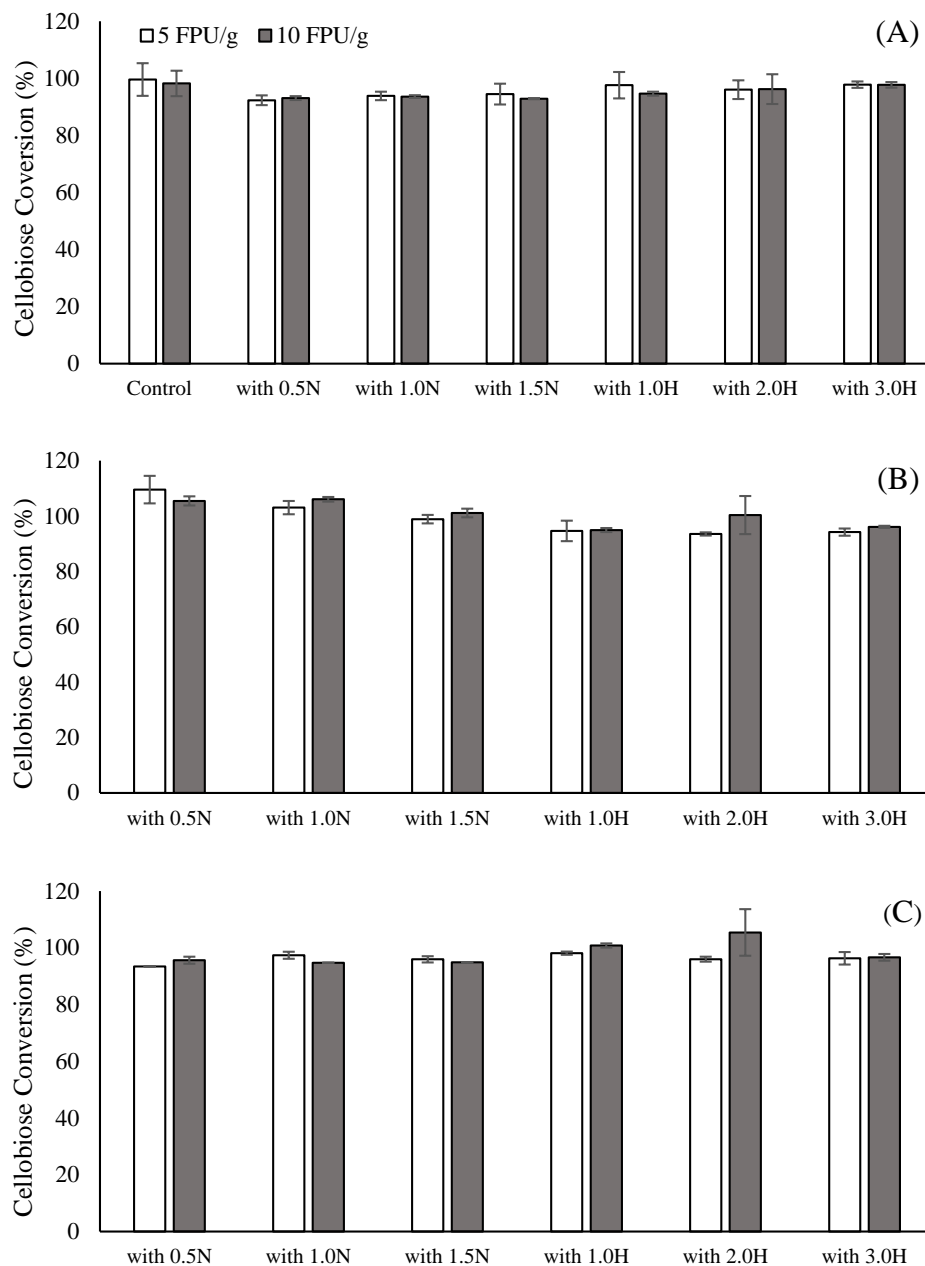


Figure 2. Cellobiose conversion during hydrolysis of lignin isolates from various pretreatments mixed with cellobiose. (A) 0.25% LI:0.5% cellobiose, (B) 0.5% LI:0.5% cellobiose, and (C) 1.0% LI:0.5% cellobiose. Control as cellobiose only.

