

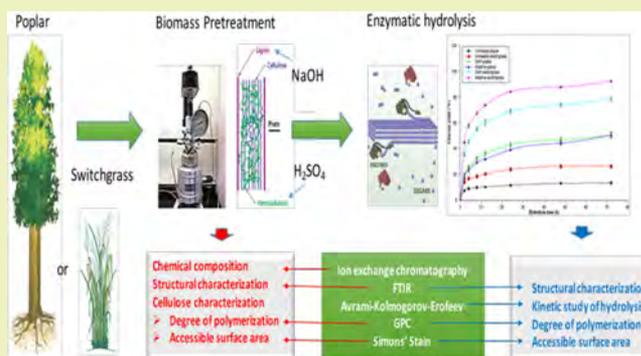
Physicochemical Structural Changes of Poplar and Switchgrass during Biomass Pretreatment and Enzymatic Hydrolysis

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S Supporting Information

ABSTRACT: Converting lignocellulosics to simple sugars for second generation bioethanol is complicated due to biomass recalcitrance, and it requires a pretreatment stage prior to enzymatic hydrolysis. In this study, native, pretreated (acid and alkaline) and partially hydrolyzed poplar and switchgrass were characterized by using Simons' staining for cellulose accessibility, GPC for degree of polymerization (DP), and FTIR for chemical structure of plant cell wall. The susceptibility of the pretreated biomass to enzymatic hydrolysis could not be easily predicted from differences in cellulose DP and accessibility. During hydrolysis, the most significant DP reduction occurred at the very beginning of hydrolysis, and the DP began to decrease at a significantly slower rate after this initial period, suggesting an existence of a synergistic action of endo- and exoglucanases that contribute to the occurrence of a "peeling off" mechanism. Cellulose accessibility was found to be increased at the beginning of hydrolysis, after reaching a maximum value then started to decrease. The fresh enzyme restart hydrolysis experiment along with the accessibility data indicated that the factors associated with the nature of enzyme such as irreversible nonspecific binding of cellulases by lignin and steric hindrance of enzymes should be responsible for the gradual slowing down of the reaction rate.

KEYWORDS: Biomass recalcitrance, cellulose accessibility, degree of polymerization, Simons' stain, peeling off, irreversible nonspecific binding



INTRODUCTION

Long-term driving forces including diminishing availability of fossil fuels and growing concerns about environmental stewardship have prompted scientists to develop renewable fuel sources, such as biomass, for biofuel production.¹ Enzymatic hydrolysis refers to a process in which cellulose chains are effectively broken down to glucose by applying a multicomponent enzyme system that typically contains endoglucanase, cellobiohydrolase, and β -glucosidase.² However, this hydrolysis process is usually very slow due to innate biomass recalcitrance which refers to the complex characteristics of lignocellulosics to protect its carbohydrates from degradation by enzymes.³ Pretreatment can increase the accessible surface area (ASA) of cellulose through altering biomass particle size, plant cell wall porosity, cellulose crystallinity, cellulose degree of polymerization, and lignin/hemicellulose distribution. The ultimate goal of pretreatment is to enhance the yields of fermentable sugars from cellulose or hemicellulose during enzymatic hydrolysis.^{4,5} To date, numerous physical or chemical pretreatments have been developed,

including dilute acid (DA), hot water, alkali, steam explosion, organic solvent, ammonia fiber expansion (AFEX), and ionic liquid (IL) pretreatments.⁶ Years of research have tried to modify different substrate factors through pretreatment and correlate these alterations to changes in biomass recalcitrance; however, much of the literature has reported conflicting trends.⁷

During enzymatic hydrolysis, the pore size and accessible surface area of the substrate are expected to change. Li et al. reported that the concentration of pores smaller than 6 nm in cotton fabric decreased after enzyme treatment as measured by size exclusion liquid chromatography, but no significant changes were observed for the concentration of pores larger than 6 nm.⁸ Santa-Maria and Jeoh reported two important observations of cellulose microstructure change during enzymatic hydrolysis: the untwisting of cellulose microfibrils

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in the early reaction at a high hydrolysis rate and the appearance of channels along microfibril length at the late stage of the hydrolysis.⁹ On the other hand at the macro-molecular fiber level, it was found that pretreated pulp fibers are hydrolyzed through a two-step mode of action, including an initial fragmentation followed by simultaneous swelling and peeling/erosion of fragmented fibers.¹⁰

It was repeatedly observed that the enzymatic hydrolysis of lignocellulosic biomass proceeded at an initial fast rate followed by a rapid decrease in the conversion rate. The exact mechanisms leading to this rate decrease are not fully understood and in most cases are controversial.^{11–13} However, it is reasonable to hypothesize that the increased recalcitrance of substrate could be one of the potential reasons causing hydrolysis rate gradually decreases. As a result, substrate characteristics such as cellulose accessibility, crystallinity, DP, biomass particle size, and enzyme related features such as enzyme inactivation, jamming effects, and diffusion constraints are all potential factors to be responsible for the slowdown of reaction rates.² Eibinger et al. proposed that the decline in enzymatic hydrolysis rates was due to (a) the depletion of hydrolyzable material within cellulose surface area accessible to a single cellulase and (b) the impropriety of enzyme to escape local restrictions.¹⁴ A majority of studies that tried to highlight these changes of cellulose structure made use of highly digestible pure cellulosic substrates such as Avicel or bleached kraft pulp, which are not truly indicative of how real heterogeneous lignocellulosic biomass might behave.^{9,13,15} In this study, poplar and switchgrass were pretreated by dilute acid and alkaline and subjected to enzymatic hydrolysis for 72 h. The cellulose accessible surface area and degree of polymerization of pretreated and incomplete hydrolyzed biomass were characterized to understand the fundamentals of biomass recalcitrance and limitations occurring during enzymatic hydrolysis of lignocellulosic substrates that might be responsible for the gradual slowing down of the reaction. The results obtained here should provide insight into the mechanism of enzymatic hydrolysis of heterogeneous lignocellulosic biomass. Along with the method by which pretreatment aids enzymatic hydrolysis, they should be extremely helpful for the selection or development of biomass pretreatment for different biomass substrates.

