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Evaluation of Wet Oxidation Pretreatment for Enzymatic Hydrolysis of Softwood

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Abstract

The wet oxidation pretreatment (water, oxygen, elevated temperature, and pressure) of softwood (*Picea abies*) was investigated for enhancing enzymatic hydrolysis. The pretreatment was preliminarily optimized. Six different combinations of reaction time, temperature, and pH were applied, and the compositions of solid and liquid fractions were analyzed. The solid fraction after wet oxidation contained 58–64% cellulose, 2–16% hemicellulose, and 24–30% lignin. The pretreatment series gave information about the roles of lignin and hemicellulose in the enzymatic hydrolysis. The temperature of the pretreatment, the residual hemicellulose content of the substrate, and the type of the commercial cellulase preparation used were the most important factors affecting the enzymatic hydrolysis. The highest sugar yield in a 72-h hydrolysis, 79% of theoretical, was obtained using a pretreatment of 200°C for 10 min at neutral pH.

Index Entries: Wet oxidation; enzymatic hydrolysis; lignocellulose; cellulases.

Introduction

Lignocellulosic residues could provide a potential, cheap source of raw material for production of ethanol and other chemicals. These include agricultural and forest residues and waste materials. Possible forest-based raw material sources are logging wastes; forestry residues, consisting of various wood species; as well as waste streams from paper mills. Compared with hardwood or agricultural residues, softwood raw materials are,

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however, not well suited for bioconversion processes, and they have been generally recognized as most difficult to be hydrolyzed *(1–3)*. Biomass originating from softwood is composed of about 40–45% (w/w) cellulose, 20–30% (w/w) hemicelluloses, and 28–30% (w/w) lignin. Cellulose is a homogeneous polymer consisting of β-1,4-linked glucose units. Hemicelluloses in spruce are mainly galactoglucomannans with a linear backbone built up by 1-4-linked β-D-glucopyranose and β-D-mannopyranose units. Mannose and glucose units are partially substituted by acetyl groups, on the average one group per three to four hexose units. Hemicelluloses are easily hydrolyzed by acids to their monomeric components: mannose, glucose, galactose, xylose, and arabinose. The acetyl groups are more easily cleaved by alkali than acid *(4)*. Hemicellulose as well as lignin forms a physical barrier that surrounds cellulosic microfibrils and resists enzymatic attack. The recalcitrance of softwood to enzymatic hydrolysis is often attributed to the high lignin content and small pore size *(5,6)*. Lignin also affects enzymatic hydrolysis by irreversibly adsorbing the cellulase enzymes *(7)*. In addition, the lower content of hemicellulose-derived acetyl groups in softwood compared with hardwood *(8)* may limit the autohydrolysis of hemicellulose, which occurs at low pH, by acetic acid liberated during pretreatment.

Processes for bioconversion of lignocellulosic materials have been studied extensively, and numerous different pretreatment techniques for lignocellulosic materials have been tested. Physical pretreatments include comminution, irradiation, steam explosion, and hydrothermolysis. Chemical pretreatments include typically dilute acid, alkali, solvent, ammonia, SO_2, CO_2, and other chemicals *(9)*. Hydrothermal pretreatment refers to the use of water and heat to treat biomass. High-temperature pretreatment without chemicals but in the presence of moisture is called autohydrolysis. Autohydrolysis is catalyzed by organic acid liberated from biomass *(9)*. Hydrothermolysis involves high-temperature cooking with water. Liberated organic acids are not the primary hydrolytic agents in this reaction; thus, it may exhibit a different reaction mechanism from autohydrolysis *(10)*. Steam pretreatment with sulfuric dioxide or H_2SO_4 impregnation is one of the most successful and often used methods that has been investigated also in pilot scale *(11)*. Steam pretreatment is usually performed at 190–230°C. Its disadvantage is the formation of degradation products from lignin and sugars, some of which are toxic and inhibit the fermentation step *(12,13)*.

Wet oxidation operates with water and oxygen or air at elevated temperature and pressure *(14)*. The wet oxidation process has been found to convert many organic polymers to oxidized compounds, such as low molecular weight carboxylic acids, or even to CO_2 and H_2O *(15)*. Industrially used wet air oxidation processes involve oxidation of soluble or suspended materials by using oxygen in aqueous phase at high temperatures (150–350°C) and high pressure (5–20 MPa). These wet air oxidation processes with or without a catalyst are commonly used to oxidize wastes with a high organic matter to intermediate products, such as low molecular

