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Enhanced Enzymatic Hydrolysis of Steam-Exploded Douglas Fir Wood by Alkali-Oxygen Post-treatment

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Abstract

Good enzymatic hydrolysis of steam-exploded Douglas fir wood (SEDW) cannot be achieved owing to the very high lignin content (>40%) that remains associated with this substrate. Thus, in this study, we investigated the use of alkali-oxygen treatment as a posttreatment to delignify SEDW and also considered the enzymatic hydrolyzability of the delignified SEDW. The results showed that under optimized conditions of 15% NaOH, 5% consistency, 110°C, and 3 h, approx 84% of the lignin in SEDW could be removed. The resulting delignified SEDW had good hydrolyzability, and cellulose-to-glucose conversion yields of over 90 and 100% could be achieved within 48 h with 20 and 40 filter paper units/g of cellulose enzyme loadings, respectively. It was also indicated that severe conditions, such as high NaOH concentration and high temperature, should not be utilized in oxygen delignification of SEDW in order to avoid extensive condensation of lignin and significant degradation of cellulose.

Index Entries: Steam explosion; Douglas fir; oxygen delignification; enzymatic hydrolysis; lignin.

Introduction

During the twentieth century, fossil fuels were the major driving force for economic growth in the world. In particular, a cheap and abundant oil supply has brought North America tremendous economic benefits, a profusion of products, and a high quality of life. However, consumption of fossil fuels on an ever-expanding scale has also led to adverse changes in

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our climate and a dramatic increase in air pollution. Mann at al. *(1)* study has suggested that the increasing concentrations of carbon dioxide and other greenhouse gases in the atmosphere during the past century have played a significant role in global warming. Detrimental compounds found in automobile emissions, such as carbon monoxide, nitrogen oxides, volatile hydrocarbons, sulfur oxides, and benzene, have been identified as the underlying cause of a number of human health problems. Thus, there are environmental and health-related incentives to develop alternative sources of energy to alleviate our dependence on fossil fuels.

The Kyoto protocol had identified the development of an alternative biofuel from biomass as one of several areas deserving of research support, since this type of renewable fuel could help reduce greenhouse gas emissions. The use of bioethanol as a viable motor fuel to replace or augment gasoline is an attractive component of an integrated strategy to reduce the release of detrimental hydrocarbons, carbon monoxide, nitrogen oxide, sulfur dioxide, and aromatics *(2–3)*.

Lignocellulosics are the most abundant renewable organic materials in the biosphere. They account for approx 50% of the total biomass in the world, with an estimated annual production of $1–50 \times 10^9$ t *(4)*. Lignocellulosic materials, particularly the residues obtained from wood processing, are usually much cheaper than sugar- and/or starch-derived feedstock, such as sugarcane and corn. They also have no competitive use as human or animal foodstuffs.

Softwoods are the predominant species of tree in Canada. In British Columbia, an estimated 2.2 million t of surplus wood residues are generated each year *(5)*, which until now have been of limited use as a commercial product. Bioconversion of these residues into biofuel ethanol and valuable chemicals provides an attractive opportunity for the sustainable development of both renewable energy and Canada's forest resources.

A typical wood-to-ethanol bioconversion process consists of at least three major steps: pretreatment, hydrolysis, and fermentation. The pretreatment stage has been shown to be the key step to providing a substrate susceptible to the subsequent hydrolysis. Steam explosion is one of the most intensively studied pretreatment methods for bioconversion of softwood materials *(6–10)*.

The steam explosion process uses high temperature and pressure followed by sudden release to separate individual fibers within the woody substrate. One of the problems with the steam explosion process is that it does not significantly delignify the wood; most of the lignin remains in the resulting cellulosic substrate. This high lignin content is one of the reasons that pretreated substrate continues to be very resistant to enzymatic hydrolysis. Our previous work *(11)* has shown that the enzymatic digestibility of steam-exploded Douglas fir wood (SEDW) can be enhanced with a peroxide posttreatment step. However, the high hydrogen peroxide consumption involved in this process makes it impractical for industrial use. There is, therefore, a need to develop a cost-effective posttreatment method capa-

ble of reducing the lignin content of the SEDW and enhancing its hydrolyzability.