As noted by [16,29], lignin concentration is not the sole factor in the hydrolysis processes. In their research, Ref. [33] tested the influence of five types of lignins on the enzymatic hydrolysis of cellulose, and their results suggested that the structure and chemistry of lignin plays a major role in inhibition. Similar results were reported by [18], who observed that lignin's inhibition of hydrolysis was related to the phenolic hydroxyl content and the hydrophobicity. Interestingly, it was noted that lignin, depending on its hydrophilicity, might even enhance the enzymatic hydrolysis. Wang et al. [29] observed a significant increase in hydrolysis efficiencies in the presence of hydrophilic lignin compared to hydrophobic lignin, and opined that hydrophilic lignins may serve as surfactants that could improve the hydrolysis processes. The role of lignin's hydrophilicity on enzymatic hydrolysis was also reported by [53]. When compared to hydrophobic lignin (derived from pine), sweet-gum-derived-lignin provided better hydrolysis yields, although the molecular weight and negative surface charges may

also have played a role. Li et al. [54] reported that the molecular weight of lignin plays a key role in the interaction between lignin and the hydrolysis enzymes. It was observed that lower molecular weight lignins tend to minimize the inhibition of cellulose hydrolysis. Additionally, the relative composition of lignin monomers (S, G, and H fractions) might also play a role in the inhibition [55]. As suggested by Li et al. [32], higher S/G ratios were negatively correlated with enzyme adsorption and positively associated with hydrolysis. Further, ref. [30] noted that a higher proportion of guaiacyl lignin tended to promote adsorption of enzymes, especially beta-glucosidase, resulting in a decrease in hydrolysis efficiency, which suggests that extraction of G-lignin may enhance hydrolysis yields. In our previous research, we observed that NaOH pretreatment increased S/G ratio from 0.64 (untreated) to 0.73–0.75 (0.5–1.5% NaOH). Additionally, NaOH was also able to selectively extract H-type lignin monomers from miscanthus, resulting in an overall increase in the enzymatic hydrolysis efficiency, which was also suggested by [55]. Similarly, ref. [33] reported that among the five different types of lignin evaluated for their effects on the enzymatic hydrolysis, the lignin with the highest S/G ratio (1.53) exhibited the lowest inhibition, suggesting that the inhibition of hydrolysis depends on the lignin's chemical characteristics. Furthermore, as suggested by [24,56], alkaline pretreatment not only depolymerizes lignin but also delinks lignin and carbohydrate, thus allowing enhanced access for the enzymes to interact with the carbohydrate units. As per [57], NaOH disrupts the hydrogen bonding between lignin, hemicellulose, and cellulose and extracts the lignin–hemicellulose complex. Subsequently, the hydroxyl ions are expected to disrupt the carbon to carbon bonds and alpha and beta-aryl bonds of the lignin molecule, resulting in fragmentation [58–60]. Similarly, H₂SO₄ treatment is known to solubilize the hemicelluloses and alter the structure of the lignin present in biomass matrices [61,62]. Furthermore, ref. [28] suggested that lignin's role as an inhibitor of hydrolysis is not relevant when enzymes have increased accessibility to cellulose sites. Thus, it appears that the pretreatment may have fragmented the lignin while simultaneously altering the miscanthus, allowing for enhanced accessibility of cellulose sites to the enzymes and resulting in substantially reduced inhibitory effects.

Avicel conversion showed similar results to the cellobiose (Figure 3). Though a higher CTec2 dose (10 FPU/g) produced significantly ($p < 0.05$) more glucose from Avicel than 5 FPU/g CTec2, the presence of lignin isolates did not significantly ($p > 0.05$) affect 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. The hydrolysis reaction time (72 h) in this study might have been enough time for free CBH and EG (or these CBH and EG bound to the lignin isolates) to convert into glucose, of which there was a similar amount from Avicel hydrolysis without lignin isolates. In one of their research reports, ref. [63] proposed that inhibition due to lignin is significantly decreased with time and is mostly eliminated around 72 h. It was theorized that the initial inhibition due to lignin accumulation on the biomass surface was overcome after lignin clusters were displaced by the enzymatic interaction [63]. Similar results were reported by [28] who reported that higher hydrolysis times in combination with higher enzyme loadings could result in increased hydrolysis yields. Our results were similar to those reported by [33], who in their research observed that when enzyme loading was increased from 10 FPU/g to 20 FPU/g, the inhibition of selected lignins dropped from 23.4–27.3% to 13.9–14.6%. Overall, it appeared that for *Miscanthus × giganteus*, the presence of lignin did not affect the hydrolysis of cellobiose and Avicel even at low enzyme loadings of 5 and 10 FPU g⁻¹.

