Delignification of Lignocellulosic Biomass and Its Effect on Subsequent Enzymatic Hydrolysis

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The effect of delignification on the enzymatic hydrolysis of biomass was investigated to determine how different delignification processes affect enzymatic hydrolysis conversion yields. Oxygen, hydrogen peroxide, and sodium chlorite treatments were performed, and the structural and chemical changes in the biomass were evaluated. Sodium chlorite delignification proved the most effective process to remove lignin in hardwood samples, followed by oxygen delignification. Hydrogen peroxide delignification was not as effective as the other two methods. As for the enzymatic conversion of carbohydrates after delignification processes on hardwood, oxygen and sodium chlorite treatments substantially improved conversion yields as the number of successive treatments was increased, compared to untreated hardwood samples. Changes in α-cellulose after delignification were less substantial than those of hardwood samples, and corresponding conversion yields were also lower. Delignification-induced structural changes in treated substrates might be responsible for the changes in carbohydrate conversion yield observed following subsequent enzymatic hydrolysis.

Keywords: Delignification; Enzymatic hydrolysis; Water retention value

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INTRODUCTION

An understanding of the changes that occur to the carbohydrate structure, of cellulose in particular, during delignification processes is essential to their utilization, not only in current chlorine-free bleaching technologies for papermaking but also in the biochemical conversion process of biomass feedstock for energy production (Himmel *et al.* 2007). Chlorite bleaching, which is known for the preparation of holocellulose, has historically been one of the most conventional methods of delignification by oxidation (Wise *et al.* 1946; Sarkanen 1962) . Since the first commercial installation over 40 years ago, the use of oxygen delignification processes has become more prominent in industry due to increased environmental concerns about chlorinated bleaching agents (Rowlandson 1971). Additionally, alkaline hydrogen peroxide has been used extensively for the bleaching of mechanical and chemical pulps (Hobbs and Abbot 1991). However, with increased degree of delignification, losses in fiber strength are expected to occur due to carbohydrate degradation (De Souza *et al.* 2002). During these delignification processes there are two primary cellulose degradation reactions that can occur: (1) peeling reactions caused by alkali-driven rearrangements of reducing end groups, and (2) chain scission caused by the oxidation of the glycosidic linkage between glucose residues (Olm and Teder 1979).

There are several different factors that can affect the enzymatic hydrolysis of carbohydrates in biomass. It has been widely accepted that physical and biochemical hindrances by lignin and the physicochemical characteristics of the carbohydrate structure are two major factors governing the bioconversion yield of lignocellulosic biomass. In this study, the effect of lignin removal on enzymatic hydrolysis was studied by using three different delignification methods (oxygen, hydrogen peroxide, and sodium chlorite delignification) with varying degrees of lignin removal. These observations were expected to help develop an understanding of how different delignification processes affect the structure of lignocellulosic biomass and the corresponding enzymatic hydrolysis conversion yields.

EXPERIMENTAL

Materials

Biomass substrates

Two types of cellulosic biomass were examined to investigate the effect of different delignification processes. Mixed hardwood (HW) chips obtained from a local pulp mill in North Carolina were air-dried and milled to particle size between 40 to 60 mesh using a Wiley mill. For the comparison, α-cellulose (Batch #: 058K0132, Sigma Aldrich, St. Louis, MO, USA) was used as a control material and was treated with the same delignification conditions used for the hardwood sample.

Methods

Delignification

For each delignification process, 5 OD (oven-dry) g of substrate was used. Oxygen delignification was done with 3% loading of sodium hydroxide on substrate at 130 °C for 1 h at an oxygen pressure of 689.47 kPa in a pressurized stainless steel bomb with a horizontal rotisserie mixer running at 100 rpm. Hydrogen peroxide delignification was performed with 10% H_2O_2 and 2% sodium hydroxide on substrate at 110 °C with the same 100 rpm mixing speed. Sodium chlorite delignification was carried out in an 80 °C water bath with the addition of 41% sodium chlorite and 8% acetic acid on substrate. The oxygen and hydrogen peroxide delignification processes were done in triplicate, and the samples generated by each repeated reaction were labeled Ox-1, 2, and 3 for oxygen delignification and HP-1, 2, and 3 for hydrogen peroxide delignification. A final digit of 0 (zero) on a sample label indicates that it is the control sample without oxygen or hydrogen peroxide treatment. During sodium chlorite delignification, the reaction mixture was successively added 6 times, once every hour of reaction time, and delignified samples were obtained after each addition of sodium chlorite-acetic acid mixture. These samples were referred to as SC-1 to SC-6.

