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Strategies to Enhance the Enzymatic Hydrolysis of Pretreated Softwood with High Residual Lignin Content

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Abstract

Pretreatment of Douglas-fir by steam explosion produces a substrate containing approx 43% lignin. Two strategies were investigated for reducing the effect of this residual lignin on enzymatic hydrolysis of cellulose: mild alkali extraction and protein addition. Extraction with cold 1% NaOH reduced the lignin content by only approx 7%, but cellulose to glucose conversion was enhanced by about 30%. Before alkali extraction, addition of exogenous protein resulted in a significant improvement in cellulose hydrolysis, but this protein effect was substantially diminished after alkali treatment. Lignin appears to reduce cellulose hydrolysis by two distinct mechanisms: by forming a physical barrier that prevents enzyme access and by non-productively binding cellulolytic enzymes. Cold alkali appears to selectively remove a fraction of lignin from steam-exploded Douglas-fir with high affinity for protein. Corresponding data for mixed softwood pretreated by organosolv extraction indicates that the relative importance of the two mechanisms by which residual lignin affects hydrolysis is different according to the pre- and post-treatment method used.

Index Entries: Softwood; bioconversion; pretreatment; steam explosion; lignin; cellulose; hydrolysis.

Introduction

Softwoods are the dominant lignocellulosic feedstocks for bioconversion in the Northern hemisphere and are viewed as a potential source of fermentable carbohydrate for fuel-ethanol production in Canada, Sweden, and the US *(1,2)*. In British Columbia, an estimated 2.2 million tonnes of surplus wood residues are generated per year *(3)*. Conversion of these residues into fuel ethanol and other commodity chemicals provides an attractive opportunity for more sustainable development of forest resources.

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A primary technoeconomic challenge in all lignocellulose-to-ethanol bioconversion processes is overcoming the recalcitrance of the cellulose fraction to enzymatic hydrolysis. Cellulose is intrinsically resistant to enzyme attack and is further protected by the surrounding matrix of lignin and hemicellulose in lignocellulose. Softwood lignocellulose is considered particularly recalcitrant for hydrolysis owing to the amount and nature of the lignin component.

Steam explosion is one of the most intensively studied pretreatment methods for bioconversion of softwood materials *(1,4–7)*. The steam explosion process uses high temperature and pressure, followed by sudden release, to separate individual fibers within the wood. Meanwhile, most of hemicellulose is hydrolyzed and removed during steam explosion, contributing to the improved susceptibility of the substrate. However, the steam-explosion process does not result in net delignification: the lignin content after steam explosion is actually increased due to the removal of hemicellulose. For example, the lignin content of steam-exploded Douglas-fir (SEDF) is typically >40%, compared to approx 30% for untreated Douglas fir. Softwood lignin also undergoes condensation reactions during steam explosion, which further complicate removal.

Our previous work indicates that enzymatic digestibility of SEDF can be significantly enhanced by alkaline peroxide *(8)* or alkali-oxygen post-treatment *(9)*. However, the high chemical consumption involved in these processes makes them uneconomic for industrial use. In addition, the extracted lignin has little value as a co-product, while post-treatment introduces the additional problem of waste stream management.

The objective of the present research was to examine new approaches to enhance the enzymatic hydrolysis of SEDF without costly delignification. Two strategies are described below. Although industrial-scale process economics remain to be evaluated, the results of this study provide useful insights into the effects of residual lignin in bioconversion processes.

Experimental

Materials

Steam-exploded Douglas-fir (*Pseudotsuga menziesii*) (SEDF) was prepared as described below. Douglas-fir wood was chipped and screened to a relatively homogeneous chip size of approx 3 × 3 × 0.3 cm. The chips were initially impregnated with 4.5% (w/w) of gaseous SO_2 and then steam-exploded in a 1-L steam gun in batches of 50 g dry chip at 195ºC for 4.5 min. Batches were combined, washed, and defibrillated on a refiner to produce a homogeneous feedstock.

Ethanol organosolv pulps (EP) were prepared from mixed softwood (spruce/pine/fir) at a pilot plant operated by Lignol Innovations, Inc.

