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Assessment of Pretreatment Conditions to Obtain Fast Complete Hydrolysis on High Substrate Concentrations

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ABSTRACT

Steam-heating of aspen wood chips improved the enzymatic digestibility of the cellulose. Scaling up the reaction vessel from 2 to 60 L had virtually no influence on the chemical composition and the accessibility of the lignocellulosic substrate. Over 85% of the cellulose could be hydrolyzed to glucose when an 8% substrate concentration was used. The residual content of alkali-insoluble lignin appeared to control the digestibility of the cellulose. Increased delignification either by prolonged steaming, oxidative posttreatment, or SO_2 catalysis improved the accessibility of the cellulose. The use of SO_2 as a catalyst also increased the recovery yield of the wood after steam-heating, with more than 70% of the original xylan recovered as monomeric xylose. Conversion yields of above 90% were achieved at low levels of filter paper activity after a relatively short incubation time. Removal of alkali-soluble lignin did not influence digestibility when the enzyme concentration was based on the cellulose content of the substrates.

Index Entries: Steam-pretreatment; SO_2-catalysis; enzymatic hydrolysis; high substrate concentration; chemical composition (alkali-insoluble lignin).

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NOMENCLATURE

For convenience, the following abbreviations will be used in referring to the pretreated substrates.

SHA-WI	water-insoluble fraction of steam-heated aspen wood
SHA-WIA	water-and-alkali-insoluble fraction of steam-heated aspen wood
SHA-WIA/H_2O_2	water-and-alkali-insoluble fraction of steam-heated aspenwood posttreated with hydrogen peroxide

Abbreviations preceded by SO_2 indicate, that SO_2 had been used as an acidic catalyst (e.g., SO_2-SHA-WIA means water-and-alkali-insoluble fraction of SO_2-catalyzed steam-heated aspen wood).

INTRODUCTION

Lignocellulosic substrates require some form of pretreatment before efficient enzymatic hydrolysis of cellulose can take place. Among a number of physical and chemical pretreatment options (*1–7*), steam-pretreatment has the advantage of being relatively inexpensive and allowing the partial fractionation of the substrate into its cellulose, hemicellulose, and lignin components. The cellulose fraction, after such a steam pretreatment of aspen wood, can be as readily hydrolyzed as commercial substrates such as Solka floc.

One of the difficulties in assessing the effectiveness of pretreatment is in distinguishing between the actual accessibility of the cellulose, and enzyme-related factors such as endproduct inhibition. Although at a 2% concentration the cellulose of steam-pretreated aspen wood could be completely hydrolyzed within 2 d, at higher concentrations both the rate and degree of hydrolysis were reduced substantially even when more enzyme was added.

Many workers (*8–17*) have studied the influence of substrate-related factors such as surface area, lignin association and crystallinity, on the enzymatic hydrolysis of cellulose. In this paper a variety of different enzyme preparations have been compared at various levels of activity to determine the influence of enzyme concentration on both the rate and completeness of hydrolysis. Various aspects of pretreatment and hydrolysis, which are important to making bioconversion of wood residues to glucose an economically viable process, have been considered.

A number of factors, such as, enzyme addition on the basis of cellulose content rather than weight of total material, the amount of residual alkali-insoluble lignin in pretreated substrates, and the use of SO_2 as a catalyst during pretreatment, all influence the interpretation of results. We have tried to identify the reasons why decreased saccharification yields are obtained when substrates are hydrolyzed in a batch fashion at high substrate concentrations.

MATERIALS AND METHODS

Steam Pretreatment

Charges of commercial never-dried chips of aspen wood (*Populus tremuloides*) with moisture contents between 36.0–42.0% (wet-wood basis) were used in the steam-treatment with or without addition of 1.6% SO_2 (dry-wood basis) as an acidic catalyst. The chips were steam-treated without rapid decompression either in a 2-L stainless steel pressure vessel using thin-walled stainless steel canisters, as described earlier (*18*) or in a 60-L pressure vessel fitted with a basket to hold the material. Both vessels were preheated to the desired reaction temperature with saturated steam immediately before use. When aspen wood chips were treated in the 60-L vessel without SO_2 impregnation, the steam temperature was 240°C with reaction times from 60 to 140 s. Pretreatments of SO_2-impregnated chips in the 2-L vessel were carried out at temperatures ranging from 190 to 220°C with a reaction time of 100 s. In all the experiments, after closing the steam inlet valve, the pressure was bled down to atmospheric at a controlled rate, without explosion. The steam-heated chips were broken up in a blender and extracted twice with distilled water by stirring for 1 h at a 5% solids concentration. The water-washed substrates were also similarly washed twice with 0.4% sodium hydroxide. In some cases, alkali-extracted material was posttreated with hydrogen peroxide, essentially as described previously (*19*).

Analytical Methods

The apparent Klason lignin contents of the various substrates were determined by TAPPI standard method T222 os-74 for "acid-insoluble" lignin. Analysis of "acid-soluble" lignin was essentially carried out according to TAPPI Useful Method 250.

HPLC, as described earlier (*20*), was used for chemical characterization of insoluble fractions resulting from water-washing, extraction with dilute alkali, and H_2O_2-posttreatment by analyzing the sugars in Klason lignin filtrates. An HPX-87P column (Bio-Rad Labs) was employed for analysis of sugars in enzymatic hydrolysates. An HPX-87H column (Bio-Rad) was used to characterize water soluble fractions of steam-heated aspen wood (*21*).