Composition analysis and water retention value (WRV) measurement

Composition analysis using two-stage sulfuric acid hydrolysis, also known as the Klason lignin method, was applied to measure the chemical composition of untreated and delignified samples. First, 100 mg from each sample were reacted with 72% H_2SO_4 at room temperature for 2 h with frequent mixing. The mixture was then diluted to 3% acid concentration with deionized (DI) water and autoclaved at 121 °C for 1.5 h. After cooling to ambient temperature, the acid hydrolyzate was filtered through a filtering crucible, and

the residue was washed with DI water, dried, and weighed to determine the Klason lignin content. The liquid hydrolyzate after filtration was subjected to acid soluble lignin (ASL) analysis (absorbance at 205 nm, extinction coefficient value of 110 L/g-cm used for calculation) using UV-Vis spectrophotometry (Lambda XLS, Perkin Elmer; USA) and sugar analysis using an HPLC apparatus (Agilent 1200 Series, Agilent Technology Inc.; USA) equipped with a Shodex Sugar SP0810 column (Showa Denko America Inc.; USA) after the liquid was adjusted to neutral pH using $CaCO_3$. Water retention values (WRV) for the prepared samples were measured according to the method described in TAPPI UM256 (1981).

Enzymatic hydrolysis for saccharification

Enzymatic hydrolysis for raw and delignified samples was carried out in 100-mM sodium acetate buffer (pH 4.8) at 5% consistency with total volume of 20 mL. The enzyme mixture consisted of cellulase (NS50013) and β-glucosidase (NS50010), which were generously provided by Novozymes North America Inc. (Franklinton, NC, USA). The enzyme loadings during the hydrolysis were 1 part cellulase with an enzyme activity of 5 FPU (filter paper unit)/OD g of substrate and 1/9 part of β-glucosidase suspension. The substrate-enzyme mixture was incubated at 50 °C in a shaking incubator (150 rpm) for 96 h before the concentration of the sugars in the liquid hydrolyzates were measured by HPLC, as described previously. Values of carbohydrate conversion in this study were calculated based on what is the percentage of sugars converted from total carbohydrate in each delignified substrate.

RESULTS AND DISCUSSION

Composition Analysis of Delignified Biomass

Composition analysis data are summarized in Table 1. For hardwood (HW) samples, the solid yields after delignification decreased as the number of process repetitions increased for oxygen and chlorite delignification. Considering the solid yield and lignin content in the delignified samples, delignification by hydrogen peroxide presented the least severe results as compared to the other two processes. After the first round of the H_2O_2-delignification reaction, samples with repeated treatments (HW-HP-2 and 3) did not show a substantial decrease in lignin content, resulting in 21% Klason lignin content with 90.4% solid yield for HW-HP-3. After three iterations of oxygen delignification, however, the solid yield was decreased to less than 75.6% and the Klason lignin content was reduced to 17.3%. Sodium chlorite delignification appeared to be more effective, with about a 33% decrease in Klason lignin content after the first treatment, and most of the lignin fraction was removed after 6 successive additions of sodium chlorite-acetic acid mixture.

Changes in each component in the HW samples after delignification (raw material basis) are plotted in Fig. 1a. It was observed that oxygen delignification removed not only the lignin fraction, but also a substantial amount of carbohydrates, especially xylan, resulting in consistent decreases in the total carbohydrate fraction as the treatment was repeated. After the third iteration of oxygen delignification, about 49% of the initial insoluble lignin fraction (Klason) was removed.

Table 1. Composition Analysis (based on each substrate) for Raw and Delignified Biomass