(Vancouver, Canada) using 50% (v/v) aqueous ethanol with sulfuric acid as catalyst *(10)*.

Enzyme preparations, Celluclast (cellulase) and Novozym 188 (β-glucosidase), were provided by Novo Nordisk. Peptone and bovine serum albumin (BSA) were from Sigma-Aldrich.

Dilute Alkali Extractions of Steam-Exploded Douglas-Fir Wood

Cold 1% NaOH extraction: A SEDF sample (equivalent to 20 g oven dry weight) was stirred with 800 mL of 1% NaOH at room temperature (approx 20ºC) for 72 h. Extracted SEDF was washed extensively with water and stored briefly at 4ºC before analysis.

Hot 1% NaOH extraction: A SEDF sample (equivalent to 20 g oven dry weight) was stirred with 800 mL of 1% NaOH at 100ºC for 7 h, then washed and stored as described above.

Alkali-Oxygen Post-treatment

SEDF (30 g/batch) was stirred in a solution of NaOH under oxygen at 0.5 MPa (see Table 3 for further details of process parameters). Magnesium sulfate (0.5% by weight of SEDF) was added to prevent cellulose degradation during oxidation. Post-treated SEDF was washed extensively with water and stored briefly at 4ºC before analysis *(9)*.

Analytical Procedures

Total cellulase activity was measured using the filter paper assay as recommended by IUPAC *(11)*. β-Glucosidase activity was measured using *p*-nitrophenyl-β-D-glucoside as substrate according to Wood and Bhat *(12)*.

Sugars in hydrolyzates were analyzed by HPLC using a Dionex DX-500 system fitted with a Dionex CarboPac™ PA1 anion exchange column and ED40 electrochemical detector. Sugars were eluted with de-ionized water at a flow rate of 1 mL/min.

Klason lignin was determined according to the TAPPI standard method T-222. The hydrolysate from the Klason lignin determination was collected and further analyzed for sugars and acid-soluble lignin. Sugars were determined as described above except that sugar standards were autoclaved in 3% H_2SO_4 at 120ºC for 1 h to correct for sugar loss during sample preparation. Acid-soluble lignin was determined by UV adsorption, as previously described *(13)*.

The number average and weight average molecular weights (M_n and M_w, respectively) of lignin samples were estimated by gel permeation chromatography (GPC), as previously described *(10)*. Phenolic hydroxyl groups were estimated by periodate oxidation *(14)*.

Enzymatic Hydrolysis

All enzymatic hydrolysis experiments were conducted with a combination of cellulase and β-glucosidase at a ratio of 1 FPU:2 CBU (filter paper units and cellobiase units, respectively). Batch hydrolysis was conducted at 2% cellulose consistency in 50 m*M* acetate buffer, pH 4.8, containing 0.004% tetracycline. Reaction mixtures were incubated at 45ºC on a rotary shaker at 200 rpm and sampled periodically for sugar analysis. Each data point is the mean of two replicates.

To examine the effect of exogenous protein on hydrolysis, substrates were preincubated at 2% cellulose consistency in 50 m*M* acetate buffer containing peptone or bovine serum album (BSA) for 16 h, 45ºC, 200 rpm, prior to enzyme addition.

Results and Discussion

Dilute Alkali Extraction

Although dilute alkali extraction has been used extensively for removing lignin from steam-exploded hardwood and agricultural residues prior to enzymatic hydrolysis *(15–17)*, it is ineffective for the delignification of steam-exploded softwood. Generally, softwoods are more highly lignified than hardwoods and agricultural residues, and their higher content of guaicyl units promotes condensation during pretreatment *(18)*. The objective of the present study was to examine the effect of limited delignification of SEDF by dilute alkali on cellulose hydrolysis to further our understanding of the mechanism by which lignin interferes with enzyme action.