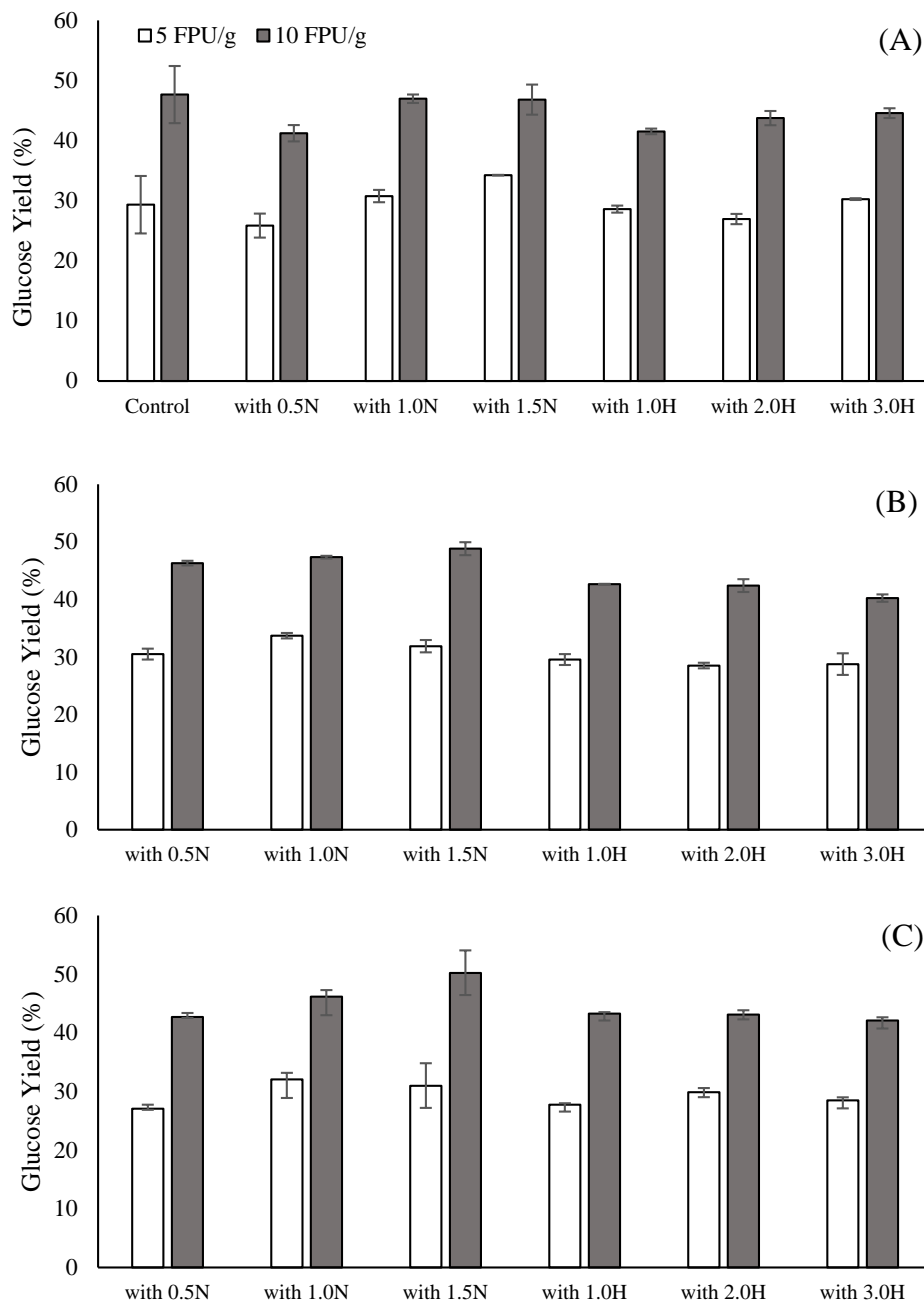


Figure 3. Avicel conversion during hydrolysis of lignin isolates from various pretreatments mixed with Avicel as (A) 0.25% LI:0.5% Avicel, (B) 0.5% LI:0.5% Avicel, and (C) 1.0% LI:0.5% Avicel. Control as Avicel only.