	Solid yield (%, raw-basis)	WRV (g/g)	Structural carbohydrates (%)					Lignin (%)		
			Glucan	Xylan	Galactan	Arabinan+Mannan	Total	Klason	Acid-soluble	Total
HW (raw)	-	0.80	41.5	14.7	2.7	7.5	66.4	25.6	3.6	29.2
HW-Ox-0	89.3	1.06	42.5	12.4	2.1	7.2	64.2	23.9	3.6	27.4
HW-Ox-1	80.5	1.17	44.3	12.7	2.9	6.8	66.6	22.9	2.5	25.5
HW-Ox-2	76.8	1.45	46.6	9.5	2.7	6.8	65.6	22.8	2.1	24.9
HW-Ox-3	75.6	1.67	48.1	9.1	2.7	6.7	66.6	17.3	2.1	19.3
HW-HP-0	92.3	0.92	41.4	12.2	2.0	7.0	62.7	23.5	3.8	27.3
HW-HP-1	90.8	0.99	42.7	11.8	2.8	6.7	64.0	23.0	2.8	25.8
HW-HP-2	90.6	1.25	43.2	12.6	2.9	6.8	65.4	22.1	2.7	24.8
HW-HP-3	90.4	1.31	43.7	10.5	2.5	6.2	63.0	21.0	2.6	23.6
HW-SC-1	91.2	0.93	42.4	9.7	2.6	8.0	62.7	18.8	5.2	24.0
HW-SC-2	86.7	1.07	43.6	9.9	2.5	8.0	63.9	11.8	6.3	18.1
HW-SC-4	70.1	1.33	51.9	15.8	3.0	7.3	78.1	2.9	7.9	10.8
HW-SC-6	64.8	1.50	54.2	16.0	3.0	7.3	80.6	0.1	6.8	6.9
αCell (raw)	-	1.06	77.2	11.2	2.1	9.0	99.6	0.0	0.4	0.4
αCell-Ox-0	92.9	1.45	80.2	2.2	2.0	7.7	92.1	0.0	0.4	0.4
αCell-Ox-1	91.2	1.51	81.5	1.8	2.0	7.8	93.2	0.0	0.4	0.4
αCell-Ox-2	93.2	1.58	82.7	0.0	2.0	7.8	92.5	0.0	0.4	0.4
αCell-Ox-3	93.0	1.34	82.9	0.0	2.0	7.8	92.7	0.0	0.4	0.4
αCell-HP-0	94.6	1.11	80.5	3.1	2.0	8.1	93.8	0.0	0.4	0.4
αCell-HP-1	93.9	1.04	80.8	4.6	2.1	8.2	95.6	0.0	0.4	0.4
αCell-HP-2	95.0	1.15	80.3	3.3	2.1	8.0	93.7	0.0	0.4	0.4
αCell-HP-3	95.0	1.39	81.4	3.0	2.1	8.0	94.4	0.0	0.4	0.4
αCell-SC-1	96.8	1.06	78.7	2.2	1.8	8.4	91.1	0.0	0.4	0.4
αCell-SC-2	97.8	1.43	75.2	2.1	1.8	8.0	87.1	0.0	0.4	0.4
αCell-SC-4	97.1	1.19	76.1	2.2	2.0	8.3	88.6	0.0	0.4	0.4
αCell-SC-6	98.2	0.93	75.6	1.6	2.5	8.3	88.0	0.0	0.3	0.3

As for α-cellulose in Fig. 1b, all the three processes caused about a 10 to 15% reduction in the carbohydrate fraction. The decrease in total carbohydrate content is mainly due to the removal of the xylan fraction from α-cellulose. It is noted that the raw material in this study (α-cellulose purchased from Sigma Aldrich) contains 11.2% xylan, 9.0% mannan, and 2.1% galactan. The difference in glucan content on a raw material basis among delignified samples was not substantial, which is consistent with that of HW samples. Therefore, the change in solid yield is attributed to the losses from the hemicellulose and lignin fractions, which are more readily affected by the delignification process.