The composition of SEDF, before and after dilute alkali extraction at room temperature or 100ºC, is shown in Tables 1 and 2. Cold alkali extraction removed approx 30% of the residual lignin in SEDF. This was accompanied by removal of approx 5% of the cellulose (based on glucose content). However, part of the glucose removal can be accounted for by loss of hemicellulose. The net result of cold alkali extraction was a decrease in lignin content by 7% and an increase in cellulose content by 8% (Table 1). In contrast, hot alkali extraction removed approx 34% of the residual lignin and 41% of the cellulose (based on glucose content) (Table 2). The net result of hot alkali extraction was an increase in lignin content by 3% and a decrease in cellulose content by 2% (Table 1). The total loss of solids was approx 15% for cold alkali and approx 39% for hot alkali (Table 2).

The enzymatic hydrolysis of SEDF, before and after cold-alkali or hot-alkali extraction, is shown in Fig. 1. Cold-alkali extraction resulted in a significant enhancement of hydrolysis, despite a reduction in total lignin content of only approx 7%. The percentage cellulose-to-glucose conversion after 72-h incubation was increased from 32% to 62% at an enzyme loading of 20 FPU/g cellulose, and from 55% to 85% at a loading of 40 FPU/g. Hydrolysis rates were markedly improved by cold-alkali extraction, especially during the early stages of the reaction (Fig. 2). Complete hydrolysis (100% conversion) could be achieved when incubation was extended to 7 d

Table 1
Percentage Weight Composition of SEDF Before and After Extraction with Cold or Hot Alkali

	Percentage dry weight		
		Alkali extraction	
Component	SEDF	Cold NaOH	Hot NaOH
Total lignin	43.2	35.5	46.4
Klason lignin	42.2	35.0	45.9
Acid-soluble lignin	1.0	0.5	0.5
Carbohydrate	63.8	69.5	59.7
Arabinose	ND	ND	ND
Galactose	0.6	ND	ND
Glucose	61.1	68.6	58.7
Xylose	0.5	0.2	0.4
Mannose	1.6	0.7	0.6

ND, not detected.

Table 2
Weight Composition of SEDF Before and After Extraction with Cold or Hot Alkali

	Dry weight (g); data in parentheses show percentage removal		
Composition	SEDF	Cold NaOH	Hot NaOH
Total solids	19.76	16.79 (15.0)	12.13 (38.6)
Total lignin	8.53	5.96 (30.1)	5.63 (34.0)
Klason lignin	8.33	5.88 (29.5)	5.57 (33.2)
Acid-soluble lignin	0.20	0.08 (57.5)	0.06 (69.3)
Carbohydrate	12.61	11.67 (7.4)	7.24 (42.5)
Arabinose	0.00	0.00 (0.0)	0.00 (0.0)
Galactose	0.12	0.00 (100.0)	0.00 (100.0)
Glucose	12.07	11.52 (4.6)	7.12 (41.0)
Xylose	0.10	0.03 (66.0)	0.05 (50.9)
Mannose	0.32	0.12 (62.8)	0.07 (77.0)

(data not shown). Hydrolysis of hot-alkali extracted SEDF at a loading of 40 FPU/g was similar to that seen for the cold-alkali extracted material under the same conditions: a small enhancement of the initial rate was apparent but the percentage conversion after 72 h was slightly reduced.

We have previously demonstrated that alkali-oxygen post-treatment is an effective method to improve the hydrolysis of SEDF *(9)*. Therefore,