Enzymatic Hydrolysis

All substrates (unless otherwise specified) were hydrolyzed with a Celluclast/Novozym enzyme preparation (NOVO Lab. Inc., USA) in a 0.05 *M* acetate buffer at pH 4.8. The flasks containing various substrate concentrations from 2 to 10% (w/v) were shaken at 140 rpm in an incubator at 45°C. Filter paper activities (FPU) and β-glucosidase activities (CBU) were determined using the procedures described previously (*22*).

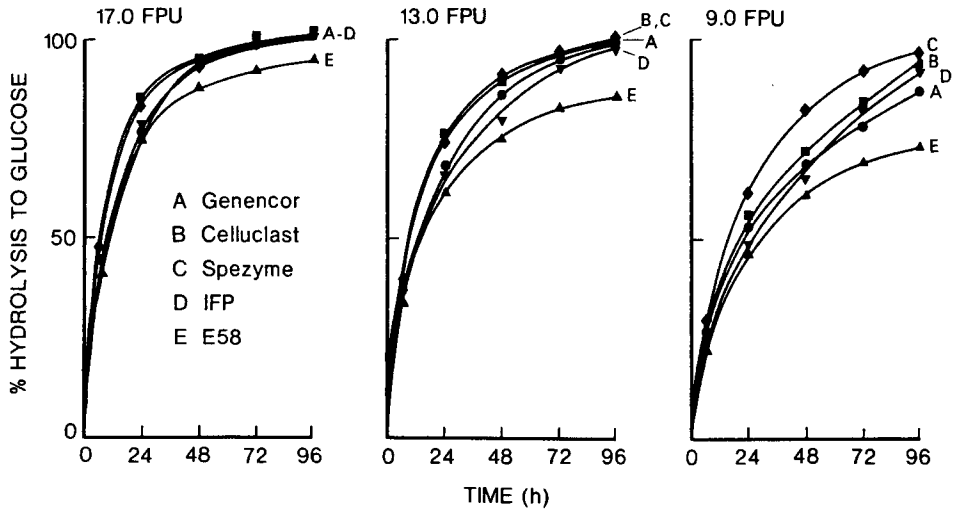

Fig. 1. Effect of enzyme concentration (FPU/g substrate) on enzymatic hydrolysis of pretreated aspen wood using various enzyme preparations supplemented with β-glucosidase (Novozym) to a constant level of cellobiase activity.

In some experiments, hydrolyses were carried out with enzyme preparations from *Trichoderma harzianum* (*9*) and *T.reesii* mutants. The cellulase preparations from *T.reesii* mutants were obtained as gifts from Genencor Inc. (USA), Finnish Sugar Co. Ltd. (Spezyme), and Institut Francais du Petrole (IFP).

RESULTS AND DISCUSSION

Five different cellulase preparations, as specified above, were compared for their ability to hydrolyze steam-pretreated aspen wood. In this comparison each preparation was supplemented with a commercial cellobiase (Novozym) to a common level of 35 cellobiase U/g of substrate. After 96 h at an enzyme loading of 17 FPU/g, all of the enzyme mixtures other than that from *T. harzianum* E58 gave complete hydrolysis of the cellulose (Fig. 1). Differences among the enzyme mixtures were apparent on reduction of the loading to 13 FPU/g, and became more pronounced at 9 FPU/g in both rate and completeness of hydrolysis. The Celluclast/Novozym mixture, which is readily available to most researchers, gave favorable results, and was used to evaluate the different pretreatments described below.

It has previously been shown (*23*), with a 2-L steam-treatment vessel, that explosion is not an essential element of steam-pretreatment to enhance enzymatic hydrolysis. To ensure that effective pretreatment could be obtained in a larger vessel, without the need for rapid decompression, aspen wood chips were heated with steam at 240°C in a 60-L vessel for various times.