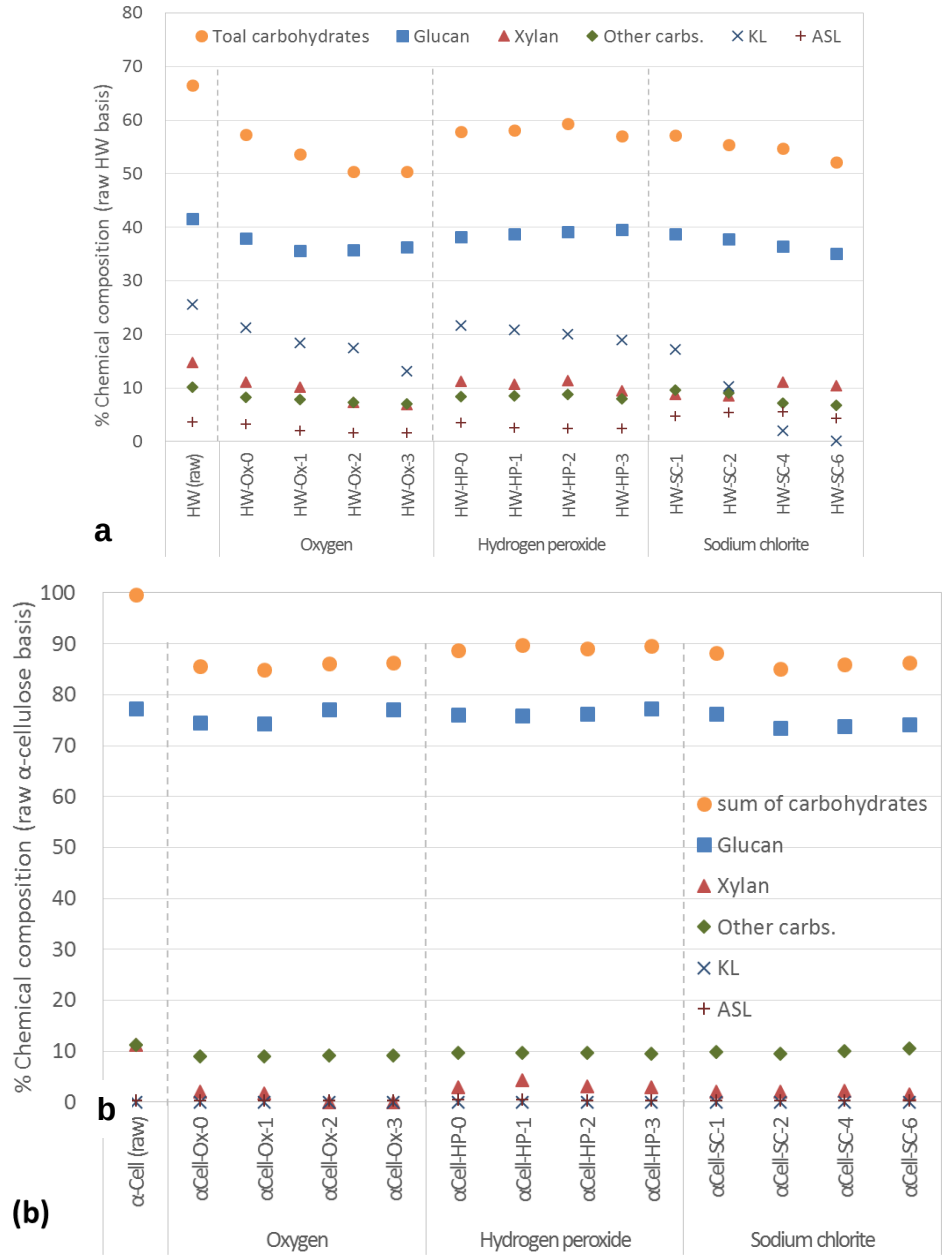

Fig. 1. Compositional analysis of raw and delignified samples. (a) HW samples (raw HW basis) and (b) α-cellulose samples (raw α-cellulose basis)

Enzymatic Hydrolysis of Carbohydrates After Delignification

Hardwood samples not subjected to delignification showed very low conversion of carbohydrates after enzymatic hydrolysis, amounting to only about 2.5% of the total carbohydrates (Fig. 2a). After the delignification processes, however, it was observed that the conversion increased substantially, and repeated treatments resulted in higher conversion yields in delignified HW samples. After oxygen delignification, even the control sample without the presence of pressurized oxygen showed an increase in glucan and xylan conversion (10.6 and 18.6%, respectively). With repeated oxygen-delignifying treatments, enzymatic conversion of carbohydrates in HW samples was strongly promoted, and HW-Ox-3 led to the complete conversion of xylan and about 78% yield for glucan.

As for hydrogen peroxide delignification, a different trend was noted. Considering the relatively low weight loss and insignificant differences in lignin content after iterated processes (which might indicate less effective removal of the lignin fraction) it can be expected that the effect of hydrogen peroxide delignification on subsequent enzymatic hydrolysis will be lower among the three different treatments. The carbohydrate conversion after 3 iterations of hydrogen peroxide delignification was about 27% for glucan and 63% for xylan. Between the HW-HP-2 and 3 samples, the xylan conversion was raised by 44 percentage points, but only about 16 percentage points for glucan. Hence, the effect of hydrogen peroxide delignification was more significant on the enzymatic hydrolysis of hemicellulose than that of cellulose. It has been reported that the cellulase enzyme cocktail (NS50013 + NS50010) can degrade xylan fraction in pretreated biomass, and the supplement of xylanase did not affect the xylan conversion yield significantly (Xu *et al.* 2011). High susceptibility to xylan degradation due to chemical and structural changes by delignification might be responsible to the high enzymatic conversion yields in delignified HW samples.

