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Enzymatic hydrolysis of loblolly pine: effects of cellulose crystallinity and delignification

Abstract: Hydrolysis experiments with commercial cellulases have been performed to understand the effects of cell wall crystallinity and lignin on the process. In the focus of the paper are loblolly pine wood samples, which were systematically delignified and partly ball-milled, and, for comparison, Whatman CC31 cellulose samples with different crystallinities. In pure cellulose samples, the percentage of cellulose hydrolysis was inversely proportional to the degree of crystallinity. For the loblolly pine samples, the extent of hydrolysis was low for the fraction with 74- to 149-μm particle size, but the ball-milled fraction was hydrolyzed easily. The impact of lignin removal was also influential as demonstrated on progressively delignified wood, i.e., the degree of saccharification increased with lignin removal. On the basis of data of 72 h hydrolysis time on materials with similar crystallinity, the cell wall was found to be eight times less hydrolyzable than Whatman CC31 cellulose. Taken together, cellulose crystallinity and composition are not as important as the ultrastructural changes caused by the disruption of the tightly packed regions of the cell wall that ensued upon acid chlorite delignification.

Keywords: ball milling, cellulase, cellulose, crystallinity, enzymatic hydrolysis, hydrolysis, lignin, loblolly pine, wood

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Introduction

For the conversion of biomass to ethanol, the pretreatment methods and the enzymatic hydrolysis of cellulose via glucose and fermentation are most practical (Sugimoto et al. 2009; Díaz et al. 2011; Kirsch et al. 2011; Schütt et al. 2011). Cellulose is an essential component of all plant materials and it occurs in pure form only in cotton fibers. To date, costly pretreatments and high dosages of cellulases are needed to achieve complete hydrolysis of the cellulose fraction of the biomass (Walker and Wilson 1991; Ragauskas et al. 2006; Zhu et al. 2009a; and quotations above) because of the inaccessibility of cellulose to enzymes within the complex cell wall matrix. In principle, wood is an excellent source of cellulose because it requires relatively less energy to grow and process, and there are different technologies available to increase the cellulose accessibility in wood fibers.

Wood fibers are typically 1–3 mm long and 10–50 μm wide. The cell walls of wood fibers are composed mainly of cellulose, hemicelluloses, and lignin, whereas pectins, proteins, and extraneous substances are the minor constituents. Accordingly, the individual cellulose microfibrils in the wood cell wall are surrounded by other polymers and they form a special supramolecular architecture (Salmén and Burgert 2009; Stevanic and Salmén 2009; Terashima et al. 2009). The most essential factors influencing the enzymatic hydrolysis of wood are crystallinity and degree of polymerization of cellulose, amounts of lignin and hemicelluloses, interactions between all essential wood components, pore size and volume, and the cell wall surface (Fan et al. 1980; Dasari and Berson 2007; Zhu et al. 2009a; Luo and Zhu 2011; Luo et al. 2011; Rahikainen et al. 2011; Wang et al. 2012).

However, the findings in this field are not always unambiguous. In case of pure cellulose, crystallinity was found to be more important than the sample surface area (Fan et al. 1980). Other research emphasized the accessibility to cellulose in pretreated biomass as an equally important factor (Jeoh et al. 2007), which in some cases is even more important than crystallinity (Rollin et al. 2010). When wood was pretreated with ionic liquids, the conversion from cellulose I to cellulose II and modification of lignin-carbohydrate interactions also influenced the enzymatic hydrolysis (Cheng et al. 2011).

In the present paper, the effects of cellulose crystallinity and lignin removal were in focus concerning the enzymatic hydrolysis of wood. Loblolly pine (*Pinus taeda* L.) as a softwood was selected for the experiments with a particle size of 74–149 μm. A part of the wood was ball-milled. The effect of lignin removal was studied on progressively delignified wood samples. A softwood was chosen for this study as these species are known to be hydrolyzable

less efficiently by cellulases and they have higher lignin contents compared to hardwoods. Whatman CC31 celluloses (with crystallinities between 78% and 0%) based on cotton linters were selected for comparison as they have in general the highest degrees of crystallinity than Avicel. Moreover, cotton linters have a simple morphology and are well suited for comparison.

