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Effects of Cell-Wall Acetate, Xylan Backbone, and Lignin on Enzymatic Hydrolysis of Aspen Wood

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ABSTRACT

Aspen wood substrates with varying degrees of deacetylation, xylan, and lignin removal have been prepared and submitted to enzymatic hydrolysis with a cellulase/hemicellulase preparation for an extended constant period of hydrolysis. Controlled deacetylation has been achieved by treating wood with various alkali metal hydroxide solutions, at various alkali/wood ratios. It has been found that samples with the same extent of deacetylation produce the same sugar yields upon enzymatic hydrolysis. Increased degree of deacetylation increases the yield of sugars obtained from enzymatic hydrolysis, all other compositional parameters held constant. The acetyl group removal is proportional to the stoichiometric relation between added base and wood acetyl content, i.e., the same number of milliequivalents of base/weight of wood remove the same extent of acetyl groups, regardless of the concentration of the base solution. No cation effects are found among Li, Na, and K alkali hydroxide solutions, suggesting that swelling is not as important a parameter as is the removal of the acetyl groups from the xylan backbone in determining the extent of hydrolyzability of the resulting sample.

Index Entries: Aspen; enzymatic hydrolysis; xylan; acetate; lignin.

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INTRODUCTION

Lignocellulosics, consisting mainly of polysaccharides (cellulose and hemicellulose) and lignin (*1–3*), are quite resistant to enzymatic attack when in the form of woody materials and agricultural residues (biomass) (*4–7*). Preliminary preparation or pretreatment of biomass is generally applied to overcome the impediments of the cell wall to enzyme digestion (*5*). Pretreatments with steam (*8*), dilute acid (*9*), alkali (*6*), alkaline peroxide (*10–13*), or other chemicals have been used in efforts towards a cost-effective solution to this problem.

Cellulose is the most important sugar source in lignocellulosics, and it is well known that the crystallinity of cellulose serves to retard the rate of production of fermentable sugars (*1,2,5,14*). However, the enzyme accessibility of cellulose in plant cell walls is governed by more than cellulose crystallinity (*1*). Although the biodegradation of lignocellulose has probably attracted more attention worldwide than any other single biochemical event outside the field of medicine, mechanisms for hydrolysis of polysaccharides in plant cell walls are not yet fully understood (*7*).

Take, for example, the case of lignin, the most important nonpolysaccharide component of biomass. There has been much work done in observing the behavior of lignin in enzyme hydrolysis of lignocellulosics, and conventionally, it is thought that the accessibility of enzymes to polysaccharides is restricted by the presence of the lignin matrix acting as a barrier (*5,6,14*). However, the actual role of lignin needs further study. Scientists have judged the role of lignin by removing it under certain conditions, but their work may not account for other changes, such as the deacetylation that does occur under the conditions employed (*15*).

PRELIMINARY STUDIES ON THE ROLE OF HEMICELLULOSE AND ACETATE

The second significant sugar source of lignocellulosics is hemicelluloses, shorter chain polysaccharides associated with cellulose (*14,16*). Sinner et al. (*17*) studied the enzymatic hydrolysis of beechwood and sprucewood holocelluloses (delignified lignocellulose), and pointed out that in order for the cellulose fibrils to be digested, hemicellulose had to be degraded. It was this and related studies that earlier suggested to one of the authors (*1*) that hemicellulose was an integral part of the resistance mechanism.

Xylans, the major hemicelluloses in hardwood cell walls, are extensively acetylated (*2,16,18*), with acetyl groups (OAc) amounting to about 3–5% of the wood substance (*18*). Concurrently, another group also funded through SERI found new information on the role of ester groups in hemicellulose. Grohmann et al. (*15*) used hydroxylamine solution to remove

OAc in aspen wood and wheat straw, and concluded that: "Ester groups play an important role in the mechanism of plant cell wall resistance to enzyme hydrolysis in both aspen wood and wheat straw."

The determination of the relative roles of cell-wall components in relation to polysaccharide accessibility is then of great importance. It should be clear that studies towards solving this problem should entail appoaches in which only one single major component is changed at a time and other primary components of the parent substrates are kept in a chemically unaltered form. This research has attempted to do so.