Sodium chlorite delignification exhibited a similar trend with oxygen delignification, with a steep rise in carbohydrate conversion for both glucan and xylan. It was also observed that HW-Ox-3 and HW-SC-6 had similar conversion yields. As shown in Fig. 1a, HW-Ox-3 retained about half of its initial lignin, but most of the lignin fraction was removed from HW-SC-6. This might indicate that the change in lignin content does not play the sole role in the increase of carbohydrate conversion *via* enzymatic hydrolysis, and that there are other factors that might affect the efficiency of carbohydrate conversion. This will be visited later in this study.

Figure 2b shows the enzymatic hydrolysis of delignified cellulosic material, α-cellulose. As for both oxygen and hydrogen peroxide delignifications, an increase of up to about 10 percentage points in the carbohydrate conversion was observed. A noteworthy observation is that there were clear differences in the changes of carbohydrate conversion between HW and α-cellulose samples. Hardwood feedstock, in which carbohydrates account for about two-thirds of the mass, initially showed only about 2.5% carbohydrate conversion after enzymatic hydrolysis, but the yield increased significantly after all three delignification processes. In contrast, α-cellulose, a nearly pure carbohydrate with high cellulose content, accounted for about 53% of the total carbohydrate conversion, but it was increased by less than 15 percentage points after oxygen and hydrogen peroxide delignification processes. This might indicate that the existence of lignin hinders enzymatic hydrolysis of lignocellulosic biomass (Mooney *et al.* 1998; Fengel and Wegener 1984) and that delignification processes can also alter the structure of carbohydrates, which can affect the subsequent carbohydrate conversion by enzymes (Gould 1984; Cara *et al.* 2006). As

for the high xylan conversion, the majority of xylan was removed during delignification, and the xylan content decreased to a few percent in delignified α-cellulose samples. It was noted that this remaining xylan portion might be easily hydrolyzed even with cellulase enzymes.

Sodium chlorite delignification on α-cellulose samples clearly showed a different trend than other processes. After more than four successive additions of sodium chlorite-acetic acid mixture, the carbohydrate conversion was substantially decreased. Considering insignificant changes in the solid yield and chemical composition (Fig. 1b), this might be related to structural alteration during the delignification process, which can physically hinder enzymatic hydrolysis.