4. Conclusions

The effect of binding between lignin isolated from miscanthus and cellulolytic enzymes in Cellic® CTec2 was investigated. In addition, the effect of the isolated lignin on the hydrolysis of carbohydrate model compounds, namely cellobiose and Avicel, was studied. While an increase in NaOH concentration resulted in an increased content of lignin in isolates, H₂SO₄ concentration did not have any significant impact on lignin content in the isolates. Of all the cellulolytic enzymes likely to comprise CTec2, β -glucosidase had a significantly higher tendency to bind to lignin isolates. Nevertheless, the addition of these isolates in various amounts during hydrolysis of cellobiose and Avicel did not significantly affect their conversion to glucose, indicating that the enzymes were

catalytically active despite being bound to lignin, suggesting that lignin content alone is not an indicator of inhibition. 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 the varying tendencies of cellulolytic enzymes towards binding on lignin.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2227-9717/7/10/755/s1>, Figure S1: SEM images of untreated miscanthus (A and B, magnification 250× and 1000×), lignin isolated from miscanthus pretreated with 1% NaOH at 121 °C for 30 min (C and D, magnification 250× and 1000×), and lignin isolated from miscanthus pretreated with 2% H₂SO₄ at 121 °C for 60 min (E and F, magnification 250× and 1000×).

Author Contributions: Conceptualization, R.S.-S. and P.K.; Methodology, W.J.; Formal analysis, W.J.; Writing—original draft preparation, W.J.; Review and Editing, R.S.-S. and P.K.

Funding: This research was funded by North Carolina State University College of Agriculture and Life Sciences. The APC was funded by NCSU College of Agricultural and Life Sciences Biological and Agricultural Engineering.

Acknowledgments: The authors acknowledge in this section you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

References

- Bauer, S.; Sorek, H.; Mitchell, V.D.; Ibáñez, A.B.; Wemmer, D.E. Characterization of *Miscanthus giganteus* lignin isolated by ethanol organosolv process under reflux condition. *J. Agric. Food Chem.* **2012**, *60*, 8203–8212. [[CrossRef](#)] [[PubMed](#)]
- Somerville, C.; Youngs, H.; Taylor, C.; Davis, S.C.; Long, S.P. Feedstocks for lignocellulosic biofuels. *Science* **2010**, *329*, 790–792. [[CrossRef](#)] [[PubMed](#)]
- Yan, J.; Chen, W.; Luo, F.; Ma, H.; Meng, A.; Li, X.; Zhu, M.; Li, S.; Zhou, H.; Zhu, W. Variability and adaptability of *Miscanthus* species evaluated for energy crop domestication. *Gcb Bioenergy* **2012**, *4*, 49–60. [[CrossRef](#)]
- Lee, W.; Kuan, W. *Miscanthus* as cellulosic biomass for bioethanol production. *Biotechnol. J.* **2015**, *10*, 840–854. [[CrossRef](#)] [[PubMed](#)]
- Brosse, N.; Dufour, A.; Meng, X.; Sun, Q.; Ragauskas, A. *Miscanthus*: A fast-growing crop for biofuels and chemicals production. *Biofuels Bioprod. Biorefin.* **2012**, *6*, 580–598. [[CrossRef](#)]
- Hodgson, E.M.; Nowakowski, D.J.; Shield, I.; Riche, A.; Bridgwater, A.V.; Clifton-Brown, J.C.; Donnison, I.S. Variation in *Miscanthus* chemical composition and implications for conversion by pyrolysis and thermo-chemical bio-refining for fuels and chemicals. *Bioresour. Technol.* **2011**, *102*, 3411–3418. [[CrossRef](#)] [[PubMed](#)]
- Kumar, D.; Murthy, G.S. Impact of pretreatment and downstream processing technologies on economics and energy in cellulosic ethanol production. *Biotechnol. Biofuels* **2011**, *4*, 27. [[CrossRef](#)]
- Li, M.; Si, S.; Hao, B.; Zha, Y.; Wan, C.; Hong, S.; Kang, Y.; Jia, J.; Zhang, J.; Li, M. Mild alkali-pretreatment effectively extracts guaiacyl-rich lignin for high lignocellulose digestibility coupled with largely diminishing yeast fermentation inhibitors in *Miscanthus*. *Bioresour. Technol.* **2014**, *169*, 447–454. [[CrossRef](#)]
- Ho, M.C.; Ong, V.Z.; Wu, T.Y. Potential use of alkaline hydrogen peroxide in lignocellulosic biomass pretreatment and valorization—A review. *Renew. Sustain. Energy Rev.* **2019**, *112*, 75–86. [[CrossRef](#)]
- Li, M.; Pu, Y.; Ragauskas, A.J. Current understanding of the correlation of lignin structure with biomass recalcitrance. *Front. Chem.* **2016**, *4*, 45. [[CrossRef](#)]
- Van der Weijde, T.; Torres, A.F.; Dolstra, O.; Dechesne, A.; Visser, R.G.; Trindade, L.M. Impact of different lignin fractions on saccharification efficiency in diverse species of the bioenergy crop *Miscanthus*. *Bioenergy Res.* **2016**, *9*, 146–156. [[CrossRef](#)]
- Vermaas, J.V.; Petridis, L.; Qi, X.; Schulz, R.; Lindner, B.; Smith, J.C. Mechanism of lignin inhibition of enzymatic biomass deconstruction. *Biotechnol. Biofuels* **2015**, *8*, 217. [[CrossRef](#)] [[PubMed](#)]

