# ENZYMATIC HYDROLYSIS OF BIOMASS: EFFECTS OF CRYSTALLINITY, PARTICLE SIZE, AND LIGNIN REMOVAL

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## ABSTRACT

Enzymatic hydrolysis of cellulose in plant and wood cell walls is expected to be affected by its chemical composition as well as structural and morphological features. In the present study, different crystallinity cellulose samples and varying size fractionated loblolly pine wood cell wall particles were hydrolyzed using a mixture of Celluclast and Novozyme 188. In pure cellulose samples of Whatman CC31, at lower crystallinity, higher hydrolysis rates and higher conversion to glucose were observed. Percent cellulose hydrolysis was linearly but inversely correlated with percent crystallinity. After 72 h, the extent of hydrolysis for highly crystalline and completely amorphous cellulose samples was 40% and 90%, respectively. In the case of loblolly pine, softwood, four different size Wiley-milled wood fractions along with a ball milled sample were hydrolyzed. Although, for the four of the five fractions, where the cellulose crystallinity was similar, there was some increase in enzyme hydrolysis with reduced particle size, most of the increased hydrolysis occurred for the ball milled wood indicating that the destruction of crystallinity and accompanying changes had a major impact. Moreover, to examine the impact of lignin removal, partially delignified pine samples of the four fractions and increasingly delignified samples of a chosen loblolly pine fraction were subjected to enzymatic saccharification. In the former case, lignin removal was found to be associated with significantly increased hydrolysis - extent of hydrolysis at 72 h for the four wood fractions increased on average by 10 fold. Results of the experiments on the increasingly delignified samples indicated higher saccharification with increased lignin removal. Lastly, when the hydrolysis data between the similar crystallinity cellulose and wood cell wall were compared, at 72 h, the cell wall conversion was found to be 4 times slower. These results taken together highlighted that in the biomass hydrolysis cellulose crystallinity and cell wall composition are not as important as the ultrastructural changes that ensued upon acid chlorite delignification.
*Keywords: enzymatic hydrolysis, biomass, cell wall, cellulose crystallinity, lignin, saccharification*

## INTRODUCTION

In the field of conversion of biomass to fuels and chemicals, an important area of research is the enzymatic hydrolysis of cellulose. As the 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.

Cell walls are composed of three main constituents – cellulose, hemicelluloses, and lignin. Pectins, proteins, and wax substances are the remaining minor constituents. The exact chemical composition is biomass specific and plays a great role in determining its properties. In the context of hydrolyzing the cellulose component, besides enzymes, a number of substrate factors are thought to be important. Based on prior research[4-6], cellulose crystallinity and degree of polymerization, lignin and hemicellulose amounts, lignin-hemicellulose interactions, and surface area or pore volume of the cell wall are all important considerations.

In the present investigation, effect of cellulose crystallinity on cellulose hydrolysis and effects of cell wall particle size and lignin removal on enzymatic hydrolysis of cell wall were investigated. The wood species chosen in the study was loblolly pine (*Pinus taeda*). To study the role of crystallinity Whatman CC31 celluloses with crystallinity in the range 80.5% to 0% were studied and the rate of hydrolysis of loblolly pine cell walls was compared with that of a similar crystallinity cellulose sample. Cellulose crystallinity was determined by the FT-Raman based method, which was recently developed by the corresponding author and his colleagues[7]. The method is thought to be more accurate compared to the traditionally used Segal-WAXS method [7].

Considering that the particle size reduction opens up, to some degree, the tightly structured cell wall and allows enzymes access to the carbohydrate polymers, role of particle size reduction was investigated by hydrolyzing four different size fractions of the Wiley-milled wood cell walls. Additionally, a ball milled fraction where cell wall is completely amorphous was included in the study. In the latter fraction, although the cellulose was non-crystalline, the chemical composition remained similar to that of the other four fractions. Lastly, to study the effect of delignification, not only all the four fractions were partially delignified but one of the selected fractions was progressively delignified. This permitted evaluation of the early stages of the delignification in the cell wall hydrolysis.

## EXPERIMENTAL

### Materials

Whatman CC31 powder was from Whatman

International Ltd., (Maidstone, UK). Lower crystallinity and the amorphous cellulose sample were generated by grinding, 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. This group of 8 samples along with the control Whatman CC31 (crystallinity 80.5%) was used in the hydrolysis experiment that has to do with investigating the effect of cellulose crystallinity.

