ETHANOL PRODUCTION FROM THE MIXTURE OF HEMICELLULOSE PREHYDROLYSATE AND PAPER SLUDGE

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Much of the hemicellulose fraction of pulp mill feedstock is released into black liquor during the pulping process, and it is combusted to recover chemicals and energy in the form of steam and electricity. It is technically feasible to recover this fraction of carbohydrates and convert it into value-added products. In this study, a portion of the hemicellulose in pulp feed was hydrolyzed to soluble sugars by hot-water treatment. The sugars (mixtures of pentose, hexose, and their oligomers) were then converted to ethanol by simultaneous saccharification and fermentation (SSF) employing pectinase and the ethanologenic microorganism, *Saccharomyces cerevisiae*. The prehydrolysate produced from wood also contained toxins, primarily lignin and sugar degradation products, which strongly inhibited the microbial and the enzymatic reactions. Detoxification of the prehydrolysates was achieved by over-liming (addition of excess CaO). The total sugar concentration in the prehydrolysate obtained from softwood was below 4 wt%, which is roughly equivalent to 2 wt% ethanol, far below the acceptable level for downstream processing. In our previous study (Kang *et al*. 2010), a certain amount of water is added to attain fluidity required for SSF operation. In this study, prehydrolysate, in place of water, was added into the bioreactor along with the sludge. The proposed scheme has proven that total sugar concentration as well as product concentration in the bioreactor can be significantly increased above that of the sludge-alone operation.

Keywords: Prehydrolysate; Softwood; Paper mill sludge; Ethanol; Simultaneous saccharification and fermentation

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INTRODUCTION

While the commercialization of cellulosic ethanol production is still emerging from the cradle, the pulp and paper industry in the United States is struggling because of shrinking paper demand in the domestic market and increased influx of foreign paper products. This industry is in dire need of ways to improve its competitiveness. An integrated bio-refinery, which can extract a part of the hemicellulose while preserving cellulose for pulp production, has been proposed as a feasible process scheme (van Heiningen 2006). If it is done properly, such a process can improve the economics without disrupting the main process of pulp and paper making.

In the United States, 80% of the total pulping is done by chemical pulping, of which 98% is kraft pulping, with the remaining 2% being sulfite pulping (American Forest & Paper Association 2002). Much of the hemicellulose fraction of pulp mill feedstock is released into black liquor during the kraft pulping process (Huang *et al.*

2010). It is burned to recover pulping chemicals and to generate steam and electricity. Once the hemicellulose is extracted and mixed into the black liquor, it is extremely difficult to separate the hemicellulose sugars from the mixture. If the hemicellulose is to be utilized, it needs to be recovered prior to pulping. Since the heating value of hemicellulose (13.6 MJ/kg) is only half that of lignin (27 MJ/kg) (Sjostrom 1993) and the quantity of hemicellulose in black liquor is much lower than lignin, the loss of heat recovery from black liquor due to pre-extraction is rather insignificant. It has been proven to be technically feasible to recover part of the hemicellulose before pulping. Hemicellulose can be selectively converted to soluble sugars by treatment with hot water (Yoon *et al.* 2008, 2010a), dilute sulfuric acid (Springer and Harris 1982), or mild alkaline hydrolysis (Al-dajani and Tschirner 2008). Hot water extraction is attractive from an economic standpoint because the process is simple and does not require reagents. The sugars produced from prehydrolysis contain mixtures of pentose, hexose, and their oligomers. Such prehydrolysate can be converted to ethanol through further hydrolysis and downstream fermentation (Walton *et al.* 2010a). Production of other value-added chemicals from hemicellulose prehydrolysate has also been promoted, such as lactic acid (Walton *et al.* 2010b) and hydrogels (Lindblad *et al.* 2001). Oligosaccharides of hemicellulose sugars are a potential source for other types of value-added products including animal feed additives (Davis *et al.* 2002; Fernandez *et al.* 2002).