Our first efforts in a SERI research project (*19*) were directed towards the development of a mild holocellulose procedure. Chlorine dioxide, which is the most selective lignin oxidant among the chlorine-based bleaching chemicals and reacts only very slowly with polysaccharides (*2*), was used to oxidize aspen wood. Even though much care was taken to monitor the quality of chlorine dioxide and the reaction conditions, analyses showed that both the lignin and hemicellulose (mainly acetyl content) changed in all samples, regardless of the degree of oxidation (*19*).

The samples prepared at different chlorine dioxide oxidation stages were then treated with dilute alkali. Results showed (*20*) that, as the xylan became increasingly deacetylated with alkali, sugar yield from enzyme digestion increased. More interestingly, OAc could be removed to various extents (including OAc-free) at very mild reaction conditions, little affecting the lignin or xylan backbone contents. Alkali has long been used to enhance cellulose accessibility and to enhance the digestibility of lignocellulosic materials for ruminants (*6,21*). Increases in accessibility are usually attributed to the swelling caused by the alkali, although such treatments also remove acetate. With these observations indicating potential for removing major cell-wall components one at a time, the study of the relative roles of such components in cell-wall enzyme accessibility entered a new stage.

MATERIALS AND METHODS

Material

The lignocellulosic substrate selected for this study was aspen wood (*Populus tremuloides*) contributed by the Solar Energy Research Institute. Since most of hemicellulose in aspen wood is xylan (*22*), the wood sugar composition is rather simple, compared to that of softwoods. The sample received was a mixture of various particle sizes and was rescreened. Some 58% passed through sieve No. 10 (2.0 mm) and was retained by sieve No. 20 (0.84 mm). This fraction was selected as the substrate.

On a dry extractive-free wood basis, the wood particles analyzed 20% lignin, 22% xylan, and 3.7% acetate. Acetate here is regarded as a "component."

Methodology

Extractive-Free Wood Preparation

Screened aspen wood was extracted with alcohol-benzene (2:1, 7 h, Soxhlet), 95% ethanol (7 h, Soxhlet), and cold water (3 times, 1 d ea., 19–22°C) (*18,23*).

Vol/Wt and Mol/Wt Ratios

The terms volume/wt ratio and mol/wt ratio are defined as alkali solution vol/wood wt, mL/g and mol alkali/wood wt, m*M*/g, respectively. Molar concentration times vol/wt ratio equals mol/wt ratio.

Partial Deacetylation with Alkali

Extractive-free wood was reacted with various concentrations of alkali solution (0.05–0.3*M*) at a constant vol/wt ratio of 5 mL/g to effect mol/wt ratios of 0.25–1.5 m*M*/g. Reaction was at room temperature (19–22°C) with shaking at 100 rpm for 24 h.

Complete Deacetylation at Mild Conditions

KOH (0.1*M*) at a vol/wt ratio of 12.5 (=1.25 mol/wt ratio) was used for complete deacetylation. Reaction was again at room temperature (19–22°C) with shaking at 100 rpm for 24 h. Using these conditions, no lignin loss or xylan backbone loss could be detected.

Delignification with Acidified Sodium Chloride

OAc-free wood was reacted with acidified sodium chloride at 75°C with 3:1 sodium chlorite:glacial acetic acid (g:mL). Fresh sodium chlorite and glacial acetic acid were added every half hour, with glacial acetic acid added first. Erlenmeyer flasks in a constant temperature bath were used as reactors. A glass cover was placed in the neck of the flask to cause condensation and refluxing so as to cut down on evaporation. The residue was washed with water, acetone, and alcohol sequentially (*18,23*). Samples of each delignifying stage were prepared separately and air-dried so that the reaction and extraction rate (the yield) could be calculated properly to obtain comparable data.

Enzymatic Hydrolysis

The various treated samples were subjected to cellulase/hemicellulase digestibility studies for assessment of the relative roles of the components in resistance to enzymatic attack. Cellulase/hemicellulase used in this work was Cytolase™ 123 from Genencor Co. (South San Francisco, CA). The following conditions were applied: time, 72 h; temperature, 50°C; substrate, 0.25 g; enzyme, 104 IFPU/g substrate; buffer, 25 mL 50 m*M* NaOAc, pH 4.8, with 40 μg/mL tetracycline and 30 μg/mL cyclohexamide (*15*). Digestions were done with a New Brunswick Rollordrum installed in a Blue M incubator. An excessive time period and amount of enzyme were used to ensure as complete hydrolysis as possible, i.e., to ensure that the

digestion results reflected the resistance of the samples. In this study, we were more interested in extent of hydrolysis than in rate of hydrolysis.

