

Solid-state NMR characterization of switchgrass cellulose after dilute acid pretreatment

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Reichel Samuel¹, Yunqiao Pu^{1,2†}, Marcus Foston¹ & Arthur J Ragauskas^{1,3†}

Background: Switchgrass (*Panicum virgatum* L.) is being considered as a potential feedstock for bioethanol production and extensive research is ongoing to establish the optimum pretreatment conditions for switchgrass. Cellulose comprises the most abundant biopolymer in the biosphere and its crystalline/ultrastructure is considered to be related to biomass recalcitrance. This study investigates the effects of dilute acid pretreatment on ultrastructural features of switchgrass cellulose. **Results:** In this study, switchgrass was pretreated in a pilot-scale reactor at 190°C (0.05 g sulfuric acid per gram of dry switchgrass) with 25% total solid loading and a reactor residence of 1 min. Cellulose was isolated from the pretreated and untreated switchgrass. The impact of pretreatment on the ultrastructure of cellulose was determined by solid-state cross polarization/magic angle spinning ¹³C NMR spectroscopy. Switchgrass demonstrated a preferable degradation of amorphous cellulose regions during dilute acid pretreatment. The pretreated switchgrass cellulose showed an 18% increase in crystallinity index when compared with the untreated switchgrass. Line-fitting analysis of the C-4 region of ¹³C NMR spectra revealed that the relative proportion of crystalline and paracrystalline celluloses in switchgrass was observed to increase after dilute acid pretreatment, accompanied with a concurrent decrease of the relative abundance of fibril surface cellulose. **Conclusion:** After dilute acid pretreatment, most of the hemicellulose in switchgrass was removed. The amorphous cellulose regions in switchgrass were degraded preferably during dilute acid pretreatment and the cellulose crystallinity index of pretreated switchgrass increased. Pretreated switchgrass had an increase in relative proportion of crystalline and paracrystalline cellulose in comparison to the starting material.

The development of low-cost, sustainable and low net carbon footprint renewable biofuels as a viable alternative to fossil fuels is a growing societal issue [1,2,101]. Fuels derived from lignocellulosic biomass, such as woody plants, forest residues and nonfood agroenergy crops are a viable alternative to fossil fuels and food-based biofuels [3–6]. Lignocellulosic biomass is a complex bioresource consisting primarily of cellulose, hemicellulose and lignin [7]. Lignin–carbohydrate associations in biomass hinder the availability of cellulose for enzymatic deconstruction and contribute, in part, to biomass recalcitrance. The biochemical conversion of lignocellulosic materials to ethanol normally includes three

processes: **pretreatment**, hydrolysis of polysaccharides to fermentable sugars and the fermentation of sugars to ethanol [8,9]. One promising way to address biomass recalcitrance is a pretreatment that may involve biological [10,11], chemical and physical treatments, such as uncatalyzed steam explosion [12,13], hot-water [14], dilute acid [15], lime [16] and ammonium fiber explosion [17].

Switchgrass is a sustainable herbaceous plant that is a promising biofuel crop due to its high mass yield per acre, broad adaptability, low agrochemical input and positive environmental effects [18–22]. Extensive research is ongoing to establish optimum pretreatment conditions for switchgrass. Suryawati *et al.* performed hydrothermolysis

[†]Author for correspondence

¹BioEnergy Science Center, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA, USA

²BioEnergy Science Center, Institute of Paper Science and Technology, Georgia Institute of Technology, Atlanta, GA, USA;

²E-mail: Yunqiao.pu@ipst.gatech.edu

³E-mail: Arthur.ragauskas@chemistry.gatech.edu

Key terms

Pretreatment: Physical, chemical or biological treatment that increases the accessibility of cellulose to enzymes and solubilizes hemicellulose sugars

Switchgrass cellulose: Most abundant polysaccharide in switchgrass

with various temperature and residence times and established that the highest concentration of ethanol (i.e., 72%) was produced from switchgrass pretreated at 210°C for 15 min using simultaneous saccharification and fermentation [23].