weight carboxylic acids, acetaldehydes, and alcohols, and finally to CO_2 and H_2O. The degree of oxidation depends on the process conditions, time, and feed composition *(16)*. In addition to the treatment of polluted soil and wastewater, wet oxidation has been successfully applied for the treatment of wheat straw and hardwood *(17,18)*. In recent studies on alkaline wet oxidation of wheat straw, the main degradation products found from hemicellulose and lignin were carboxylic acids, CO_2, and H_2O. About a 65% degree of delignification could be achieved with wheat straw *(19)*. Wet oxidation of wood material has been shown to dissolve mainly the hemicellulose *(14,17)*. One reported advantage of the wet oxidation process is the lower production of furfural and 5-hydroxymethylfurfural, which are potential inhibitors in the fermentation step *(20)*. In addition, wet oxidation of biomass is a rapid reaction and takes places at lower temperatures (150–200°C) than steaming. Wet oxidation of softwood has not been previously reported, and the aforementioned advantages make the process attractive for a potential pretreatment for softwood.

Presently, enzymatic hydrolysis is considered the most promising technology for converting biomass into sugars, although the slow rate of the process and the high cost of enzymes are still factors limiting commercialization *(21)*. Several enzymes are needed for the efficient degradation of cellulose fraction in lignocellulose. *Trichoderma reesei*, one of the most extensively studied cellulose-degrading fungi, is able to produce all the enzymes that are needed to degrade crystalline cellulose and hemicelluloses. Several commercial cellulase preparations produced by *Trichoderma* are on the market. However, the commercial cellulase mixtures are not generally designed for lignocellulose hydrolysis, and their efficiency in the hydrolysis of lignocellulose varies significantly. In the present work, the chemical composition and enzymatic conversion of the solid fraction after different wet oxidation pretreatment conditions were determined. The composition of the liquid fraction was also examined. The hydrolytic efficiencies of two commercial cellulase preparations, Celluclast and Multifect, on wet-oxidized lignocellulose were compared. In addition, a comparison to the commonly used steam pretreatment was made.

Materials and Methods

Substrates

Fresh, chipped, bark-free Norway spruce (*Picea abies*) was obtained from Glumsoe sawmill, Denmark. The wood chips were dried at room temperature for 7 d and ground with a hammer mill to 5-mm size. This chip size has been previously found applicable in steam pretreatment *(22)*. Chips were stored in plastic bags at room temperature. The same batch of raw material was used for both the wet oxidation and the steam pretreatment. The pretreated substrates were stored frozen and not dried before use in the hydrolysis experiments.

Enzymes

The cellulase mixtures used in the screening of the different wet oxidation batches were Celluclast 1.5 L and the β-glucosidase Novozym 188, provided by Novozymes. In addition, Multifect (Genencor) was used to study hydrolysis efficiency further. The enzymes were dosed according to the filter paper unit (FPU) activity, which was assayed using Whatman no. 1 filter paper as substrate *(23)*. Endoglucanase activity against hydroxyethyl cellulose (HEC) was assayed using 1% hydroxyethyl cellulose (Fluka 54290) as substrate *(23)*. Xylanase activity was determined using 1% glucuronoxylan (Roth 7500) as substrate *(24)*. Mannanase activity was determined using locust bean gum (G-0753; Sigma, St. Louis, MO) as substrate *(25)*. The accessory hemicellulase activities were determined as follows: β-xylosidase activity was assayed using *p*-nitrophenyl-β-D-xylopyranose (N-2132;Sigma) as substrate *(26)*. α-Arabinosidase activity was assayed using 2 m*M p*-nitrophenyl-α-L-arabinofuranoside (N-1381; Sigma) as substrate *(27)*. α-Galactosidase activity was assayed using 1 m*M p*-nitrophenyl-α-galactopyranoside (N-0877; Sigma) as substrate *(28)*. β-Mannosidase activity was assayed analogously to α-galactosidase activity using 1 m*M p*-nitrophenyl-β-D-mannopyranose as substrate. β-Glucosidase activity was assayed using 1 m*M p*-nitrophenyl-β-D-glucopyranose as substrate *(29)*.

Wet Oxidation Pretreatment

Wet oxidation was carried out in a 2-L loop reactor constructed at Risoe National Laboratory *(20)*. Samples of air-dried spruce chips (60 g dry wt) were mixed with 1000 mL of tap water. A statistical fractional factorial design was used to screen the process parameters in the wet oxidation. Oxygen pressure (12 bars) and raw material concentration (60 g/L) were kept constant in the experiments, and the reaction temperature (two levels), pH (three levels), and time (two levels) were varied, giving six different pretreatments. The experiments were carried out in randomized order to avoid systematic errors. Randomized order was generated by a computer using random sampling numbers.