MATERIALS AND METHODS

Feedstocks and Chemicals. Poplar (*Populus trichocarpa x deltoides*) and switchgrass were harvested in 2012 from area 0800 at Oak Ridge National Laboratory (ORNL), TN. Poplar sample was debarked, and the size was reduced in a Mini Wiley Mill (Thomas Scientific, Swedesboro, NJ) to pass a 0.841 mm screen. Direct dyes were obtained from Pylam Products Co. Inc. (Garden City, NY). Enzymes, protease, and other chemicals used in this study were all purchased from Sigma-Aldrich and used as received.

Biomass Pretreatment. Dilute acid pretreatment was applied on poplar and switchgrass using 1% (w/w) H₂SO₄ at 120 °C for 60 min with a starting pH 0.92. The biomass samples (~20 g) were transferred to a 1 L Parr reactor with 1% sulfuric acid solution at 5% dry solids loading. The reactor was then heated to 120 °C over ~20 min, held at this temperature (±2 °C) for 60 min, and then quenched in an ice bath for ~5 min. The pretreated slurry was filtered to remove the solid material and washed with an excess of deionized water. Alkaline pretreatment was done at a similar procedure using 1% (w/w) NaOH at 120 °C for 60 min with a starting pH 13.2. Never-dried samples were stored in a refrigerator at 4 °C.

Carbohydrate and Lignin Composition. Extractives were removed by placing the biomass samples into an extraction thimble

in a Soxhlet apparatus (Foss, Soxtec 2050). The extraction flask was filled with dichloromethane and then refluxed with a boiling rate of 24 solvent cycles per h for ~8 h. The extractive-free solids were air-dried overnight in a fume hood, and sealed in bags and stored in a refrigerator before composition analysis. The chemical composition of the substrates was determined according to NREL analytical procedures as shown in Supporting Information S1—chemical composition analysis.¹⁶ The resulting solution was diluted, filtered and injected into a high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using Dionex ICS-3000 (Dionex Corp., USA) with a conductivity detector, a guard CarboPac PA1 column (2 × 50 mm, Dionex), a CarboPac PA1 column (2 × 250 mm, Dionex), an AS40 automated sampler, and a PC 10 pneumatic controller at room temperature. 0.20 and 0.40 M NaOH was used as the eluent and postcolumn rinsing effluent. The total analysis time was 70 min, with a flow rate 0.40 mL/min. Calibration was performed with standard solutions of glucose, xylose, arabinose, mannose, and galactose, and fucose was used as an internal standard.

Enzymatic Hydrolysis. Enzymatic hydrolysis was performed at a consistency of 1% (w/v) in 50.0 mM citrate buffer (pH 4.80) with cellulase (*Trichoderma reesei* ATCC 26921) and β-glucosidase (Novozyme 188) loadings of 20 FPU/g and 40 CBU/g, respectively. The mixture was incubated at 50 °C under continuous agitation at 150 rpm for 72 h with antibiotic antimycotic solution (Sigma A5955) 1% (v/v) added to avoid microbiological contamination. Hydrolysis liquid (1.00 mL) was withdrawn after 2, 4, 8, 12, 24, 48, and 72 h, quenched by submersion for 10 min in a boiling water bath, and then immediately frozen to -20 °C until analysis on an Agilent 1200 series HPLC system equipped with an auto sampler and an Aminex HPX-87H column and precolumn (Biorad Laboratories). The analysis was carried out at 65 °C using 10 mM nitric acid as the eluent at a flow rate of 0.6 mL min⁻¹ and with refractive index detection. The kinetics of hydrolysis were studied using the Avrami–Kolmogorov–Erofeev (AKE) model and the theoretical enzymatic hydrolysis yield based on the calculated AKE kinetic parameters was also determined and compared with the experimental data.

Fourier Transform Infrared (FTIR) Spectroscopy. The samples were dried at ~45 °C in a vacuum oven overnight prior to FTIR analysis. A Spectrum One FTIR system (PerkinElmer, Wellesley, MA) with a universal attenuated total reflection (ATR) accessory was used to characterize the changes of functional groups in the biomass samples. Each sample was pressed uniformly against the diamond surface using a spring-loaded anvil. FTIR spectra were obtained by averaging 64 scans from 4000 to 800 cm⁻¹ at 4 cm⁻¹ resolution. Baseline and ATR corrections for penetration depth and frequency variations were carried out using the Spectrum One software.

Gel Permeation Chromatography Analysis. Cellulose isolation and tricarbanilation was done according to previously published literature with its detail shown in Supporting Information S2—cellulose isolation and derivatization for GPC analysis. Prior to GPC analysis, the cellulose derivative was dissolved in tetrahydrofuran (1.00 mg/mL), and the solution was filtered through a 0.45 μm polytetrafluoroethylene (PTFE) filter, and placed in a 2 mL autosampler vial. The molecular weight was analyzed by an Agilent GPC SECurity 1200 system equipped with four Waters Styragel columns (HR0.5, HR2, HR4, HR6), a refractive index (RI) detector, and a UV detector (270 nm). Tetrahydrofuran was used as the mobile phase (1.00 mL/min) and the injection volume was 30.0 μL. Data collection and processing was performed by Polymer Standards Service WinGPC Unity software (Build 6807). The molecular weight was calculated by the software relative to the universal polystyrene calibration curve.

Protease Treatment. Cellulases hydrolyzed samples were taken from enzymatic hydrolysis system after different residence times (2, 4, 8, 12, 24, 48, and 72 h), washed with DI water and heat treated in boiling water for 10 min. The samples were then incubated overnight at 37 °C in phosphate buffer (50 mM, pH 7), containing 1 U/mL of protease (Sigma, USA) to hydrolyze any remaining cellulases. The samples were thoroughly washed with DI water and sodium chloride, heat treated, and stored in a refrigerator prior to further testing.