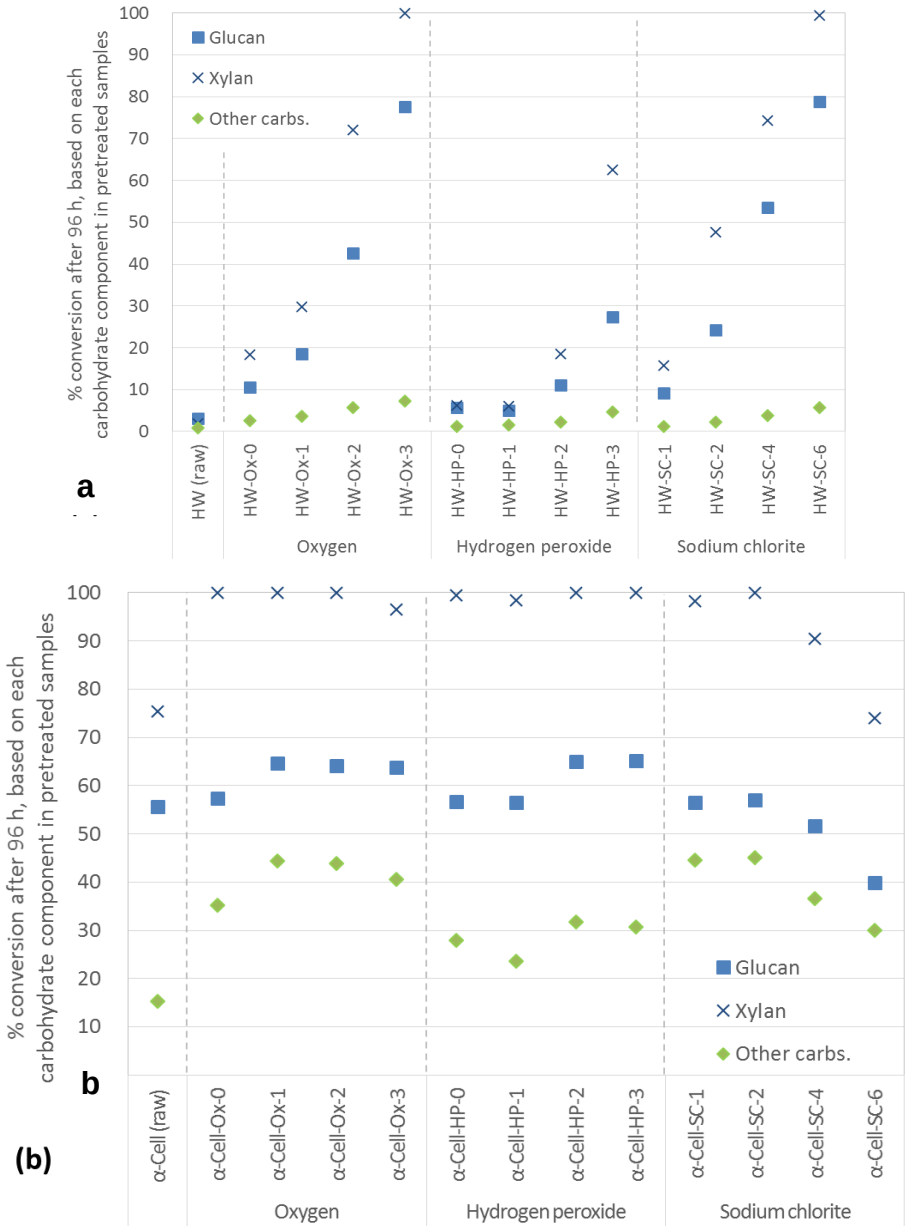

Fig. 2. Carbohydrate conversion after 96 h of enzymatic hydrolysis, based on each carbohydrate component in (a) HW samples and (b) α-cellulose samples

Water Retention Value and Enzyme Accessibility

It is widely accepted that the removal of lignin by various delignification processes can enhance the enzymatic hydrolysis of lignocellulosic biomass by reducing physical and biochemical hindrances from lignin and by making pores more accessible for the effective mass transfer of enzymes (Yu *et al.* 2011, 2014). The increase in carbohydrate conversion after delignification has also been confirmed in this study, but it will be necessary to explain the high carbohydrate conversion in oxygen-delignified HW samples, which still contain a considerable amount of lignin. The decreasing trend in enzymatic hydrolysis in sodium chlorite-delignified cellulose also requires further elucidation. It is likely that those observations might be due to the structural alteration that occurs during the delignification processes. To evaluate the structural change in biomass, a simple technique – water retention value (WRV) measurement – has been proposed (Zhu *et al.* 2009) and can provide information about the total pore volume inside biomass, which is directly related to the digestibility of biomass.

Table 1 summarizes WRVs for all samples following each delignification process. All the HW samples showed a substantial increase in WRV after delignification processes, indicating the formation of a more porous structure by removing the lignin fraction and some carbohydrates. Figure 3a demonstrates strong linear correlations between weight loss during enzymatic hydrolysis and measured WRV. Oxygen delignification showed a moderate weight loss after hydrolysis and a substantial increase in WRV, which was more than two-fold compared to that of the untreated HW. Sodium chlorite delignification presented the highest weight loss during enzymatic hydrolysis, and the consequent increase in WRV was also quite large, close to that of HW-Ox-3. Hydrogen peroxide delignified samples yielded the least increase in weight loss from enzymatic hydrolysis and WRV among the three processes. This implies that the removal of the lignin fraction can effectively increase the amount of internal pores, resulting in the formation of a larger cellulase-accessible surface.