The major part of softwood hemicellulose is an *O*-acetyl-galactoglucomannan (15 to 25% of the wood), which is beta-1,4-linked mannose and glucose residues in the ratio 3:1, often with alpha-1,6-galactose as a side group (Timell 1967; Meier H. 1961; Katz 1965). Because softwood hydrolysates contain much more mannose than xylose, the xylose-fermenting organism is not required in the bioconversion process. For fermentation of softwood hydrolysates, *S. cerevisiae* has a distinct advantage over ethanologenic bacteria, since *S. cerevisiae* has higher tolerance to toxins and to ethanol. *Saccharomyces cerevisiae* was therefore used in this work to convert the hexoses in the prehydrolysate into ethanol. The prehydrolysate produced from lignocellulosic biomass contains toxic components such as furan, organic acids, and various phenolic compounds that inhibit the fermentation process (Klinke *et al.* 2004; Palmqvist and Hahn-Hägerdal 2000a; Palmqvist and Hahn-Hägerdal 2000b) as well as the enzymatic hydrolysis (Kothari and Lee 2011). The prehydrolysate therefore needs to be detoxified for efficient bioconversion. Several detoxification processes have been attempted for removal of the inhibitory components from wood hydrolysates with varying degree of success. The methods include over-liming (Martinez *et al.* 2001), treatments with ammonium hydroxide (Alriksson *et al.* 2005), ion exchange resins (Fein *et al.* 1984), active charcoal (Roberto *et al.* 1991), enzymatic detoxification (Jönsson *et al.* 1998), extraction (Fein *et al*. 1984), and adsorption (Roberto *et al.* 1991). Identification of a detoxification method suitable for pine wood prehydrolysate was an important part of this investigation.

It is essential that the proposed partial pre-extraction is done in such a way that it does not affect the quality or the yield of pulp. Yoon *et al.* (2010a) proved that it can be done if the hemicellulose removal is limited to a certain level. The pre-extraction by hot water brings the concentration of sugars below 4 wt%, which is roughly equivalent to 2 wt% ethanol, far below the level acceptable as distillation feed. In our previous study, the sludge was diluted with water to attain the fluidity required to operate the fermentation

(Kang *et al.* 2010). If the solid feedstock (sludge) is mixed with prehydrolysate, in place of water, then the end-product concentration is increased. In this study, a new scheme of bioconversion process was explored whereby two different feed streams available from pulp mills are utilized simultaneously. The primary objective of this investigation is to determine whether such a process is technically feasible and to assess the potential benefits of using two feed streams.

EXPERIMENTAL

Materials

Feedstock

Southern pine chips were obtained from Rock-Tenn Company in Demopolis, Alabama. Chips with major defects including bark, knots, and decayed parts were removed prior to screening on a CHIP CLASS™ laboratory screen equipped with a stack from top to bottom of 45-mm round screens, 8-mm bar screens, 6-mm bar screens, and 4-mm round screens. The wood fraction passing 45-mm round screens and 8-mm bar screens and retained on 6-mm bar screens was collected, well mixed, and air-dried before use.

The paper primary sludge (PS) was collected from the primary wastewater clarifier unit of a Kraft paper mill, Boise Paper Company (Jackson, AL, USA). The sludge was washed with tap water three times to remove undesirable odors and possible toxic chemicals, and further thickened to 38% consistency using a vacuum filter, and stored at 4ºC.

The primary sludge and southern pine chips were analyzed for carbohydrates, moisture content, and ash content according to the National Renewable Energy Laboratory (NREL) standard procedure (NREL 2008). Primary sludge was analyzed to contain 44.5% glucan, 9.9% xylan, 8.1% lignin, 1.5% others (mannan, arabinan, acetyl group, and other unknown chemicals) and 36.0 wt. % ash, which includes 26.0% acid-soluble ash and 10.0% acid-insoluble ash. Southern pine chip was analyzed to contain 40.6 % glucan, 7.8% xylan, 2.2% galactan, 1.5% arabinan, 8.8% manna, 32.8% lignin and 0.32 wt. % ash, and 5.7% others (protein, acetyl group and other unknown chemicals).

Enzymes

Cellulase (Spezyme CP, Lot No. 301-00348-257, 59 FPU/mL, 123 mg protein/mL), xylanase (Multifect Xylanase, Lot No. 301-04021-015; 42 mg protein/mL), and pectinase (Multifect Pectinase PE, Lot No. A21-03356-001, 82 mg protein/mL), along with their protein content/activities were generously provided by Genencor Division of Danisco US, Inc. (Palo Alto, CA, USA). β-Glucosidase (Novozyme188, Cat. No. C-6150, 665 CBU/mL, 140 mg protein/mL) was purchased from Sigma (St. Louis, MO, USA). The activities in commercial enzyme preparations are presented in Table 1, which was based on the publication by Dien *et al.* (2008) and Berlin *et al.* (2007).