Oxygen delignification has been widely used in the pulp and paper industry to remove lignin in both pulping and bleaching processes *(12)*. A typical commercial oxygen delignification operation is able to remove about 50% of the lignin present in a Kraft pulp without significant impact on pulp yield and strength. There is little information in the existing literature describing the application of oxygen delignification to high-lignin-containing substrates, such as SEDW, and those few studies that were carried out were generally unsuccessful *(13)*. Thus, the aim of the present work was to evaluate the potential of using oxygen delignification as a posttreatment to enhance the susceptibility of SEDW.

Materials and Methods

Preparation of SEDW

Douglas fir (*Pseudotsuga menziesii*) 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) gaseous SO_2 and then steam exploded in a 1-L steam gun in batches of 50 g of dry chips at 195°C for 4.5 min, as previously described by Boussaid et al. *(14)*. The steam-exploded wood samples of each batch were collected, combined, washed, and finally defibrillated on a refiner to produce a homogeneous feedstock.

Oxygen Delignification

Oxygen delignification of SEDW was carried out in a 1-L steel reactor equipped with a motorized stirrer. The SEDW (30 g for each batch), NaOH, and water was premixed in a beaker and transferred to the reactor. The amount of NaOH and water were adjusted according to the reaction conditions (alkali dosage and consistency; *see* Table 1). All experiments were conducted under an oxygen pressure of 0.5 MPa. Magnesium sulfate (0.5% on SEDW [w/w]) was added to prevent the cellulose from degrading during oxidation. At the end of the reaction, the suspension was removed from the reactor. The oxygen-delignified SEDW in the suspension was separated from waste liquor using filtration, washed with distilled water, then stored in a refrigerator for subsequent analysis and hydrolysis.

Enzyme Preparations

The enzyme preparations were provided by Novo Nordisk. Cellulase (Celluclast) possessed 115 filter paper units (FPU)/mL; β-glucosidase (Novozym 188) had 570 cellobiose units (CBU)/mL.

Enzymatic Hydrolysis

All hydrolysis experiments were conducted with a combination of cellulase (Celluclast) and β-glucosidase (Novozym 188) at a ratio of 1:2

Table 1
Oxygen Delignification of SEDW

		Oxygen-delignified SEDW[a]					
Sample ID	SEDW	O-1	O-2	O-3	O-4	O-5	O-6
Delignification conditions							
NaOH (% on oven dry pulp)		20	20	15	15	15	10
Consistency (%)		12	10	10	5	5	5
Temperature (°C)		140	110	110	110	100	110
Time (h)		1.5	1.5	1.5	3	3	3
Pulp yield, %	100.0	59.6	66.5	71.5	58.5	61.8	71.7
Total lignin (%)	42.8	47.1	49.0	49.1	12.0	14.8	23.8
Klason lignin (%)	41.7	46.7	48.6	48.7	11.5	14.3	23.3
Acid-soluble lignin (%)	1.1	0.4	0.4	0.4	0.5	0.5	0.5
Carbohydrate composition (%) [b]	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Arabinose	0.5	ND	ND	ND	ND	ND	ND
Galactose	0.7	ND	0.5	0.5	ND	ND	ND
Glucose	87.7	93.3	93.2	92.6	96.8	96.6	96.9
Xylose	8.3	5.0	4.5	5.1	1.4	1.5	1.3
Mannose	2.8	1.7	1.8	1.8	1.8	1.9	1.8

[a] ND, not detected.
[b] Percentage of sugars in total carbohydrates..

(FPU/CBU). β-Glucosidase was added to avoid end-product inhibition owing to cellobiose accumulation. Batch hydrolysis experiments were conducted at 2% consistency of cellulose in 50 m*M* acetate buffer, pH 4.8, with 4 mg of tetracycline/100 mL of buffer as antibiotic. The hydrolysis incubation was performed at 45°C on a rotary shaker at 150 rpm. Samples of the supernatants were taken at assigned intervals for sugar analysis. All hydrolysis experiments were performed in duplicate, and the averages were reported.