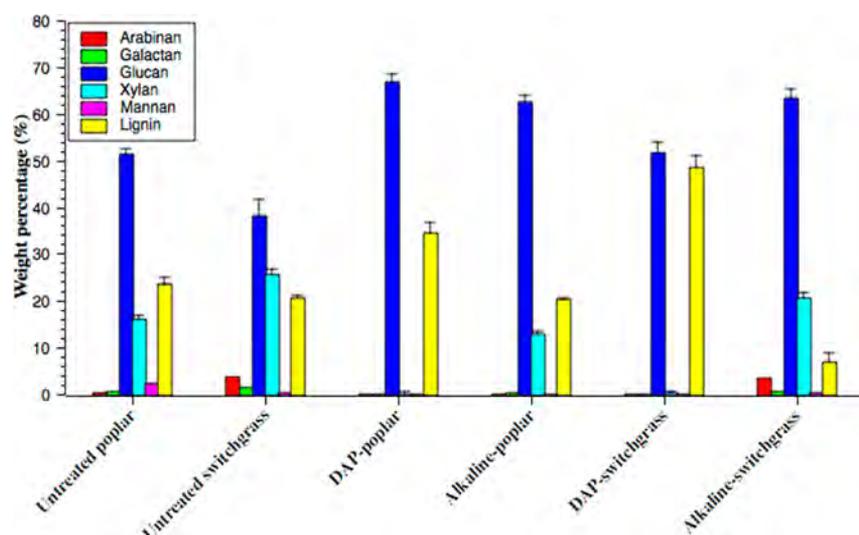


Figure 1. Carbohydrate and Klason lignin content of the untreated, DAP, and dilute alkaline pretreated poplar and switchgrass.

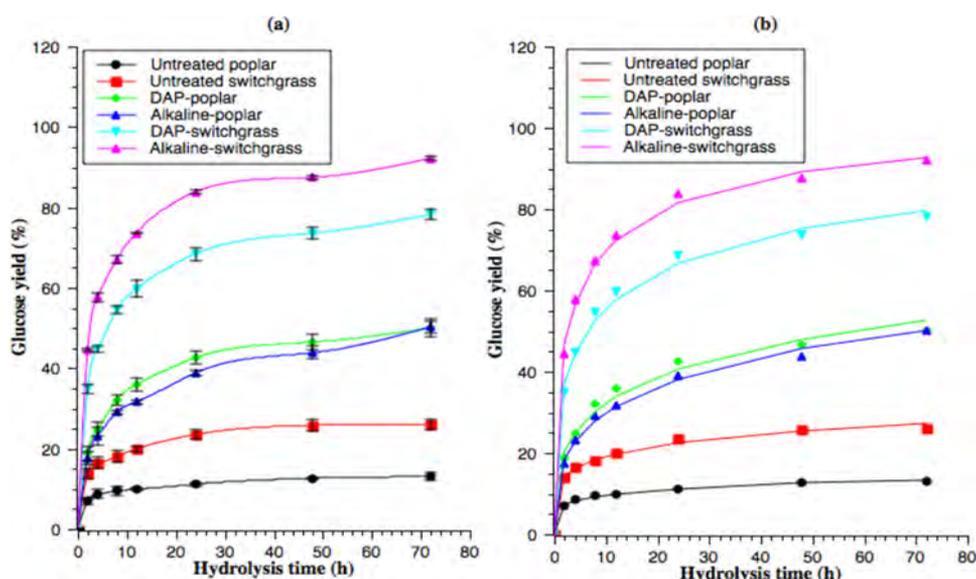


Figure 2. Enzymatic hydrolysis of the untreated, DAP, and alkaline pretreated poplar and switchgrass. (a) Experimental yield. (b) Theoretical yield (solid line) simulated by AKE kinetic model.

Simons' Stain Analysis. Simons' staining was performed according to a modified procedure by Chandra et al. with detail procedure shown in Supporting Information S3—Simons' stain for accessibility test.¹⁷ For staining of cellulases treated biomass, ~10 mg of the samples instead of ~100 mg was used due to the limited amount of sample available, and the amount of dye and phosphate buffered saline solution added to the solution were also scaled down accordingly.

Uninterrupted and "Restart" Cellulose Hydrolysis. The samples produced after 12 h of enzymatic hydrolysis of pretreated biomass were isolated, protease treated, and chosen as the starting materials for the uninterrupted and "restart" experiments. The uninterrupted batch hydrolysis experiment was performed as described previously at a consistency of 1% (w/v) in 50 mM citrate buffer (pH 4.8) with cellulase (*Trichoderma reesei* ATCC 26921) and β -glucosidase loadings of 20 FPU and 40 CBU, respectively. Hydrolysis liquid was withdrawn and analyzed after 1, 2, 3, 4, 24, and 25 h using the equipment noted previously. For "restart" hydrolysis experiments, the hydrolysis system was interrupted and the substrates produced at those times were recovered by filtration followed by protease treatment as previously described. After hydrolyzing the remaining

enzyme from surface, the solid residue was mixed with 50 mM fresh buffer (pH 4.8) at 1% (w/v) loading, and fresh enzyme was added to achieve a loading of 20 FPU and 40 CBU as stated above. The new enzymatic hydrolysis experiment was then performed for 1 h by the same protocol. The 1 h hydrolysis rate was measured and calculated for both uninterrupted and restart cellulose hydrolysis based on the amount of cellulose hydrolyzed in 1 h as well as the total amount of cellulose available at the beginning of that period.

Error Analysis. Error bars shown in each figure represent standard error which is the standard deviation of the sampling distribution of a statistic, defined as the standard deviation divided by the square root of sample sizes—three independent assays unless otherwise specified.

RESULTS AND DISCUSSION

Compositional Analysis. The chemical composition of the untreated and pretreated substrates is shown in Figure 1. The untreated poplar had a higher glucan and lignin content, 51.3% and 23.4% respectively, whereas the untreated switchgrass had a higher xylan content (25.5%). Xylan was almost completely removed after dilute acid pretreatment (DAP) for both poplar

and switchgrass, and only ~37% and ~67% of xylan was removed after alkaline pretreatment for poplar and switchgrass, respectively. Partial delignification was observed after alkaline pretreatment, and much more lignin was removed for switchgrass than poplar (86% vs 34%).

Enzymatic Hydrolysis Yields and Rate. Figure 2 shows the time course of glucose conversion of various samples during 72 h of enzymatic hydrolysis. Switchgrass always had a higher glucose yield when compared with poplar before and after pretreatment. As to be expected, the glucose yields for the untreated materials were low for both poplar (<15%) and switchgrass (~26%) after 72 h. Significantly higher glucose yields were obtained for the pretreated poplar and switchgrass. DAP poplar exhibited slightly higher glucose yield during the 48 h period. However, they had almost identical glucose yield around ~50% conversion after 72 h. Unlike poplar, alkaline pretreated switchgrass always has higher glucose yield from very beginning of the hydrolysis all the way to the end of hydrolysis, e.g., ~92% vs ~78% for alkaline pretreated and DAP switchgrass after 72 h.