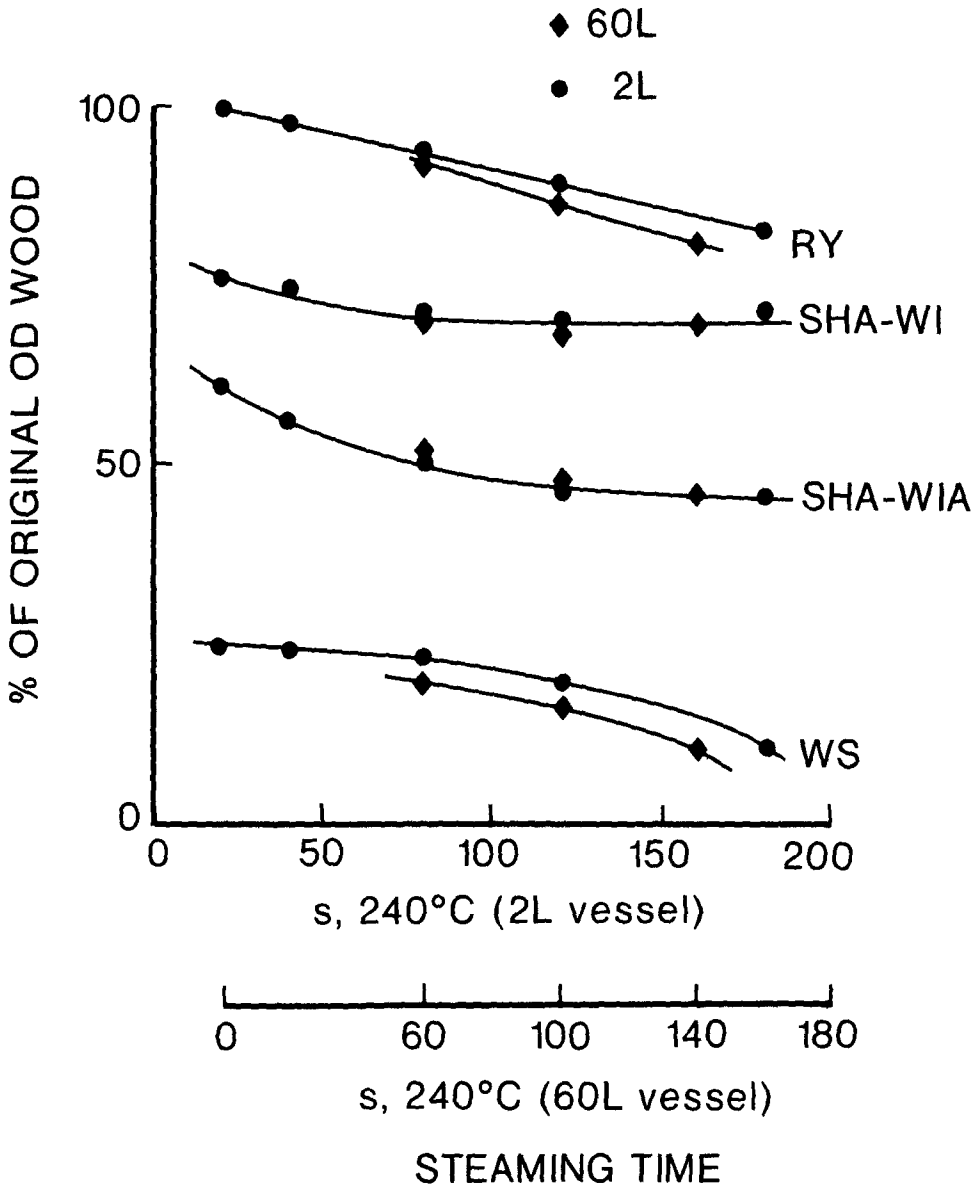

Fig. 2. Effect of vessel size (60 and 2-L) on recovery yields (RY), water-insoluble fractions (SHA-WI), water- and alkali-insoluble fractions (SHA-WIA) and water-soluble fractions (WS) obtained after steam-heating of aspen wood for various times at 240°C.

Figure 2 shows that, when the time scale for the 60-L data was displaced by 20 s, the recovery-yield curves for the water insolubles and the water-and-alkali insolubles were virtually identical for the 60 and 2-L vessels. This displacement adjusts for the longer time required to close and to pressurize the larger vessel, during which some preheating of the charge occurred. Pressurization of the 60-L vessel required 8 s to 450 psi, and an additional 10 s to 470 psi, whereas the 2-L vessel required a total of

Table 1
Comparison of the Chemical Composition of SHA-WIA Obtained after Steam-Heating at 240°C in Reaction Vessels of Different Sizes[a]

Reaction vessel 60 L				Reaction vessel 2 L			
	% of OD substrate				% of OD substrate		
Reaction time, s	TAL[b]	Cell	Xylan	Reaction time, s	TAL[b]	Cell	Xylan
60	11.3	85.4	1.6	80	9.0	86.6	1.7
100	5.3	92.6	0.8	120	3.9	92.5	1.0
140	3.2	91.9	—	180	2.4	91.8	—

[a]Composition of original wood (ethanol-benzene-extracted); TAL = 21.79%, cellulose and other glucan = 48.08%, Xylan = 19.59%.
[b]TAL = Total Apparent Lignin (Klason lignin + acid soluble lignin).

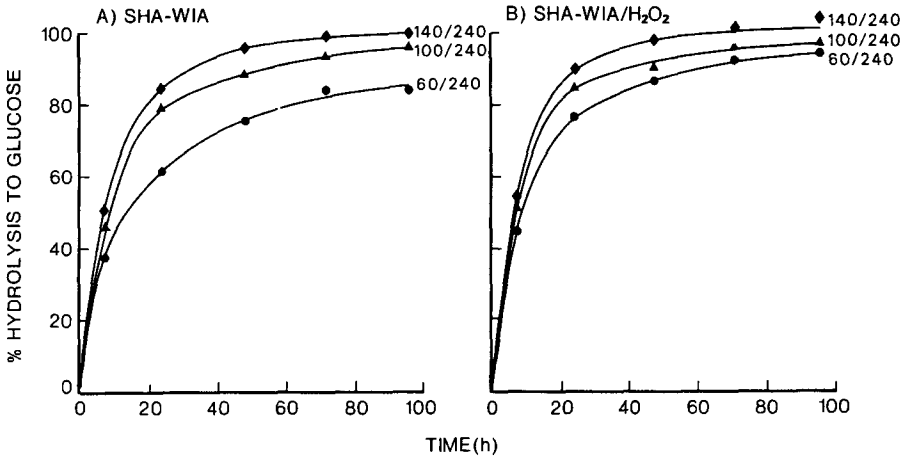

Fig. 3. Enzymatic hydrolysis of A) SHA-WIA and B) SHA-WIA/H_2O_2 using a mixture of Celluclast/Novozym at an enzyme concentration of 17.0 FPU/g substrate; substrates obtained after steam-heating of aspen wood in the 60-L vessel at 240°C for 60, 100, and 140 s.

