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Variations in Cellulosic Ultrastructure of Poplar

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Abstract A key property involved in plant recalcitrance is cellulose crystallinity. In an attempt to establish the typical diversity in cellulose ultrastructure for poplar, the variation and distribution of supramolecular and ultrastructural features, including the fraction of crystalline cellulose forms I_α and I_β, para-crystalline cellulose and amorphous cellulose content were characterized. In this study, the percent crystallinity (%Cr) and lateral fibril dimensions of cellulose isolated from poplar were determined for 18 poplar core samples collected in the northwestern region of the USA.

Keywords Cellulose · Poplar · Solid-state NMR

Introduction

Biomass recalcitrance describes a complex combination of material properties that result in the plant cell wall's ability to resist breakdown into its constituent units. Over the past few decades, a tremendous amount of research has focused on determining the exact nature and source of this complex property [1, 2]. A more comprehensive understanding of recalcitrance will enable more effective deconstruction of lignocellulosic substrates via enzymes, microbes, and/or other chemical/mechanical processes. High-yield, low-cost, efficient deconstruction of biomass is essential to the widespread use of renewable energy sources such as second-generation biofuels, e.g., cellulosic ethanol [1].

Various models of lignocellulosic substrates and their recalcitrance have been proposed; many, in particular, describe cellulase enzyme activity and the potential effect of substrate characteristics such as crystallinity, degree of polymerization (DP), specific surface area, lignin distribution, etc. [3–5]. Much of the information stated in the literature pertains to the effect of crystallinity on enzymatic glucose production. Overall, these studies have conflicting results mainly due to varying substrates (pure cellulose or cellulose within natural lignocellulosics), varying enzymes whose mechanism of deconstruction are different, and/or the use of chemical or physical treatments employed to create variability in crystalline index, which will invariably alter other substrate characteristics [4].

One of the few studies that directly evaluated cellulose ultrastructure impact on cellulase including the relative fraction of cellulose I_α, I_β, *para*-crystalline, and amorphous was the work by Pu et al. [6]. Using solid-state NMR, a faster decrease was observed in amorphous and *para*-crystalline cellulose contents when compared with the other crystalline allomorphs during enzymatic deconstruction. Although some early research has gone into genetic manipulation of recalcitrance, it would be particularly useful to first determine to what extent these ultrastructure components vary in native biomass. This knowledge could then be used in the long term to develop new genetic clones and/or growing conditions that may influence the cellulose ultrastructure.

In this study, we present the use of ^{13}C cross-polarization magic angle spinning (CP/MAS) NMR on isolated cellulose

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as means of evaluating the lignocellulosic supramolecular and ultrastructure in poplar.

Materials and Methods

Substrates

Poplar (*Populus trichocarpa*) samples were harvested in the Rainier, Washington state area between 2007 and 2008 by Oak Ridge National Lab (ORNL) as part of a populus association study. The poplar tree core samples were taken cross-sectionally, at breast height (~1.3 m), avoiding proximity to branches or stem defects whenever possible. The sampled trees were taken from a maximum diversity of ages/establishment cohorts purposefully in order to truly sample the population of poplar in the area. The biomass was sized-reduced in a Wiley mill to pass through a 20-mesh screen. Extractives were subsequently removed by placing the biomass into an extraction thimble in a Soxhlet extraction apparatus. The extraction flask was filled with a 2:1 (*v*/*v*) ethanol/benzene mixture (~150 mL) and then refluxed at a boiling rate which cycled the biomass for at least 24 extractions over a 4-h period.

Sample Preparation for NMR

Following well-cited procedures, holocellulose was isolated from the extracted samples by exposure to $NaClO_2$ (1.30 g/1.00 g lignocellulosic dry solids) in acetic acid (375 mL, 0.14 M) at 70°C for 2 h [6–12]. The samples were then collected by filtration and rinsed with an excess of deionized (DI) filtered water. This was repeated to ensure complete removal of the lignin component, typically twice. Cellulose was isolated from the holocellulose samples (1.00 g) by hydrolysis of the hemicellulose via reaction with 2.5 M HCl following literature procedures [6–12]. The isolated cellulose samples were then collected by filtration, rinsed with an excess of DI filtered water, and left at ambient room conditions to allow the moisture content to equilibrate.

