DOI: 10.1002/cssc.201402752

The Effect of Alkaline Pretreatment Methods on Cellulose Structure and Accessibility

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The effects of different alkaline pretreatments on cellulose structural features and accessibility are compared and correlated with the enzymatic hydrolysis of *Populus*. The pretreatments are shown to modify polysaccharides and lignin content to enhance the accessibility for cellulase enzymes. The highest increase in the cellulose accessibility was observed in dilute sodium hydroxide, followed by methods using ammonia soaking and lime ($Ca(OH)_2$). The biggest increase of cellulose accessibility occurs during the first 10 min of pretreatment, with further increases at a slower rate as severity increases. Low temperature ammonia soaking at longer residence times dissolved a major portion of hemicellulose and exhibited higher cellulose accessibility than high temperature soaking. Moreover, the most significant reduction of degree of polymerization (DP) occurred for dilute sodium hydroxide (NaOH) and ammonia pretreated *Populus* samples. The study thus identifies important cellulose structural features and relevant parameters related to biomass recalcitrance.

In recent years, renewable energy resources such as wind, solar, and biomass have become of particular interest as a way to lower consumption of fossil fuels and to meet the ever-increasing demand for energy.[1] Amongst the various renewable sources being explored, bioethanol is being pursued as one of the most promising solutions to complement the usage of conventional fuels.[2] However, biomass recalcitrance is the biggest obstacle in the development of large-scale second-generation cellulosic ethanol production and use. Biomass recalcitrance hinders the effectiveness of enzymes during the bioconversion process due to lack of accessibilty, and mostly arises from the complex structure and ultrastructure of lignocellulosics, predominantly composed of a cellulose, hemicellulose, and lignin network matrix.[3] Hence, biomass requires pretreatment before it can be enzymatically deconstructed into simple sugars. Pretreatment has several aims, such as disrupting the physical structure of the biomass by breaking the lignin barriers, disrupting cellulose crystallinity, and removing noncellulosic components in order to increase the cellulose accessibility.[4] Previous studies indicate that structural parameters of the cellulose, mainly its degree of polymerization (DP) and its crystallinity, affect the biomass recalcitrance and subsequent enzymatic saccharification.[5] However, in many cases it is not clear how these features limit the enzymatic hydrolysis of cellulose.[6] The accessibility of biomass cellulose to cellulase is essential for the efficient deconstruction of cellulose and is believed to play a major role in influencing the rates of enzymatic deconstruction and glucose yield.[7]

Over the past few decades, various pretreatment methods, such as mechanical, physiochemical, and chemical treatments, often using alkali, acid, organosolv, ionic liquids, or steam, have been developed with the goal of increasing the enzymatic digestibility of biomass.[8] With respect to alkaline pretreatment, widely used reagents are sodium hydroxide,[9] ammonia,[10] and lime (i.e., calcium hydroxide; $Ca(OH)_2$).[11] Pretreatment with sodium hydroxide (NaOH) is the one of the most common methods and has been extensively studied in the bioconversion of lignocellulosics. NaOH treatment is very effective in increasing the digestibility of hardwood and agricultural residues with low lignin content.[12] Furthermore, Xu et al. reported that NaOH pretreatment offers great potential because it works at reduced temperatures and also exhibits a remarkable delignification capacity relative to its severity.[9b] Another effective alkaline process is pretreatment with lime. Lime pretreatment removes lignin, which improves the enzymes effectiveness because that eliminates nonproductive adsorption sites and increases access to cellulose and hemicellulose.[13] Ammonia pretreatment is an alternative alkaline pretreatment process, and involves the use of an ammonia solution either at high pressures (AFEX process) or in ambient conditions, by soaking biomass in aqueous ammonia.[14] All of these alkaline pretreatment conditions have a common effect: they increase the digestibility of the lignocellulosics. This is achieved by either changing the complex lignin–hemicellulose network or by increasing lignin removal.