Analytical Procedure

Sugar concentrations of hydrolysates were determined using a Dionex high-performance liquid chromatography system (DX-500) equipped with an AS3500 autosampler, a GP40 gradient pump, an anion-exchange column (Dionex CarboPac™ PA1), and an ED40 electrochemical detector. Deionized water was used as an eluent at a flow rate of 1 mL/min; NaOH (1 *M*) was used to equilibrate the column after elution of sugars. To optimize baseline stability and detector sensitivity, 0.2 *M* NaOH was added postcolumn. After being filtered through 0.45-µm nylon syringe filters (Chromatographic Specialties), a 20-µL sample was injected.

Klason lignin determination of substrates was carried out according to TAPPI standard method T-222. The hydrolysate from the Klason lignin determination was collected and analyzed for sugars and acid-soluble lignin. Sugars were determined as just described, with the exception that the mixture of sugar standards was autoclaved at 120°C for 1 h to compensate for sugar changes during autoclaving. Acid-soluble lignin was determined by ultraviolet spectra, as described by Dence *(15)*.

Results and Discussion

Oxygen Delignification Operations

Oxygen delignification, a delignification method using combined oxygen and alkali, has been widely utilized in the bleaching of pulps in the paper industry. In most cases, the lignin content of a brown stock prior to bleaching is about 5% (w/w). Because of the poor selectivity of oxygen, the extent of oxygen delignification is typically limited to 45–50% lignin removal in order to avoid the undesired degradation of cellulose and resulting negative impact on pulp strength *(12)*.

The main process parameters of an oxygen delignification operation include alkali (NaOH) charge, temperature, pulp consistency, reaction time, and oxygen pressure. Previous work *(16)* has shown that approx 1% NaOH (per oven dry pulp) is required to remove each percentage of lignin from the pulp. An increase in temperature will accelerate the delignification rate; however, there is a simultaneous increase in the risk of cellulose degradation. In general, higher consistency produces improved delignification at a fixed NaOH loading. Typical oxygen delignification operations are carried out at 90-115°C, at 10–20% pulp consistency, over 30–80 min of reaction time and 0.5–0.7 MPa of oxygen pressure.

Distinct from kraft pulp, SEDW has a very high lignin content (about 40%), and the lignin present in SEDW has been shown to be highly condensed and less reactive than is found in commercial pulps *(17)*. To assess the potential of oxygen delignification on SEDW lignin removal, a series of oxygen delignification experiments was designed with different reaction conditions (Table 1). The severity of the treatments was controlled by a combination of reaction parameters including temperature, substrate consistency, and alkali charge. The severities of different oxygen delignification experiments vary, with the O-1, O-2, and O-3 group representing relatively severe treatment conditions, and the O-4, O-5, and O-6 group corresponding to relatively mild treatment conditions.

The chemical composition of oxygen-delignified SEDW at different conditions was determined and compared with the control, untreated SEDW. There was a significant loss in the sample yield, varying from 58.5 to 71.7%, after oxygen delignification. The glucose fraction of the total carbohydrate increased in all treated samples, when compared to untreated SEDW, probably owing to the removal of the hemicellulose present in the substrates. Among the different hemicellulose sugars, arabinose and galac-