NMR Analysis

The NMR samples were prepared with ground isolated cellulose placed into 4-mm cylindrical ceramic MAS rotors. Repetitive steps of packing sample into the rotor were performed to fully compress and load the maximum amount of sample. Solid-state NMR measurements were carried out on a Bruker Advance-400 spectrometer operating at frequencies of 100.55 MHz for ^{13}C in a Bruker double-resonance MAS probe head at spinning speeds of 10 kHz. CP/MAS experiments utilized a 5-μs (90°) proton pulse, 1.5-ms ramped contact pulse, 4-s recycle delay, and 4–8 K scans. All spectra were recorded on wet samples whose moisture content was adjusted to 60–80%, and the line fitting analysis of spectra was performed using NUTS NMR data processing software (Acorn NMR, Inc.).

Statistical Relevance

In order to provide a means to determine statistical relevance, five samples from the same batch of poplar baseline material were processed and tested according to the above procedures. The standard deviation (σ) of the data from these five control samples was calculated for percent crystallinity (%Cr) and lateral fibril dimensions (LFD). The 3σ values are 1.71% and 2.1 nm, respectively. Any variation greater than 3σ was deemed statistically significant and not the result of variation due to experimental technique or instrumentation.

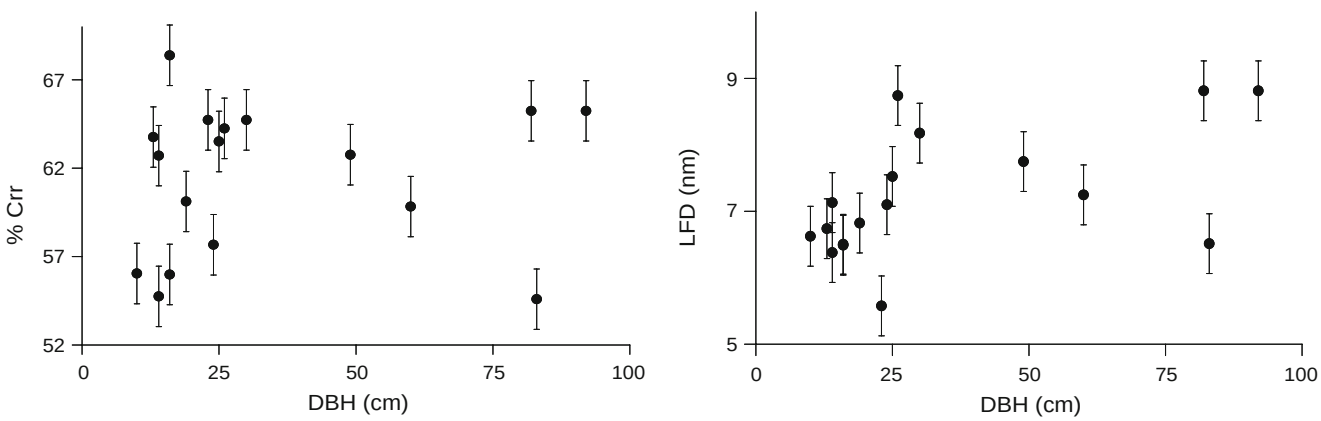

Fig. 1 Percent crystallinity plotted and lateral fibril dimensions against diameter at breast height

Table 1 Cellulose crystallinity measures by NMR for various hardwoods

Hardwood species	%Cr
Picea (Spruce) [9]	48
Betula (Birch) [9]	36
Tectona grandis (common teak) [10]	51
Pseudowintera axillaris (Heropito) [10]	52
Eucalyptus regnans (mountain ash) [10]	53
Beilschmiedis tawa [10]	55
Castanea sativa (sweet chestnut) [10]	55
Quercus robur (English oak) [10]	57
Populus trichocarpa × *deltoids*[a]	63

[a] Poplar BESC baseline sample, a clone produced for long-term consistent comparisons to wild or genetically altered poplar within the bioenergy center

Results and Discussion

The sampling of 18 poplar tree samples was accomplished from a poplar planted stand located in the northwestern region of the USA within approximately 265 km of one another. The maximum variation in growth site altitude was approximately 114 m. Although growth site latitude and altitude do not completely or explicitly describe all pertinent environmental growth factors, board variations in location can be used to represent geographically driven variation in a number of climate- and soil substrate-related factors such as mean average temperature, precipitation, and soil nutrient availability [13].

