ON THE CONFLICTING FINDINGS OF ROLE OF CELLULOSE-CRYSTALLINITY IN ENZYME HYDROLYSIS OF BIOMASS

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Introduction

In the field of conversion of biomass to ethanol, an important area of research is the enzymatic hydrolysis of cellulose. Once cellulose is converted to glucose, it can be easily fermented to ethanol. As the cellulosic ethanol technology stands now, costly pretreatments and high dosages of cellulases are needed to achieve complete hydrolysis of the cellulose fraction of the biomass.[1-3] This is mostly due to the inability of the enzymes to fully access the cellulose fraction given the complex plant cell wall structure. Many different technologies are being developed to modify the biomass to make cellulose more accessible for enzymatic saccharification.

Of the various feedstocks for cellulose bioethanol, wood appears to be an excellent energy source because it requires relatively less energy to grow and process. The exact chemical composition is wood-species specific and plays an important role in determining the material properties. In the context of hydrolyzing the cellulose component, a number of substrate factors other than enzymes are thought to be important. Based on prior research,[3-9] crystallinity and degree of polymerization of cellulose, amounts of lignin and hemicellulose, interactions between lignin, hemicellulose, and cellulose, pore volume, and the cell wall surface area are all important considerations.

However, the research findings on some of these topics have not always been unambiguous. For instance, crystallinity was found to be more important than the sample surface area[4] in pure cellulose samples. As an example, based on our work, enzyme hydrolysis data of varying-crystallinity Whatman CC31 samples is shown in Fig. 1.

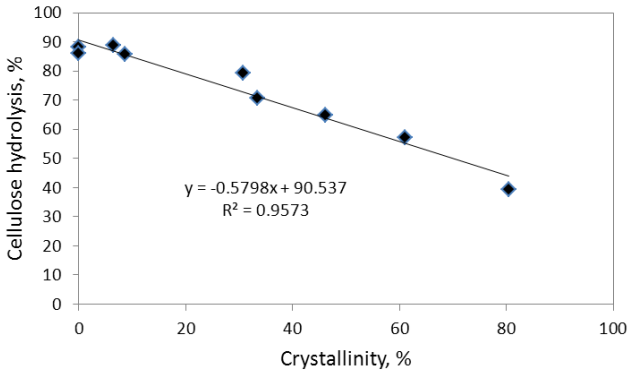

Figure 1. Correlation between cellulose crystallinity (Whatman CC31) and enzyme hydrolysis. Percentage hydrolysis data is for the 72-h duration (for more information see ref. 12)

The results clearly indicated that high crystallinity samples were difficult to hydrolyze. On the contrary, other research suggested that in pretreated biomass (composites of cellulose, hemicellulose, and lignin), accessibility to cellulose is an equally important factor[10] and in some cases, is more important[11] than crystallinity. Similarly, our research on partially- and mostly-delignified (acid chlorite, 70º C) loblolly pine (LP) indicated that cellulose crystallinity of wood was not a detrimental factor in enzyme hydrolysis[12] (Table 1) and accessibility to cellulose was more important. When ionic liquid was used as a pretreatment to evaluate the role of crystallinity, difficulty from conversion to cellulose II and modification of lignin-carbohydrate interactions were found to be the additional complicating factors.[13] Therefore, based on these findings it is hypothesized that the original crystallinity of biomass-materials does not play a negative role. And in cases where the thermochemical pre-treatments are performed (to increase the accessibility of enzymes to cellulose), the treatments themselves lead to increase in substrate crystallinity which then becomes a problem. The best approach for improved biomass hydrolysis seems to lie in discovering approaches that remove non-cellulose components without increasing the substrate crystallinity.

Table 1. Comparison of percentage cellulose conversion in various samples.

Hydrolysis duration	Whatman CC31 5-min BM[a]	LPControl[b]	LPDelig6[b]	LPBall-milled[c]
12 h	49.7	6.2	73.9	88.5
72 h	64.9	7.6	93.3	92.0

[a]Cellulose crystallinity of 5-min ball-milled Whatman CC31 sample is 46.1% (ref. 12, Table 1).
[b]LPControl is 149 to 74 µ control Wiley-milled wood fraction.LPDelig6 is mostly delignified (Klason lignin 5.5%) wood fraction 149 to 74 µ.
[c]Cellulose crystallinities of pine fractions LPControl and LPBall-milled are 46.7 and 0%, respectively (Table 1 of ref. 12).