Most recently, Ying *et al.* studied dilute acid pretreatment on switchgrass germplasms and observed that hemicellulose solubilization depended on pretreatment intensity and no significant delignification occurred [24]. Asli *et al.* reported simultaneous saccharification and fermentation followed by aqueous ammonia pretreatment and observed a 40–50% delignification, a 50% decrease in hemicellulose and an unchanged cellulose content in the pretreated switchgrass [25]. Alkali pretreatment coupled with radiofrequency-based dielectric heating techniques was reported to increase sugar yield from switchgrass in both pretreatment and enzymatic hydrolysis stages [26]. Cheng *et al.* reported that 26% xylan and 29% lignin were solubilized as a result of lime pretreatment of switchgrass; however, there was no significant glucan solubilization observed [27]. Wu and Lee investigated a two-stage dilute acid percolation on switchgrass with a low acid concentration (0.078 wt% sulfuric acid) under moderate temperature (145–170°C) and observed that hemicellulose was completely solubilized and the lignin content in switchgrass increased after pretreatment [28]. In the present study, we investigated the effects of dilute acid pretreatment on ultrastructures of **switchgrass cellulose** using solid-state cross polarization/magic angle spinning (CP/MAS) NMR spectroscopy.

Materials & methods

All chemicals used in this study were purchased either from VWR International or Aldrich and used as received. The lowland cultivar Alamo switchgrass used in this study was received from National Renewable Energy Laboratory (NREL) and harvested from Ardmore, Oklahoma on November 2007. It was ground to pass a 20-mesh screen.

▪ **Acid pretreatment**

Dilute sulfuric acid pretreatment was carried out at NREL following a published procedure [29,30]. In brief, pretreatment was performed in a pilot-scale reactor at 190°C (0.05 g sulfuric acid per gram of dry switchgrass) with 25% total solid loading with a reactor residence of 1 min. The pretreated material was filtered and stored below 0°C before use. The frozen pretreated switchgrass was thawed to room temperature, filtered through a Buchner funnel and

washed with deionized water, until the effluent was pH neutral, and air-dried overnight. In order to remove the low-molecular weight extractives, the switchgrass sample was Soxhlet-extracted with benzene/ethanol (2:1 v/v) and ethanol for 24 h each [31]. The extracted residue was air-dried overnight prior to chemical characterization.

▪ **Carbohydrate analysis**

Carbohydrate analysis of extractive-free untreated and pretreated switchgrass was performed according to a procedure established in the literature [32,33]. In brief, the extracted switchgrass sample was treated with 72 wt% sulfuric acid for 1 h at 30°C and then diluted to 4 wt% sulfuric acid using deionized water, and subsequently autoclaved at 121°C for 1 h. The resulting solution was cooled to room temperature and the precipitate was filtered. The filtrate was used for the detection of sugar composition by high-performance anion-exchange chromatography with pulsed amperometric detection. The sugar composition was measured using a Dionex DX-500 Ion Chromatograph system with a GP-40 gradient pump, PC10 pneumatic controller, AS40 autosampler, ED40 electrochemical detector. A Dionex PA-10 column was used. 2.00-mM NaOH was used as the eluent and 0.2-M NaOH as the postcolumn rinsing effluent. Fucose was used as an internal standard. The flow rate was 1.0 ml/min. The results are shown in **Table 1**.

▪ **Holocellulose pulping & cellulose isolation**

Holocellulose was isolated from extractive-free biomass using a modified procedure from the literature [34,35]. To a Kapak sealing pouch was added extractive-free switchgrass (8 g), deionized water (260 ml), sodium chlorite (2.40 g) and glacial acetic acid (2 ml). The sealed pouch was placed in a shaking water bath, set at 75°C for 2 h. After 2 h, another batch of sodium chlorite and glacial acetic acid were added and the mixture was heated in the water bath for a further 2 h. This process was repeated until the holocellulose turned white. The treated switchgrass was cooled, filtered through a Buchner funnel, washed several times with deionized water and air-dried in a fume-hood. Yields of holocellulose from untreated and pretreated switchgrass were approximately 78 and 67%, respectively. Cellulose was isolated from holocellulose sample by an acid treatment following a procedure from the literature [36,37]. In

Table 1. Carbohydrate content in switchgrass before and after pretreatment (based on dried biomass).