The pH was adjusted to 3.5 with H_2SO_4 or to 10.5 by adding 6.5 g of Na_2CO_3. An oxygen pressure of 12 bars was applied before heating. The temperature was kept within ±2°C of the set value during the reaction. The pretreated suspension was filtered, and the solid fraction was washed with a small amount of tap water. The pH of the liquid fraction and the weight of the wet solid fraction were measured. The dry mass of filter cakes was determined at 105°C with a Mettler Toledo HA73 halogen moisture analyzer. The composition of each fraction was analyzed.

Steam Pretreatment

Steam pretreated spruce (SPS) was prepared at optimal conditions (SO_2 concentration of 2%, temperature of 215°C, and residence time of 5 min) for enzymatic hydrolysis as described by Stenberg et al. *(22)*. SPS was kindly provided by Professor Guido Zacchi (Chemical Engineer-

ing, Lund University, Sweden). The slurry after the pretreatment was filtered, and the solid material was washed with a small amount of tap water.

Analysis of Solid Fiber Fraction

The composition of the solid samples was analyzed according to the gravimetric methods described by Browning *(30)*. The sample was first extracted with a mixture of toluene, acetone, and ethanol (4:1:1) in a Soxhlet apparatus for 5 h to remove extractives. The sample was then briefly washed with acetone and dried overnight at 105°C. It was subsequently delignified with an acidified solution of sodium chlorite at 80°C for 4 h. Next, the sample was washed with cold water and acetone and dried overnight at 105°C. Finally, the α-cellulose fraction was isolated by extracting the hemicelluloses with a mixture of 10% NaOH and 15% $Na_2B_4O_7$ for 2 h at room temperature. The solid residue (α-cellulose) was washed thoroughly with hot water and dried overnight at 105°C. The ash content was determined from the unextracted wood by heating the sample at 550°C for 3 h.

Analysis of Solid and Liquid Fractions by Acid Hydrolysis

In addition to the gravimetric method, the composition of solid materials was also analyzed by acid hydrolysis. The dried sample (100 mg) was incubated with 70% (w/w) H_2SO_4 (1 mL) at 30°C for 1 h. The mixture was transferred to a 50-mL measuring flask in 28 mL of water and autoclaved at 120°C for 50 min. The flask was filled with distilled water to 50 mL before taking a sample for analysis.

The content of sugars in the liquid fraction was measured after acidic hydrolysis of the polysaccharides. The filtrate after wet oxidation was first treated with 4% H_2SO_4 at 121°C for 10 min. The samples were filtered (0.45-μm filter) and neutralized by adding $Ba(OH)_2$. The supernatant was purified by ion-exchange chromatography using Dowex MR-3 (Fluka). The recoveries in the purification procedure were determined for glucose, mannose, xylose, galactose, and arabinose by standard addition of sugars before addition of $Ba(OH)_2$.

The carbohydrate content was determined as monosaccharides by high-performance anion-exchange chromatography. A Dionex CarboPac PA-1 column at 30°C in a Dionex DX 500 series chromatograph equipped with pulse amperometric detection (Dionex ED 40) was used as described by Tenkanen and Siika-aho *(31)*. The content of polysaccharides in the acid hydrolysates was calculated from glucose, mannose, and xylose concentrations. The following molar ratios for the components of softwood were used in the calculations: (Xyl:MeGluA:Ara) = 8:1.6:1 for xylan *(8)* and (Man:Glc:Gal) = 4:1:0.5 for glucomannan. The following equations were used to estimate the amount of cellulose and hemicellulose in the samples:

$$m_{\text{xylan}} = \frac{132}{150} \cdot \left(m_{\text{Xyl}} + \frac{1 \cdot n_{\text{Xyl}}}{8} \cdot M_{\text{Ara}} \right) + \frac{190}{208} \cdot \left(\frac{1.6 \cdot n_{\text{Xyl}}}{8} \cdot M_{\text{MeGlc}} \right)$$

$$m_{\text{glucomannan}} = \frac{162}{180} \cdot \left(m_{\text{Man}} + \frac{1 \cdot n_{\text{Man}}}{4} \cdot M_{\text{Glc}} + \frac{0.5 \cdot n_{\text{Man}}}{4} \cdot M_{\text{Gal}} \right)$$

$$m_{\text{cellulose}} = \frac{162}{180} \cdot \left(m_{\text{Glu}} - \frac{1 \cdot n_{\text{Man}}}{4} \cdot M_{\text{Glc}} \right)$$

in which M is molar mass (g/mol), m is the amount of the component in the sample (g/g dry material), and n is the mass of components as moles (mol). The total amount of hemicellulose was calculated as a sum of xylan and glucomannan.