13. Sammond, D.W.; Yarbrough, J.M.; Mansfield, E.; Bomble, Y.J.; Hobdey, S.E.; Decker, S.R.; Taylor, L.E.; Resch, M.G.; Bozell, J.J.; Himmel, M.E. Predicting enzyme adsorption to lignin films by calculating enzyme surface hydrophobicity. *J. Biol. Chem.* **2014**, *289*, 20960–20969. [[CrossRef](#)] [[PubMed](#)]
14. Liu, H.; Sun, J.; Leu, S.; Chen, S. Toward a fundamental understanding of cellulase-lignin interactions in the whole slurry enzymatic saccharification process. *Biofuels Bioprod. Biorefin.* **2016**, *10*, 648–663. [[CrossRef](#)]
15. Nakagame, S.; Chandra, R.P.; Saddler, J.N. *The Influence of Lignin on the Enzymatic Hydrolysis of Pretreated Biomass substrates in Sustainable Production of Fuels, Chemicals, and Fibers from Forest Biomass ACS Symposium Series*; American Chemical Society: Washington, DC, USA, 2011.
16. Saini, J.K.; Patel, A.K.; Adsul, M.; Singhania, R.R. Cellulase adsorption on lignin: A roadblock for economic hydrolysis of biomass. *Renew. Energy* **2016**, *98*, 29–42. [[CrossRef](#)]
17. Pareek, N.; Gillgren, T.; Jönsson, L.J. Adsorption of proteins involved in hydrolysis of lignocellulose on lignins and hemicelluloses. *Bioresour. Technol.* **2013**, *148*, 70–77. [[CrossRef](#)]
18. Yang, Q.; Pan, X. Correlation between lignin physicochemical properties and inhibition to enzymatic hydrolysis of cellulose. *Biotechnol. Bioeng.* **2016**, *113*, 1213–1224. [[CrossRef](#)]
19. Kellock, M.; Rahikainen, J.; Marjamaa, K.; Kruus, K. Lignin-derived inhibition of monocomponent cellulases and a xylanase in the hydrolysis of lignocellulosics. *Bioresour. Technol.* **2017**, *232*, 183–191. [[CrossRef](#)]
20. Rahikainen, J.L.; Evans, J.D.; Mikander, S.; Kalliola, A.; Puranen, T.; Tamminen, T.; Marjamaa, K.; Kruus, K. Cellulase–lignin interactions—The role of carbohydrate-binding module and pH in non-productive binding. *Enzym. Microb. Technol.* **2013**, *53*, 315–321. [[CrossRef](#)]
21. Mosier, N.; Wyman, C.; Dale, B.; Elander, R.; Lee, Y.Y.; Holtzapple, M.; Ladisch, M. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2005**, *96*, 673–686. [[CrossRef](#)]
22. Hassan, S.S.; Williams, G.A.; Jaiswal, A.K. Emerging technologies for the pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **2018**, *262*, 310–318. [[CrossRef](#)] [[PubMed](#)]
23. Mood, S.H.; Golfeshan, A.H.; Tabatabaei, M.; Jouzani, G.S.; Najafi, G.H.; Gholami, M.; Ardjmand, M. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. *Renew. Sustain. Energy Rev.* **2013**, *27*, 77–93. [[CrossRef](#)]