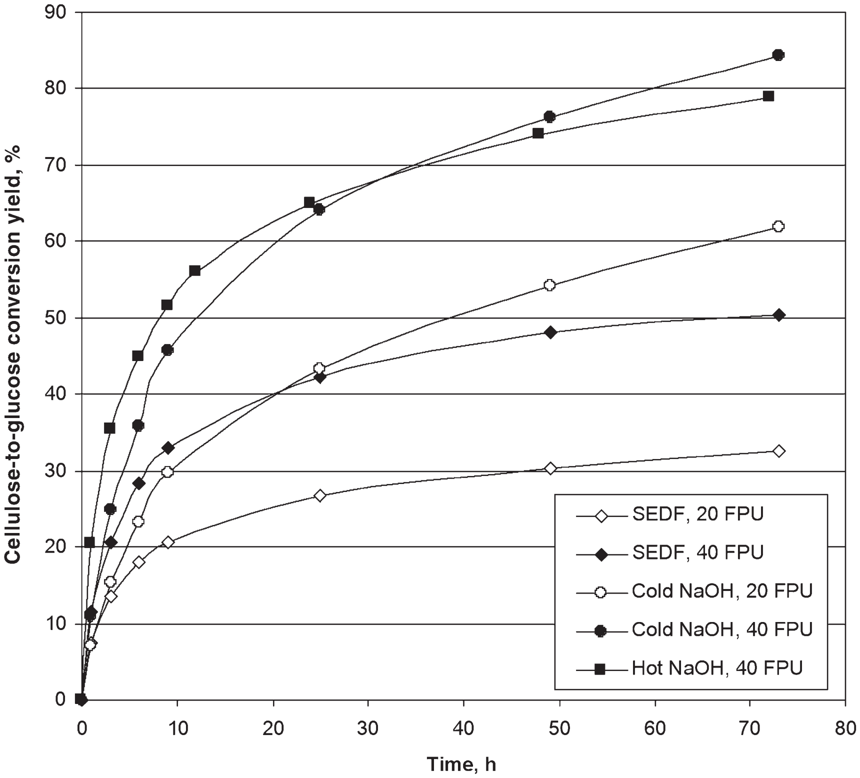

Fig 1. Enzymatic hydrolysis of alkali-extracted SEDF.

we also compared effects of cold alkali extraction and alkali-oxygen post-treatment on hydrolysis, as summarized in Table 3. The overall efficiency of delignification is expressed as the ratio of the increase in percentage conversion to the decrease in lignin content at 48 h (Δ conversion/Δ lignin). Cold NaOH extraction increased cellulose conversion by approx 24% and decreased lignin content by approx 8% (efficiency of delignification = 3.1). Oxygen-alkali removed considerably more lignin (approx 28 and 31%, depending on the temperature of the post-treatment; Table 3) and increased cellulose conversion by approx 71 and 90%, respectively. However, the efficiencies of delignification (Δ conversion/Δ lignin) for oxygen delignification (1.4 and 1.9, respectively) were significantly lower than that for cold alkali extraction (3.1).

These results suggest that cold alkali selectively removes a fraction of the total lignin that exerts a particularly strong effect on enzymatic hydrolysis, although other effects of cold alkali, such as disruption of cellulose structure, cannot be discounted at this time. It is possible that the cold-alkali-extracted fraction corresponds to material that is largely responsible for presenting a physical barrier in SEDF that restricts enzyme access to the cellulose component. Alternatively, this fraction may have a particularly high affinity for protein, resulting in non-productive binding of enzymes