5 s to 470 psi. Bleed-down of pressure required 30 s and 15 s for the 60 and the 2-L vessel, respectively. The recovery of water solubles from the 60-L vessel was lower than from the 2-L and was reflected in the slightly lower total recovery yield.

Chemical analyses (Table 1) of the water- and alkali-insoluble factions, from the 60-, 100-, and 140-s treatments in the 60-L vessel, were similar to those from the 80-, 120-, and 180-s treatments in the 2-L vessel, respectively. Table 1 also shows that the content of residual alkali-insoluble lignin continued to decrease significantly with increasing steaming time from 60 to 140 s in the 60-L vessel. In contrast, the corresponding decrease in the already low xylan content of 1.6% was negligible after 60 s.

We found that the rate and extent of enzymatic hydrolysis, of these same alkali-washed products of Table 1, increased with increased time of steaming in the 60-L vessel (Fig. 3a). Similar increases have also been ob-

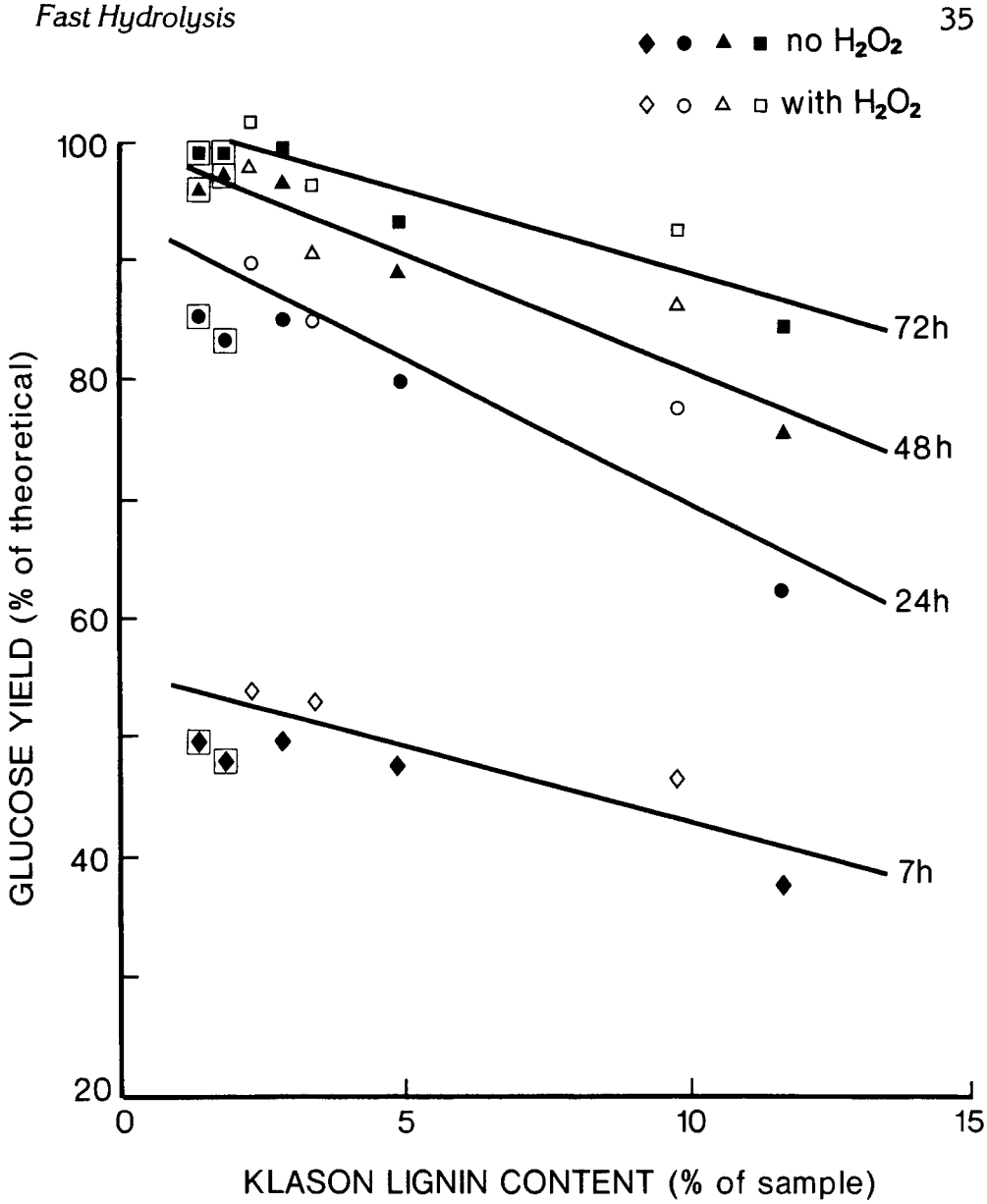

Fig. 4. Correlation between saccharification yield and Klason lignin content after 7, 24, 48, and 72 h of enzymatic hydrolysis; ◆ ● ▲ ■ SHA-WIA, ◇ ○ △ □ SHA-WIA/H_2O_2, ◈ ◉ ◭ ▣ SO_2-SHA-WIA.

tained with the 2-L vessel. Posttreatment of the alkali-washed products of Table 1, with H_2O_2, further reduced the lignin contents but did not reduce the already low xylan contents. The resulting improvement in the hydrolysis curves is shown in Fig. 3b.