The kinetic of enzymatic hydrolysis was also studied using the equation of Avrami–Kolmogorov–Erofeev (AKE):¹⁸

$$\ln(1 - \alpha) = -kt^n$$

where α is the conversion degree of cellulose, representing the glucose yield as shown in Figure 2 (y axis); k is the effective rate constant; n is the effective reaction order, and t is the hydrolysis time. The linear logarithmic form of AKE model, $\ln F = \ln k + n \ln t$, where F is $-\ln(1 - \alpha)$ allows determination of the coefficient n and the effective rate constant k . The hydrolysis kinetic parameters of all the samples are shown in Table 1. The

Table 1. AKE Kinetic Parameter of Enzymatic Hydrolysis of Untreated and Pretreated Poplar and Switchgrass

samples	rate constant (k)	effective order (n)
untreated poplar	0.068	0.177
untreated switchgrass	0.133	0.204
DAP-poplar	0.182	0.331
DAP-switchgrass	0.365	0.346
alkaline-poplar	0.160	0.344
alkaline-switchgrass	0.482	0.396

AKE quantitative analysis clearly showed that the hydrolysis of switchgrass occurred at a much higher reaction rate than poplar. It also showed that DAP is slightly more effective than alkaline pretreatment for poplar, while alkaline pretreatment is much more effective than DAP for switchgrass. The effective reaction orders are all less than 1, suggesting the hydrolysis is strongly limited by the diffusion-adsorption of enzymes. In addition, the theoretical enzymatic hydrolysis curve based on the calculated AKE kinetic parameters is shown in Figure 2b along with the actual experimental data, and the results confirmed the adequacy of AKE kinetic model.

Structural Characterization of Untreated, Pretreated, and Cellulases Hydrolyzed Biomass. The chemical fingerprinting of untreated, pretreated, and cellulases treated poplar and switchgrass were characterized by ATR-FTIR, and their spectra are presented in Figure 3. Table S1 shows some common FTIR absorption bands from biomass. The peak near 3550 cm^{-1} represents OH stretching of the hydrogen bonds of cellulose, and the band position at 2900 cm^{-1} is attributed to C—H stretching within the methylene of cellulose. The width

of bands between 3000 and 3600 cm^{-1} became narrower after the DAP and alkaline pretreatment, suggesting the pretreatment could destroy the intermolecular hydrogen bonds in cellulose.¹⁹ This could be also possibly due to the fact that the pretreatment disrupted the lignin–hemicellulose matrix and made it easier for the water trapped in the plant cell wall to escape via evaporation. The peak centered around 1740 cm^{-1} has been proposed to be associated with the alkyl ester of acetyl group in hemicellulose, and the intensity significantly decreased after both the alkaline and DA pretreatment of poplar and switchgrass, due to the hemicellulose removal by DA and acetyl group removal by NaOH.²⁰ A reduction at the 1595 and 1513 cm^{-1} band after alkaline pretreatment, which is assigned to the lignin aromatic ring stretching and vibrations, is attributed to lignin removal.²¹ Guaiacyl ring-related IR spectra could be observed in all untreated materials at around 1505–1510 cm^{-1} , and this peak is decreased after NaOH pretreatment due to the lignin removal, but still can be seen after the DA pretreatment and enzymatic hydrolysis.²² The bands around 1460 cm^{-1} could be attributed to C—H methyl and methylene deformation of syringyl lignin common in hardwoods such poplar, and the band at 1315 cm^{-1} is attributed to C—O absorption of syringyl rings in lignin of poplar.^{23,24} The presence of syringyl units in switchgrass is evident from the bands at 1315 cm^{-1} , which is well-defined in the untreated, pretreated and enzyme hydrolyzed material. The peak around 1708 cm^{-1} became sharper after all the pretreatment indicating the presence of an unconjugated carbonyl group probably in the lignin fraction.²⁵ The peak around 1640 cm^{-1} represents C=O groups in the alkyl groups of lignin side chains that conjugate with the aromatic structure, and the intensity of this peak decreased after the DAP and alkaline pretreatment due to either partial delignification caused by alkaline pretreatment or lignin redistribution caused by acid-catalyzed reaction.²⁶

Change of Cellulose Degree of Polymerization during Pretreatment and Enzymatic Hydrolysis. Although the effect of cellulose DP on enzymatic hydrolysis is still under debate, it is generally believed that cellulose with lower DP which has more reducing ends available to cellulases to begin cleavage favors enzymatic hydrolysis.²⁷ Figure 4 illustrated the effect of different pretreatments on the weight-average cellulose degree of polymerization (DP_w) determined by GPC. Native poplar has a DP_w around 5400, whereas switchgrass has a DP_w of ~4600. A substantial reduction in DP_w was found for both poplar and switchgrass after pretreatment. However, the extent of reduction for DAP is much larger than that of alkaline pretreatment. Different substrates decreased to a similar DP_w upon pretreatment with acid or alkaline. The DP_w of cellulose decreased ~27% and ~25% after the alkaline pretreatment for poplar and switchgrass, respectively. After DAP, ~89% and ~88% reduction of cellulose DP_w was observed for poplar and switchgrass, respectively. In an effort to determine the effect of cellulose DP_w on biomass digestibility, 72 h enzymatic hydrolysis yield was plotted versus the DP_w of untreated and various pretreated poplar and switchgrass (Figure 4). It has been reported that pretreatment increases cellulose digestibility partially due to the reduction of cellulose molecular weight.²⁸ However, the alkaline pretreated switchgrass has a very similar cellulose DP compared to poplar, but the sugar release of switchgrass is much higher. Furthermore, the alkaline-switchgrass gave much higher glucose yields than the DAP-switchgrass even though the alkaline switchgrass has a significantly higher DP number. The alkaline-switchgrass has