Moreover, this contradiction in the hydrolysis-behavior of the native (e.g., in wood) and isolated plant celluloses (e.g. avicel) can be explained by our recent finding on the ultrastructure of natural cellulose which showed that cellulose in wood is ordered but not crystalline [14]. In other words, compared to wood-cellulose avicel and Whatman CC31 are difficult to hydrolyze (even at lower crystallinities) because, compared to wood-cellulose, they are significantly more ordered and crystalline. This discovery of native plant celluloses being non-crystalline is likely to have important implications for research and development activity in the field of biofuels. Here we present evidence that indicated that disorder. In particular, the existence of the disorder with respect to the conformation of the $-CH_2OH$ group in otherwise organized cellulose chains in the native state.

Experimental

Materials

Whatman CC31 powder was from Whatman International Ltd., (Maidstone, UK). Lower crystallinity and amorphous cellulose samples were generated by milling, for various durations, Whatman CC31 in a vibratory mill using steel balls. The milling was conducted in a cold room (5° C) for 2.5, 5, 10, 15, 30, 45, 60, and 90 min. The crystallinities of these cellulose samples were 61.1, 46.1, 33.4, 30.7, 8.7, 6.5, 0, and 0%, respectively. Both the 60- and 90-min ball-milled samples had 0% crystallinity indicating that they were completely non-crystalline. Cellulose crystallinity was determined by the univariate FT-Raman method.[15] This method has been correlated to the Segal-18-WAXS and Segal-21-WAXS methods.[15,16] This group of 8 samples along with the control Whatman CC31 (crystallinity 80.5%) were used in the hydrolysis experiment that has to do with the role of cellulose crystallinity.

Other cellulose samples used were as follows. The name stated in the parentheses identifies the researcher who gifted the sample to us – Tunicate (Dr. A. Isogai), Cellotriose, cellotetraose, cellopentose, and cellodextrin (Dr. R. Atalla), Valonia macrophysa (Prof. N. Terashima), Cladophora Glomerata (Dr. S. Chundawat). Alkali-extracted-holocellulose (Jack pine) and cellulose II and cellulose III from avicel were prepared in our laboratory.

The 149 to 74 μ size sieved wood fraction (passed by the 149 μ screen but retained by the 74 μ screen) was used and was isolated from the acetone:water (9:1) extracted Wiley-milled loblolly pine ground wood that passed a 1-mm (1,000-micron) screen. The reasoning behind choosing the 149 to 74 μ sized fraction was to have a faction whose cellulose crystallinity was similar to one of the ball milled Whatman CC31 samples.[12]

Additionally, a ball-milled fraction (particle size < 10 microns) was used in the study. Ball-milled wood was prepared as follows. 200 grams of extracted, Wiley-milled loblolly pine was dried over P_2O_5. A Sieman's rotating vibratory ball mill was programmed to run for 30 min of each hour to allow for cooling, for a total time of 44 h with 22 h of actual milling time. The sample was dry milled. The ball milled wood fraction was found to be completely non-crystalline.[12]

Other chemical compounds listed in Table 2 were from Sigma-Aldrich or were obtained from the researchers at the Forest Products Laboratory, Madison.