This Communication examines the effects of various alkaline pretreatment methods on cellulose structure and its accessibility in milled hybrid *Populus* (*Populus trichocarpa x deltoids*). This study not only reveals the changes that occur in cellulose structure and accessibility upon a variety of low-cost and mild

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Supporting Information for this article is available on the WWW under http://dx.doi.org/10.1002/cssc.201402752.

Table 1. Alkaline pretreatment conditions.

T [°C]	*t*	Conditions	Sample name
120	2 min	2.0% sodium hydroxide	NaOH 2 min
120	10 min	2.0% sodium hydroxide	NaOH 10 min
120	60 min	2.0% sodium hydroxide	NaOH 60 min
120	10 min	0.10 M calcium hydroxide (lime)	$Ca(OH)_2$ 10 min
120	60 min	0.10 M calcium hydroxide (lime)	$Ca(OH)_2$ 60 min
25	5 days	soaking in 30% ammonia solution	SA 5 days
75	24 h	soaking in 30% ammonia solution	SA 24 h

alkaline treatments, but also determines some of the key factors responsible for biomass recalcitrance. The pretreatment conditions were chosen according to literature reports,[8a,10a,15] and were optimized for enzymatic release of sugars from *Populus* (Table 1).

The amounts of carbohydrates in untreated and alkaline-pretreated *Populus* samples are given in Figure SI1 (Supporting Information). There was a significant increase in glucan content, 12%, 35%, and 40%, after 2, 10, and 60 min of sodium hydroxide pretreatment, respectively. Pretreatment with lime for 10 min increased the glucan content by 12%, while soaking in ammonia for 5 days increased the glucan content by 14%. However, pretreatment by soaking in ammonia remained the most effective method for solubilizing most of the hemicellulose (ca. 41%). For untreated *Populus*, the klason lignin content was 29.9% of the total biomass, which agrees with the typical lignin content found in *Populus* species (20–30%).[16] For pretreated samples, the content varied from 19.5% to 26.0%. Samples pretreated with sodium hydroxide showed the lowest content of klason lignin (ca. 19–21%), indicating that the pretreatment is fairly effective at removing the majority of lignin under the conditions studied (Supporting Information, Figure SI2). All of the pretreatment methods also decreased the acid-soluble lignin content (Figure SI2).

Measurements of the molecular weights of the cellulose samples indicated that the highest level of degradation occurred in samples pretreated with sodium hydroxide, and increasing the severity of the pretreatment further degrades the cellulose. Pretreatment with sodium hydroxide for 2 min led to a ca. 8% decrease in cellulose DP_w, and as the residence time was increased to 10 min and 60 min degradation in cellulose DP increased to 61 and 76%, respectively (Table 2). For samples pretreated with lime for about 10 min, the DP_w of cellulose exhibited a decrease of 7% while a pretreatment time of 60 min caused more cellulose degradation, leading to a decrease in DP_w of 15%. For samples pretreated in ammonia at room temperature and a residence time of 5 days, the observed decrease in cellulose DP_w was 23% whereas, for a relatively shorter time of 24 h but a higher temperature, 65% decrease in cellulose DP_w was observed.

Table 2. Molecular characterization of pretreated *Populus* samples.[a]

Sample	DP_w	DP_n	PDI	CrI
Untreated	2504	342	7.3	55
NaOH 2 min	2312	173	13.3	52
NaOH 10 min	963	115	8.3	51
NaOH 60 min	578	77	7.5	54
$Ca(OH)_2$ 10 min	2312	154	15.0	51
$Ca(OH)_2$ 60 min	2119	169	12.5	50
SA 5 days	1926	134	14.3	50
SA 24 h	867	115	7.5	52

[a] DP_w = weight-average degree of polymerization, DP_n = number-average degree of polymerization, PDI = polydispersity index.