24. Kumar, P.; Barrett, D.M.; Delwiche, M.J.; Stroeve, P. Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Ind. Eng. Chem. Res.* **2009**, *48*, 3713–3729. [[CrossRef](#)]
25. Yang, Y.; Sharma-Shivappa, R.; Burns, J.C.; Cheng, J.J. Dilute acid pretreatment of oven-dried *Switchgrass* germplasms for bioethanol production. *Energy Fuels* **2009**, *23*, 3759–3766. [[CrossRef](#)]
26. Kim, J.S.; Lee, Y.Y.; Kim, T.H. A review on alkaline pretreatment technology for bioconversion of lignocellulosic biomass. *Bioresour. Technol.* **2016**, *199*, 42–48. [[CrossRef](#)]
27. Haque, M.A.; Barman, D.N.; Kang, T.H.; Kim, M.K.; Kim, J.; Kim, H.; Yun, H.D. Effect of dilute alkali pretreatment on structural features and enhanced enzymatic hydrolysis of *Miscanthus sinensis* at boiling temperature with low residence time. *Biosyst. Eng.* **2013**, *114*, 294–305. [[CrossRef](#)]
28. Kumar, L.; Arantes, V.; Chandra, R.; Saddler, J. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. *Bioresour. Technol.* **2012**, *103*, 201–208. [[CrossRef](#)]
29. Wang, W.; Zhu, Y.; Du, J.; Yang, Y.; Jin, Y. Influence of lignin addition on the enzymatic digestibility of pretreated lignocellulosic biomasses. *Bioresour. Technol.* **2015**, *181*, 7–12. [[CrossRef](#)]
30. Ko, J.K.; Ximenes, E.; Kim, Y.; Ladisch, M.R. Adsorption of enzyme onto lignins of liquid hot water pretreated hardwoods. *Biotechnol. Bioeng.* **2015**, *112*, 447–456. [[CrossRef](#)]
31. Haven, M.Ø.; Jørgensen, H. Adsorption of β -glucosidases in two commercial preparations onto pretreated biomass and lignin. *Biotechnol. Biofuels* **2013**, *6*, 165. [[CrossRef](#)]
32. Li, X.; Zheng, Y. Lignin-enzyme interaction: Mechanism, mitigation approach, modeling, and research prospects. *Biotechnol. Adv.* **2017**, *35*, 466–489. [[CrossRef](#)] [[PubMed](#)]
33. Zhang, H.; Wu, S.; Xie, J. Evaluation of the effects of isolated lignin on enzymatic hydrolysis of cellulose. *Enzym. Microb. Technol.* **2017**, *101*, 44–50. [[CrossRef](#)] [[PubMed](#)]
34. Berlin, A.; Balakshin, M.; Gilkes, N.; Kadla, J.; Maximenko, V.; Kubo, S.; Saddler, J. Inhibition of cellulase, xylanase and β -glucosidase activities by softwood lignin preparations. *J. Biotechnol.* **2006**, *125*, 198–209. [[CrossRef](#)] [[PubMed](#)]
35. Rahikainen, J.; Mikander, S.; Marjamaa, K.; Tamminen, T.; Lappas, A.; Viikari, L.; Kruus, K. Inhibition of enzymatic hydrolysis by residual lignins from softwood—Study of enzyme binding and inactivation on lignin-rich surface. *Biotechnol. Bioeng.* **2011**, *108*, 2823–2834. [[CrossRef](#)] [[PubMed](#)]