	Arabinan (%)	Galactan (%)	Glucan (%)	Mannan (%)	Xylan (%)
Untreated switchgrass	3.1	1.1	45.6	0.5	26.1
Pretreated switchgrass	0.4	0.5	54.8	0.2	7.2

brief, holocellulose (0.6 mg) was treated with aqueous 2.5-M HCl (60 ml) at 98–100°C for 4 h, cooled to 0°C, filtered in a Buchner funnel, washed several times with deionized water and air-dried in a fume-hood. The isolated cellulose was then conditioned with water vapor before solid-state NMR measurements were taken.

▪ Solid-state NMR analysis

NMR samples were prepared with ground cellulose packed into a 4-mm cylindrical Zirconia MAS rotor. Solid-state NMR measurements were carried out on a Bruker Avance-400 spectrometer operating at frequencies of 100.59 MHz for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS ^{13}C NMR experiments were carried out with a 90° proton pulse, 1.5-ms contact pulse, 4-s recycle delay and 8192 scans. All spectra were recorded on moist samples (40–60% water content). The line-fitting analysis of spectra was performed using NUTS™ NMR data-processing software (Acorn NMR Inc.). The cellulose crystallinity index (CrI) was determined from the areas of the crystalline and amorphous C-4 signals using the following formula [38]:

$$\frac{A_{86-92 \text{ ppm}}}{(A_{79-86 \text{ ppm}} + A_{86-92 \text{ ppm}})} \quad \text{Equation}$$

Results & discussion

The results of chemical analysis indicated that glucan and xylan were the predominant carbohydrates in switchgrass. Table 1 illustrates the carbohydrate content of pretreated and untreated switchgrass. The glucan proportion in the carbohydrate was observed to increase and other carbohydrates showed a decrease in content. It was noticed that, as a result of pretreatment, there was approximately a 72% decrease in relative xylan content and approximately a 20% increase in relative glucan content. The data suggest that the hemicellulose in switchgrass samples was preferentially degraded with respect to the cellulose when subjected to acid pretreatment.

Solid-state CP/MAS ^{13}C NMR was used to study the switchgrass cellulose ultrastructure. Figure 1 represented the CP/MAS ^{13}C NMR spectra of cellulose isolated from untreated and pretreated switchgrass. The peaks at δ 61.9 and δ 64.8 ppm are assigned to the C-6 glucopyranosyl repeating units in cellulose (δ 61.9 ppm for amorphous cellulose and δ 64.8 ppm for crystalline cellulose). The cluster of resonances around the peaks at δ 72.2 and δ 75.8 ppm are assigned to C-2, C-3 and C-5. The peaks at δ 84.4 and δ 89.0 ppm are attributed to C-4 and the absorption peak at δ 105.0 ppm is assigned to C-1 of glucose in cellulose [36–40]. The pretreated switchgrass showed