The crystallinity index (CrI) of cellulose isolated from alkaline-pretreated *Populus* samples was determined by ^{13}C CP/MAS measurements. The alkaline-pretreated cellulose showed significant change in the CrI as compared to cellulose isolated from untreated *Populus*, however, not much variation in CrI was observed among various pretreatment methods. The CrI data for alkaline-pretreated *Populus* samples ranged from 50% to 54%, which is slightly lower than that of the untreated *Populus* exhibiting 55% of cellulose crystallinity. The percent decreases in cellulose crystallinity in various alkaline-pretreated samples with respect to untreated cellulose sample were 5–7%, 7–9%, and 5–9% in sodium hydroxide, lime, and ammonia pretreatment, respectively. This indicates that the alkaline pretreatment methods may slightly disrupt the crystalline cellulose structure. The slight increase in crystallinity of *Populus* cellulose at longer pretreatment time is presumably due to the dissolution of amorphous cellulose, which is more susceptible to hydrolysis; however, at shorter pretreatment time the different pretreatment conditions appeared to have no notable preference of alkaline hydrolysis of cellulose amorphous regions. As reported earlier, high-severity conditions induce the thermochemical changes by breaking hydrogen bonds of cellulose and making amorphous cellulose more amenable to dissolve at higher temperature or longer residence time.[17] Further, treatment of pure cellulose samples with NaOH and ammonia usually alters their crystalline structures, which has also been shown to impact their enzymatic digestibility. While treatment with NaOH produces less-crystalline cellulose II, liquid ammonia pretreatment transforms cellulose I to the cellulose III allomorph.[18] In present study, the singlet (C-1) at 105.0 ppm in ^{13}C CP/MAS spectra of isolated cellulose samples indicates that the cellulose is predominantly in cellulose I form in all alkaline-pretreated *Populus* samples (see Supporting Information, Figure SI3).

The Simon's stain technique was used to evaluate the porosity of the biomass. The method involves the use of a dye mixture comprised of direct blue 1 (DB), which has a molecular diameter of ca. 1 nm, and direct orange 15 (DO),[19] with a molecular diameter in the range of 5–36 nm. The DB has a low affinity to cellulose while DO has a high affinity to cellulose initially. In general DB enters all the pores with a diameter larger than 1 nm, while DO only populates the larger pores. An increase of pore size for *Populus* would facilitate the DO dye gaining

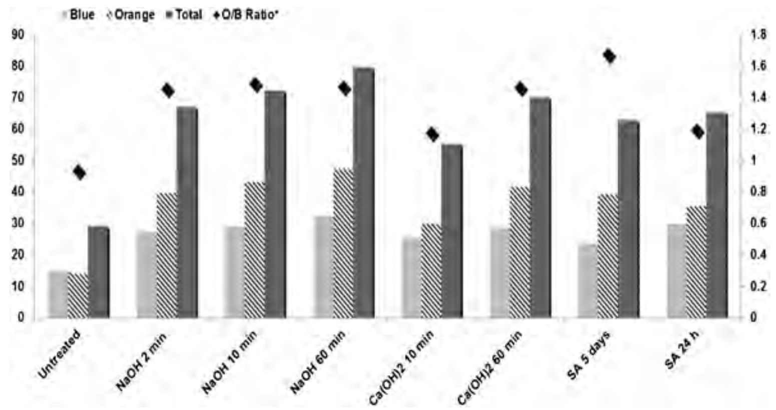

Figure 1. Dye adsorption diagram for NaOH-, $Ca(OH)_2$-, and ammonia-pretreated *Populus*.

report, employing an NMR relaxometry technique, on increasing accessibility by dilute acid pretreatment.[7]

The glucan and xylan yields (Figure 2) obtained from *Populus* by different pretreatments could be directly correlated to its compositional analysis data and other structural parameters of the cellulose, such as DP, crystallinity, and cellulose accessibility. The sodium hydroxide pretreatment resulted in a three- to four-fold increase of the cellulose-to-glucose conversion yield as compared to untreated *Populus*. Ammonia pretreatment resulted in a two-fold increase in glucose yield, as well as a four- to five-fold increase in xylose yield.