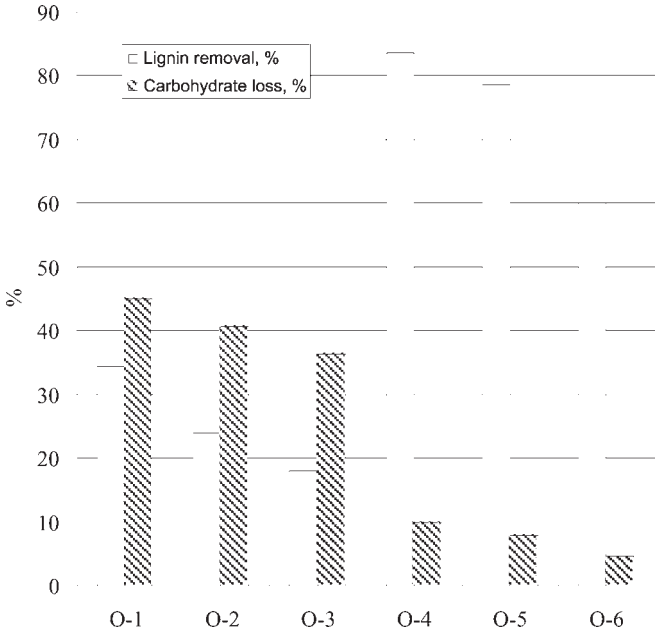

Fig. 1. Effect of oxygen delignification conditions on lignin removal and carbohydrate loss.

tose were totally removed and a considerable decrease in the amount of xylose and mannose was detected. However, the changes in the lignin content after oxygen delignification were somewhat unexpected. The lignin content increased after oxygen treatments under relatively severe conditions (O-1, O-2, and O-3), whereas there was a significant removal of lignin under milder conditions (O-4, O-5, and O-6). The reason for substantial delignification under mild conditions was further investigated.

Effect of Oxygen Delignification on Removal of Lignin and Yield of Carbohydrates

The degree of delignification was plotted against the loss of carbohydrates after each treatment. As shown in Fig. 1, there was a significant loss of carbohydrates, from 37–44.9%, under severe treatment conditions (O-1, O-2, and O-3). Conversely, mild treatment conditions (O-4, O-5, and O-6) resulted in less degradation of the carbohydrates. It was anticipated that higher alkalinity combined with higher temperature would increase delignification. The results from our experiments proved otherwise, showing that mild conditions removed much more lignin than severe conditions. These results can be postulated by the reaction mechanisms of carbohydrate and lignin during oxygen delignification.

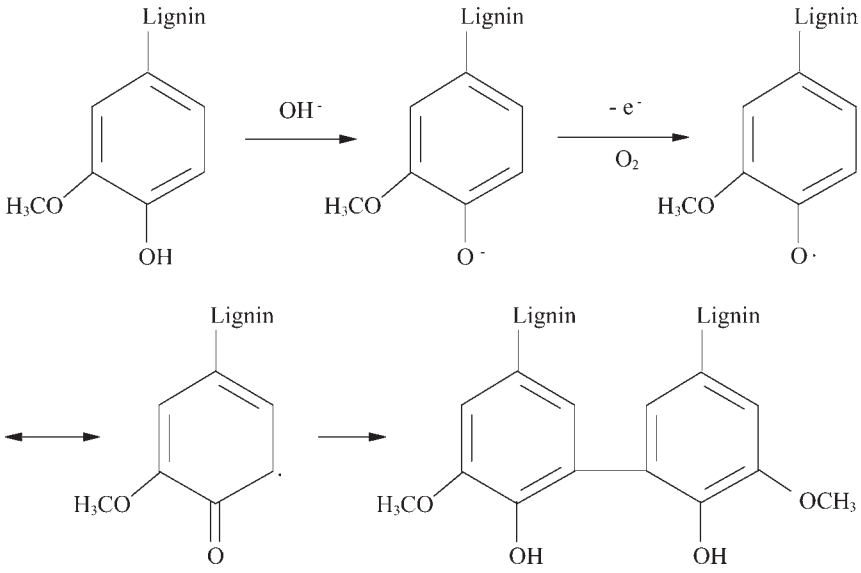

Fig. 2. Condensation reaction of lignin during oxygen delignification.

In general, there are two types of reactions that would lead to cellulose degradation during oxygen delignification *(12)*. The first is random chain cleavage (alkaline hydrolysis), which occurs at any point along the cellulose chain. The first step in this chain cleavage process is the oxidation of a hydroxyl group to a carbonyl group. The enol form of the ensuing carbonyl-containing unit will then undergo a β-elimination reaction to cleave the glycosidic bond and break the cellulose chain *(18)*. The second is the "peeling" reaction, during which the reducing units (containing the carbonyl group) on the end of the chain are attacked and successively removed. Random chain cleavage will promote the peeling reaction by producing new reducing ends *(12)*. Both higher temperature and alkaline conditions will typically enhance random chain cleavage and the peeling reaction.