The relationship between enzymatic hydrolysis of water- and-alkali-washed steamed aspen wood, and the residual contents of alkali-insoluble lignin, is shown in Fig. 4. Approximately straight lines, of glucose yield vs alkali-insoluble-lignin content, were obtained at each hydrolysis time. Furthermore, the data with and without H_2O_2 treatment lay along the

same lines. This relationship strongly indicates that it was the reduction in the amount of alkali-insoluble lignin, regardless of how the reduction was obtained, that increased enzymatic digestibility. The relationship was valid over a range of lignin contents from at least 12–2%. Also included in Fig. 4 are several points from SO_2-catalyzed steam treatments. Although slightly lower enzyme loadings were used, these points also lie close to the same lines.

In a comparable series of steam-treatments with the 2-L vessel and *T. harzianum* enzyme, a wider range of steam-treatment times (20–180 s) was used. This extension resulted in correspondingly wider ranges of residual contents of both alkali-insoluble lignin (from 15.5 to 0.9%), and xylan (from 11.3 to 0.6%). Again, with and without H_2O_2-posttreatment, a similar series of straight lines was obtained. In contrast, when the glucose yields were plotted against the content of residual xylan, instead of against residual lignin, the resulting curves, after H_2O_2-treatment, lie well above those without H_2O_2-treatment (Fig. 5). The H_2O_2 curves are also almost horizontal over much of the xylan range showing virtually no effect of xylan content. They then rise steeply between 1.7 and 0.6% xylan, where the accompanying removal of a substantial amount of lignin was apparently the cause.

It is concluded that, for water- and-alkali-washed steam-treated aspen wood, over the range of residual lignin contents from at least 15–0.9%, and of residual xylan contents from 11 to 0.6%, it is the alkali-insoluble lignin content and not the xylan content that governs accessibility. It is important to recognize that it is not the total lignin content that influences the enzymatic hydrolysis. It is only the alkali-insoluble fraction, which presumably is more closely associated in the cell wall and restricts swelling. Accordingly, removal of the lignin fraction that is soluble in dilute alkali does not alter enzymatic hydrolysis of water-washed steam-treated aspenwood, provided the enzyme loading per gram of cellulose is the same. This conclusion appears to be not entirely in accordance with the observation by Chum and coworkers (*24*). In their study of organosolv pulping of poplars, they found that the higher the residual xylan content, the smaller was the proportion of fast-hydrolyzable glucans and the lower was the ultimate glucan digestibility.

Economic studies (*25*) have shown that substrate concentration has a major impact on process costs. The effect of substrate concentration from 2 to 8%, with an enzyme loading of 17.6 FPU/g was accordingly examined. The substrate used was aspen wood chips, steam-treated in the 60-L vessel for 100 s at 240°C then washed with water and dilute alkali and subsequently treated with H_2O_2. The results presented in Fig. 6, show that although both hydrolysis rate and yield decreased with increasing substrate concentration, high glucose yields were obtained at all concentrations. The analyses of the original substrate, and of the insoluble residues remaining after saccharification at 5% and 7% concentration, are listed in Table 2. These analysis are in agreement with the high yields and provide a mass