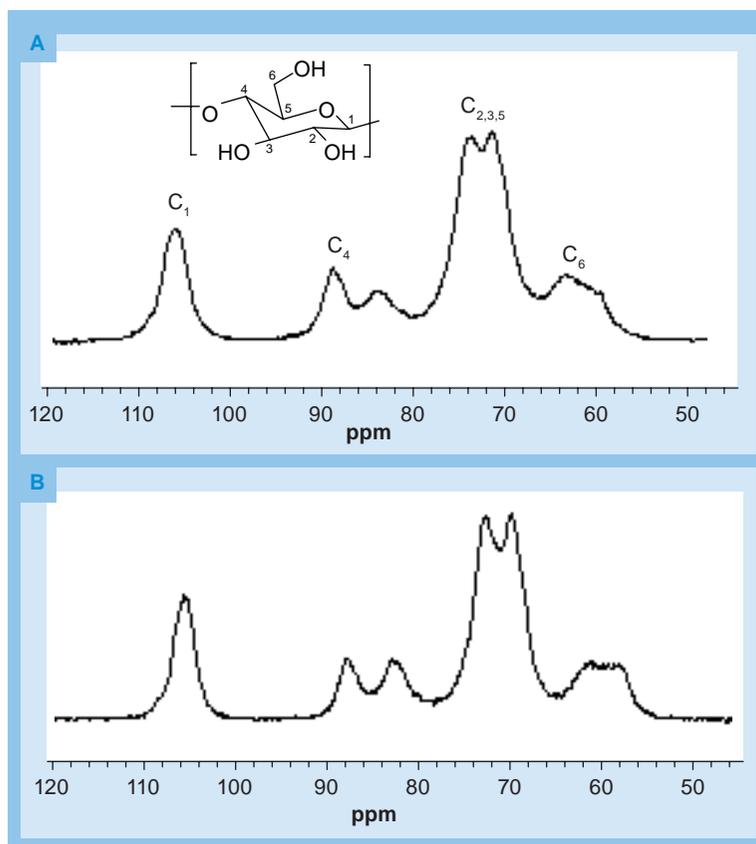


Figure 1. Cross polarization/magic angle spinning ^{13}C NMR spectra of cellulose isolated from untreated and pretreated switchgrass. (A) Pretreated; (B) untreated.

a decrease in signal intensities at both the C-6 and C-4 peaks of amorphous cellulose (Figure 1), indicating that the switchgrass cellulose underwent a preferred degradation of amorphous regions during dilute acid pretreatment. Cellulose CrI was determined from the crystalline and amorphous signal areas of the C-4 region. The cellulose crystallinity index of untreated

Table 2. Crystallinity index of lignocellulosic biomass before and after pretreatment.

	Untreated	Pretreated
Switchgrass	0.44	0.52
Loblolly pine ^a	0.63	0.70
<i>Buddleja davidii</i> ^b	0.55	0.53
Rice straw ^c	0.60	0.64
Rice straw ^d	0.55	0.60

^aTwo-stage dilute acid pretreatment (crystallinity index measured using cross polarization/magic angle spinning NMR) [32].

^bEthanol organosolv pretreatment at 180°C for 60 min, 1.25% sulfuric acid and ethanol 50% (crystallinity index measured using CP/MAS NMR) [41].

^cSolid-state NaOH pretreatment (crystallinity index measured using X-ray) [42].

^dFungal pretreatment by *Phanerochaete chrysosporium* (crystallinity index measured using x-ray) [10].

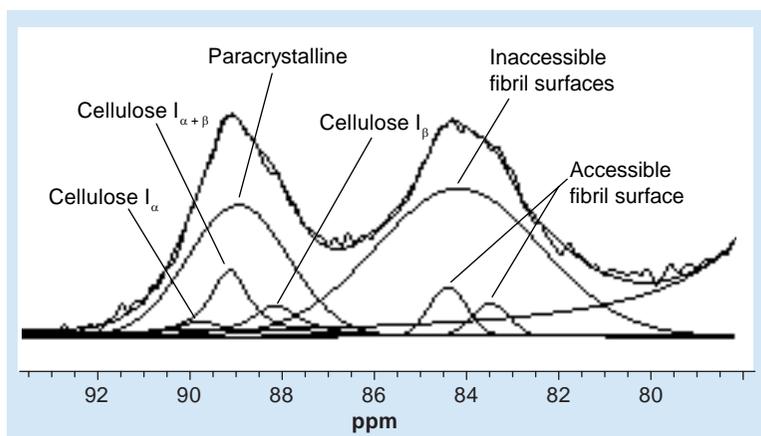


Figure 2. Spectral fitting for the C-4 region of cross polarization/magic angle spinning ^{13}C NMR spectrum of cellulose isolated from untreated switchgrass.

switchgrass cellulose was determined to be 0.44. After dilute acid pretreatment, the CrI of switchgrass cellulose was increased to 0.52, which is an increase of 18% (Table 2). Loblolly pine was observed to show an increase of CrI by approximately 11% after two-stage dilute acid pretreatment (Table 2) [32]. Hallac *et al.* isolated cellulose from *Buddleja davidii* after ethanol organosolv pretreatment and measured the CrI index using CP/MAS ^{13}C NMR [41]. There was no noticeable change observed for the CrI of *Buddleja davidii* cellulose after ethanol organosolv pretreatment. Rice straw pretreated respectively with solid-state sodium hydroxide and the fungus *Phanerochaete chrysosporium* demonstrated a small increase in CrI (Table 2) [10,42]. The effects of pretreatment on lignocellulosic biomass cellulose CrI appear to be dependent on the biomass species and pretreatment methods.