Enzymatic Hydrolysis of Solid Fractions

The enzymatic conversion of cellulose in the different WOS samples (20 g/L dry matter [DM]) was evaluated using Celluclast (30 FPU/g DM) and Novozym 188 (500 nkat of β-glucosidase/g of DM) in 0.05 *M* sodium acetate buffer (pH 5.0). (The softwood samples after different pretreatment conditions are designated as WOS, and they are numbered from 1 to 6, depending on the pretreatment conditions. The steam-pretreated sample is designated as SPS.) The suspension (5 mL) was incubated in glass tubes with small magnetic stirrers in a water bath at 40°C. After hydrolysis (24, 48, or 72 h), the samples were cooled on ice, centrifuged, and the hydrolysate was boiled for 10 min. Boiling was performed using glass-capped test tubes, and the final volume was unchanged. Reference experiments were performed without addition of enzymes or substrates, and reported values were obtained after subtraction of control experiment values. The release of soluble reducing sugars in the hydrolysates was monitored by the dinitrosalicylic acid (DNS) method *(32)* with glucose as a standard. The percentage of polysaccharides (cellulose and hemicellulose) converted to sugars was calculated using the following equation:

$$\text{Conversion} = 100\% \cdot \left[m_{\text{reducing sugars}} / \left(\frac{180}{162} \cdot m_{\text{cellulose}} + \frac{150}{132} \cdot m_{\text{hemicellulose}} \right) \right]$$

Two commercial cellulase mixtures (Celluclast and Multifect) with different loadings (5, 10, and 30 FPU/g of DM) were compared in the hydrolysis of wet-oxidized and steam-pretreated softwood.

Results

Wet Oxidation

The pretreatment conditions (Table 1) were chosen by utilizing previous experience obtained in the optimizations with wheat straw *(18)*. The filter cakes from the duplicate experiments had almost identical dry

Table 1
Pretreatment Conditions, and Final pH and Mass Yield After Wet Oxidation and Steam Pretreatment

	Conditions				
Sample	Temperature (°C)	Time (min)	pH	Final pH	Yield of insoluble material (% of original)
WOS-1	185	10	3.5	2.7	71
WOS-2	185	20	7	2.7	67
WOS-3	185	10	10.5	7.5	81
WOS-4	200	20	3.5	2.5	58
WOS-5	200	10	7	2.8	64
WOS-6	200	20	10.5	4.8	66
SPS	215	5	[a]	1.9	68

[a]2% SO_2 added.

masses (variation of 1–3%; result not shown), indicating a good reproducibility of the pretreatment process.

The yield of solid material was dependent on the reaction temperature; as expected, more solid was degraded at higher temperature (Table 1). The highest weight loss, 42% of the DM, was detected at the most severe reaction conditions (WOS-4: 20 min, 200°C, pH 3.5). The pH of the WOS samples decreased during the pretreatment (Table 1), most probably owing to release of acetic acid from hemicellulose.

Fractionation and Carbohydrate Recovery

The parameters chosen for the pretreatment resulted in very different compositions of fractions. The solid fraction after wet oxidation contained 58–64% cellulose, 2–16% hemicellulose, and 24–30% (w/w) lignin (Table 2). As expected, the relative content of cellulose of the WOS substrates increased owing to the solubilization of hemicellulose and part of the lignin fraction. At low pH, hemicellulose is known to be autohydrolyzed. Also, in these experiments the low pH enhanced solubilization of hemicellulose. The high original pH of the reaction liquid seemed, on the other hand, to prevent autohydrolysis, resulting in very low solubilization of hemicellulose, especially the mannose fraction, as shown in Table 3.

The solubilization of lignin was clearly highest at elevated pH. The amount of extractives in the samples varied significantly and decreased along with increased treatment pH. Obviously, part of the low molecular weight lignin fraction was solubilized during the extraction by acetone-ethanol-toluene solution and was regarded as extractives in some of the samples. Thus, the treatment resulted in an especially high apparent extractive content of SPS (Table 2).

The hemicellulose values obtained by gravimetric analysis were generally slightly lower and α-cellulose values higher than with acid hydrolysis (Table 2). To elucidate whether the gravimetric analysis had caused the

Table 2
Compositions of Pretreated Softwood Chips by Gravimetric Analysis and Acid Hydrolysis
(% of dry wt in Pretreated Material)

	Composition (% dry wt)						
	α-Cellulose		Hemicellulose				
Sample	Gravimetric	Acid hydrolysis	Gravimetric	Acid hydrolysis	Lignin (gravimetric)	Extractives and dissolved lignin (gravimetric)[a]	Ash
Untreated wood	43.8	40.0	18.1	21.0	28.5	0.7	0.3
WOS-1	60.9	56.1	4.3	7.6	30.1	4.7	0.1
WOS-2	64.3	64.7	2.7	4.0	28.8	4.1	0.2
WOS-3	58.5	52.5	16.1	21.8	25.5	0.0	1.0
WOS-4	58.5	58.1	4.9	0.8	27.8	8.8	0.3
WOS-5	63.7	64.3	1.8	2.4	27.1	7.5	0.1
WOS-6	62.9	55.5	9.4	11.7	24.2	3.6	0.5
SPS	42.9	40.1	4.7	3.0	31.5	19.6	0.2

[a]Acetone-ethanol-toluene extracts.