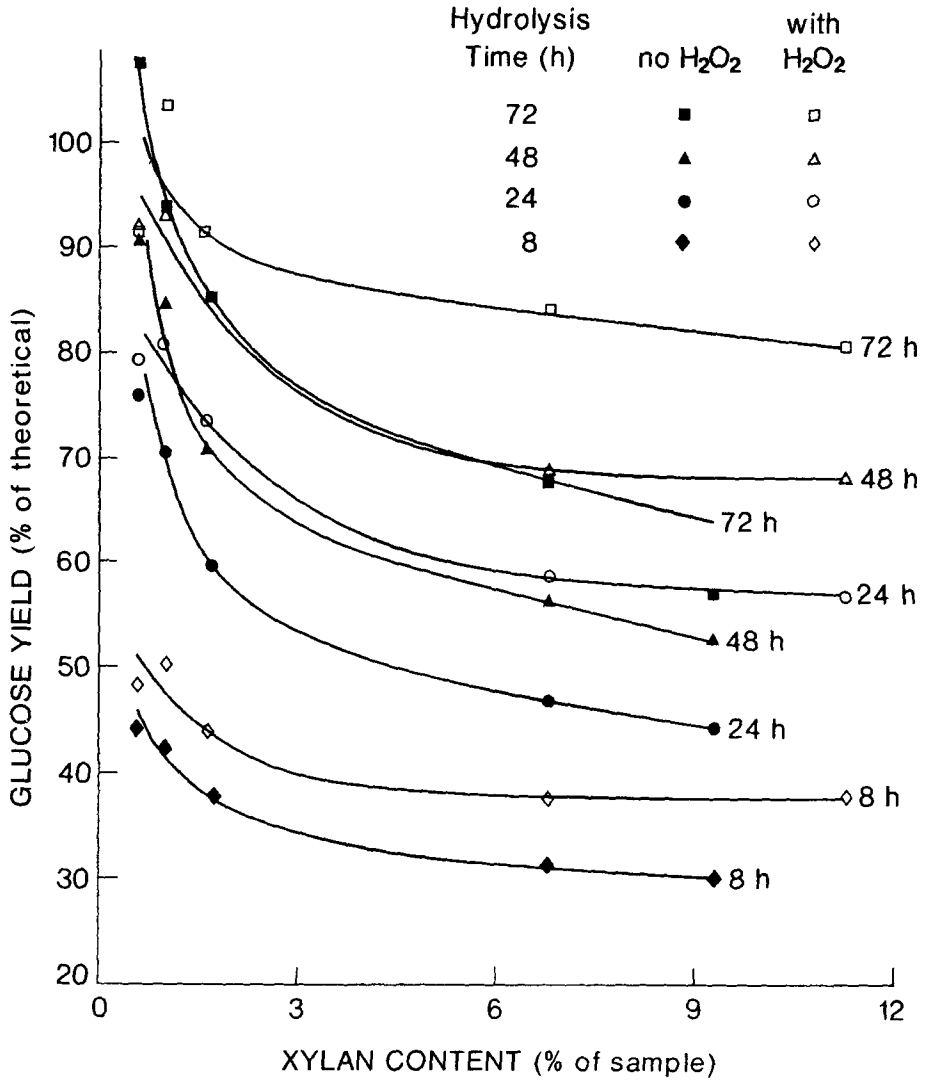

Fig. 5. Correlation between saccharification yield and Xylan content after 7, 24, 48, and 72 h of enzymatic hydrolysis; ◆ ● ▲ ■ SHA-WIA, ◇ ○ △ □ SHA-WIA/H_2O_2.

balance. Only 3.1% and 4.9%, respectively, of the original cellulose remained in the residues from hydrolyses at these two substrate concentrations.

Although steam-treated aspen wood could be enzymatically hydrolyzed at high substrate concentrations in high yields, considerable decomposition of the hemicellulose-derived products resulted from the relatively long steaming time. Mackie et al. (*26*) have shown that impregnation of aspen wood chips with SO_2, prior to steam-treatment, catalyzes hydrolysis while reducing the relative extent of decomposition of the hemicellulose-derived sugars. The effect of steam temperature, during SO_2-catalyzed treatments, was, therefore, examined on recovery yield, fractionation, chemical composition, and enzymatic hydrolysis.

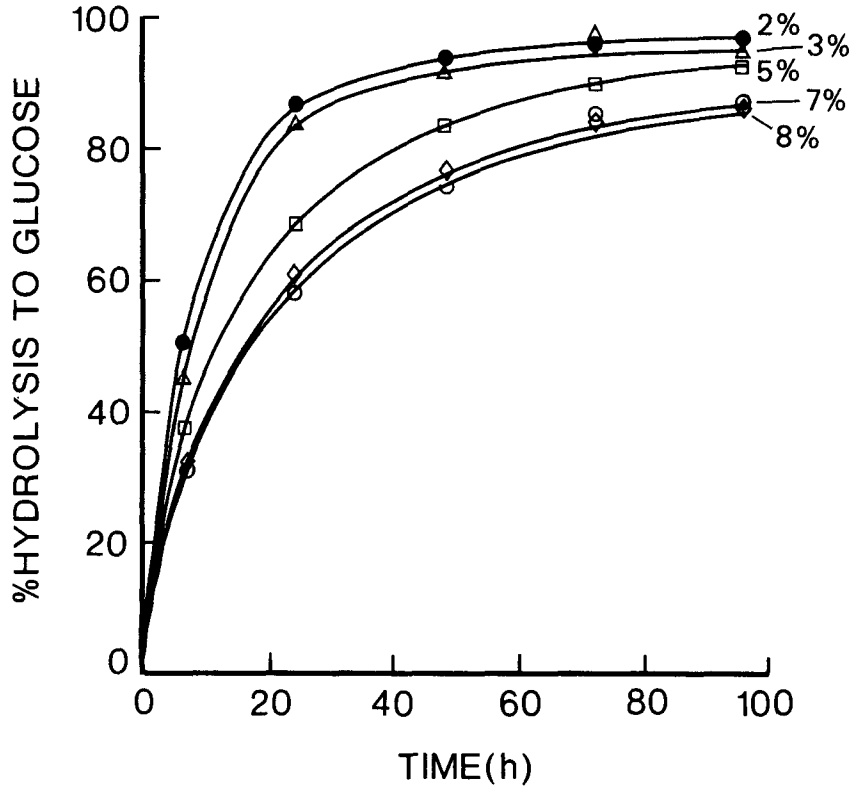

Fig. 6. Effect of concentration of substrate (SHA-WIA/H_2O_2) on enzymatic hydrolysis with a mixture of Celluclast/Novozym at an enzyme concentration of 17.6 FPU/g substrate.

Table 3 shows that more than 95% of the original wood could be recovered after treatment at 190 and 200°C, with more than 70% of the xylan recovered as monomeric xylose. Subsequent enzymatic hydrolysis of the water-insoluble and of the dilute-alkali-insoluble fractions, resulted in little difference in the rate and extent of hydrolysis, when an enzyme loading of 18.6 FPU/g of total substrate was used. When a range of lower enzyme loadings from 6.0 to 15.1 FPU/g of total substrate was used, the glucose yields from the alkali-insoluble fractions were lower than those from the corresponding water-insoluble fractions. Subsequent treatment with hydrogen peroxide then raised the yields again (Fig. 7).