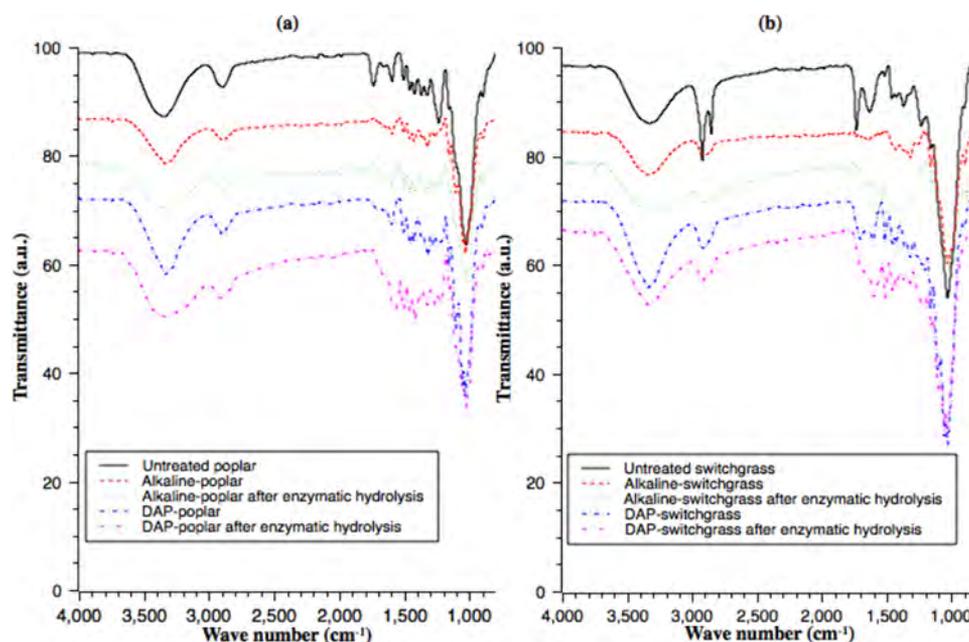


Figure 3. FTIR spectra of untreated, pretreated, and enzymatic hydrolyzed poplar (a) and switchgrass (b) in the fingerprint region (800–4000 cm^{-1}).

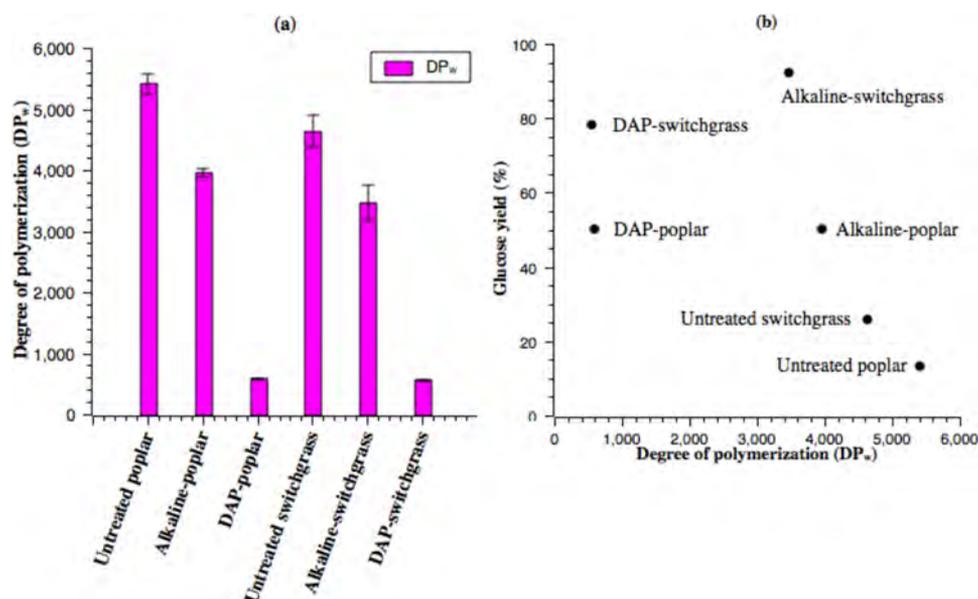


Figure 4. Effect of pretreatment on cellulose DP_w (a) and relationship between cellulose DP_w and 72 h substrate digestibility (b).

the lowest lignin content among all the pretreated samples, which is probably the most likely reason causing the dichotomy. Thus, the results obtained here suggested that cellulose DP_w was probably not the main factor affecting the cellulose saccharification.

The change of cellulose degree of polymerization (DP_w) upon enzymatic hydrolysis for the untreated and pretreated poplar and switchgrass is shown in Figure 5. Obviously, the DP_w of cellulose decreased after enzymatic hydrolysis. For example, the DP_w of cellulose decreased from 3950 to 2124 and from 3467 to 1341 after 72 h enzymatic hydrolysis of the alkaline pretreated poplar and switchgrass, respectively. It is also noted that the most significant DP_w variation occurred at the very beginning of the enzymatic hydrolysis (0 to 4 h). After this initial period, the DP_w begin to decrease at a significantly

slower rate (4 to 12 h), and then remained approximately constant. For example, $\sim 42\%$ reduction of the DP_w of cellulose isolated from the alkaline pretreated poplar was observed after the first 4 h enzymatic hydrolysis, while only $\sim 20\%$ of reduction was observed from 4 to 12 h. The results obtained here are consistent with the work by Del Rio et al., who reported an initial substantial (60%) decrease in cellulose DP during the early stages of enzymatic hydrolysis of organosolv-pretreated lodgepole pine followed by a much slower decrease in cellulose DP.²⁹ This could be explained by the existence of the hydrolytic cleavage of internal glucosidic linkages catalyzed by endoglucanase, which has a much more pronounced effect on the decrease of cellulose DP at the beginning of hydrolysis.³⁰ After that, the hydrolysis was probably dominated by a “peeling-off” mechanism of the newly generated chain ends