Four wood fractions - A, B, C, and D of acetone: water (9:1) extracted Wiley milled loblolly pine ground wood that passed the 1 mm screen were used. The fractions A, B, C, and D were distinguished as follows - passed (p) 590 microns (m) sieve but retained (r) by 420 m sieve, p 149 m r 74 m, p 74 m r 53 m, and p 53 m, respectively. Additionally, a ball milled fraction called sample E (particle size < 10 microns) was used in the study. The idea behind choosing A B C was to see the effect of increasing surface area on cellulose hydrolysis (crystallinity was roughly same). Fraction D showed slightly reduced crystallinity whereas sample E was completely amorphous.

### Partial Delignification

The acid chlorite treatment of the fractions and other samples was carried out by using the procedure recommended by Browning[8]. In accordance with this procedure, at about 70° C, glacial acetic acid and sodium chlorite were added to the water suspended wood samples and the addition of chemicals was repeated over several hours until a desired degree of delignification was achieved. The extent of delignification achieved was measured by Klason method and/or by FT-Raman spectroscopy.

### 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, Massachusetts) at 200 r/min. A mixture of Celluclast 1.5 L with an activity loading of 20 FPU/g cellulose and Novozyme 188 with an activity loading of 22.5 CBU/g cellulose was used for enzymatic hydrolysis. Excessive Novozyme 188 (activity loading of 22.5 CBU/g cellulose) was used to prevent cellobiose accumulation. Hydrolysates were sampled periodically for glucose analysis. Each data point was the average of duplicate experiments.

### Compositional Analysis

Wood samples were analyzed chemically to quantitatively determine the amounts of lignin (Klason method) and carbohydrates (Sugar analysis).

### Crystallinity Determination

Cellulose crystallinities of samples were determined by the univariate FT-Raman method[7]. The method is dependent upon the band intensity ratio 380/1096 obtained from the FT-Raman spectrum of the sample.

## RESULTS AND DISCUSSION

### Cellulose Crystallinity

To determine the role of crystallinity in cellulose hydrolysis, differing-crystallinity samples of Whatman CC31 were hydrolyzed. Both the rate and extent of hydrolysis had a strong dependence on the substrate crystallinity (extent of 72 h hydrolysis plotted in Fig. 1). The data in Fig. 1 shows an inverse linear relationship between % cellulose hydrolyzed and crystallinity. Higher was the cellulose crystallinity smaller was the amount of cellulose that was saccharified. This result is not new; similar observation has been made in previously published research[4].

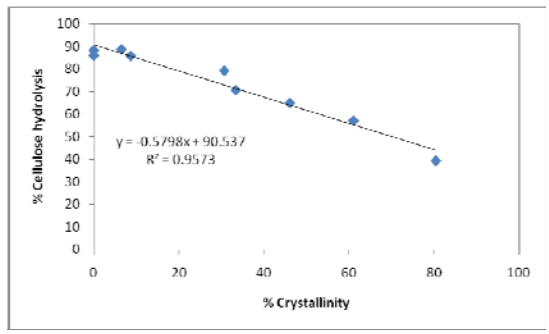


**Fig. 1. Correlation between cellulose crystallinity and enzyme hydrolysis. % hydrolysis data is for the 72 h duration**

### Particle size

Results of enzymatic hydrolysis of the different pine fractions and ball milled sample are shown in Fig. 2. It was expected that the higher specific surface area present in smaller particles will have considerable impact on saccharification. From Fig. 2 it can be noted that in accordance with the expectation the smaller particles had higher rates and conversions. However, both these parameters depended only weakly on the particle size. For instance, after 72 h, fraction D (size, <53 microns) hydrolyzed about 5 times more compared to fraction A (size, 590 to 420 microns), but the total conversion was only 14.5%. Nevertheless, compared to the ball milled case (sample E), where the conversion was 91% the fraction D conversion was quite low (Fig. 1). High conversion of 91% is likely to have been brought about by the fact that the cell wall has been rendered amorphous and, although lignin and hemicelluloses were still present, its original structure was significantly disrupted in a beneficial manner. The nature of the physicochemical changes that occur in cell wall

upon ball milling remain poorly understood[9] and further insights need to be developed to better understand how ball milling brings about greatly enhanced hydrolysis.