Table 3
Sugar Composition of Solid Fraction of Pretreated Spruce Chips[a]

Solid fraction	Glucose (g/100 g)	Mannose (g/100 g)	Xylose (g/100 g)	Galactose (g/100 g)	Arabinose (g/100 g)
Untreated wood	47.0	11.8	5.0	1.8	1.0
WOS-1	63.1	3.0	3.1	0.3	0
WOS-2	72.2	1.1	2.1	0	0
WOS-3	61.7	13.6	3.9	1.3	0.7
WOS-4	64.6	0.1	0.5	0	0
WOS-5	71.6	0.5	1.4	0	0
WOS-6	63.8	8.5	0.9	0.2	0
SPS	45.0	1.6	0.8	0.2	0.1

[a]The monosaccharide amounts were determined by high-performance liquid chromatography (HPLC)-analysis of the acid hydrolyzed substrate.

error, a hemicellulose-rich WOS-3 was first isolated according to the gravimetric analysis method, and subsequently the residual α-cellulose was hydrolyzed by acid and the monosaccharides were determined. The sample contained 99.9% glucose, 0.6% xylose, and 2.9% mannose, showing that only a slight fraction of hemicellulose remained with the cellulose. Thus, the higher cellulose values from gravimetric analysis were not owing to residual, undissolved hemicellulose. Most probably, the crude substrates were not properly degraded in the acid hydrolysis process, which should be carefully optimized for this purpose in the future. The lignin content of the untreated spruce analyzed as Klason lignin was 28.7%. The gravimetric analysis gave a very similar value, 28.5%.

To evaluate the recovery of wood components in the wet oxidation process, the monosaccharides in the liquid fractions were determined. The total yield of monomeric sugars after acid hydrolysis in the liquid fraction of WOS samples varied from 4 to 15% (w/w) from the original raw material (Fig. 1). The solubilization of mannose seemed to be the lowest when high pretreatment pH was applied. A significant part of the cellulose fraction in the SPS was dissolved during the steam pretreatment process. This resulted in the high glucose content of the liquid fraction, as seen in Fig. 1.

The monosaccharide composition of the acid-hydrolyzed WOS revealed which components of carbohydrates were retained in the solid fraction (Table 3). WOS-2 and WOS-5, both treated under neutral conditions, had the highest glucose content. The main hemicellulose-derived sugar component, mannose, was highest in WOS-3 and WOS-6, which were treated under alkaline conditions. In addition, minor amounts of xylose, arabinose, and galactose remained in the solid fraction.

The yields of substrate components (cellulose, hemicellulose, lignin) as g/100 g of original dry wood in the solid and liquid phases after the pretreatments are presented in Table 4. The sugar values were based on the HPLC analysis of the acid-hydrolyzed substrate. Lignin was calculated

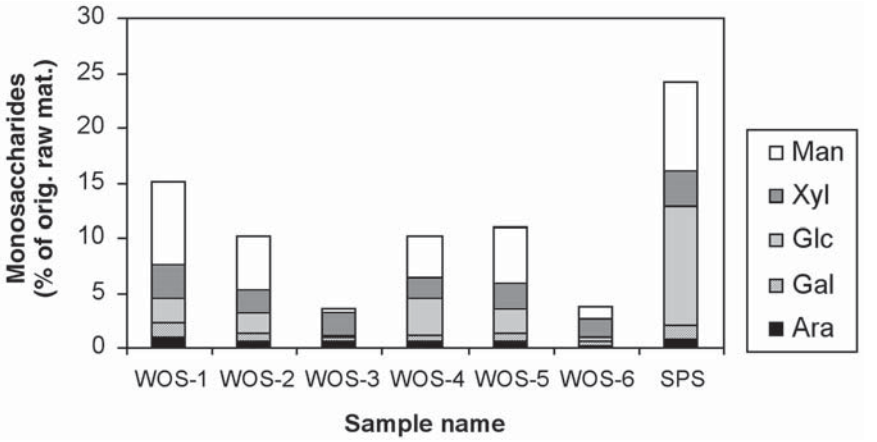

Fig. 1. Monosaccharide composition of liquid fraction after wet oxidation reaction. Sugar composition was determined by HPLC after acid hydrolysis of liquid and presented as % (w/w) of monosaccharides produced from the original raw material. The pretreatment conditions of the samples are described in Table 1.