Table 2. CH_2OH conformation in, chemicals, models and celluloses

Sample	Band positions cm^{-1}	*gg*	*gt*	*tg*
Ethanol	1480			X
	1460	X		
Ethylene Glycol	1460	X		
Ethylene Glycol-d2	1460	X		
D-Glucose	1461	X		
Methyl-α-D-glucopyranoside (solid)	1476			X
	1458	X		
Methyl-β-D-glucopyranoside (solid)	1478			X
	1457	X		
Ethyl diamine	1459	X		
Cellobiose	1457	X		
Cellotriose	1459	X		
	1468		X	
Cellotetraose	1461	X		
Cellopentose	1461	X		
Cellodextrin (DP 15)	1461	X		
Cellulose II	1462	X		
Cellulose III	1463	X		
Cellulose IV	1454	X		
Amorphous cellulose	1463	X		

Raman

All samples were analyzed with a MultiRam spectrometer (Bruker Instruments Inc., Billerica, MA, USA). This Raman system is equipped with a 1064 nm 1000 mW CW diode pumped Nd:YAG laser. There were three sampling modes used in recording spectra. First, as a pellet, approximately 0.1−0.2 g of each sample was pressed into a pellet with a hydraulic press. Some dry samples were also analyzed in the NMR tube. Lastly, samples were analyzed in never-dried state in H_2O or D_2O in the NMR tube. The laser power used for sample excitation was between 600 and 900 mW, and for most samples 1024 scans were accumulated. Some samples where the signal was very weak spectra were obtained over a longer duration. Bruker's OPUS software program was used to find peak positions and process the data. Processing of the spectra included, among other things, selecting a specific region, baseline correction, normalization, and spectral subtraction.

Enzymatic hydrolysis

Enzymatic hydrolyses of the substrates were carried out at substrate consistency of 1% (w/v) in 50-mL sodium acetate buffer (pH 4.8) at 50 °C using a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 200 rpm. A mixture of Celluclast 1.5 L with an activity loading of 20 FPU/g cellulose and Novozyme 188 with an activity loading of 30 CBU/g cellulose was used for enzymatic hydrolysis. Excessive Novozyme 188 (activity loading of 30 CBU/g cellulose) was used to prevent cellobiose accumulation. Hydrolysates were sampled periodically for glucose analysis. Glucose analysis was done using a commercial glucose analyzer (YSI 2700S, YSI Inc.,Yellow Springs,Ohio). Each data point was the average of duplicate experiments. Based on the replicate analyses, the standard deviation of the procedure was ± 2.5%. For clarity purposes, the error bars are not shown in the Figures.

Crystallinity determination

Cellulose crystallinities of the samples were determined by the univariate FT-Raman method.[15,16] The method is dependent upon the band intensity ratio 380/1096 obtained from the near-IR (1064 nm) FT-Raman spectrum of the sample. Approximately 0.2 g of air-dried sample was pressed into a pellet that was analyzed using a Bruker MultiRam spectrometer (Bruker Instruments Inc., Billerica, MA). The sample percentage of crystallinity was calculated using the Raman band peak intensities at 380 and 1096 cm^{-1} as indicated below and was corrected for the instrument-dependent intensity differences between RFS-100 and MultiRam (both FT-Raman instruments from Bruker).

$$Cr_{Raman} = [(I_{380}/I_{1096}) - 0.0286]/0.0065$$

Results and Discussion

The celluloses (tunicate, valonia macrophysa, cladophora glomerata, avicel, and alkali-extraced holocellulose) were studied in their native- and dried-states by Raman spectroscopy. The band positions of the majority of the spectral features in these spectra were similar to cellulose I. Nevertheless, upon detailed examination it was found that in some cases the features showed important differences. These consisted of differences in shapes, intensity, and frequency of bands (Fig. 2 – Fig. 4).

In particular, the region 1450 to 1480 cm^{-1} (Fig. 3) was found to be the most informative. The contributions of the five cellulose I samples differed in this region (Fig. 3). This wavenumber region is known to represent the CH_2 scissor mode in celluloses and was found to be conformationally sensitive. Based on the literature and Raman investigation of a number of chemical compounds, including cellulose models and cellulose polymorphs, contributions in this region were assigned to the $–CH_2OH$ group in *tg*, *gg*, and *gt* conformations. For non-cellulose I samples (Table 2) that were analyzed in Raman the $–CH_2$ scissor mode band positions and their assignment in terms of the *gg*, *gt*, and *tg* rotamers are listed in Table 2. Compared to data in Table 2, contributions of cellulose I samples showed more complexity. For the latter, such differences are indications of the presence of significant amounts of *gg*, *gt*, and *tg* conformations. The kind and extent of $–CH_2OH$ conformation was