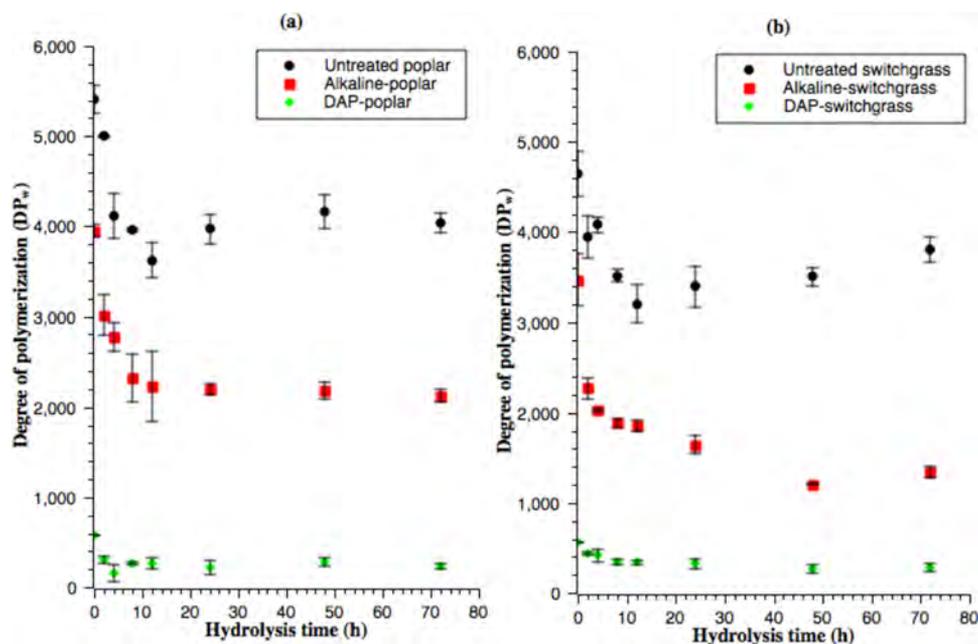


Figure 5. Changes of DP of cellulose isolated from untreated and pretreated poplar (a) and switchgrass (b) during 72 h enzymatic hydrolysis.

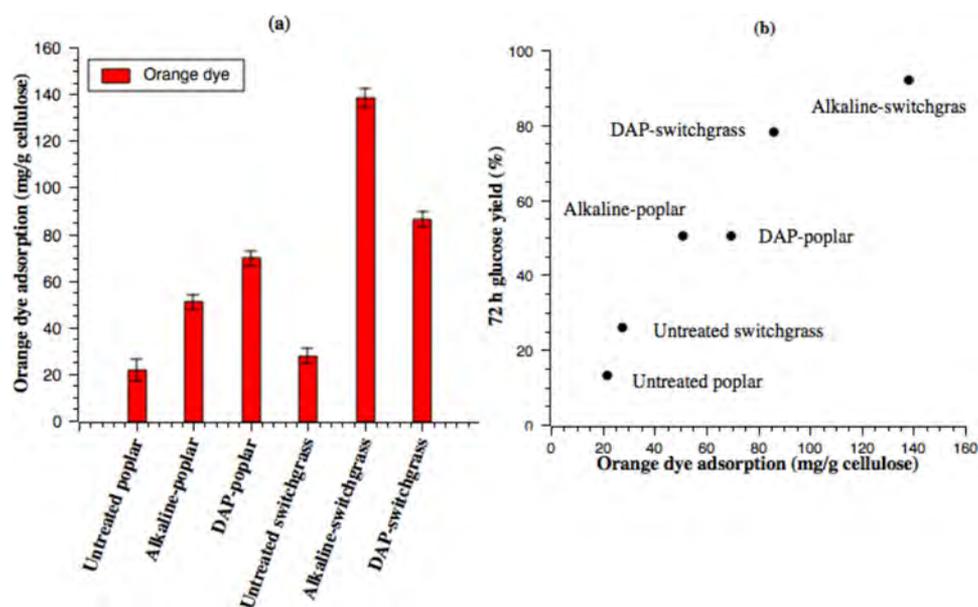


Figure 6. Effect of pretreatment on cellulose accessibility based on orange dye adsorption during Simons' staining (a) and relationship between cellulose accessibility and 72 h substrate digestibility (b).

by exoglucanase action.³¹ The relatively constant DP at the late stage of hydrolysis could be due to the enzyme becoming inhibited or inactivated.

Cellulose Accessibility Change during Pretreatment and Enzymatic Hydrolysis. Cellulose accessibility has been shown to be one of the most important factors affecting substrate digestibility.^{4,32} Because of the orange dye's approximate molecular size similarity to typical cellulases, the adsorption of orange dye during Simons' stain could be used to determine the accessible surface area of cellulose.³³ Figure 6a shows the adsorption of orange dye during Simons' staining (mg dye/g cellulose) of the untreated and various pretreated lignocellulosic biomass. The results indicated the pretreated switchgrass always had higher accessible surface area than the

pretreated poplar. The DAP poplar had an orange dye adsorption of 69.5 mg dye/g cellulose whereas the adsorption for the alkaline pretreated poplar was 50.9 mg, indicating the DAP of poplar is slightly more effective than the alkaline pretreatment in terms of cellulose accessible surface area increase. In contrast, the alkaline pretreated switchgrass had much more accessible surface area than the DAP switchgrass. Figure 6b shows a strong general positive relationship between the cellulose accessibility and substrate digestibility. The two samples that are not quite following the perfect linear pattern are the DAP and alkaline pretreated poplar. The glucose yields and the kinetics are quite similar for these two pretreatments on poplar, while the cellulose accessible surface area of DAP-poplar is actually slightly higher than the alkaline-poplar. The