from the gravimetric analysis. The recoveries of cellulose and hemicellulose were calculated in order to estimate the losses in the wet oxidation reactions (Table 4). The calculation was based on the mass balances of substrate components, which were detected in both the liquid and solid phases after pretreatment. The recoveries of hemicellulose and cellulose were the lowest when low pH, higher temperature, and longer treatment time were applied (WOS-4). Because of the extreme treatment conditions, the sugars were most probably decomposed and converted into other products. Recovery of hemicellulose was generally lower than that of cellulose. Very high recovery of hemicellulose in the liquid fraction, 86%, was obtained in WOS-1, pretreated at low pH and lower temperature (Table 4). Surprisingly, about 30% of the cellulose fraction in SPS was solubilized and partly degraded in the steam pretreatment, resulting in significantly lower cellulose content of solid fraction compared to WOS substrates. In this experiment, the recovery of cellulose in SPS was only 87%. The recovery of hemicellulose in SPS was relatively high (65.6%).

Enzymatic Hydrolysis

The cellulase and hemicellulase activities of the commercial cellulases used, Celluclast and Multifect, were determined (Table 5). The activities, standardized to 10 FPU/g of substrate, are expressed as units/gram of substrate used. Table 5 shows that Cellulast had a 10% lower HEC activity, a 20% lower mannanase activity, and a 40% lower xylanase activity compared with those of Multifect. The activities of the accessory hemicellulases were rather equal.

The enzymatic hydrolysis of WOS substrates was first compared using the commercial cellulase preparation, Celluclast (30 FPU/g). The solid

Table 4
Recoveries of Substrate Components After Pretreatment[a]

	Yield of substrate components (g/100 g original wood)					Recovery (% of original wood)	
	Solid fraction			Liquid fraction		Solid and liquid fractions	
Sample	Cellulose	Hemicellulose	Lignin	Cellulose	Hemicellulose	Cellulose	Hemicellulose
Untreated wood	40.0	21.0	28.5	—	—	—	—
WOS-1	39.8	5.4	25.7	0.3	9.5	100	71
WOS-2	43.5	2.7	21.0	0.5	5.9	110	41
WOS-3	42.6	17.7	20.9	0.1	0.5	107	86
WOS-4	33.7	0.4	23.9	2.2	4.7	90	25
WOS-5	41.1	1.5	21.3	0.7	6.3	105	37
WOS-6	36.9	7.7	21.8	0.2	1.4	93	44
SPS	27.5	2.1	21.5	7.5	11.5	87	66

[a]The cellulose and hemicellulose contents were based on the HPLC analysis of acid-hydrolyzed substrates. The lignin was calculated from the gravimetric analysis. Recoveries of cellulose and hemicellulose were calculated from the monosaccharides determined in acid hydrolysates of both liquid and solid fractions.

Table 5
Hemicellulase and Cellulase Activities of Enzyme Preparations Used in Hydrolysis Experiments, Standardized to 10 FPU/g of Substrate[a]

Hemicellulase activity	Multifect + Novozym 188	Celluclast + Novozym 188
FPU (FPU/g)[a]	10	10
HEC (nkat/g)[a]	3350	3060
Mannanase (nkat/g)	750	600
Xylanase (nkat/g)	6750	4100
β-Glucosidase (nkat/g)	580	470
β-Mannosidase (nkat/g)	6	7
β-Xylosidase (nkat/g)	60	50
α-Arabinosidase (nkat/g)	50	80
α-Galactosidase (nkat/g)	70	70

[a]About 500 nkat β-glucosidase (Novozym 188)/g of substance was added in the reaction mixture. The activity of Novozym 188 is not included.

Table 6
Hydrolysis of Pretreated Substrates With Celluclast (30 FPU/g)[a]

	Hydrolysis yield (% of carbohydrates in pretreated substrate)	
Sample	24 h	72 h
Untreated	6	Not tested
WOS-1	37	62
WOS-2	42	74
WOS-3	23	67
WOS-4	53	76
WOS-5	50	79
WOS-6	47	72
SPS	77	80

[a]The reducing sugars released in hydrolysis were determined by the DNS method, and the carbohydrate content (cellulose + hemicellulose) was calculated from gravimetric analysis results. Hydrolysis conditions were as follows: sodium acetate buffer, pH 5.0; 40°C; 20 g/L of substrate; 5-mL reaction volume.

fraction was most efficiently hydrolyzed after pretreatment at the higher reaction temperature, 200°C (Table 6). The maximum sugar production of WOS-4 in 24-h hydrolysis was about 21 g/100 g of original softwood, corresponding to a hydrolysis yield of 53% of carbohydrates. The maximum hydrolysis yields after 72 h, 76 and 79% of carbohydrates, were obtained in WOS-4 and WOS-5, respectively. The hydrolysis yield was considerably