^{13}C CP/MAS NMR spectroscopy experiments were conducted to determine the relative intensity of crystalline and non-crystalline ultrastructural components of cellulose [8, 14] and how those relative intensities vary across the sampled poplar trees. The C_4 region in spectra of isolated cellulose, which extends over a chemical shift range of $\delta \sim$ 80–92 ppm, is used to determine crystallinity by integration of non-crystalline domains which appear as broad signals from $\delta \sim$ 80–85 ppm [12] and crystalline domains at $\delta \sim$ 85–92 ppm [7]. By interpreting intensity of the non-crystalline domains as fibril surfaces and fibril-to-fibril contacts, while also utilizing a simple fibril model in which the fibril cross-section is square and the cross-sectional area of a cellulose chain is 0.55 chains/nm^2, the cellulose crystalline LFD can also be determined following literature methods [11].

Figure 1 seems to show a weak positive correlation between tree maturity, as measured by diameter at breast height and LFD, which is directly correlated to relative amount of fibril surfaces and thus related to crystallinity. The correlation, though weak, is supported by observations made by Jahan et al. [15]. In their study, Nalita trees were sampled at the age of 12, 18, 24, and 30 months from the Dhaka region of Bangladesh. The isolated cellulose from the tree at different stages of development was then studied by X-ray diffraction and dilute solution viscometry. The resulting data suggested that the proportion of crystallinity, crystal size, and DP increased with tree age.

Wood is a complex and heterogeneous material. Variations can exist in the chemical structure, pore distribution, structure and type of cells, as well as the content of cell wall fractions. All these factors, along with variations in cellulose morphology within a microfibril, can potentially affect the degree of cellulose crystallinity and the subsequently observed recalcitrance. Table 1 shows the percent crystallinity of poplar and various hardwoods complied from the literature. This would indicate that there are differences in crystallinity between different hardwoods and, along with the observations in Fig. 1, also within the same sample species.

The data in Table 1 indicate that poplar has a comparatively high %Cr while also providing further suggestions that genetics may play an important role in determining cellulose morphology. Although the sampled population was not large enough to generate a model to accurately describe natural genetic variation on cell wall morphology, the sampled poplar has an average crystallinity of ~61.4% with a standard deviation of ~4.2%. A histogram plot of the %Cr for the sampled poplar population, seen in Fig. 2, shows a relatively wide distribution of crystallinities. This, along with the appreciable scattering in the plot of %Cr and DHB, suggests that some combination of genetic and/or environmental growth factors determine the resulting cellulosic ultrastructure for poplar. Given this, it would then be possible through either genetic screening or a select choice of growth locations to generate large quantities of poplar whose ultrastructure has a higher cellulosic reactivity and lower recalcitrance.

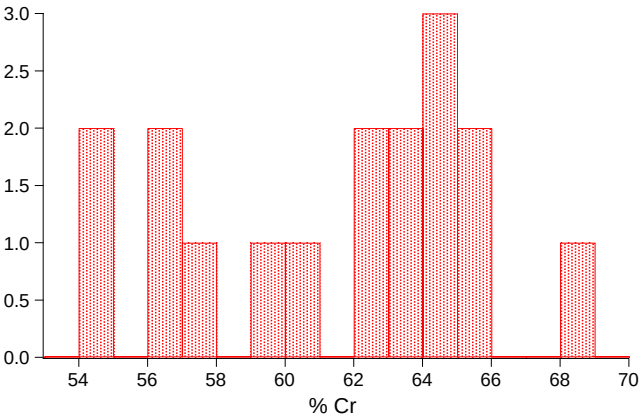

Fig. 2 Percent crystallinity for the sample population plotted as a histogram

Table 2 Average values for the nonlinear least-squared spectral fitting results of the C_4 region for ^{13}C CP/MAS spectra of isolated cellulose for the population of poplar association samples