Materials and methods

Materials

Whatman CC31 powder (crystallinity, Cr 78%) was from Whatman International Ltd. (Maidstone, UK). Lower degrees of Cr up to the amorphous state were generated by milling of this cellulose in a vibratory mill equipped with steel balls. Milling times and Cr data are listed in Table 1, ranging from 78% (unmilled control) to 0% (milled for 60 or 90 min).

The wood meal (74- to 149-μm fraction, which passed by the 149-μm screen but was retained by the 74-μm screen) was prepared by acetone/water (9:1) extraction of Wiley-milled loblolly pine particles that passed a 1-mm screen (Figure 1). The various sieved fractions of the wood had different crystallinities, and fraction 74–149 μm was selected as its Cr was similar to that of a fraction of the ball-milled Whatman cellulose (Table 1).

A ball-milled fraction (particle size <10 μm) of wood was also studied. It was prepared as follows: 200 g of extracted Wiley-milled wood was dried over P_2O_5 and milled in the dry state (without suspension). A Siemen's rotating vibratory ball mill (Munich, Germany) was programmed to run for 30 min of each hour to allow for cooling so that within 44 h in the mill the real milling time was 22 h. The ball milled wood was completely noncrystalline (Table 1).

Sample	Ball-milling time (min)	Fraction size (μm)	Cr (%)
$Whatman_{control}$	0	n.d.	78
Whatman	2.5	n.d.	61.1
Whatman	5	n.d.	46.1
Whatman	10	n.d.	33.4
Whatman	15	n.d.	30.7
Whatman	30	n.d.	8.7
Whatman	45	n.d.	6.5
Whatman	60	n.d.	0
Whatman	90	n.d.	0
$Wood_{420–590}$	–	420–590	51.8
$Wood_{74–149}$	–	74–149	46.7
$Wood_{53–74}$	–	53–74	39.2
$Wood_{<53}$	–	<53	33.6
$Wood_{ball-milled}$	1320	<10	0

Table 1 Parameters of sample preparation and cellulose crystallinity (Cr) of Whatman CC31 cellulose samples and samples of Wiley-milled and sieved loblolly pine wood (Wood).
n.d., not determined.

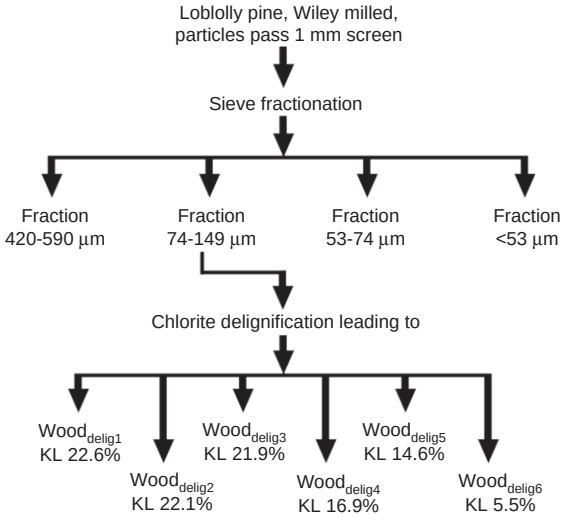

Figure 1 Outline of sample preparation via sieve fractionation and progressive delignification of the fraction 74–149 μm. KL refers to the Klason lignin contents. See also Table 1.

Progressive delignification

The acid chlorite treatment of the pine wood meal (fraction 74–149 μm) was carried out according to Ahlgren and Goring (1971): at about 70°C, glacial acetic acid and sodium chlorite were added to the water-suspended wood meal and the addition of chemicals was repeated over several hours. Sample aliquots were removed periodically so that partially delignified samples ($wood_{delig1}$ to $wood_{delig6}$ with lignin contents between 23% and 5.5%; Figure 1 and Table 2) were obtained. The Klason lignin (KL) determination (TAPPI 1983) was applied. All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Enzymatic hydrolysis

Substrate consistency was 1% (w/v) in 50-ml sodium acetate buffer (pH 4.8) during hydrolysis at 50°C in a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA, USA) at 200 rpm. The enzymes (from Novozymes, Franklinton, NC) used were a mixture of Celluclast 1.5 l with an activity loading of 20 filter paper units (FPU) g^{-1} cellulose and Novozyme 188 with an activity loading of 30 cellobiase (CBU) g^{-1} cellulose. Excessive Novozyme 188 (activity loading of 30 CBU g^{-1} cellulose) was used to prevent cellobiose accumulation. Hydrolysates were sampled periodically for glucose analysis, which was done by means of a glucose analyzer (YSI 2700S, YSI Inc., Yellow Springs, OH, USA). Each data point is the average of duplicate experiments with standard deviation (SD) of ±2.5%. For clarity of the figures, the error bars are not shown.