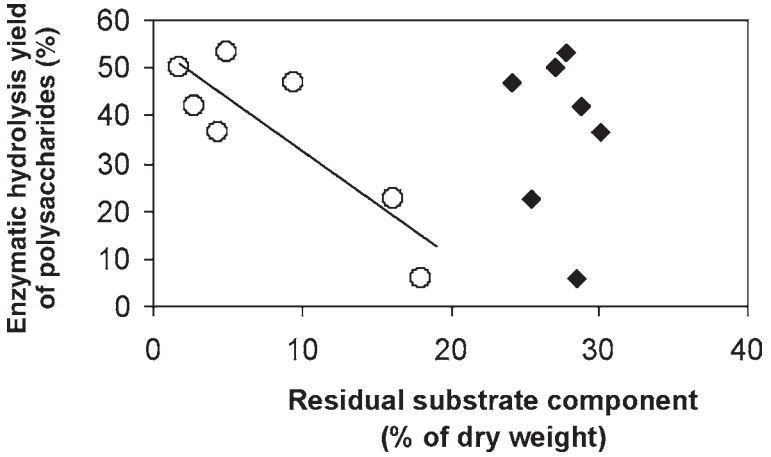

Fig. 2. Dependency of hydrolysis yield (from Table 4) on lignin (◆) and hemicellulose (○) contents (from Table 2) of wet oxidized softwood. Untreated starting material is included, giving the lowest hydrolysis yield.

lower when the wet oxidation pretreatment conditions at the lower temperature and shorter time were applied (WOS-1, WOS-3). The residual hemicellulose appeared to limit the hydrolysis considerably, as seen in Fig. 2, in which the hydrolysis yields are plotted as a function of the amount of residual hemicellulose. The lowest hydrolysis yields were obtained with the samples with the highest amount of residual hemicellulose (WOS-1, WOS-3, WOS-6). No clear correlation between the hydrolysis yield and lignin content could be observed (Fig. 2). The variations in lignin content were, however, very small.

Enzymatic hydrolysis was further investigated using WOS-5 (treated at 200°C, neutral pH, 10 min) and WOS-3 (treated at 185°C, elevated pH, 10 min). WOS-5 was among the best in this pretreatment series. WOS-5 had the lowest hemicellulose content and the second highest α-cellulose content. The enzymatic accessibility of WOS-5 was among the best ones (together with WOS-4). In addition, WOS-5 was filtered quickly and easily, whereas the samples with H_2SO_4 or sodium carbonate additions were slow to filter after the wet oxidation reaction. To investigate the limiting effect of hemicellulose on hydrolysis, WOS-3, with the highest residual hemicellulose content, was chosen as a comparison.

Both Celluclast and Multifect were efficient in the 24-h hydrolysis of WOS-5 and SPS, giving almost equal yields (Table 7). As expected, the action of cellulases was slightly less efficient on the hemicellulose-containing WOS-3. Multifect, with a higher xylanase activity, was more efficient in the hydrolysis of WOS-3. Celluclast (10 FPU/g) was able to produce only 11 g of sugars/100 g of original raw material, corresponding to a maximum conversion of 26% of polysaccharides in WOS-3. The maximum conversions of WOS-5 and SPS in 24-h hydrolysis with 10 FPU/g of Multifect were about 34 and 48% of polysaccharides, respectively. The maximum reducing

Table 7
Enzymatic Hydrolysis (24 h, 40°C, pH 5.0, 2% [w/w]) of SPS, Cellulose-Rich WOS-5, and Hemicellulose-Rich WOS-3 as Analyzed by Reducing Sugars[a]

Enzyme	Amount of enzyme (FPU/g DM)	Reducing sugars (g/100 g original wood)		
		SPS	WOS-5	WOS-3[a]
Celluclast	5	14.0	10.9	NT
	10	20.5	17.7	11.2
	30	27.8	23.5	NT
Multifect	5	10.7	8.8	NT
	10	17.5	15.9	17.4
	30	25.9	25.7	NT

[a]The cellulose and hemicellulose contents of substrates were determined by gravimetric analysis. NT, not tested.

sugars, using the highest enzyme loads, calculated from original wood were about 26 and 28 g from 100 g in WOS-5 and SPS, respectively. The cellulose content of SPS was lower than that of WOS-5, and, thus, the production of reducing sugars from original wood was in the same range for both WOS-5 and SPS (Table 5). The conversions of polysaccharides in WOS-5 were about 69 and 76% (w/w) with Celluclast and Multifect, respectively, when a longer, 48-h incubation time with 30 FPU/g was applied (result not shown).