36. Jung, W. Characterization of Lignin Change Due to Chemical Pretreatment of Herbaceous Biomass. Ph.D. Thesis, North Carolina State University, Raleigh, CA, USA, 2016.
37. Sluiter, A.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D. Determination of extractives in biomass. *Lab. Anal. Proced. (LAP)* **2005**, *1617*.
38. Sluiter, A.; Hames, B.; Ruiz, R.; Scarlata, C.; Sluiter, J.; Templeton, D.; Crocker, D. Determination of structural carbohydrates and lignin in biomass. *Lab. Anal. Proced.* **2008**, *1617*, 1–16.
39. Belmokhtar, N.; Habrant, A.; Ferreira, N.L.; Chabbert, B. Changes in phenolics distribution after chemical pretreatment and enzymatic conversion of *Miscanthus × giganteus* internode. *Bioenergy Res.* **2013**, *6*, 506–518. [[CrossRef](#)]
40. Yamamura, M.; Noda, S.; Hattori, T.; Shino, A.; Kikuchi, J.; Takabe, K.; Tagane, S.; Gau, M.; Uwatoko, N.; Mii, M. Characterization of lignocellulose of *Erianthus arundinaceus* in relation to enzymatic saccharification efficiency. *Plant Biotechnol.* **2013**, *30*, 25–35. [[CrossRef](#)]
41. Zanchetta, A.; dos Santos, A.C.F.; Ximenes, E.; Nunes, C.D.C.C.; Boscolo, M.; Gomes, E.; Ladisch, M.R. Temperature dependent cellulase adsorption on lignin from sugarcane bagasse. *Bioresour. Technol.* **2018**, *252*, 143–149. [[CrossRef](#)]
42. Yarbrough, J.M.; Mittal, A.; Mansfield, E.; Taylor, L.E.; Hobdey, S.E.; Sammond, D.W.; Bomble, Y.J.; Crowley, M.F.; Decker, S.R.; Himmel, M.E. New perspective on glycoside hydrolase binding to lignin from pretreated corn stover. *Biotechnol. Biofuels* **2015**, *8*, 214. [[CrossRef](#)]
43. Chauve, M.; Mathis, H.; Huc, D.; Casanave, D.; Monot, F.; Ferreira, N.L. Comparative kinetic analysis of two fungal β -glucosidases. *Biotechnol. Biofuels* **2010**, *3*, 3. [[CrossRef](#)] [[PubMed](#)]
44. Hui, J.P.; Lanthier, P.; White, T.C.; McHugh, S.G.; Yaguchi, M.; Roy, R.; Thibault, P. Characterization of cellobiohydrolase I (Cel7A) glycoforms from extracts of *Trichoderma reesei* using capillary isoelectric focusing and electrospray mass spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **2001**, *752*, 349–368. [[CrossRef](#)]
45. Medve, J.; Lee, D.; Tjerneld, F. Ion-exchange chromatographic purification and quantitative analysis of *Trichoderma reesei* cellulases cellobiohydrolase I, II and endoglucanase II by fast protein liquid chromatography. *J. Chromatogr. A* **1998**, *808*, 153–165. [[CrossRef](#)]
46. Nakagame, S.; Chandra, R.P.; Kadla, J.F.; Saddler, J.N. The isolation, characterization and effect of lignin isolated from steam pretreated Douglas-fir on the enzymatic hydrolysis of cellulose. *Bioresour. Technol.* **2011**, *102*, 4507–4517. [[CrossRef](#)]
47. Vinzant, T.B.; Adney, W.S.; Decker, S.R.; Baker, J.O.; Kinter, M.T.; Sherman, N.E.; Fox, J.W.; Himmel, M.E. Fingerprinting *Trichoderma reesei* hydrolases in a commercial cellulase preparation. *Appl. Biochem. Biotechnol.* **2001**, *91*, 99–107. [[CrossRef](#)]
48. Chirico, W.J.; Brown, R.D., Jr. Purification and characterization of a β -glucosidase from *Trichoderma reesei*. *Eur. J. Biochem.* **1987**, *165*, 333–341. [[CrossRef](#)]
49. Nakagame, S.; Chandra, R.P.; Saddler, J.N. The Influence of Lignin on the Enzymatic Hydrolysis of Pretreated Biomass Substrates. Ph.D. Thesis, University of British Columbia, Vancouver, BC, Canada, 2010.
50. Dong, D.J.; Fricke, A.L.; Moudgil, B.M.; Johnson, H. Electrokinetic study of kraft lignin. *Tappi J. (USA)* **1996**, *79*, 191–197.
51. Lou, H.; Zhu, J.Y.; Lan, T.Q.; Lai, H.; Qiu, X. pH-Induced lignin surface modification to reduce nonspecific cellulase binding and enhance enzymatic saccharification of lignocelluloses. *ChemSusChem* **2013**, *6*, 919–927. [[CrossRef](#)]
52. Klemm, D.; Heublein, B.; Fink, H.; Bohn, A. Cellulose: Fascinating biopolymer and sustainable raw material. *Angew. Chem. Int. Ed.* **2005**, *44*, 3358–3393. [[CrossRef](#)]
53. Lai, C.; Tu, M.; Shi, Z.; Zheng, K.; Olmos, L.G.; Yu, S. Contrasting effects of hardwood and softwood organosolv lignins on enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* **2014**, *163*, 320–327. [[CrossRef](#)]
54. Li, X.; Li, M.; Pu, Y.; Ragauskas, A.J.; Klett, A.S.; Thies, M.; Zheng, Y. Inhibitory effects of lignin on enzymatic hydrolysis: The role of lignin chemistry and molecular weight. *Renew. Energy* **2018**, *123*, 664–674. [[CrossRef](#)]
55. Dos Santos, A.C.; Ximenes, E.; Kim, Y.; Ladisch, M.R. Lignin–enzyme interactions in the hydrolysis of lignocellulosic biomass. *Trends Biotechnol.* **2018**, *37*, 518–531. [[CrossRef](#)] [[PubMed](#)]
56. Agbor, V.B.; Cicek, N.; Sparling, R.; Berlin, A.; Levin, D.B. Biomass pretreatment: Fundamentals toward application. *Biotechnol. Adv.* **2011**, *29*, 675–685. [[CrossRef](#)] [[PubMed](#)]