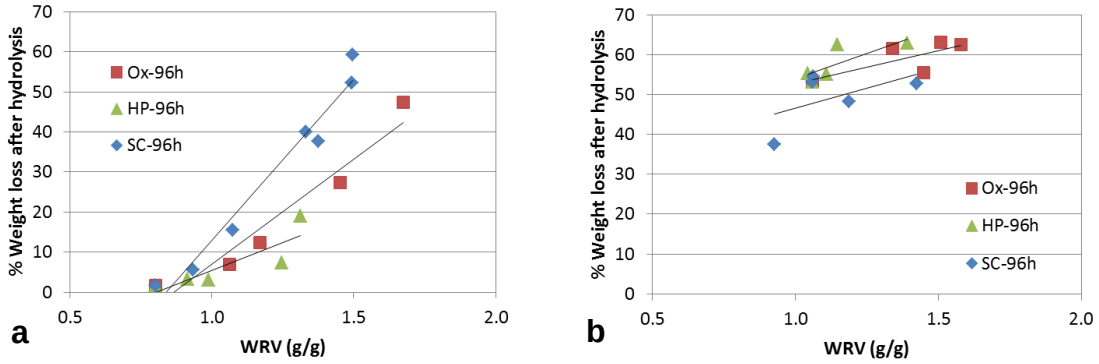

Fig. 3. Weight loss by enzymatic hydrolysis after 96 h of incubation *vs.* water retention value: (a) HW samples and (b) α-cellulose samples

Compared to HW samples, α-cellulose samples did not exhibit strong correlations between weight loss and WRV as shown in Fig. 3b. In the case of oxygen- and hydrogen peroxide-delignified α-cellulose samples, WRV was increased to 49% and 31%, respectively, with slight weight loss after hydrolysis. However, WRV for sodium chlorite-delignified samples were increased at the beginning, but decreased after four and six iterations of the process. αCell-Ox-3 also showed decreased WRV, compared to αCell-Ox-2. This can be explained by the hornification of the samples. During the delignification

process, pores can be developed by the removal of lignin and hemicellulose. This phenomenon makes it easier to form crosslinking within cellulose structure by organized hydrogen bonding between adjacent cellulose fibers (Maloney and Paulapuro 1999; Fernandes Diniz *et al*. 2004). In the case of α-cellulose, the partial removal of hemicellulose and amorphous cellulose might cause such hornification, resulting in the decrease of WRV for sodium chlorite-delignified samples. This might be the reason for the similar yield of carbohydrate conversion in HW-SC-6 with HW-Ox-3, despite the fact that the lignin content is as low as 6.9% after 6 successive iterations of sodium chlorite delignification, compared to 19.3% in HW-Ox-3.

The correlation between carbohydrate conversion yields with different incubation time and WRV was a close linear trend, as shown in Fig. 4, but the characteristics of each feedstock were quite different. As for the HW samples, the dependency of carbohydrate conversion on WRV was much stronger, as the slope of the 96-h plot was substantially steeper than that of the 12-h plot. This implies that samples with lower WRVs could not develop more cellulase-accessible area as enzymatic hydrolysis progressed. On the contrary, samples with higher WRVs could effectively open up more pores within their cellulosic material, resulting in substantial increases in carbohydrate conversion yield. As for α-cellulose samples, this dependency on WRV was not clear. In Fig. 4b, it can be observed that all three plots of different incubation times shifted vertically without a notable increase in slope. This means that the increase in carbohydrate conversion yield was at the similar rate with a lapse in incubation time, regardless of differences in WRVs. This might be because these α-cellulose samples were unaffected by the inherent nature of lignin on enzymatic hydrolysis, such as a physical barrier to decrease the number of substrate sites susceptible to cellulase and nonspecific binding with enzymes, which can hinder cellulolytic activity. Without lignin, their carbohydrate conversion yields were only dependent on structural characteristics, and they increased linearly, with nearly parallel plots for different incubation times.

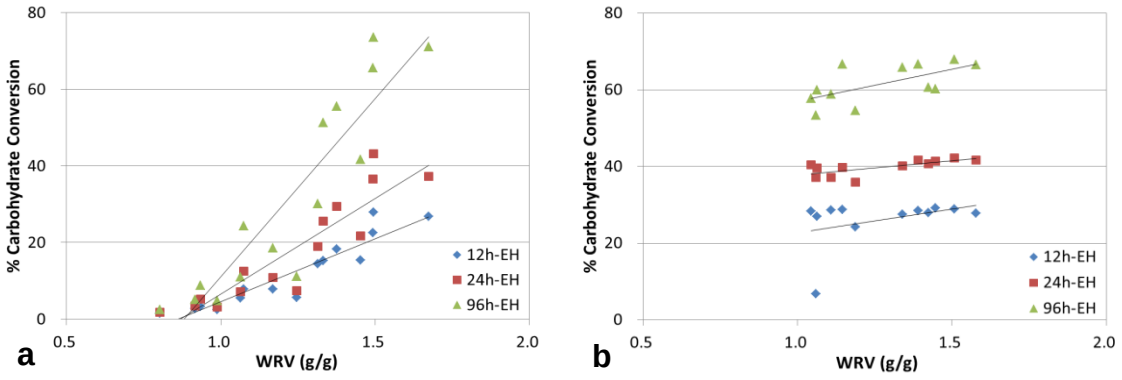

Fig. 4. Carbohydrate conversion yields, based on carbohydrates in delignified substrate, with different incubation times *vs*. water retention value: (a) HW samples and (b) α-cellulose samples