dependent upon the source of the cellulose and in not a single case was restricted to the *tg* conformation. This observation is contradictory to the literature report where the conformation of the cellulose I $-CH_2OH$ group is essentially reported to be *tg*.[17] The conformation-variability detected in Raman has important implications for cellulose ultrastructure and reactivity because the $-CH_2OH$ group is involved not only in intra- and inter-molecular H-bonding but also is the principal site of chemical and biological modifications when cellulose is used technologically. In the $-CH_2OH$ scissor mode region, the most drastic change is observed between the spectra of the tunicate and cladophora glomerata samples (Fig. 3) although smaller differences exist between the spectra of other celluloses as well (Fig. 2). Presence of different rotamers implies presence of disorder and non-uniformity at the molecular level. This we believe is the primary reason why native state of plant celluloses is non-crystalline. In this context, it's worth noting that the biomass cellulose has been shown to be capable of being irreversibly transformed to a state of higher crystallinity[18] and morphologically changed, leading to biomass-cellulose dehydration[19], upon isolation at elevated temperatures.

Although not discussed here, Raman spectra in the 250 to 750 cm^{-1}, 750 to 1450 cm^{-1}, and 1700 to 3500 cm^{-1} regions (Fig. 2 and Fig. 4) further supported the observations of molecular level disorder in native celluloses. The appearance of specific bands in some spectra indicated that in these cellulose-samples the conformation of the chains had more than one conformations, some resembling even cellulose II chain conformation.