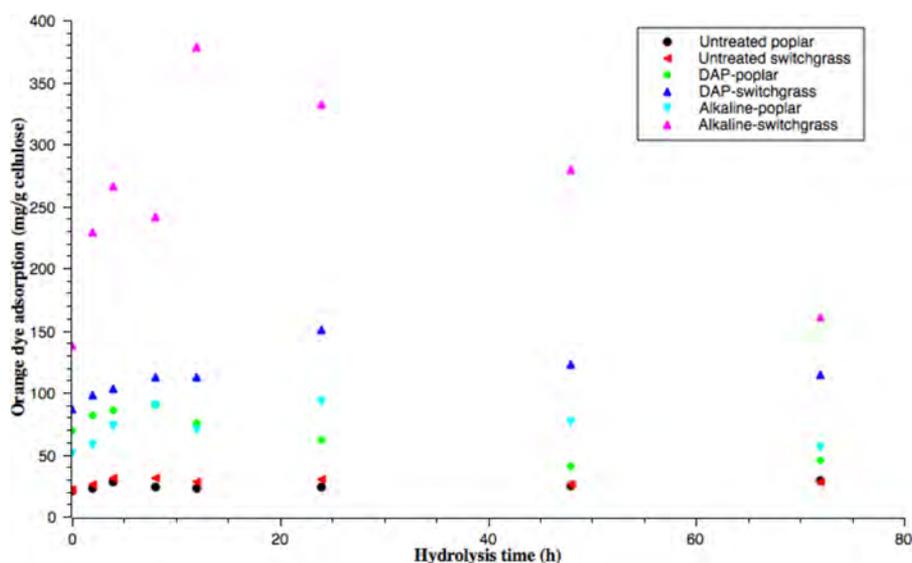


Figure 7. Changes of cellulose accessibility measured by Simons' stain of untreated and pretreated poplar and switchgrass during 72 h enzymatic hydrolysis.

likely reason could be the observation that lignin can not only affect the cellulose accessibility but also bind to the cellulases unproductively, therefore reducing the efficiency of hydrolysis.³⁴ Herein, delignification by alkaline did not increase the cellulose accessibility to the extent that DAP did, but the negative binding effect of lignin could be potentially decreased to some extent during the subsequent hydrolysis causing a similar sugar release for the DAP and alkaline pretreated poplar.³⁵ The removal of hemicellulose, specifically xylan (~99%), from the poplar after DAP improved the cellulose accessibility and glucose yield by about 220% and 37%, respectively as compared to the untreated poplar. Relatively same amount of xylan could be removed from switchgrass after DAP as well, and the cellulose accessibility and glucose yield was increased by ~210% and ~52%. Therefore, it indicated that the xylan removal had a much more important effect on the substrate digestibility of switchgrass than that of poplar. On the other hand, ~15% of lignin from poplar was removed after the alkaline pretreatment, causing the cellulose accessibility and the glucose yield increased by ~136% and ~37% whereas ~66% of lignin from switchgrass after the alkaline pretreatment improved the cellulose accessibility and glucose yield by about 400% and 66%. However, at the same level of delignification, e.g., 1% of lignin removal, the increase of accessibility and glucose yield for poplar was actually larger than that for switchgrass. Therefore, lignin likely plays a more important role in recalcitrance in poplar than switchgrass. DeMartini et al. also reported the xylan removal from switchgrass resulted in materials that achieved nearly 100% glucose yields at a high enzyme loading in subsequent enzymatic hydrolysis, whereas chlorite extractions that reduced the lignin content had the most beneficial effect in poplar.³⁶

The sample aliquots were taken from the enzymatic hydrolysis system at different time intervals, and the change of cellulose accessibility during enzymatic hydrolysis was tracked by Simons' stain after protease treatment to remove the bound enzymes (Figure 7). The cellulose accessibility was found to be increased at the beginning of hydrolysis, after reaching a maximum value then starting to decrease until the end of enzymatic hydrolysis. For the native materials treated

with cellulases, both poplar and switchgrass increased cellulose the accessibility during the first 4 h. The DAP poplar increased the cellulose accessibility during the first 8 h, whereas the DAP switchgrass increased the cellulose accessibility during the first 24 h. ~174% increase of the cellulose accessibility was observed during the first 12 h for the alkaline pretreated switchgrass, and only 76% increase was noticed during the first 8 h for the alkaline pretreated poplar. The increase of cellulose accessibility at the beginning of enzymatic hydrolysis could be due to the increase of porosity as enzyme further opened up the structure. During the initial stage of enzymatic hydrolysis, rather than the sequential shaving of cellulose fibrils from the outside, highly ordered regions of microfibrils could be delaminated and disrupted causing individual cellulose chains more accessible for interactions with cellulases.¹¹ This initial increase of internal surface area has been termed as amorphogenesis by Coughlan in 1985.³⁷ The drop in cellulose accessibility could be explained by the successive breakdown of pore walls thus again leading to smaller values.³⁸ Fan et al. showed that the specific surface area drastically increased from 3.9 to 11.6 m²/g cellulose during the first 6 h of enzymatic hydrolysis of a hammer-milled sulfite pulp, and a slow increase was observed until 24 h after this period until leveled off at ~12.2 m²/g cellulose, whereas the total cellulose surface area increased during the first 6 h of hydrolysis then started to decrease until the end of hydrolysis.³⁹ Similarly, Buschle-Diller et al. found porosity of the hemp fibers increased to approximately 270% for the first 4 h of treatment, then continuously dropped to about the original value of the untreated sample after 24 h of incubation.³⁸ Lee et al. reported the specific surface area increased from 8.50 to 9.34 m²/g cellulose during the first h of the hydrolysis due to the particle size reduction, followed by a slow decrease to 7.70 m²/g due to the hydrolysis of amorphous cellulose resulting in a larger crystalline fraction that is highly ordered with a lower specific surface area, and then gradually leveled off.¹² Although the decrease of cellulose accessibility could be a potential reason causing the decline of hydrolysis rate, it is interesting to note that cellulose accessibility is still pretty good after 72 h of enzymatic hydrolysis or even greater as compared to the starting material before enzymatic hydrolysis. For example,