The results from enzymatic hydrolysis and WRV were plotted as a function of the lignin content of the HW samples to check how those values changed as the degree of delignification increased (Fig. 5). At the same lignin content, samples subjected to three different delignification processes had different carbohydrate conversion values and WRVs. In Fig. 5a, it is clear that the samples with the oxygen delignification method had higher carbohydrate conversion compared to the other two samples with same lignin

contents. With a 20% lignin content, the oxygen-delignified sample presented more than twice the conversion of the sample subjected to sodium chlorite delignification. Despite the fact that sodium chlorite delignification resulted in a lower lignin content with repeated treatments, it could reach a similar level of carbohydrate conversion at around 10% lignin content, which is much lower than that of oxygen-delignified hardwood. The trends in measured WRV in Fig. 5b also match those of the carbohydrate conversion. At the same level of lignin content, oxygen-delignified samples presented higher WRV values up to about 1.7 g/g, and the sodium chlorite-delignified sample with 6.9% lignin content, which was significantly lower than the 19.3% lignin content after three iterations of the oxygen delignification processes, could reach only about 1.5 g/g of WRV. This can be expected by considering the positive correlation between carbohydrate conversion and WRV shown in Fig. 4a.

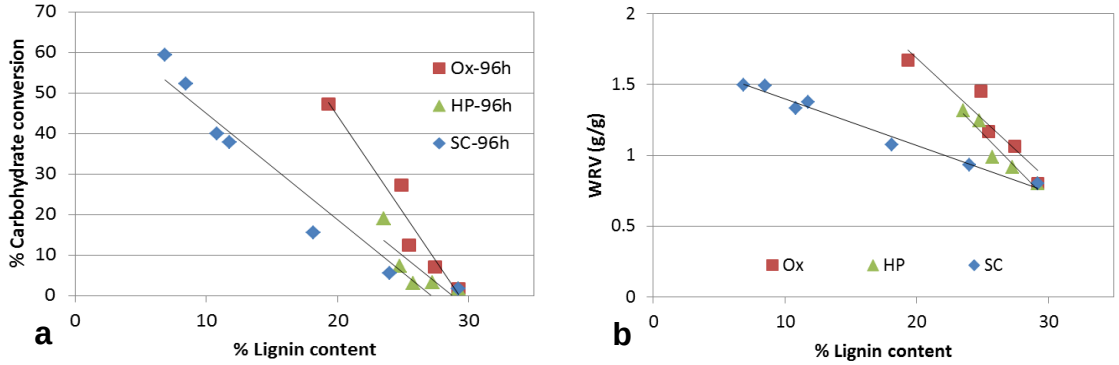

Fig. 5. (a) Carbohydrate conversion yields, based on carbohydrates in delignified substrate, at 96 h of incubation times and (b) WRV *vs.* lignin contents of HW samples

CONCLUSIONS

1. In delignification on hardwood, samples with higher WRVs presented more increase in carbohydrate conversion yield with increasing incubation time, compared to those with low WRVs. By contrast, α-cellulose with different WRVs did not show substantial changes in hydrolysis rate, which can be due to the fact that the feedstock is free of the inherent effect of lignin on enzymatic hydrolysis.
2. Lignin content in hardwood samples was found to be inversely proportional with WRV and carbohydrate conversion yield, and the trends were different among three delignification methods. Sodium chlorite delignification was observed to be the most effective way to remove lignin fraction and achieve high carbohydrate conversion yield, followed by oxygen and hydrogen peroxide delignification processes.
3. Oxygen and sodium chlorite treatments yielded substantial improvements in conversion yields as the number of successive treatments increased, but the former was able to achieve more than two-fold of yield at the same level of lignin content. None of the delignification processes presented significant increases in conversion yields of α-cellulose.

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