DNS Reducing Sugar Analysis

The total reducing sugar yields were obtained with DNS reducing sugar analysis (*24–26*). Enzyme digestion yields are compared to an OAc-free basis, that is, the sugar yield on a treated sample basis was converted to OAc-free sample basis to make the sugar yield comparable. For example, 5% KOH was used to extract OAc-free sample. Extraction loss with 5% KOH was 12.2%, and sugar yield on extracted sample was 47.5%. Then, sugar yield on OAc-free wood basis was obtained by 47.5% x (1–0.122).

Sugar Analysis

Quantitative glucose and xylose analysis was by LC, using Aminex HPX-87C and HPX-87P columns (Bio-Rad Laboratories) in tandem, water as mobile phase, flow rate 0.4 mL/min, and temperature 85°C.

Other Analytical Methods

(A) WCL-10—preparation of extractive-free wood; WCL-11—lignin content (Klason acid insoluble); WCL-12—preparation of holocellulose (acid-chlorite method); WCL-13—extraction of xylan from holocellulose (*23*). (B) Acetyl group analysis using NaOMe (*18*).

RESULTS AND DISCUSSION

Acetyl Content

Extracted wood was treated with varying concentrations of KOH (0.05–3.0*M*) and vol/wt ratios of 5 and 10 mL/g to give mol/wt ratios of 0.25–1.5 m*M*/g, and with selected NaOH and LiOH concentrations and vol/wt ratios to premit comparisons of the effects of

1. KOH concentration;
2. Mol/wt ratio KOH; and
3. Type of alkali used on subsequent acetyl, xylan, and lignin contents as well as sugar yields upon enzymatic hydrolysis.

Results are summarized in Table 1, which shows that

1. OAc can be removed with dilute alkali without lignin or xylan backbone loss at alkali concentrations below 0.1*M*;
2. Regardless of concentration, vol/wt ratio, or type of alkali used, OAc content and DNS sugar yield are comparable at all mol/wt ratios below 1 m*M*/g; and
3. Sugar yields increase with decreasing acetyl content.

At KOH concentrations $>0.1M$, sugar yields increase more rapidly.

Table 1
Effect of Alkali Deacetylation
of Aspen Wood upon Subsequent Enzymatic Hydrolysis

Exp. no.	Alkali used	Molarity, *M*	Vol/wt ratio, mL/g	Mol/wt ratio, m*M*/g	Lignin content, %	Xylan loss, %	OAc content, %	DNS sugar yield, %
1	KOH	0.150	10	1.50			0.00	46.1
2	KOH	0.300	5	1.50	18.7	1.31	0.00	46.4
3	KOH	0.250	5	1.25	19.2	0.95	0.00	47.6
4	KOH	0.100	10	1.00			0.14	41.6
5	KOH	0.200	5	1.00	20.0	0.43	0.17	40.1
6	KOH	0.075	10	0.75			0.81	29.8
7	KOH	0.150	5	0.75	20.0	ND	0.77	29.0
8	NaOH	0.150	5	0.75			0.74	29.6
9	LiOH	0.150	5	0.75			0.78	29.2
10	KOH	0.050	10	0.50			1.62	17.9
11	KOH	0.100	5	0.50	20.0	ND	1.55	18.4
12	KOH	0.075	5	0.38	20.0	ND	2.05	15.6
13	NaOH	0.075	5	0.38			2.05	15.1
14	LiOH	0.075	5	0.38			2.09	15.7
15	KOH	0.050	5	0.25	20.0	ND	2.45	13.8
16	NaOH	0.050	5	0.25			2.47	13.8
17	None	0.000	0	0.00	20.0	0.00	3.60	10.8

OAc loss and sugar yield are plotted against mol/wt ratio in Fig. 1 (only those below 1 m*M*/g plotted to reflect only that state in which there is no lignin or xylan loss detected in treatment). The same mol/wt ratio, regardless of concentration, alkali used, or vol/wt ratio used, results in the same OAc loss and sugar yield. The relationship with acetyl content is nearly linear. Stoichiometric calculation shows that 56.1 g KOH will remove 43 g OAc for stoichiometric removal ratio OAc removed to KOH used of 0.77. For sample No. 7, the actual ratio is 0.74, suggesting that nearly all KOH used was consumed in OAc removal.