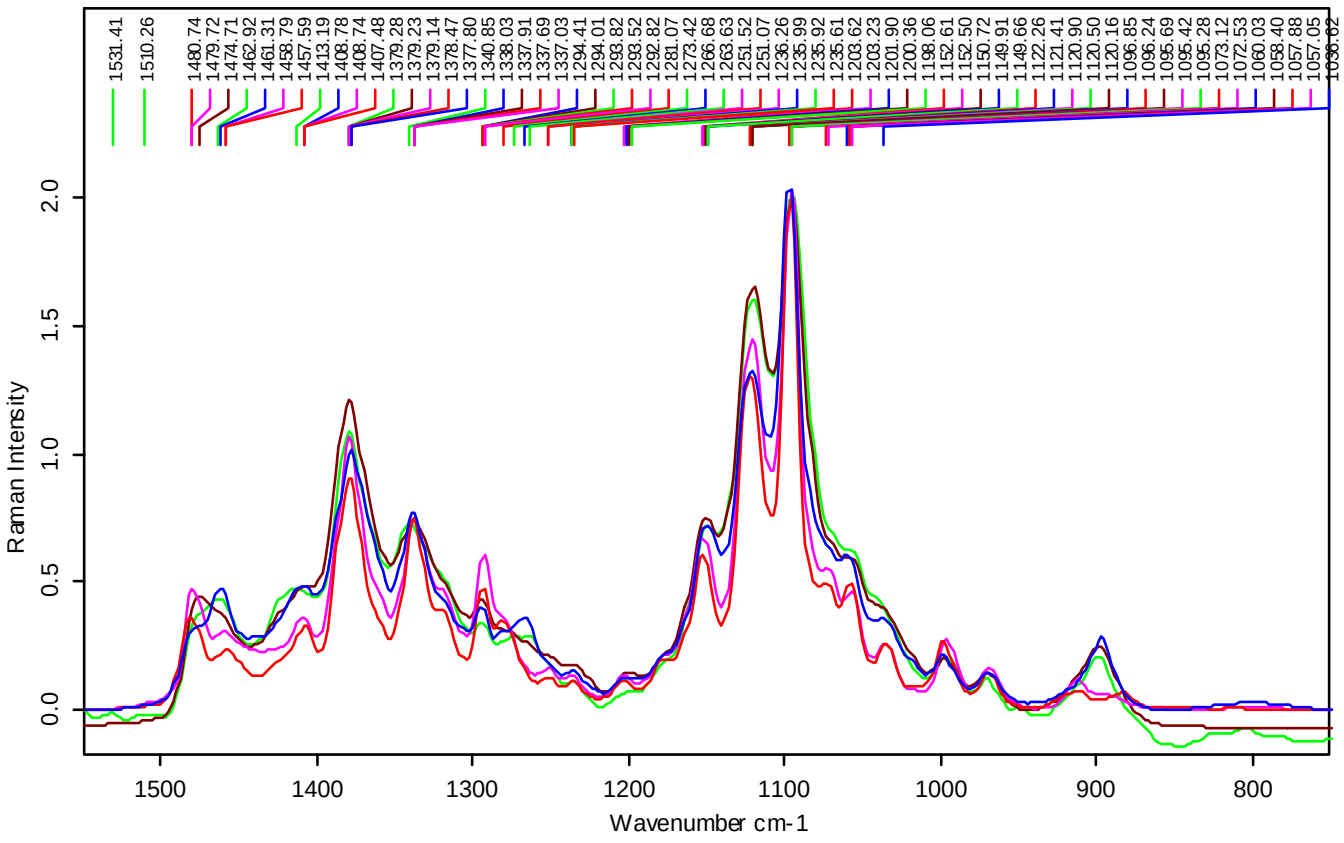

Figure 2. Raman spectra in 750 to 1550 cm^{-1} region of cladophora glomerata (blue), valonia macrophysa (red), tunicate (magenta), avicel (brown), alkali-extracted-Jack-pine holocellulose (green).

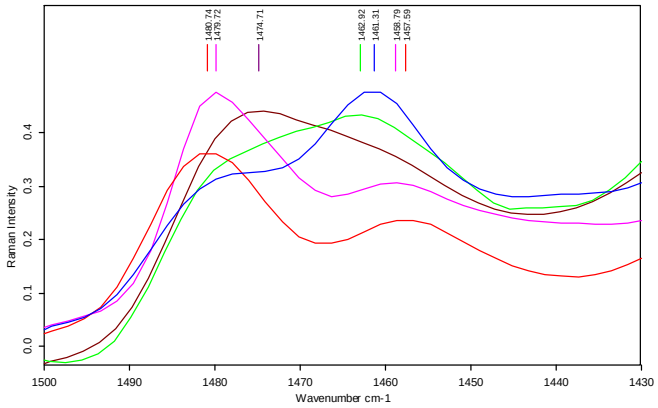

Figure 3. Raman contributions indicating presence of different amounts of *tg* (1480), *gt* (1470), and *gg* (1460) in different celluloses; cladophora (blue), valonia macrophysa (red), tunicate (magenta), avicel (brown), Jack-pine holocellulose (green).

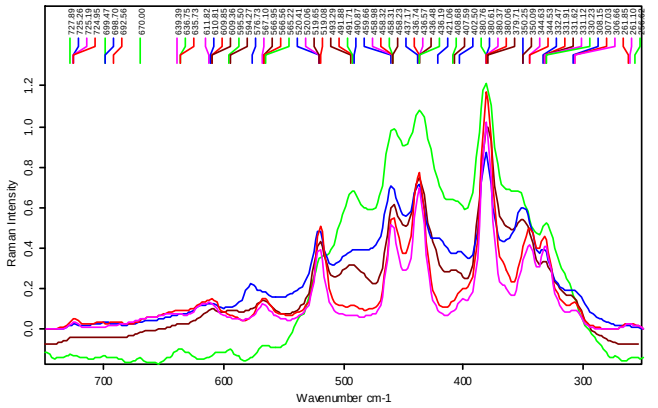

Figure 4. Variations of Raman contributions in the region 250 to 750 cm^{-1} in different celluloses; cladophora (blue), valonia macrophysa (red), tunicate (magenta), avicel (brown), Jack-pine holocellulose (green).

Conclusions

The research carried out in this report provided an explanation why the crystallinity of the delignified loblolly pine wood has no negative influence on the enzyme hydrolysis. More specifically, based on Raman analysis, the explanation has to do with the non-crystalline nature of wood-cellulose. This has important implications for future work in the field of biofuels. Our work suggests that removal of the non-cellulose components of the cell wall should proceed in ways that do not result in increasing its crystallinity.

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