Compositional analysis

The SD for KL determination was ±0.4% and the SD for carbohydrate analysis (only glucan, xylan, and mannan are reported) was <1%

Sample	KL (%)	Acid sol. lignin (%)	Glucan (%)	Xylan (%)	Mannan (%)	Yield of enzyme conversion (%) after 12 h Hydr. time	Yield of enzyme conversion (%) after 72 h Hydr. time
Wood[a]	30.3	1.1	38.2	5.6	8.5	6.2	7.6
$Wood^{b}_{ball\text{-}milled}$	28.8	1.2	39.4	5.9	8.7	88.5	92.0
$Wood^{c}_{delig1}$	22.6	2.8	43.1	6.8	10.6	8.0	9.5
$Wood_{delig2}$	22.1	3.5	41.8	6.3	9.3	8.8	10.1
$Wood_{delig3}$	21.9	3.6	43.4	6.8	10.2	9.0	10.5
$Wood_{delig4}$	16.9	4.8	46.3	7.7	11.0	14.9	20.4
$Wood_{delig5}$	14.6	4.3	40.2	7.0	9.7	31.6	49.5
$Wood_{delig6}$	5.5	1.4	53.6	8.6	6.3	73.9	93.3

Table 2 Compositional analysis and enzymatic hydrolysis data of control and delignified loblolly pine wood samples.
[a]Wood meal, 74- to 149-μm sieve fraction.
[b]$Wood_{ball\text{-}milled}$, wood was ball-milled for 22 h in dry state.
[c]$Wood_{delig1}$ to $wood_{delig6}$ are wood meals (74–149 μm) progressively delignified by chlorite treatment.

(Davis 1998). In Table 2, the total mass of the unreported material adds up to 7–8%; it consists of arabinan, galactan, and rhamnan.

Cellulose crystallinity

The univariate Fourier transform (FT)-Raman method (Agarwal et al. 2010, 2011) was applied, the results of which were correlated to the Segal-18-WAXS and Segal-21-WAXS methods (Agarwal et al. 2010, 2011). The Raman method relies on the band intensity ratio 380:1096 obtained from the near-infrared (1064 nm) FT-Raman spectrum of the sample. Approximately 0.2 g of air-dried sample was pressed into a pellet. A Bruker MultiRam spectrometer (Bruker Instruments Inc., Billerica, MA, USA) was used. The calculated crystallinities (Cr_{Raman}) were corrected for the instrument-dependent intensity differences between RFS-100 and MultiRam (both FT-Raman instruments from Bruker).

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

Scanning electron microscopy (SEM)

Samples were dispersed in water on polished aluminum mounts, air-dried, and then placed on mounts with double-stick silver tape. Samples were gold-coated by means of a Denton Desk-1 sputter coater (Cherry Hill, NJ, USA) and examined and photographed with a Zeiss EVO 40 scanning electron microscope (Carl Zeiss SMT Inc., Thornwood, NY, USA).

Results and discussion

Cellulose crystallinity (Cr)

Both the rate and the extent of hydrolysis of the Whatman cellulose samples have a strong dependence on the substrate Cr. The Cr data of 72-h hydrolysis are plotted in Figure 2, and these show an inverse linear relationship between the yield of hydrolysis and Cr, i.e., the higher the Cr the lower the amount of cellulose that can be saccharified.

Fan et al. (1980) have reported three decades ago similar results concerning the dependence of hydrolysis on the crystallinity index (CrI) of Solka Floc SW 40 (sulfite pulp, Brown Co., Berlin, NH, USA) and microcrystalline cellulose (Sigma cell 50). These findings were confirmed many times. In the quoted study, the increased surface area created by ball milling was not very high. Thus the interpretation could be limited to the pure influence of CrI.