Sugar Analysis of Hydrolysate

Hydrolysates of the various deacetylated samples were analyzed for glucose and xylose. Results, summarized in Fig. 2, indicate that (1) glucose and xylose ratios in the hydrolysate do not change appreciably with acetate content, and (2) the ratios are similar to that which would be expected if neither cellulose or xylan was preferentially hydrolyzed. Apparently, the

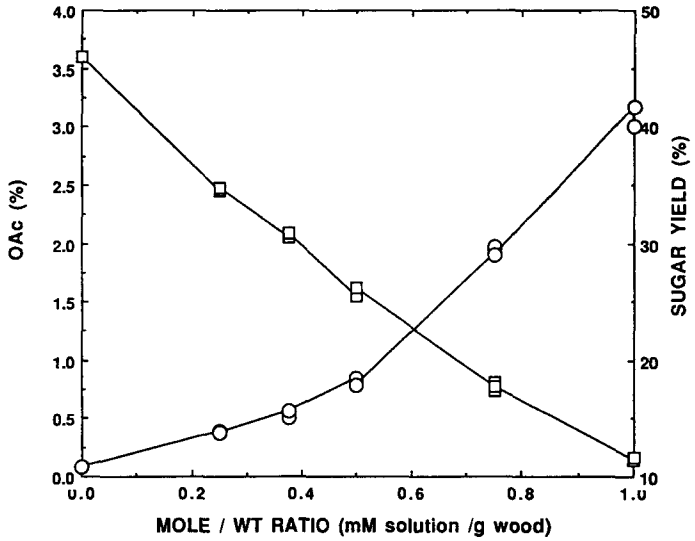

Fig. 1. Relationships between mol/wt ratio alkali used in treatment of aspen wood, subsequent acetyl content of treated wood, and DNS sugar yields upon enzymatic hydrolysis. Different alkalis, concentrations, and vol/wt ratios represented. —□— OAc; —○— sugar yield.

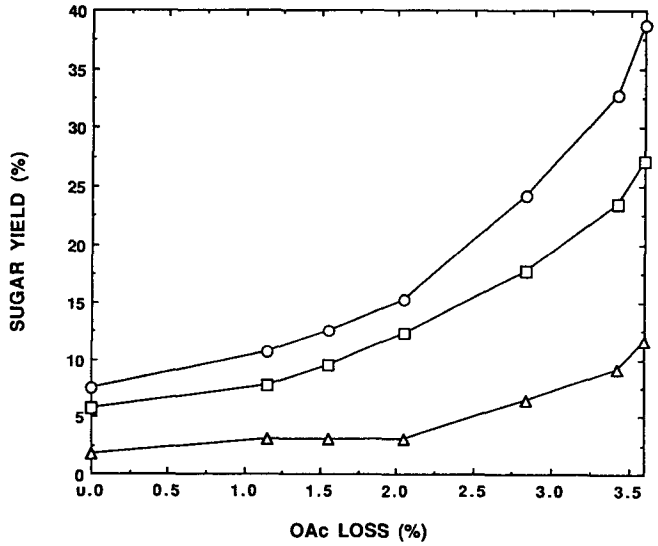

Fig. 2. Xylose and glucose compositions of enzyme hydrolysates of aspen wood deacetylated to various extents. —○— glucose + xylose; —□— glucose; —△— xylose.

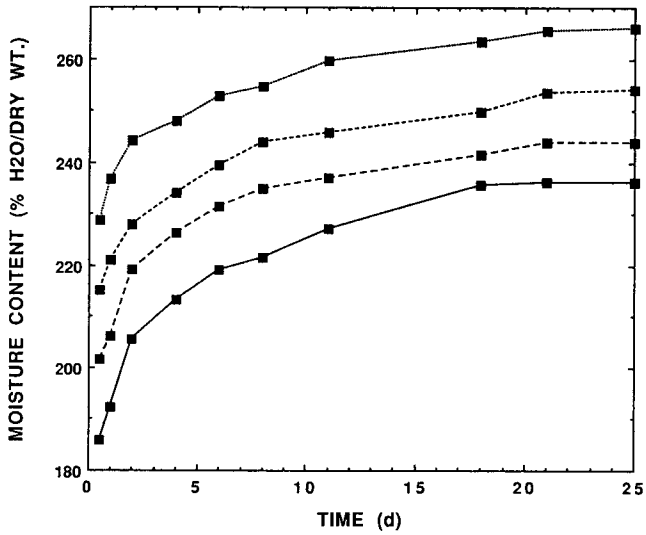

Fig. 3. Water uptake of partially acetylated aspen wood samples. ···■··· 0.0% OAc; ---■--- 0.9% OAc; --■-- 2.3% OAc; —■— 3.6% OAc.

enzyme system used is not selective. More importantly, these observations suggest that, at least here, improving accessibility does not favor access to either cellulose or xylan.