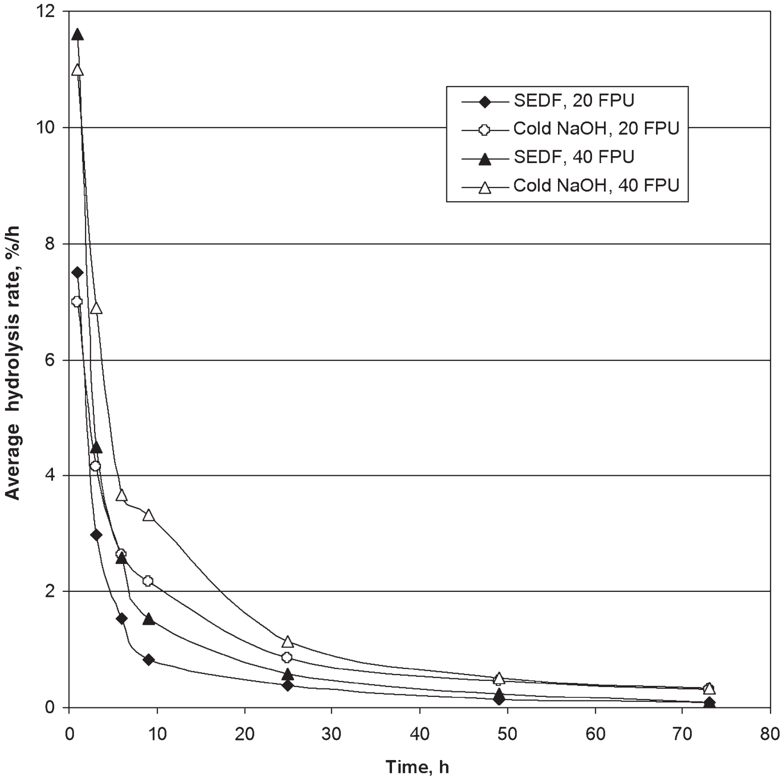

Fig. 2. Effect of cold NaOH extraction on average hydrolysis rate (cellulose-to-glucose conversion/incubation time, %/h) of SEDF.

Table 3
Effect of Lignin Removal on Enzymatic Hydrolysis of SEDF

Delignification method	Lignin content (%)	Δ Lignin (%)	48 h cellulose-to-glucose conversion[a] (%)	Δ Conversion (%)	Δ Conversion/ Δ Lignin
SEDF	43.2		30.4		
Cold NaOH	35.5	7.7	54.2	23.8	3.1
Oxygen-alkali (O-5)[b]	14.8	28.4	71.2	40.8	1.4
Oxygen-alkali (O-4)[b]	12.0	31.2	90.4	60.0	1.9

[a] Enzyme loading: 20 FPU/g cellulose.

[b] Oxygen delignification conditions *(9)* were as follows: O-5: 15 g NaOH/100g SEDF; 5% SEDF consistency; 100°C: 3 h; O-4: 15 g NaOH/100 g SEDF; 5% SEDF consistency; 110°C: 3 h.

in the cellulase complex. Preliminary data indicate that the lignin extracted by cold alkali has a low molecular weight (M_n = 1160; M_w = 1200) and a high content of phenolic hydroxyl groups (approx 1.3 mmol/g lignin). To evaluate whether this fraction has a high affinity for cellulases, we

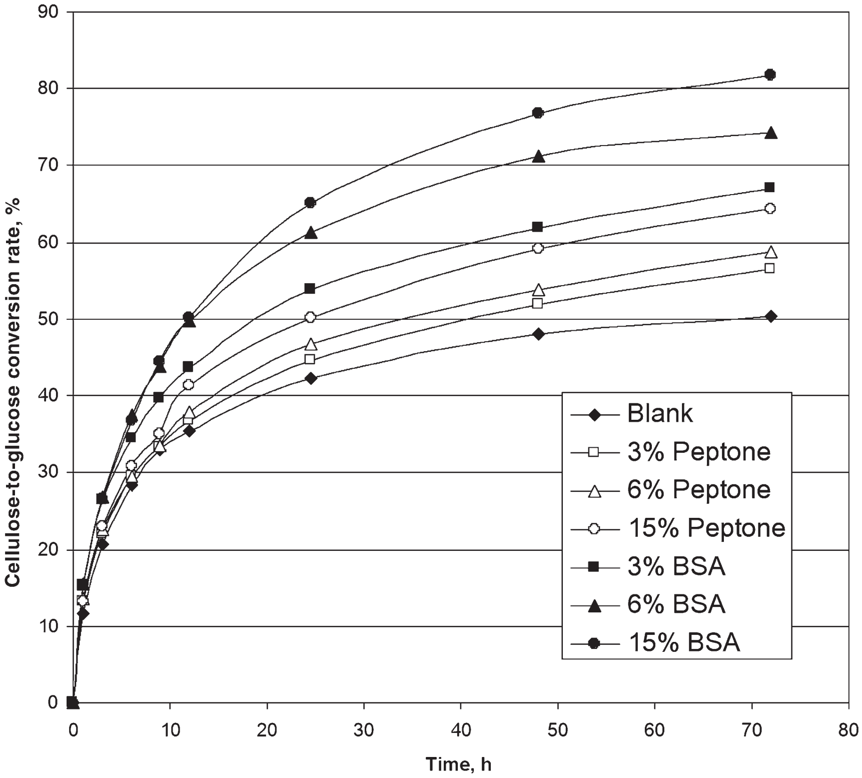

Fig. 3. Effect of exogenous protein on enzymatic hydrolysis of SEDF.

examined the effect of exogenous protein addition on enzymatic hydrolysis, as described below.