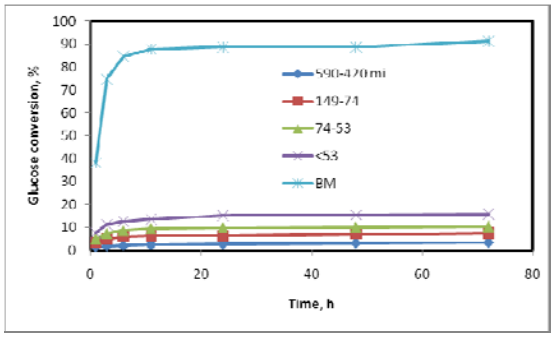


**Fig. 2. Hydrolysis of the four loblolly pine fractions: A (590~420 microns), B (149~74 microns), C (74~53 microns), D (< 53 microns) and the ball milled (BM) sample**

## Mostly Delignified

**Table 1. Compositional analysis of the control and the mostly delignified loblolly pine wood fractions**

| Sample | Klason lignin /% | Acid Soluble lignin /% | Glucan /% | Total Carbohydrates /% |
|---|---|---|---|---|
| Control | 30.2 | 1.0 | 39.4 | 59.2 |
| A-Delig. | 7.0 | 0.8 | 55.0 | 71.2 |
| B-Delig. | 5.5 | 1.4 | 53.6 | 71.1 |
| C-Delig. | 5.7 | 3.6 | 46.4 | 69.0 |
| D-Delig. | 8.6 | 3.2 | 46.1 | 67.0 |

To evaluate how lignin removal impacted the cellulose hydrolysis, the four fractions that were partially delignified were subjected to enzyme hydrolysis. The selected compositional analysis data (Klason lignin, acid soluble lignin, glucan, and total carbohydrates content) on the delignified samples is reported in Table 1. The hydrolysis data is plotted in Fig. 3. It is clear that three of the four pine fractions (B-Delig., C-Delig., and D-Delig.) had the similar saccharification behavior and, compared to the corresponding control samples (control B, C, and D), the glucose conversion yields have gone up very significantly (Fig. 2 vs. Fig. 3). This was also true, albeit to a lesser extent, for the larger particle size fraction sample A-Delig. The lower saccharification efficiency for the latter may have to do with fraction A's larger particle size. The compositional differences between these samples (A-Delig. vs. B-Delig., C-Delig., and D-Delig.; Table 1) are not significant enough to explain the slower rate and reduced conversion in the case of A-Delig. It is known that lignin removal from cell walls causes ultrastructural changes and formation of pores that make the cellulose more accessible to the hydrolytic enzymes[10].

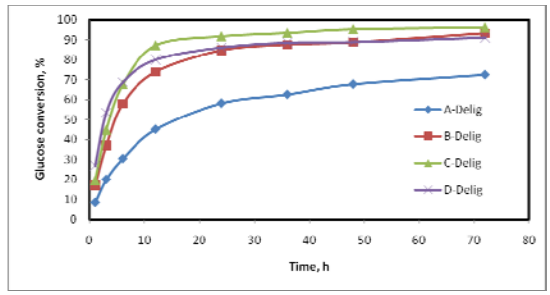


**Fig. 3. Hydrolysis of the four partially delignified loblolly pine fractions; A-Delig. (590 - 420 microns), B-Delig. (149 - 74 microns), C-Delig. (74 – 53 microns), and D-Delig. (< 53 microns)**

## Progressive Delignification

**Table 2. Compositional analysis of the control and the progressively delignified wood fraction B**

| Sample | Klason lignin /% | A.S. lignin /% | Glucan /% | Tot. Carbo. /% |
|---|---|---|---|---|
| Control, B | 30.3 | 1.1 | 38.2 | 55.9 |
| B, 0.5 h | 22.6 | 2.8 | 43.1 | 64.6 |
| B, 1 h | 22.1 | 3.5 | 41.8 | 61.2 |
| B, 2 h | 21.9 | 3.6 | 43.4 | 63.5 |
| B, 4 h | 16.9 | 4.8 | 46.3 | 68.1 |
| B, 7 h | 14.6 | 4.3 | 40.2 | 60.7 |