Acetyl Content vs Swelling

The extent of external volumetric swelling of wood in water increases quite rapidly with pH increase after it reaches 8 (*27*). It is known that cellulose accessibility is related to this alkali swelling (e.g., *1,21*). With temperature and alkali type constant, swelling of fiber in alkali increases with alkali molarity (*10*). Furthermore, it is known that swelling with alkalis of various alkali metals is related to the crystal ionic radii of the metal ions. The decreasing order of cellulose swelling in alkali solution and the corresponding crystal ionic radii of the alkali metal ions are listed as follows (*10,28*): Extent of swelling LiOH > NaOH > KOH > RbOH > CsOH; radii of alkali metal ions (Å) $0.68 < 0.97 < 1.33 < 1.47 < 1.67$.

The results of our experiments indicate that samples treated with the same mol/wt ratio of KOH, NaOH, or LiOH result in similar OAc losses and similar sugar yields, confirming that increases in accessibility are directly related to OAc content, and not with the associated swelling phenomenon. Alkali breaks the ester bonds that control water absorption and swelling, thereby increasing enzyme accessibility. Water retention tests (rate and extent of water weight gain by soaking in water) on the samples with varying acetyl contents show (*20*) that, as the xylan fraction becomes deacetylated, extent of water retention increases (*see* Fig. 3). Interestingly, acetylation of wood reduces water swelling and increases wood resistance to biodegradation (*29*).

Table 2
Effect of Xylan Backbone Removal
on Sugar Yields in Enzymatic Hydrolysis of Deacetylated Aspen Wood

Exp. no.	KOH concentration, %	Xylan loss, %	Lignin loss, %	DNS sugar yield, %	Sugar yield adjusted for xylan loss, %
X1	0	0.0	0.0	53.5	54.5
X2	3	5.1	0.6	48.0	53.1
X3	4	8.0	0.9	45.8	53.8
X4	5	10.9	1.9	41.7	52.6

Xylan Backbone

OAc-free wood was treated with 3–5% KOH to remove xylan backbone (*2,18,23*). The effect of xylan backbone removal on sugar yield is shown in Table 2. Lignin losses were small. Sugar yields decreased with increasing KOH concentration and were identical when adjusted for xylan losses. Removal of xylan backbone does not facilitate enzyme hydrolysis. It is now clear that it is acetate, a component of hemicellulose, and not the hemicellulose backbone itself, that plays an important role in enzymatic hydrolysis resistance.

Lignin

Lignin in OAc-free wood was oxidized with warm acidified sodium chlorite, using a method similar to the holocellulose preparation procedure (*18,23*). Data on lignin removal experiments are given in Table 3. With increasing lignin removal, sugar yields increase until lignin content is $<3.4\%$. It is known that ClO_2 attacks carbohydrate at low lignin contents, causing a reduction in sugar source, here reflected in decreasing sugar yields upon enzymatic hydrolysis.

Removal of lignin facilitates enzymatic digestion even after complete deacetylation resulted in sugar yields increasing from about 10% (extractive-free wood) to some 50% (OAc-free wood). Delignification of the OAc-free wood to a lignin content of 4% increases sugar yields by an additional 20%. Since it was shown above that the removal of xylan backbone did not improve sugar yields upon hydrolysis, sugar yield increases shown in Table 3 should be attributed to lignin removal.