It is known that free-radical reactions are also involved in oxygen delignification *(19)*. Free phenolic groups on the lignin have been shown to play a key role in these reactions. Under alkaline conditions, the phenolic groups can be ionized to form the phenoxide ion, which has a high electron density. The phenoxide ions are very susceptible to molecular oxygen and form phenoxy radicals. The propagation of this radical reaction will result in the consecutive rupture of the lignin structure, formation of degraded compounds, and consequent removal of lignin. However, throughout the lignin degradation reactions, the phenoxy radicals can also undergo coupling (condensation) reactions (Fig. 2) that lead to the formation of new carbon-carbon bonds between lignin subunits *(20)*. Severe treatment conditions, such as high temperature and high alkali concentration, can facilitate these coupling and condensation reactions.

The SEDW substrate has a very high lignin content and therefore has the potential to form a considerable number of phenoxy radicals in high alkaline concentration and high temperatures. It is probable that the more severe treatment conditions (O-1, O-2, and O-3) caused a significant condensation of the SEDW lignin, thereby limiting its removal. Meanwhile, the milder treatments encountered in the O-4 and O-5 treatments were more favorable to the lignin degradation. However, the insufficient alkali charge also led to limited lignin removal under O-6 treatment.

It is apparent that an increase in alkalinity and temperature during oxygen delignification of SEDW does not correlate with the extent of delignification. Different treatment parameters need to be optimized in order to minimize lignin condensation and carbohydrate degradation. Among the tested conditions, a relatively mild treatment (O-4) with 15% NaOH for 3 h at 110°C was shown to optimize the delignification of SEDW at a 5% substrate consistency. This treatment removed 83.6% of the lignin from SEDW, originally comprising 42.8% lignin. The carbohydrate yield after oxygen delignification (O-4) was 90%. The loss of carbohydrates was mainly owing to the removal of hemicellulose sugars. The glucose yield of O-4 was 99%. Our previous research *(11)* showed that the carbohydrate yield after steam explosion was 88%. Therefore, the overall yield of carbohydrates (steam explosion + oxygen delignification) was 79.2%.

Enzymatic Hydrolyzability of Oxygen-Delignified SEDW

The hydrolyzability of untreated SEDW was determined at several enzyme loadings, from 20 to 80 FPU/g of cellulose (Fig. 3). Poor enzymatic hydrolyzability was observed on the SEDW substrate even at a high enzyme loading. A 72-h-incubation resulted in approx 35, 58, and 75% cellulose-to-glucose conversions at Celluclast loadings of 20, 40, and 80 FPU/g of cellulose, respectively. Complete cellulose hydrolysis was not observed even after extended incubation for another 2 d.

The hydrolyzability of SEDW after oxygen delignification was also determined and compared with controls (Figs. 4 and 5). An enzyme loading at 20 FPU/g of cellulose was used to hydrolyze the delignified SEDW. As shown in Fig. 4, after more severe treatments (O-1, O-2, and O-3), SEDW demonstrated improved digestibility over untreated SEDW, with an increase in cellulose-to-glucose conversion yields from 8 to 21%. However, note that the O-1-, O-2-, and O-3-treated samples contained higher lignin when compared to the untreated SEDW. Although the amount of lignin present has been shown to correlate with the digestibility of a lignocellulosic substrate, it is also recognized that the nature and distribution of lignin within the substrate can also have a detrimental effect on enzyme accessibility to the substrate and subsequent hydrolysis efficiency *(21)*. Besides acting as a physical barrier to hinder enzyme accessibility, lignin has been shown to be able to irreversibly adsorb cellulase enzymes and impair the efficiency of enzymatic hydrolysis. Free phenolic groups on lignin have also been shown to play a critical role in the adsorption of