Effect of Exogenous Protein on Cellulose Hydrolysis

It has been shown previously that the negative impact of purified lignin on the enzymatic hydrolysis of filter paper is counteracted by addition of exogenous protein (ovalbumin or gelatin). Presumably, these proteins bind to sites on lignin and reduce their interaction with cellulolytic enzymes. Polyvinylpyrrolidene and polyethylene glycol, agents with high affinities for phenolic groups, exert a similar effect *(19)*. Consequently, we examined the effect of exogenous proteins on the hydrolysis of SEDF. Addition of 3–15% BSA had a dramatic effect on the enzymatic hydrolysis of SEDF, increasing cellulose conversion by up to 30%; the effect of peptone was similar but less pronounced (Fig. 3). These results indicate that enzyme–lignin interactions, in addition to the physical barrier imposed by the lignin matrix, play a critical role in reducing enzyme efficiency. In contrast to the effect seen with SEDF, hydrolysis of cold-alkali-extracted SEDF was only marginally improved by protein addition; a similar result was seen for hot-alkali-extracted SEDF (Fig. 4). These results provide additional support for the hypothesis that cold

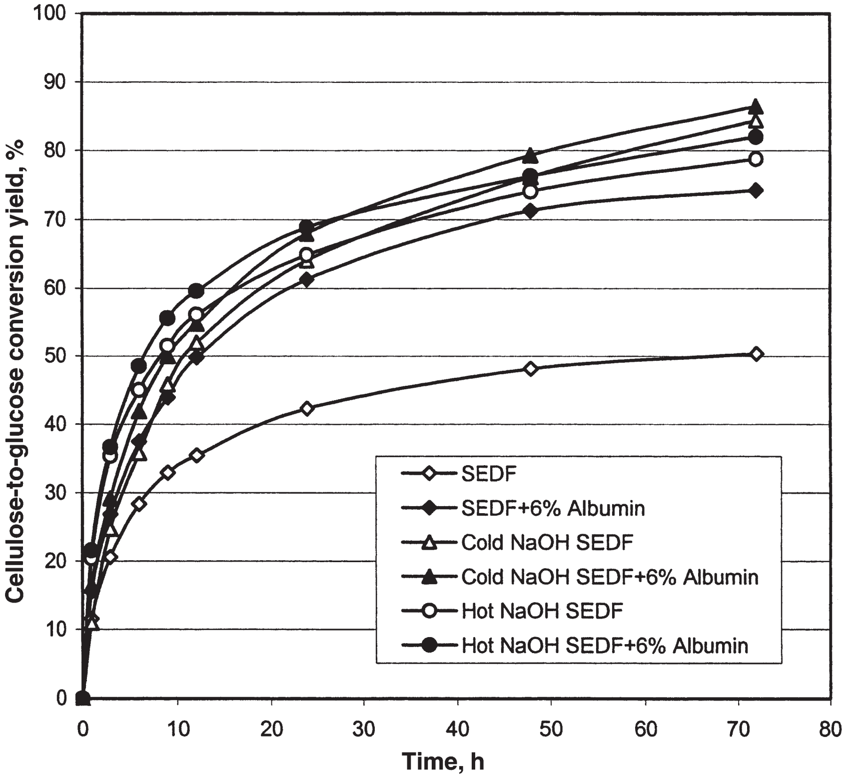

Fig. 4. Effect of exogenous proteins on enzymatic hydrolysis of alkali-extracted SEDF.

alkali extraction selectively removes a lignin fraction with particularly high affinity for cellulolytic enzymes.

To investigate this phenomenon further, we examined the effect of BSA addition on two mixed softwood samples (EP-A and EP-B) with different Klason lignin content, prepared by ethanol organosolv pretreatment (Fig. 5). Ethanol organosolv is a very effective pretreatment for softwood, resulting in a high degree of cellulose conversion. Addition of 6% BSA significantly enhanced the initial hydrolysis rate of EP-A (17.9% lignin); however, by 72 h, ≥97% cellulose conversion was achieved with or without exogenous protein. More dramatic effects were seen for EP-B (27% lignin): here, BSA greatly enhanced the initial hydrolysis rate and improved the percentage cellulose conversion by 10%.