access to enlarged pores and displacement of DB because of the higher affinity of DO for the cellulose hydroxyl groups. The ratio and amount of DO and DB absorbed into the biomass indicates the number of large pores and small pores and subsequently cellulose accessibility in lignocellulosic biomass for enzymatic deconstruction.[7,20] The amount of dye adsorbed by the substrates as well as the orange/blue (O/B) ratio is shown in Figure 1. All of the alkaline pretreatments significantly increased cellulose accessibility, as revealed by the increased amount of orange dye adsorbed as well as the O/B ratio. As pretreatment severity extended, so did cellulose accessibility. Such analysis based solely on the O/B ratio may skew data interpretation, as larger amounts of blue dye adsorbed by a substrate can cause a decrease in the overall O/B ratio.[7] In this particular study, the amount of orange dye adsorbed alone might provide an even simpler and better indicator of the cellulose accessibility. In addition, although Simons' stain has successfully been utilized to assess the accessible surface area of cellulose in pretreated substrates, the specificity of the dyes for cellulose, when compared to lignin, still needs to be more fully resolved.

For dilute sodium hydroxide-pretreated *Populus*, although the O/B ratio did not increase as the pretreatment time increased, there was an obvious increase in the amount of orange dye adsorbed, which indicates increased cellulose accessibility. Sodium hydroxide was found to be much more effective than lime in terms of increased cellulose accessibility. At equivalent pretreatment times, dilute sodium hydroxide-pretreated *Populus* always adsorbed larger amounts of orange dye, and in fact pretreatment with sodium hydroxide for 10 min was more effective than pretreatment with lime for 60 min. Pretreatment by soaking in ammonia at room temperature and with long treatment times (i.e., ~5 d) is slightly more effective than pretreatment for 24 h at higher temperatures, as indicated by the amount of orange dye adsorbed. In addition, the biggest increase in cellulose accessibility upon alkaline pretreatment occurs in the first 10 min the pretreatment, although the accessibility continues to increase through the remaining 50 min of pretreatment but at a significantly slower rate. The exact same trend was also reported in a previous published

The results of characterization experiments demonstrated that significant changes had occurred regarding the DP and crystallinity of the cellulose—both of these parameters are considered as very important for effective enzymatic conversion of cellulose to glucose.[5b,18] A lower DP is a sign of an increased number of cellulose reducing ends and, consequently, a higher exoglucanase activity can be expected during enzyme hydrolysis. This in turn exposes further sites for endoglucanase attack[4b] and it weakens the networks to permit better access for the enzymes, making the cellulose more amenable to enzymatic deconstruction.[21] In the present study, the *Populus* cellulose samples pretreated with dilute sodium hydroxide and ammonia show significantly reduced DP_w and DP_n molecular weights, resulting in a notable reduction of the biomass recalcitrance of these samples.

In addition, the alkaline pretreatment methods studied herein reduced the crystallinity of the cellulose, although only a very slight change was found in the sample pretreated by sodium hydroxide for 60 min (Supporting Information, Figure SI3). Short residence times (e.g., 2 min or 10 min) in NaOH, lime, and ammonia revealed a reduced crystallinity, indicating possible decrystallization of the cellulose,[8a] while 60 min of treatment in dilute NaOH had a very slight change. However, in contrast, the sample pretreated with sodium hydroxide for 60 min released the highest amount glucose (after 48 and 72 h), which indicates that crystallinity may not be playing a very important role in determining sugar release. The role of crystallinity in effective cellulose enzymatic hydrolysis remains a subject of debate, and the lignin content, DP, and crystallinity are considered to influence the biomass recalcitrance to some extent.

As revealed by the modified SS technique, sodium hydroxide pretreatment led to the highest increase in cellulose accessibility and was more effective than any other tested alkaline pretreatment method. Lignin removal has been shown to increase the yield of enzymatic hydrolysis, however, the direct effect of lignin removal on cellulose accessibility is still not fully clear