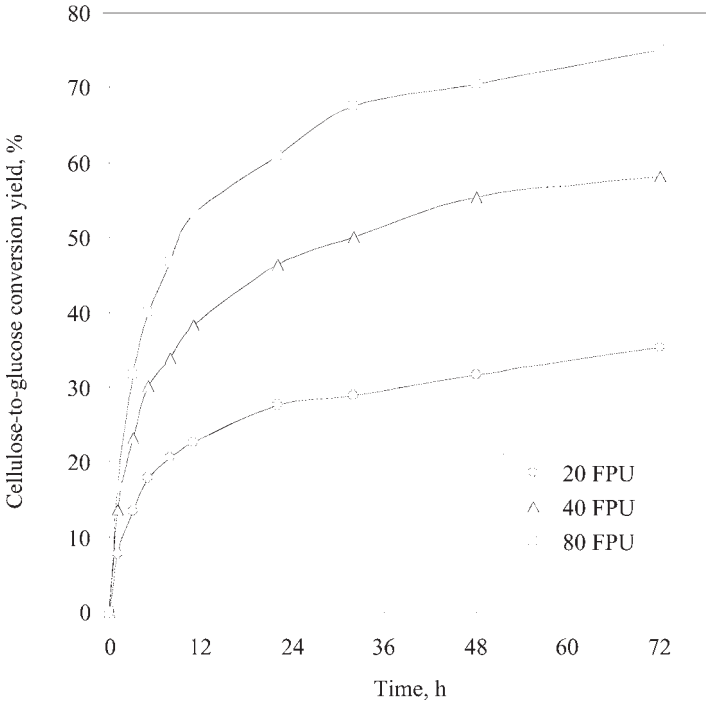

Fig. 3. Enzymatic hydrolysis of untreated SEDW.

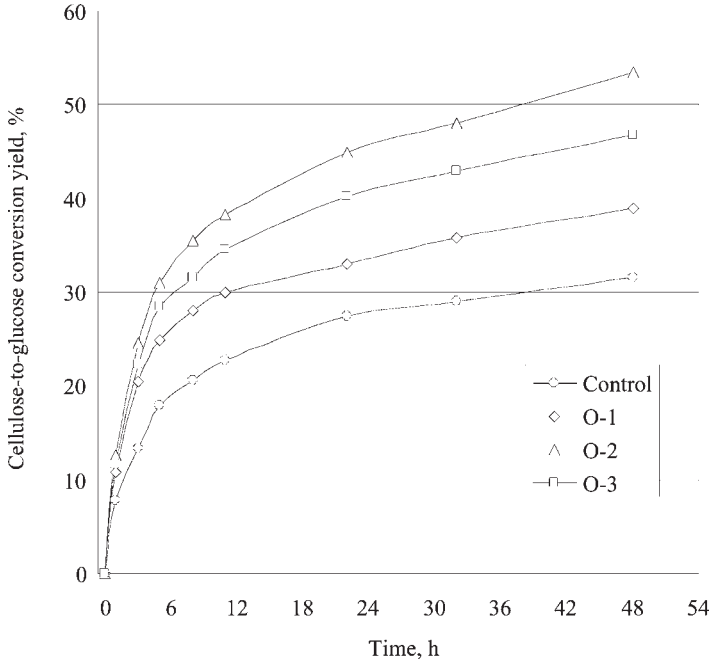

Fig. 4. Enzymatic hydrolysis of SEDWs oxygen delignified under severe conditions (cellulase loading: 20 FPU/ g of cellulose).