The chemical compositions of these three substrates are listed in Table 4, together with the actual enzyme loadings in FPU/g of total substrate, and with the corresponding calculated values in FPU/g of cellulose. When the actual glucose yields obtained after each time of hydrolysis were plotted as functions of enzyme loading, the calculated glucose yields listed in Table 5 were read from the resulting smooth curves, in order to obtain comparisons at equal FPU/g of cellulose. Table 5 accordingly shows that the water-insoluble fraction and the alkali-insoluble fraction gave similar calculated hydrolysis-yield profiles at each of 16.3, 13.3, and 10.2 FPU/g

Table 2
Chemical Composition of SHA-WIA/H_2O_2[a] and Analysis of the Insoluble Residues Isolated from Enzymatic Hydrolysates after Hydrolysis of SHA-WIA/H_2O_2 at High Substrate Concentration

Slurry concentration		Weight, g	Composition in g			Cellulose hydrolyzed after 96 h, g
			KL	ASL	Cell	
5	Before hydrolysis	10.00	0.35	0.05	9.36	—
	After hydrolysis, 96 h	0.83	0.43	0.01	0.29	8.66
7	Before hydrolysis	14.00	0.50	0.07	13.11	—
	After hydrolysis, 96 h	1.56	0.69	0.02	0.64	11.36

[a] Water-and-alkali-insoluble fraction of aspen wood after steam heating for 100 s at 240°C, posttreated with H_2O_2.

Table 3
Recovery Yields and Composition of Water-Soluble Fractions of SO_2-Impregnated[a] Aspen Wood after Steam Pretreatment for 100 s at Various Temperatures

Reaction temperature, °C	Percent of O.D. orig. wood						
	RY[b]	SO_2-SHA-WI	SO_2-SHA-WIA	WS	Xylose[c]	Glucose[c]	Acetyl[c]
190	96.4	63.0	46.4	33.4	15.0	1.8	2.8
200	95.2	62.3	41.8	32.9	14.2	3.8	2.8
210	89.5	56.9	35.3	32.6	11.8	7.4	3.0
220	86.4	49.9	26.2	36.6	7.6	12.9	3.2

[a] SO_2-impregnation: 1.6% SO_2 (oven-dry wood basis).
[b] RY = recovery yield consisting of a water-soluble fraction (WS) and a water-insoluble fraction (SHA-WI).
[c] Obtained by HPLC-analysis of the water-soluble fractions.

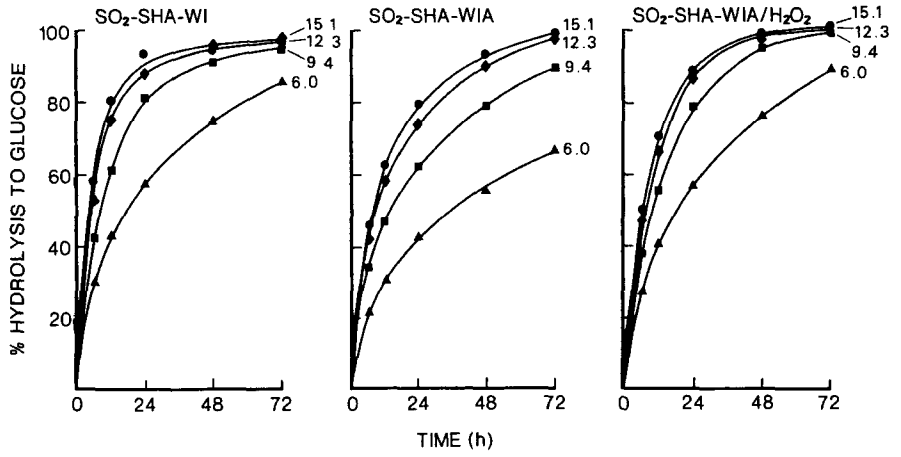

Fig. 7. Comparison of effect of enzyme concentration, when expressed as FPU/g of total substrate, on enzymatic hydrolysis of SO_2-SHA-WI, SO_2-SHA-WIA, and SO_2-SHA-WIA/H_2O_2.

Table 4
Chemical Composition of Fractions of Steam Pretreated Aspen Wood,[a] and Comparison of Filter Paper Activities (FPU) Expressed/g of Total Substrate and /g of Cellulose

				FPU/g of substrate			
	Composition in Percent			15.1	12.3	9.4	6.0
Substrate	TAL	Cell	Xylan	FPU/g of cellulose			
SO_2-SHA-WI	30.3	68.3	0.64	22.1	18.0	13.8	8.8
SO_2-SHA-WIA	5.4	92.4	0.67	16.3	13.3	10.2	6.5
SO_2-SHA-WIA/H_2O_2	2.2	94.6	0.73	16.0	13.0	9.9	6.3

[a]Water-washed, alkali-washed and H_2O_2-posttreated fractions of aspen wood were obtained after steam heating impregnated chips (1.6% SO_2 on an O.D. wood basis) for 120 s at 210°C.

of cellulose. Table 5 also shows that, on this same basis of comparison, H_2O_2 treatment significantly improved the hydrolysis, especially at the lower enzyme loadings.