Cellulose supramolecular structure was characterized and compared by nonlinear least-square line-fitting analysis of the C-4 region of CP/MAS ^{13}C NMR spectra of cellulose [37,38,40]. Lorentzian lines were used for crystalline regions and Gaussian lines for

the amorphous regions according to literature methods [38,40]. These analyses were employed to estimate the relative abundance of cellulose I_α , and I_β , paracrystalline cellulose and cellulose at solvent-accessible and solvent-inaccessible fibril surfaces [43,44]. Figure 2 represents the spectral fitting for the C-4 region of the CP/MAS ^{13}C NMR spectrum of cellulose isolated from untreated switchgrass and the line-fitting analysis results are illustrated in Table 3. Inaccessible fibril surface cellulose was the largest fraction observed. The relative intensity of crystalline cellulose (i.e., I_α , I_β and $\text{I}_{\alpha+\beta}$) and paracrystalline celluloses increased in the cellulose extracted from acid-pretreated sample, accompanied with a concurrent decrease in the relative intensity of accessible and inaccessible fibril surface cellulose. There was approximately a 12% decrease in the relative proportion of inaccessible fibril surface cellulose after diluted acid pretreatment. This was likely due, in part, to a susceptible degradation of cellulose in the amorphous region for switchgrass samples in the present study. Josefsson *et al.* reported that aspen wood after steam explosion at high temperature resulted in a severe cellulose degradation, accompanied by a decrease in fibril dislocations and an increase in crystalline and/or paracrystalline cellulose [45].

Conclusion

Cellulose was isolated from untreated and dilute acid pretreated switchgrass and analyzed by solid-state CP/MAS ^{13}C NMR spectra. After pretreatment, most of the hemicellulose in switchgrass was removed and the cellulose CrI increased by approximately 18%. Based on the line-fitting analysis of the C-4 region of cellulose solid-state ^{13}C NMR spectra, the relative proportion of crystalline and paracrystalline cellulose increased after dilute acid pretreatment, while the relative abundance of fibril surface cellulose decreased.

Future perspective

The results of this study contribute to a fundamental understanding of cellulose ultrastructure and how pretreatment can impact this important parameter. Cellulose crystallinity is well recognized to be a significant contributing factor to the ease of enzymatic deconstruction of cellulose and is often overlooked in pretreatment technologies. This study has shown that chemical pretreatment impacts cellulose ultrastructure and additional research is needed to optimize reaction conditions to maximize cellulose reactivity with cellulase. From the viewpoint of biofuel production, our study provides an insight into future research, which needs to improve pretreatment technologies that can effectively reduce biomass recalcitrance.

Table 3. Assignment and results from line-fitting analysis of the C-4 region of cross polarization/magic angle spinning ^{13}C NMR spectra of switchgrass cellulose.

Assignment	Chemical shift (ppm)	Relative proportion (%)		Line type
		Untreated	Pretreated	
Cellulose I_α	89.9	2.3	3.6	Lorentz
Cellulose $\text{I}_{\alpha+\beta}$	89.1	8.0	10.1	Lorentz
Paracrystalline cellulose	89.0	27.3	32.7	Gauss
Cellulose I_β	88.3	4.8	5.7	Lorentz
Accessible fibril surface	84.7/83.8	6.2	2.8	Gauss
Inaccessible fibril surface	84.4	51.3	45.2	Gauss

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

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Executive summary

- The effect of dilute acid pretreatment was investigated by solid-state crosspolarization/magic-angle spinning ¹³C NMR spectra.
- As a result of pretreatment, the cellulose crystallinity index increased by around 18%.
- Hemicellulose-related sugars showed a significant decrease in relative content after pretreatment.
- The relative proportion of cellulose I_α and I_β and paracrystalline celluloses were increased and, concurrently, the relative abundance of fibril surface cellulose decreased.

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