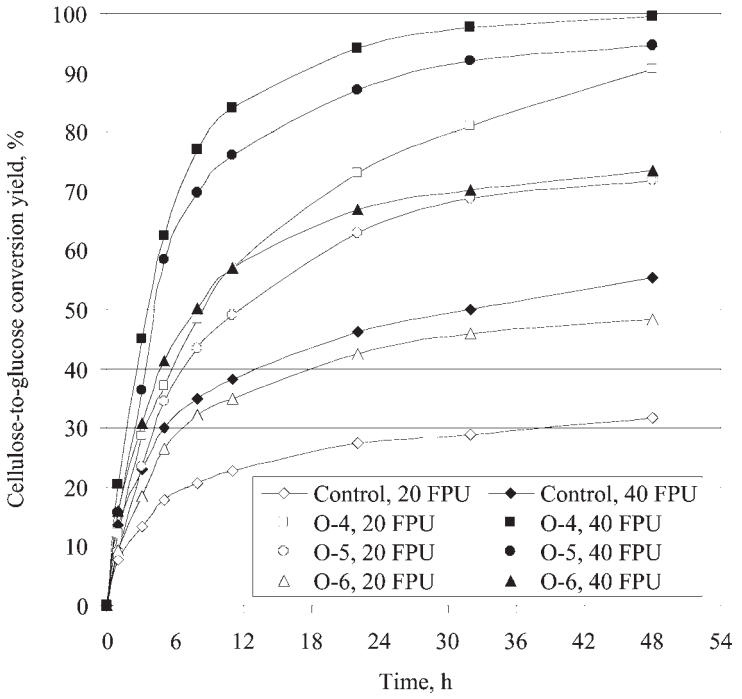

Fig. 5. Enzymatic hydrolysis of SEDWs oxygen delignified under mild conditions.

enzymes by mediating the binding between lignin and proteins *(22,23)*. Oxygen treatment reduced the number of phenolic groups present in the lignin, producing a more condensed structure *(12,24,25)*. Therefore, it is conceivable that the increased hydrolyzability of the O-1, O-2, and O-3 samples is owing to the modifications in the lignin structures making lignin less detrimental to the cellulase enzyme.

When the digestibility of O-4-, O-5-, and O-6-treated samples was compared to the control SEDW at both enzyme loadings of 20 and 40 FPU/g of cellulose, significant increases in cellulose-to-glucose conversion yield were observed (Fig. 5). A completed hydrolysis was obtained with the O-4-treated sample after 48 h of incubation with an enzyme loading of 40 FPU/g of cellulose. At the same enzyme loading, the hydrolysis of O-5 and O-6 samples produced 95 and 73% cellulose-to-glucose conversion, respectively. At the lower enzyme loading (20 FPU/g of cellulose), the O-4-treated SEDW was still able to reach a 90% conversion yield after 48 h of hydrolysis, compared with a 70% conversion yield on O-5-treated samples and a close to 50% conversion yield when the O-6-treated sample was used. It is apparent that significant decreases in the lignin content, resulting from oxygen delignification under mild conditions, were the main reason for the enhanced hydrolyzability of treated samples. It is also likely that the modifications to

the nature of steam-exploded lignin contributed to some improvement in the digestibility of the O-4-, O-5-, and O-6-treated samples.

The overall glucose yield of SEDW was 91%, as reported previously *(11)*. As mentioned earlier, the glucose yield of O-4 after oxygen delignification was 99%. The cellulose-to-glucose conversion yields after 48 h of enzymatic hydrolysis of sample O-4 were 90% after an enzyme loading of 20 FPU/g of cellulose, and 100% after an enzyme loading of 40 FPU/g of cellulose. Thus, the overall wood-to-glucose conversion yield of the whole process (steam explosion pretreatment + oxygen posttreatment + enzymatic hydrolysis) was between 81 and 90%, which is considerably higher than has been reported previously *(26)*.

Conclusion

High-lignin-containing SEDW behaved differently to normal low-lignin-containing pulps during oxygen delignification. Severe reaction conditions (high NaOH concentration and high temperature) resulted in the condensation of lignin and destruction of cellulose rather than delignification. However, relatively mild conditions resulted in substantial delignification of SEDW without significant degradation of cellulose. The optimal conditions tested were 15% NaOH, 5% consistency, 110°C, and 3 h, which facilitated the removal of approx 84% of lignin in SEDW with only 10% loss of carbohydrates.

Untreated SEDW had poor hydrolyzability, and a complete hydrolysis was not observed even at a high enzyme loading. However, the oxygen-delignified SEDW showed a significant improvement in hydrolyzability. Cellulose-to-glucose conversion yields of 90 and 100% could be achieved within 48 h with enzyme loadings of 20 and 40 FPU/g of cellulose, respectively.

Acknowledgments

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