Wood samples

SEM images of the Wiley-milled wood meal (74–149 μm) and its ball-milled fraction are presented in Figure 3a and b, respectively. Compared with $wood_{74–149\ \mu m}$ with the large-size fragments from the original cell wall, the $wood_{ball\text{-}milled}$ has globular aggregates of various sizes (mostly <10 μm) without undamaged cell wall fragments. Expectedly, the higher-magnification SEM results in $wood_{74–149\ \mu m}$

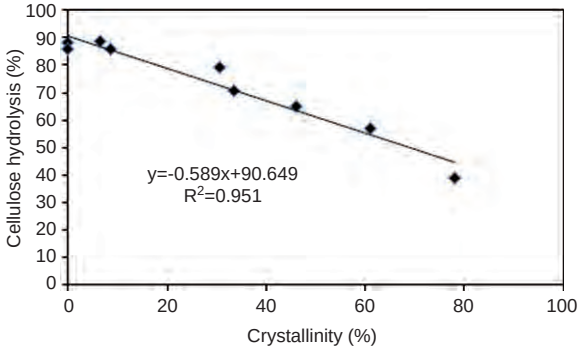

Figure 2 Correlation between cellulose crystallinity and the extent of enzymatic hydrolysis after 72 h hydrolysis time.

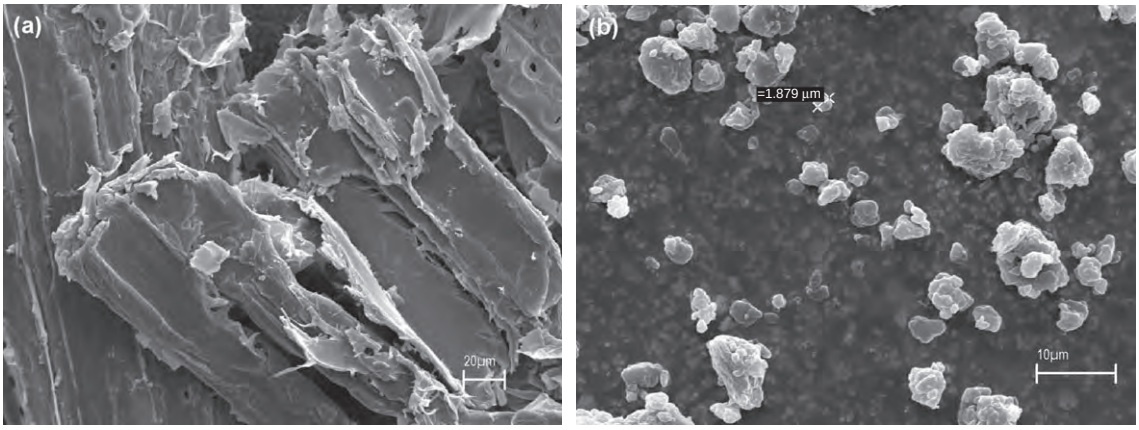

Figure 3 SEMs of the Wiley-milled sieve fraction 74–149 µm (a) and ball-milled pine wood (b).

(Figure 3a) show that the cell walls remained unfibrillated and intact (Fukazawa et al. 1982).

The chemical compositions of all nondelignified wood fractions are very similar (Table 2). Results of enzymatic hydrolysis after 12 and 72 h of $wood_{74–149\ µm}$ and $wood_{ball\text{-}milled}$ are presented in Figure 4 and Table 2. Hydrolysis results of the former show a very low level of saccharification, for example, 7.6% after 72 h. Small cell wall particles did not lead to high yield conversion to glucose. This confirms the results of Zhu et al. (2009b) obtained from disk- and hammer-milled samples. However, the hydrolysis of $wood_{ball\text{-}milled}$ was very rapid (Figure 4 and Table 2). The amorphous cell wall gives rise to 92% conversion after 72 h. Figure 4 also reveals for $wood_{ball\text{-}milled}$ that 96% of the total hydrolysis took place in the first 12 h of the enzyme treatment. Ball milling destroys the cell wall mechanically; the original supramolecular architecture is destroyed and the polymers are partly depolymerized, but the physicochemical changes occurring during the process are not completely understood (Fukazawa et al. 1982; Maurer and Fengel 1992; Guerra et al. 2006; Terashima et al. 2009). Nevertheless, ball milling increases the cellulose accessibility regardless of the presence of the other polymers.