These results indicate that the effects of residual lignin on cellulose hydrolysis differ considerably depending on the pretreatment regime. The lignin remaining after steam-explosion pretreatment plus cold-alkali extraction appears to reduce hydrolysis predominantly by forming a barrier to enzyme attack. Enzyme–lignin interactions, which appear to be significant before alkali extraction, are less critical because exogenous protein has only a relatively small effect on hydrolysis after alkali extraction. The high degree

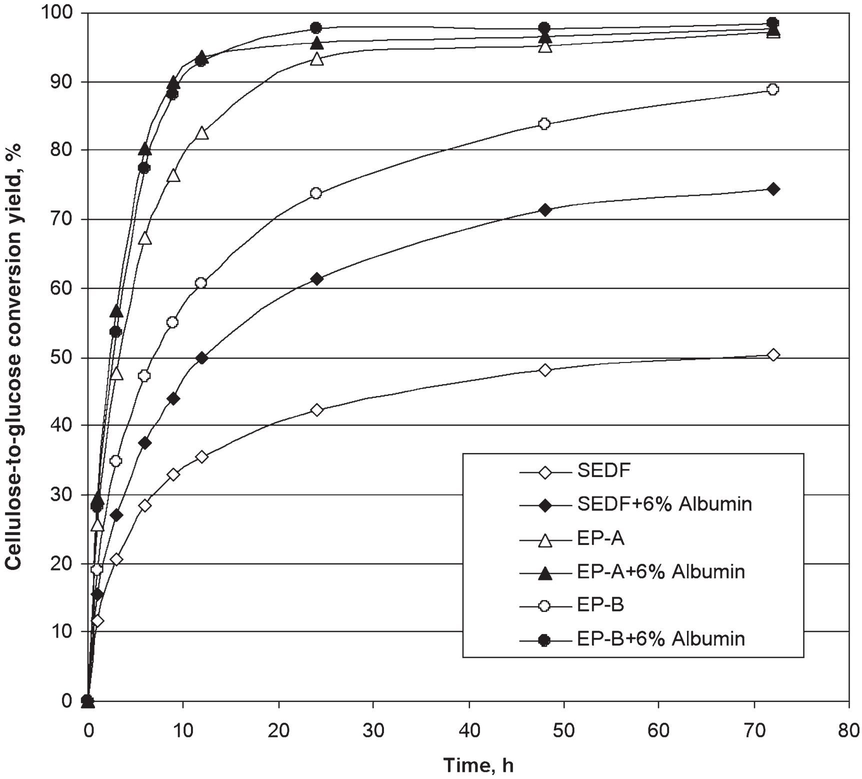

Fig. 5. Effect of exogenous protein on enzymatic hydrolysis on SEDF and ethanol organosolv-pretreated mixed softwood.

of cellulose conversion achieved with ethanol organosolv pretreated softwood indicates that this process effectively reduces the barrier to enzyme attack. However, the improvement in hydrolysis rates seen after exogenous protein addition suggests that enzyme–lignin interactions continue to exert an inhibitory effect, even when the residual lignin content is reduced below 20%.

Conclusion

Data presented above support the hypothesis that the lignin remaining after pretreatment of softwood influences subsequent enzymatic hydrolysis of cellulose by two distinct mechanisms. Some lignin components appear to act predominantly by forming a physical barrier that impedes or prevents attack by cellulases; others by binding cellulases non-productively. The relative importance of these mechanisms appears to differ, depending on the pre- and post-treatment regime used to prepare material for hydrolysis.

The applicability of alkali extraction and protein addition steps to industrial bioconversion processes will require further technoeconomic analysis. However, better understanding of enzyme–lignin interactions should allow pretreatment regimes to be optimized on a more rational basis. In the longer term, engineering of cellulases with reduced affinity for lignin may provide an effective strategy for process improvement.

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