Appearance of Digestion Residues

The residues of oxidized samples were carefully examined after enzymatic digestion. Particles of the residues were gradually destroyed with lignin content reduction. For samples at the lower lignin contents, particles were not visible after hydrolysis, that is, all wood became soluble

Table 3
Effect of Lignin Removal
on Sugar Yields in Enzymatic Hydrolysis of Deacetylated Aspen Wood

Exp. no.	Reagent rate Na chlorite/ wood, g/g	Reaction time, h	Lignin content, %	Lignin loss, %	DNS sugar yield, %	DNS sugar yield increase, %
L1	0.00	0.0	21.3	0.0	50.7	0.0
L2	0.10	0.5	18.6	2.7	56.9	6.2
L3	0.20	0.5	16.4	4.9	63.2	12.5
L4	0.30	0.5	11.2	10.1	66.9	16.2
L5	0.45	1.0	7.1	14.2	69.6	18.9
L6	0.60	1.5	4.3	17.0	70.1	20.0
L7	0.75	2.0	3.4	17.9	69.1	18.4
L8	0.90	2.5	2.8	18.5	67.1	16.4
L9	1.05	3.0	2.2	19.1	66.3	15.6
L10	1.20	3.5	1.4	19.9	63.3	12.6
L11	1.35	4.0	1.1	20.2	64.3	13.6

in enzyme solution. The appearance of digested samples then confirms that lignin removal favors enzymatic hydrolysis. On the contrary, all of the residues of samples in the xylan backbone removal experiments were particular and looked the same after digestion. It can be judged intuitively that removal of the xylan backbone did not facilitate enzyme digestion. Both the delignified samples and the samples with xylan backbone removed were prepared from OAc-free wood, so that OAc content was not a factor in these determinations, as opposed to some previous work in which the OAc role had been unfortunately ignored.

Hydroxylamine vs Dilute Alkali

Grohmann et al. (*15*) used hydroxylamine solution for 10 d to remove acetate from aspen wood and wheat straw to study the role of acetate in plant cell-wall enzyme hydrolysis. There are some questions that hydroxylamine solution extraction may cause extra swelling beyond the normal water swelling and that solutions with different concentrations might cause swelling to different extents. In addition, the Klason lignin loss in aspen wood with the hydroxylamine method is about 3% (percent total wood dry wt before pretreatment), and that for wheat straw reached 5% (*15*). In the preparation of OAc-free samples with 0.1*M* KOH and a vol/wt ratio of 12.5 mL/g, Klason lignin and xylan backbone losses in aspen wood are not detected. Further, in contrast to the hydroxylamine method, dilute KOH pretreatment is simpler, faster, and safer (hydroxylamine is toxic and mutagenic (*15*)).

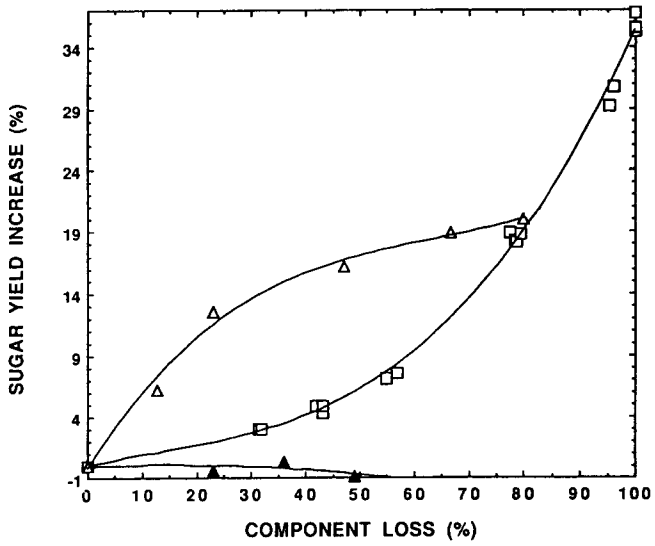

Fig. 4. Separate effects of OAc, lignin, and xylan backbone component losses on DNS sugar yield increases in enzymatic hydrolysis of aspen wood. Data for lignin and xylan backbone are on deacetylated wood. □ OAc; △ lignin; ▲ xylan backbone.

CONCLUSIONS

Figure 4 provides a comprehensive picture of the effects of the three major components. Component loss percentage was calculated in the following way: If the lignin content of the OAc-free sample is 21.3% and lignin in the oxidized sample is 7.1% (OAc-free wood basis), then lignin loss is 66.7% obtained by (21.3–7.1%)/21.3% × 100. Sugar increase percentage was determined by sugar yield of the oxidized samples (OAc-free wood basis) subtracted by the sugar yield of the OAc-free sample. For the xylan backbone and OAc, similar calculations were used. It is readily seen that for aspen wood both acetyl group and lignin are important barriers to enzyme hydrolysis; however, the xylan backbone is not.

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