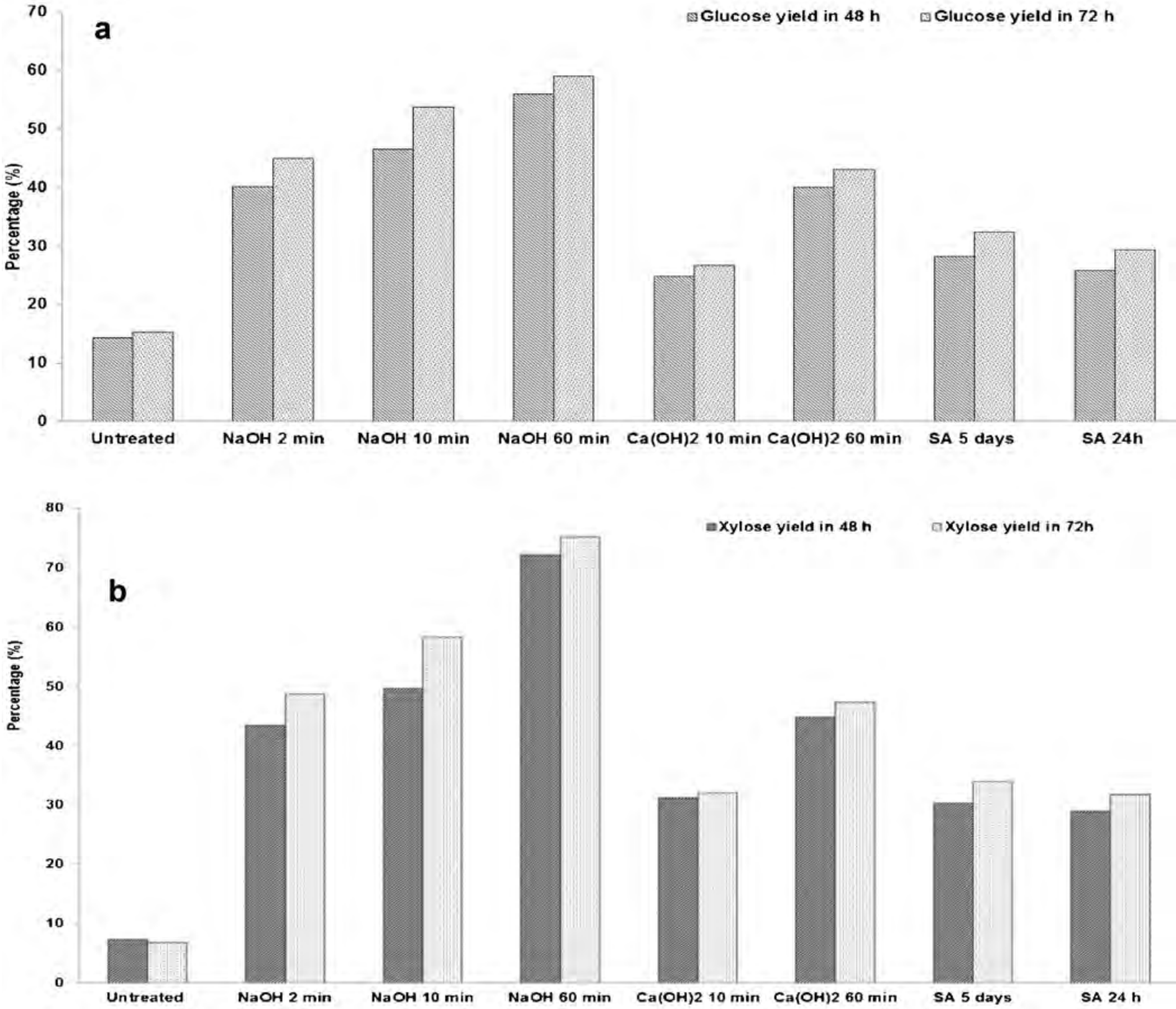

Figure 2. a) Glucose yields after 48 and 72 h. b) Xylose yields after 48 and 72 h from enzymatic hydrolysis of various *Populus* samples pretreated with alkali.