Assignment	Chemical shift (ppm)	Relative intensity (%)	
		Avg.	σ[a]
I_α	90.5	5.0	2.8 (1.0)
$I_{\alpha+\beta}$	89.4	14.2	5.1 (1.3)
Para-crystalline	88.8	31.1	3.5 (2.1)
I_β	88.2	19.8	5.5 (2.2)
Accessible surface	84.6	5.0	1.2 (0.5)
Inaccessible surface	84.2	18.3	3.3 (0.7)
Accessible surface	83.8	5.2	2.0 (0.7)

[a] Values in parentheses represent the standard deviation of five poplar baseline control samples

In addition, line shapes whose parameters of relative intensity and line width are varied were applied to the carbon signals attributed to domains of cellulose $I_{\alpha+\beta}$, I_α, I_β, *para*-crystalline cellulose, inaccessible, and accessible fibril surfaces. The average results of this fitting procedure on the sampled population poplar are compiled in Table 2 and provide an example to the type of diversity seen in poplar for the relative proportions of each cellulosic ultrastructural component. The values in parentheses are the standard deviations of five poplar baseline control samples for each ultrastructural component and represent the variation due to experimental technique. Again, these results suggest that a promising approach to developing low-recalcitrant poplar is to identify the natural diversity of cellulose crystallinity in poplar and employ this knowledge to develop the next-generation poplar.

One might expect, particularly for environmentally linked factors, that there may exist common factors affecting the content and nature of the various lignocellulosic components. Figure 2 was plotted in an attempt to indirectly determine whether any common influence exists in the biosynthesis of lignin and cellulose. The percent lignin data were determined at NREL using analytical pyrolysis, and a similar plot was generated using the ratio of syringyl-like lignin structures to guaiacyl-like lignin structures. As the random scattering in Fig. 2 illustrates, there is no appreciable link between percent lignin and %Cr, nor was there one with S/G ratio, suggesting the lack of a strong factor affecting the formation of both lignin and cellulose (Fig. 3).

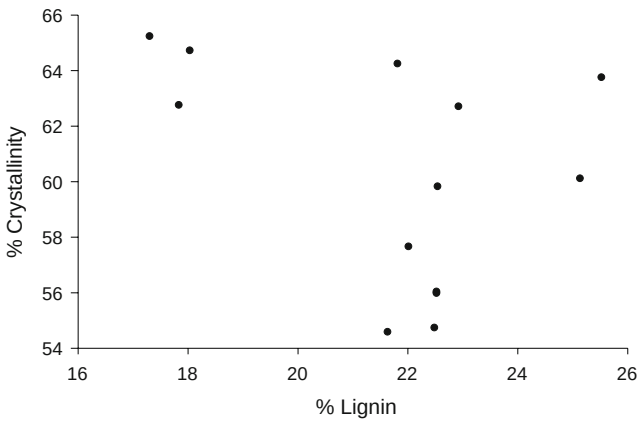

Fig. 3 Percent lignin [16] plotted against percent cellulose crystallinity

Conclusions

The NMR analysis indicates that there is no significant correlation between site location and any of the measured material properties for both cellulose and lignin. This in turn suggests that the major factors affecting lignocellulosic structure and morphology is genotypic. However, there seemed to be a small positive correlation between cellulose LFD with tree maturity. There also was no correlation between measured material properties for cellulose and lignin, indicating the lack of strong common influences. The results clearly indicate a need for an expanded sample set population and sampling from a common greenhouse to not only improve the statistics but also to more explicitly deconvolute the effect of the environment for that of genetics. Determining these correlations and whether lignocellulosic structure and morphology is influenced by environmental or genotypic factors can have important implications for our understanding of biomass recalcitrance and offer promise for the development of models that can predict the environmental and genetic impact on cell wall structure. These models would be particularly useful in any strategy to overcome recalcitrance.

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