The effect of higher concentrations of the insoluble fractions from SO_2-impregnated, steam-heated aspen wood on enzymatic hydrolysis with a relatively low enzyme concentration of 10.0 FPU/g cellulose is shown in Fig. 8. It can be seen that the rate and completeness of the reaction for SO_2-SHA-WI and SO_2-SHA-WIA substrates at a concentration of 2% are virtually identical. This could be expected, since the filter paper activity was based on the cellulose content, and the results are in agreement with those shown in Table 5. It also confirms that high saccharification yields

Table 5
Effect of Alkali-Washing (SO_2-SHA-WIA), and of Alkali-Washing Followed by H_2O_2-Treatment (SO_2-SHA-WI/H_2O_2) of the Water-Insoluble Fraction (SO_2-SHA-WI) of SO_2-Catalyzed[a] Steam-Heated[b] Aspen Wood on the Enzymatic Hydrolysis at Constant Enzyme Concentration, Expressed/g Cellulose

Substrate	FPU/g of cellulose	Saccharification yield, % of theoretical				
		6 h	12 h	24 h	48 h	72 h
SO_2-SHA-WI	16.3	49.5	71.3	86.4	94.0	97.2
	13.3	41.8	59.7	80.2	90.1	95.4
	10.2	33.7	48.0	66.0	81.4	89.7
SO_2-SHA-WIA	16.3	46.0	63.1	80.0	93.3	99.4
	13.3	42.5	58.1	74.7	90.0	98.8
	10.2	34.1	46.8	62.6	78.6	89.4
	6.5	21.9	31.0	42.8	55.7	66.8
SO_2-SHA-WIA/H_2O_2	16.3	50.3	71.0	89.2	98.7	101.6
	13.3	47.9	67.3	88.1	98.5	101.7
	10.2	39.2	56.3	80.0	95.2	101.2
	6.5	27.7	41.1	57.2	77.0	90.4

[a]SO_2-impregnation: 1.6% SO_2 (oven-dry wood basis).
[b]Steam treatment conditions: 120 s at 210°C.

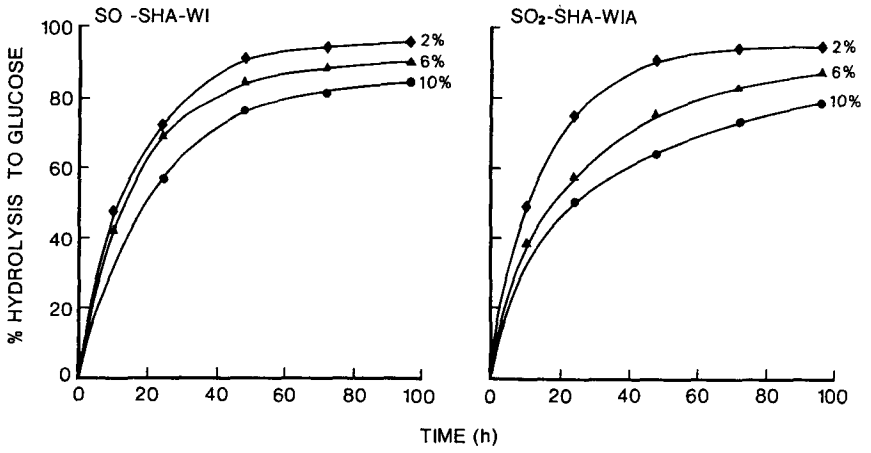

Fig. 8. Effect of concentration of substrate (SO_2-SHA-WI and SO_2-SHA-WIA) on enzymatic hydrolysis using a mixture of Celluclast/Novozym at an enzyme concentration of 10.0 FPU/g of cellulose.

can be achieved when the alkali-insoluble lignin content (i.e., 2.2% of the substrate) is low. However, when the substrate concentration was increased to 6 and 10%, both the rate and completeness of hydrolysis of the water- and alkali-extracted substrate decreased. Although this would seem to add weight to the theory that the lignin is redistributed during alkali extraction, it is more likely that the observed drop in saccharification yield can be attributed to the different cellulose content of the water-insoluble (64.1%) and the water- and alkali-insoluble (94.7%) substrates. As a result, the glucose concentrations in the batch hydrolysis of SO_2-SHA-WIA are substantially higher. Whereas the final glucose concentration after a 96 h hydrolysis of the water-insoluble fraction was 60.6 mg/mL, the glucose concentration after hydrolysis of the water- and alkali-insoluble fraction was 82.3 mg/mL. It is probable that the factor responsible for decreasing conversion yields at higher substrate concentration is increasing endproduct inhibition, rather than inaccessibility of the substrate to the enzyme complex.

It is concluded that under any feasible conditions of steam-treatment of aspen wood, which solubilize one-half or more of the original xylan content, the accessibility of the cellulose is governed primarily by the residual content of alkali-insoluble lignin. Prolonged steaming, SO_2 catalysis, and oxidative post-treatments improve accessibility by reducing the content of this lignin fraction. Scaleup of vessel size from 2 to 60-L had no significant effect on chemical composition or on enzymatic hydrolysis. Hydrolysis yields well above 90% were obtained at substrate concentrations of up to 5%. With SO_2 catalysis, recovery yields of more than 95% of the wood were obtained, with more than 70% of the original xylan recovered as monomeric xylose. Saccharification yields above 90% of theoretical were obtained within 48 h, when a filter paper loading of 10 FPU/g of cellulose was used. Alkali extraction of soluble lignin had no significant effect on subsequent hydrolysis at equal enzyme loading/g of cellulose.

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