because lignin also binds to cellulases unproductively, and the relative contributions of these two roles of lignin have not yet been fully defined. In this study, the Simons' stain method indicated that substrates pretreated with sodium hydroxide for 10 min and 60 min had the lowest lignin content and highest cellulose accessibility. However, samples pretreated with ammonia had the highest lignin content among all the alkaline pretreatments but still showed higher cellulose accessibility data compared to samples pretreated with lime for 10 min. This is likely due to the fact that soaking ammonia pretreatments are much more effective at removing xylan. The effect of xylan removal on cellulose accessibility could also be explored by comparing these two soaking ammonia pretreatments. Soaking ammonia pretreatments at lower and higher temperature showed very similar lignin contents (26.0% and 25.7%), however, pretreatment at lower temperature was more effective than that at higher temperature in terms of xylan removal, thereby leading to higher cellulose accessibility. This suggests that hemicellulose, which is normally found on the outer surface of fibers as well as in interfibrillar spaces, is another physical barrier that limits cellulose accessibility. Nevertheless, a strongly positive relationship between cellulose accessibility and sugar release could be established (see Supporting Information, Figure SI4.)

Decreasing the degree of polymerization of cellulose, removal of lignin, and altered cellulose accessibility contributes to a reduced recalcitrance of the biomass and results in cellulose that can be more easily digested by enzymes. However, it is difficult to evaluate the effect of these factors independently because pretreatment modifies many parameters. Nonetheless, the present study provides key insight into biomass recalcitrance, specifically aspects associated to cellulose structure and accessibility arising from various alkaline pretreatment methods.

Experimental Section

Baseline *Populus* (*Populus trichocarpa x deltoides*) sample used in this work was harvested in 2012 at Oak Ridge National Laboratory, TN. Poplar was ground to a 0.814 mm particle size using a Wiley mill and stored in a cold room at 4 °C. Samples were extracted with dichloromethane (6×70 mL) in a Foss Soxtec unit (Soxtec 2050) heated at 80 °C following a 4-step extraction procedure. The experimental conditions used for the alkaline pretreatments are summarized in Table 1. The detail pretreatment process, compositional analysis and structural characterization of cellulose by Gel Permeation Chromatography (GPC), solid-state nuclear magnetic resonance (NMR) for crystallinity measurement and enzymatic hydrolysis were given in supporting information (SI5). The procedure for the Simons' stain (SS) was taken from Chandra et al.[20] using the Direct Blue 1 (DB, molecular diameter of approx. 1 nm, $C_{34}H_{28}N_6O_{16}S_4$) and Direct Orange 15 (DO,molecular diameter of 5–36 nm, condensation product of 5-nitrotoluenesulfonic acid in aqueous alkali) dyes and summarized in Supplementary Information (SI3).

Acknowledgements

This research was supported, in part, by the Genomic Science Program, Office of Biological and Environmental Research, U. S. Department of Energy, under Contract FWP ERKP752. The research at Oak Ridge National Laboratory's Center for Structural Molecular Biology (CSMB) was supported by the Office of Biological and Environmental Research under Contract FWP ERKP291, using facilities supported by the Office of Basic Energy Sciences, U.S. Department of Energy. This work was also supported, in part, by the Office of Biological and Environmental Research in the DOE Office of Science through the BESC, a DOE Bioenergy Research Center. Oak Ridge National Laboratory is managed by UT-Battelle, LLC, for the U. S. Department of Energy under Contract DE-AC05-00OR22725. Finally, Q.S. wishes to acknowledge Paper Science & Engineering fellowship program and J.D. for NSF-REU support.

Keywords: biomass · cellulose · crystallinity · enzyme catalysis · hydrolysis

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Received: July 25, 2014
Revised: September 30, 2014
Published online on ■■ ■■, 0000

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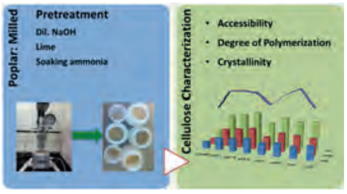

Back to basics: Biomass recalcitrance is the biggest obstacle in the development of large-scale second-generation use of cellulosic resources. The effects of different alkaline pretreatments on cellulose structural features and accessibility are compared and correlated with the enzymatic hydrolysis of *Populus*, revealing important cellulose structural features and parameters relevant to biomass recalcitrance.