Sample B (149 – 74 microns) was delignified progressively and 5 sample aliquots were removed at 0.5, 1, 2, 4, and 7 h of the acid chlorite treatment. The samples were analyzed chemically (Table 2) and subsequently, were hydrolyzed enzymatically (Fig. 4). Data in Table 2 indicated that after initial delignification (0.5 h sample), where the Klason lignin content dropped to 22.6%, the Klason lignin content remained constant for the samples that were taken at 1 and 2 h duration (about 22% in Table 2). Nevertheless, samples removed at 4 and 7 h durations showed further delignification and their Klason lignin contents were 16.9 and 14.6 %, respectively. The results of cellulose hydrolysis showed that at the significantly reduced lignin content of 14.6%, the extent of glucose conversion was only 50% (at 72 h). This is significantly lower compared to the 90% conversion for the B-Delig. sample (Fig. 3; Klason lignin 5.5%, Table 1). In summary, conversion to glucose was inversely dependent upon the extent of lignin removal in the cell walls. Moreover, the data suggested that more than 50% of the initially present lignin needs to be removed to achieve 90% cellulose conversion (at 72 h hydrolysis duration).

As far as removal of hemicelluloses during acid chlorite delignification is concerned, it is known that the procedure

is selective in removing lignin during the first 60% of delignification[11]. At higher % delignification, increasing losses in glucomannan, galactan, xylan, and araban are expected[11]. Therefore, hemicellulose removal does not seem to be an important factor in the context of the present investigation.

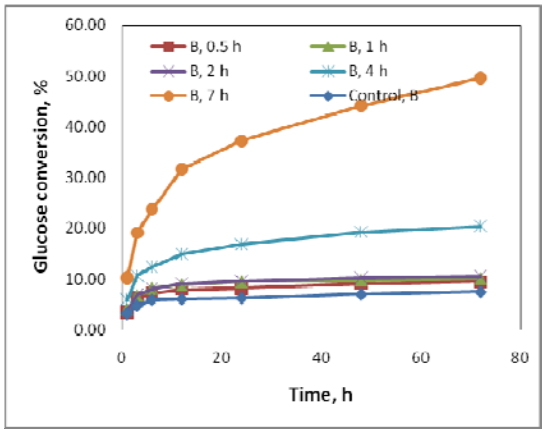


**Fig. 4. Hydrolysis of the control sample B and four of its aliquots removed at different stages of delignification: control, B; B, 0.5 h; B, 1 h; B, 2 h; B, 4 h; B, 7 h**

### Comparing Conversion in Various Samples

**Table 3. Comparison of % cellulose conversion in various samples**

| Hydrolysis Duration | Whatman CC31, 5-min BM[a] | Loblolly Pine, Fraction D[b] | D-Delig. Fraction | Loblolly Pine, Sample E |
|---|---|---|---|---|
| 72 h | 65 | 16 | 88 | 91 |
| 12 h | 50 | 8 | 72 | 88 |

[a]Whatman CC31 sample with 46% cellulose crystallinity.
[b]Loblolly pine cellulose crystallinity is assumed to be 46%.

Table 3 compares extent of conversion to glucose for various samples used in this study. The comparison is carried out for two hydrolysis durations, 12 and 72 h. A couple of observations can be made from the data in Table 3. First, considering the 72 h conversion data, compared to same % crystallinity pure cellulose pine cell wall was found to be 4 times slower to hydrolyze (6 times slower if 12 h data are compared). Nevertheless, the same cell wall increases its hydrolysability, compared to the pure cellulose, once it is significantly delignified (Table 3, D-Delig. fraction, has 8.6% Klason lignin). This implies that cellulose crystallinity of the cell wall is not as important as the changes brought about by the delignification. That's because even when the crystallinity of the cell wall did not change upon delignification, the delignified wood showed higher saccharification. The other observation has to do with the ball milled pine sample (sample E in Table 3). Its hydrolysis behavior clearly indicated that even though lignin was present in this sample it was the only sample that was most easily hydrolyzed at both the durations - 12 and 72 h.

## CONCLUSIONS

In this investigation, the enzymatic hydrolysis of wood cell wall was studied. More specifically, the roles of cellulose crystallinity, cell wall particle size, and lignin removal were evaluated. It was found that the factors that modify the ultrastructure of the cell wall are more important than the cellulose crystallinity, although the latter is important in the hydrolysis of pure cellulose samples. It is suggested that factors like lignin removal (not same as lignin content) indirectly influence the hydrolysis because removal of lignin causes the physicochemical modification of the cell wall structure that in turn makes enzymatic access to cellulose easier.

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