Discussion

We evaluated the suitability of wet oxidation for pretreating softwood. It has been well recognized that softwood substrates are generally more recalcitrant to enzymatic hydrolysis than other lignocellulosic material. The recalcitrant nature of softwood was observed also in our work on wet oxidation pretreatment. The pretreatment method, however, showed potential for enhancing the enzymatic hydrolysis of this challenging substrate. Compared with hardwood or agricultural residues, higher temperature was needed to pretreat softwood. In addition, the level of delignification was lower. In earlier studies with wheat straw, the maximum degree of delignification was 65% of the original lignin content *(18)*. In the present study, only from 24 to 42% of lignin in softwood was dissolved. The residual hemicellulose content varied greatly, and the high residual hemicellulose content was shown to correlate with the low yield in the enzymatic hydrolysis. The more inaccessible structure of the substrate owing to the residual hemicellulose seems to be a major barrier to efficient hydrolysis. Obviously, this can be partially overcome by adjusting the pretreatment conditions or by using an enzyme preparation with high hemicellulase content.

In the hydrolysis, Multifect was able to degrade the hemicellulose-containing WOS-3 as efficiently as the two other substrates, SPS and WOS-5.

It can be expected that the somewhat higher hemicellulase activities (shown in Table 5) were one reason for the better performance of Multifect compared with that of Celluclast. However, both the mannanase and β-mannosidase activities in the tested cellulase preparations were obviously too low to ensure efficient and complete hydrolysis of the residual hemicellulose.

Early investigations with wheat straw have shown that the addition of alkali in the wet oxidation pretreatment could improve enzymatic hydrolysis and reduce the formation of inhibitors *(20)*. Therefore, the addition of Na_2CO_3 was also tested in our experiments. However, no improvement in hydrolysis could be detected. It is known that hemicellulose is solubilized in alkali. In our studies, the addition of Na_2CO_3 resulted in slightly increased solubilization of lignin, but higher retention of hemicellulose. Obviously, the pH was not high enough during the entire pretreatment to promote the solubilization of hemicellulose. The pH decreased in the pretreatment obviously owing to liberation of acetic acid. On the other hand, the best hydrolysis yields were obtained when the pH was neutral or slightly acidic. It seems that autohydrolysis of the hemicellulose plays an important role in improving cellulose hydrolysis of softwood.

Other investigations have shown that the recovery of hemicellulose-derived sugars can be enhanced by process optimizations. More than half of the hemicellulose was degraded to products other than sugars under the wet oxidation conditions in which the hydrolysis was most efficient. On the other hand, a high recovery of hemicellulose in some of the treatments resulted in poor yield in the enzymatic hydrolysis. In the steam pretreatment of softwood, the recovery of a maximal amount of sugars from both hemicellulose and cellulose has been shown to require a two-step pretreatment procedure *(33)*. Recovery of monosaccharides can be optimized, e.g., by posthydrolysis of solubilized hemicellulose after steam pretreatment, if more severe process conditions would lead to sugar losses *(34)*.

The best yield of sugars in 24-h hydrolysis (with 30 FPU/g of cellulase) of WOS was 26% of original wood, which corresponds to 55% conversion of polysaccharides. The best yield from SPS was 28% of original wood. According to Tengborg et al. *(35)*, a 60% cellulose conversion was obtained in 48-h hydrolysis of washed steam-pretreated softwood (with SO_2 impregnation) with 10 FPU/g of substrate. Thus, it can be concluded that the hydrolysis yield obtained by wet oxidation pretreatment was only slightly lower than the corresponding yields obtained in the steam pretreatment process. Future optimizations of this method would expectedly lead to further improvements.

Conclusion

Wet oxidation seems to offer an attractive alternative to the more thoroughly investigated methods (e.g., dilute-acid hydrolysis and steam pretreatment). The optimal wet oxidation conditions for enzymatic hydrolysis

were 10 min at 200°C with the addition of 12 bars of oxygen. These conditions ensured a high yield in the enzymatic hydrolysis as well as rapid filtration after the pretreatment. The lignin fraction of substrate remained mainly undissolved, and thus available for energy production by combustion.

Previously, it was proposed that this method would be especially suitable for the removal of lignin during pretreatment, but according to the present study, the improvement was owing to removal of hemicellulose. Thus, the role of residual hemicellulose was clearly important. By adding enzymes hydrolyzing the residual hemicellulose in the hydrolysis, an increased yield of fermentable sugars could be obtained. The total recovery of carbohydrates was high, and improvement of the enzymatic hydrolysis of both cellulose and hemicellulose offers a challenge for future work.

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