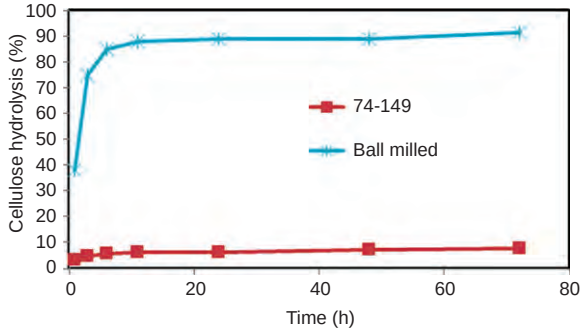

Figure 4 Hydrolysis yield of the sieve fraction 74–149 µm and ball-milled loblolly pine as a function of enzymatic hydrolysis time.

Influence of lignin

The process of lignin removal up to 60% by the acid chlorite delignification is selective (Ahlgren and Goring 1971). Glucomannan is removed only beyond this point. The results of hydrolysis of the six delignified fractions are compiled in Table 2, in which the data concerning the contents of KL, acid-soluble lignin, glucan, xylan, and mannan are listed. Clearly, xylan and mannan are present in the samples in significant amounts.

Based on the normalized Raman spectra in Figure 5 (basis band at 1096 cm^{-1}), the Cr of $wood_{74–149\ µm}$ samples did not change upon delignification; i.e., in the spectra of the delignified samples, the 380:1096 peak height ratio is unchanged. The hydrolysis results are plotted in Figure 6, and data are also partly listed in Table 2. After initial delignification ($wood_{delig1}$), in which the KL dropped from 31% to 22.6%, the lignin content remained constant for up to 2 h (22% for $wood_{delig2}$ and $wood_{delig3}$). The lignin contents

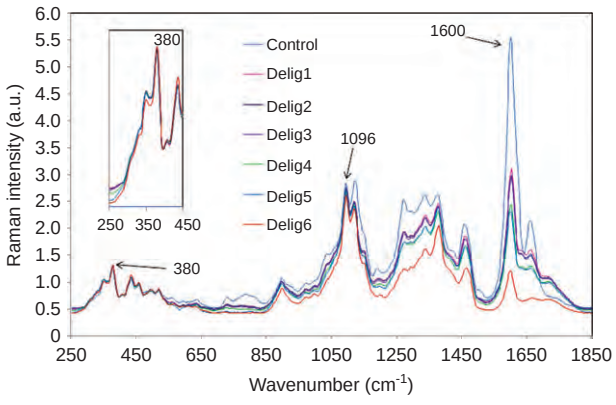

Figure 5 Raman spectra between 250 and 1850 cm^{-1} of the samples $wood_{74–149\ µm}$ and $wood_{delig1}$ to $wood_{delig6}$. Spectra are normalized on the 1096 cm^{-1} band. Inset shows the expanded 380 cm^{-1} region crucial for calculation of crystallinity.

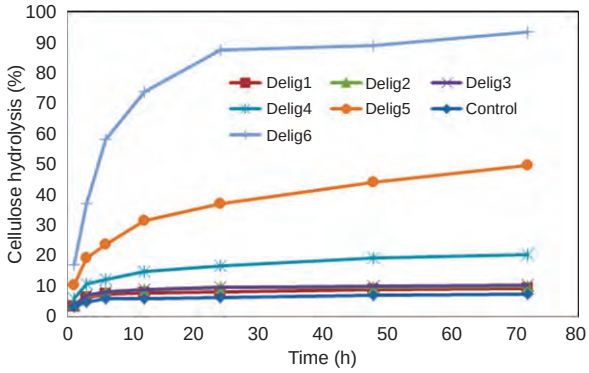

Figure 6 Plots of hydrolysis yields vs. hydrolysis time of the samples indicated in the figure.