57. Si, S.; Chen, Y.; Fan, C.; Hu, H.; Li, Y.; Huang, J.; Liao, H.; Hao, B.; Li, Q.; Peng, L. Lignin extraction distinctively enhances biomass enzymatic saccharification in hemicelluloses-rich *Miscanthus* species under various alkali and acid pretreatments. *Bioresour. Technol.* **2015**, *183*, 248–254. [[CrossRef](#)]
58. Harmsen, P.; Huijgen, W.; Bermudez, L.; Bakker, R. *Literature Review of Physical and Chemical Pretreatment Processes for Lignocellulosic Biomass*; Wageningen UR-Food & Biobased Research: Wageningen, The Netherlands, 2010.
59. Xu, J.; Sun, R. Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery. In *Recent Advances in Alkaline Pretreatment of Lignocellulosic Biomass*; Elsevier: Amsterdam, The Netherlands, 2016; pp. 431–459.
60. Zhao, X.; Zhang, L.; Liu, D. Biomass recalcitrance. Part II: Fundamentals of different pre-treatments to increase the enzymatic digestibility of lignocellulose. *Biofuels Bioprod. Biorefin.* **2012**, *6*, 561–579. [[CrossRef](#)]
61. Brodeur, G.; Yau, E.; Badal, K.; Collier, J.; Ramachandran, K.B.; Ramakrishnan, S. Chemical and physicochemical pretreatment of lignocellulosic biomass: A review. *Enzym. Res.* **2011**. [[CrossRef](#)]
62. Wyman, C.E.; Dale, B.E.; Elander, R.T.; Holtzapple, M.; Ladisch, M.R.; Lee, Y.Y. Coordinated development of leading biomass pretreatment technologies. *Bioresour. Technol.* **2005**, *96*, 1959–1966. [[CrossRef](#)]
63. Li, H.; Pu, Y.; Kumar, R.; Ragauskas, A.J.; Wyman, C.E. Investigation of lignin deposition on cellulose during hydrothermal pretreatment, its effect on cellulose hydrolysis, and underlying mechanisms. *Biotechnol. Bioeng.* **2014**, *111*, 485–492. [[CrossRef](#)]



© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).