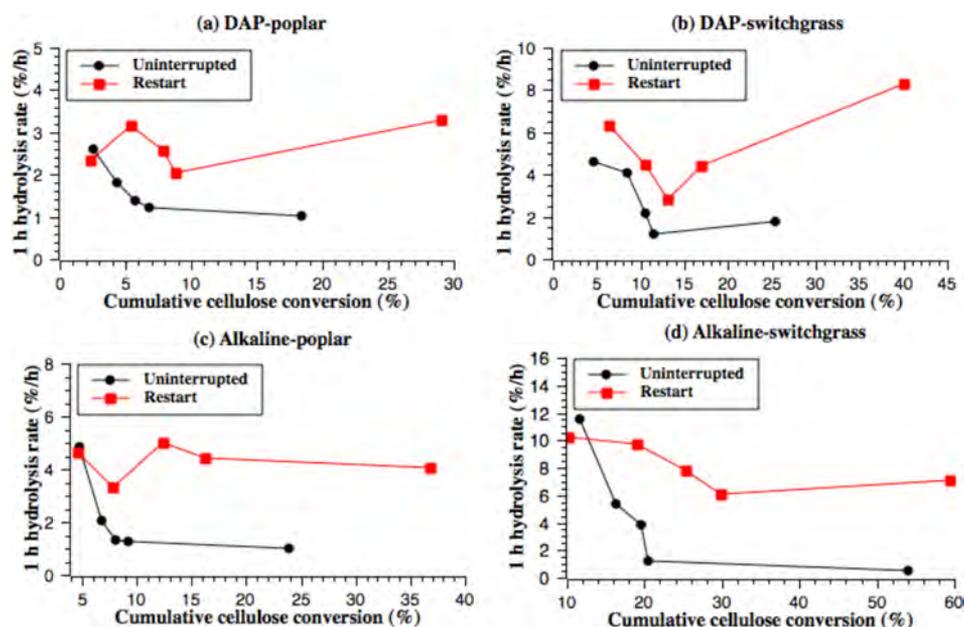


Figure 8. One-hour hydrolysis rate versus cumulative cellulose conversion for uninterrupted and restarted hydrolysis of DAP poplar (a), DAP-switchgrass (b), alkaline-poplar (c), and alkaline-switchgrass (d).

cellulose accessibility of alkaline pretreated switchgrass after 72 h enzymatic hydrolysis is 161.2 mg orange dye/g cellulose, whereas it is only 138.4 mg orange dye/g cellulose before enzymatic hydrolysis. Chandra et al. compared fed-batch and batch hydrolysis experiments which involved adding substrates to the hydrolysis reaction system at various specified times as opposed to adding the entire substrate at the beginning of the reaction.⁴⁰ Their results showed that the one-step batch reaction had higher overall hydrolysis yield compared to fed-batch reaction, suggesting that enzymes were likely unable to efficiently transfer from the initial hydrolyzed material to the freshly added substrate due to the irreversible binding of the enzymes to lignin. Furthermore, Simons' stain and water retention methods also indicated that the substrates retained a relatively high level of accessibility and an increase of swelling while the substrate was hydrolyzed. Enzyme related features including enzyme inhibition, jamming effects, diffusion constraints, clogging, and imperfect processivity might also be responsible for the slowdown of reaction rate.⁴¹ Cellulases might exhibit enhanced adsorption with progressing cellulose conversion. However, the activity of adsorbed cellulases might also decrease concomitantly, and at some point the lowering of efficiency of adsorbed cellulases will exceed the potential gain in hydrolysis rate due to the enhanced adsorption.¹⁴

One-hour Uninterrupted and "Restart" Cellulose Hydrolysis Rate. "Restart" hydrolysis, which involves disruption of batch hydrolysis and addition of fresh buffer/enzyme, provides a promising tool to assess if substrates lose their reactivity with conversion. To understand better if the hydrolysis extent was affected by the substrate reactivity during enzymatic hydrolysis, the 1 h hydrolysis rate versus cumulative cellulose conversion for both uninterrupted and restart experiment is shown in Figure 8. The incremental 1 h hydrolysis rate of all pretreated substrates decreased rapidly as the hydrolysis proceeded for uninterrupted batch hydrolysis. By contrast, the 1 h hydrolysis rate for restart hydrolysis system was either relatively stable or decreased at a significantly slower rate with an increasing of cellulose conversion. Interestingly,

there always exists at least one point where the 1 h hydrolysis rate is actually increased somehow for all the pretreated poplar and switchgrass. Furthermore, the cumulative cellulose conversion of restart hydrolysis is much higher than that of uninterrupted batch hydrolysis. Thus, these pretreated substrates probably did not lose their reactivity as it was converted over time, and in fact the accessibility clearly showed that the surface could become even more accessible later in the hydrolysis process. In addition, in light of the fact that the hydrolysis was performed at such a low solid loading (1%), it is reasonable to argue that end production inhibition by cellobiose or glucose was not postulated to be responsible for the continuous drop off of hydrolysis rate. The factors associated with the nature of enzymes such as enzyme inactivation or the enzyme steric hindrance should be responsible for the reduction of hydrolysis rate.

CONCLUSION

The action of pretreatment and cellulases on various characteristics of cellulosic fractions obtained from poplar and switchgrass was investigated. Enzymatic hydrolysis resulted in a rapid decrease in the degree of polymerization of cellulose. However, the susceptibility of the pretreated substrates to enzymatic hydrolysis could not be easily predicted from the differences in their cellulose DP. It is also difficult to relate the increase in recalcitrance of substrates to the structural modifications of cellulose during hydrolysis. However, the analysis of cellulose DP and accessibility is helpful in determining the mode of enzymes actions on the untreated and pretreated substrates. The DP analysis suggested a synergistic action of endo- and exoglucanases that contributes to the occurrence of a "peeling off" mechanism. Tracking the changes in cellulose accessibility during the course of enzymatic hydrolysis showed that the limited accessible surface area of cellulose is probably not a major limiting factor that causes the decline of hydrolysis rate in its late stage. Restart hydrolysis experiment suggested that enzyme related factors such as enzyme inactivation or steric hindrance should be responsible

to the reduction in hydrolysis rate given the large size of cellulase enzymes. In addition, the enzymatic hydrolysis of the different pretreated poplar and switchgrass strongly suggested different strategies should be applied when trying to engineer different plants for reduced recalcitrance.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.6b00603.

Experimental details regarding compositional analysis, cellulose isolation and derivatization, and Simons' stain, and all the actual data used to prepare Figures 1–7 are shown in Tables 2–8 (PDF).

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Notes

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■ ABBREVIATIONS

ATR-FTIR: Attenuated total reflectance Fourier transform infrared spectroscopy
 AFEX: Ammonia fiber expansion
 AKE: Avrami–Kolmogorov–Erofeev
 ASA: Accessible surface area
 DAP: Dilute acid pretreatment
 DP: degree of polymerization
 DP_w: Weight-average degree of polymerization
 HPAEC-PAD: High-performance anion-exchange chromatography with pulsed amperometric detection
 IL: Ionic liquid
 GPC: Gel permeation chromatography
 ORNL: Oak Ridge National Laboratory
 PEF: Polytetrafluoroethylene
 RI: Refractive index

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