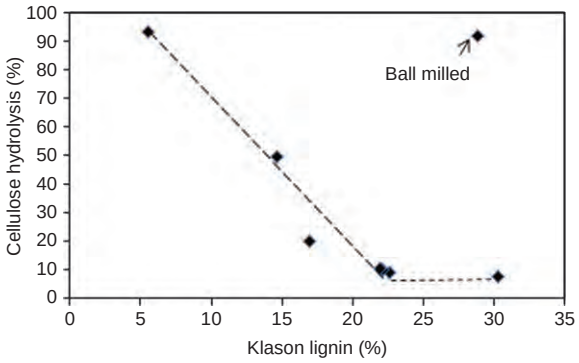

Figure 7 Plots of hydrolysis yields after 72 h hydrolysis time as a function of the KL content of the delignified samples and that of the ball-milled sample.

of $wood_{delig4}$, $wood_{delig5}$, and $wood_{delig6}$ dropped to 16.9%, 14.6%, and 5.5%, respectively. The cellulose hydrolysis (Figure 6) increased proportionally to the degree of delignification. For instance, when comparing $wood_{delig5}$ (KL 14.6%) and $wood_{delig6}$ (KL 5.5%) after 72 h hydrolysis time, the extent of glucose conversion in the former was only 49.5% and in the latter was 93.3%. Compared to $wood_{non\text{-}delig}$, similar behavior was observed for $wood_{delig4}$, a sample that was delignified to a lower extent (Table 2 and Figure 6). The hydrolysis plots (Figure 6) with 22–23% KL were similar to that of $wood_{non\text{-}delig}$. In summary, conversion to glucose was directly dependent on the degree of delignification and independent of the cellulose Cr. More than 50% of the initial lignin content needs to be removed to achieve 90% cellulose conversion at a 72-h hydrolysis level.

The results can be easily interpreted by the ultrastructural changes that ensued upon lignin removal, mainly those leading to the creation of new pores and enlargement of the present pores. These changes contribute to the increment of internal surfaces, which also include the opening of the lamellar cell wall structure (Stone and Scallan 1965). The newly created internal surfaces are not necessarily accessible to enzymes (Mooney et al. 1998; Kojiro et al. 2010), but pores opened beyond 50% delignification are certainly accessible to enzymatic hydrolysis as demonstrated in the present paper.

In Figure 7, the 72-h hydrolysis data are plotted against the KL content. A steep correlation can be observed in the range of 5–23% KL content. Then, the dependency flattens. Clearly, the hydrolysis data of $wood_{ball\text{-}milled}$ are very different from those of the other samples. A similar relationship was found for the 12-h hydrolysis data (plot not shown). The plot in Figure 7 is a clear demonstration of the peculiar effect of ball milling, which destroys the cell wall and affords 0% Cr and 90% hydrolyzability in the presence of the whole lignin content.

Whatman cellulose in comparison to wood

The hydrolysis behavior of Whatman cellulose and pine wood are compiled in Table 3 at the hydrolysis time levels of 12 and 72 h. Compared to pure Whatman cellulose (5 min ball-milled), pine wood cellulose within the cell wall (both with similar Cr) shows eight times less extensive hydrolysis for both hydrolysis times. Nevertheless, an essential delignification (e.g., $wood_{delig6}$ with 5.5% KL) renders possible a high hydrolysis yield of 73.9–93.3%. Accordingly, the crystallinity of the cell wall is not as important as the changes brought about by the change in the porosity followed by delignification. The hydrolysis behavior of $wood_{ball\text{-}milled}$ demonstrates that wood can be hydrolyzed within the shortest time even in the presence of all lignin in the cell wall because its original 3D structure is opened during the physical impact. Milling has an effect similar to intensive delignification beyond 50%.

Hydrolysis time (h)	Cellulose conversion (%)			
	Whatman 5-min ball-milled[a]	Wood[b]	Wood delig6	Wood ball-milled[c]
12	49.7	6.2	73.9	88.5
72	64.9	7.6	93.3	92.0

Table 3 Comparison of cellulose conversion in various samples with various crystallinities (Cr).
[a]Cr 46.1%.
[b]Wood is a meal fraction of 74–149 µm of Wiley-milled loblolly pine with Cr of 46.7%.
[c]Cr 0% (Table 1).

Conclusions

The factors that modify the ultrastructure of the wood cell wall are more important for the hydrolysis of cellulose embedded in the cell wall matrix than the cellulose crystallinity, although the lowering of the latter also contributes to the hydrolysis of pure cellulose samples. Lignin removal entails the creation of pores and increases the internal surfaces, and these effects facilitate the access of enzymes to cellulose.

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