Table 1. Enzyme Activities Detected in Commercial Enzyme Preparations

Activity (units/mg)	Spezyme CP	Novozyme 188	Multifect Xylanase	Multifect Pectinase PE
Cellulase (FPU) *	0.47	0.06	0.02	0.05
β-Glucosidase	1.04	4.75	0.85	4.22
Xylanase (OSX) **	21.32	0.88	600.07	20.29
α-Arbinofuransoidase	0.18	0.21	0.22	22.71
β-Xylosidase	0.06	0.12	0.54	2.27
α-Galactosidase	0.00	0.83	0.06	0.39
Feruloyl esterase	0.00	0.00	0.00	0.12
p-Coumaroyl esterase	nm ***	nm ***	0.03	0.26
* Filter paper unit				
** Oat spelt xylan				
*** nm: Not measured.				

Microorganism and Medium

The microorganism used in the SSF was *Saccharomyces cerevisiae* ATCC-200062 (NREL-D5A). The growth medium was YPD broth (Sigma, Y1375).

Pre-Extraction

Hot water treatment has been shown to be effective for extraction of hemicellulose from wood (Werpy and Petersen 2004). Three stage extractions with recycling of liquid were done to increase the sugar content in the extracted liquor and simulate the continuous extraction process. The extractions were conducted using a 500 mL cylindrical stainless steel bomb container placed inside a M/K laboratory digester filled with water as a heat transfer fluid. In every water-extraction stage, 70 grams of oven-dried untreated softwood chips were used in the bomb digester at a liquor-to-wood ratio of 5.8 to 1. The digester temperature was ramped from room temperature to a preset maximum temperature of 170°C. At the end of the digester operation, each bomb was quenched in a cold water bath. Extraction times at the preset extraction temperature (170°C) varied from 0 to 90 min to attain wood weight loss levels ranging from 0 to 15% of initial dry wood. After completion of water extraction, 70% of the total liquor was recovered. The liquor used in the first stage was deionized (DI) water. In the second stage, the recovered liquor was mixed with make-up DI water having 30% volume of the total first stage liquid to keep the total liquid at the same level same as total first stage liquid. The same procedure was applied for the third stage. Fresh chips were used in all stages. As wood chips are extracted, their weight decreases due to components dissolved and diffused from the wood into the extraction liquor (termed as prehydrolysate). The sugar contents of the extract were determined according to methods published by the National Renewable Energy Laboratory (NREL 2008). The total amount of cellulose and hemicelluloses, which led to these sugar concentrations were then calculated using the following equations derived by Yoon and van Heiningen (2010b),

$$\text{Cellulose} = Glu * \left(\frac{162}{180}\right) - \frac{\text{Man}}{\text{b}} * \left(\frac{162}{180}\right) \quad (1)$$

where b = 4.15, the average value for number of mannose units per glucose unit in hemicellulose of pine/spruce wood, as determined by Janson (1974).

$$\text{Hemicellulose} = (Ara + Xyl) * (\frac{132}{150}) + (Gal + Glu + Man) * (\frac{162}{180}) - Cellulose \quad (2)$$

In order to determine the severity of the pre-extraction, the combined effects of time and temperature were measured in terms of the H-factor (H). The H-factor has been defined so that 1 hour at 100 °C is equivalent with an H-factor of 1 (Grace and Malcolm, 1989):

$$H = \int_0^t (\exp (43.19 - \frac{16113}{T}))\, dt \quad (3)$$

where t is reaction time (hour) and T is reaction temperature (^oK).

Composition Analysis of Prehydrolysate

The composition analysis of the extracted liquor was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL 2008). The extracted liquor was centrifuged to separate the solid part from liquid. The liquid portion was put through secondary hydrolysis (incubation with 4% sulfuric acid at 121°C in an autoclave) to convert oligomers to monomers. The liquid was neutralized with $CaCO_3$, and the total amount of sugar monomers were analyzed by HPLC. The concentration of oligosaccharides with a degree of polymerization 2 and larger than 2 was calculated as follows:

$$Hexose\_Oligomer\ (g/L) = \frac{Hexose\_After\_Acid\_Hydrolysis\ (g/L) - Hexose\_Before\_Acid\_Hydrolysis\ (g/L)}{1.11} \quad (4)$$

$$Pentose\_Oligomer\ (g/L) = \frac{Pentose\_After\_Acid\_Hydrolysis\ (g/L) - Pentose\_Before\_Acid\_Hydrolysis\ (g/L)}{1.136} \quad (5)$$

Oligomers of individual sugar components are denoted as: mannose oligomer (Man 2), galalactose oligomer (Gal 2), glucose oligomer (Glu 2), arabinose oligomer (Ara 2), and xylose oligomer (Xyl 2). Hexose here is interpreted as mannose (Man), galactose (Gal), and glucose (Glu). Pentose here is interpreted as arabinose (Ara), and xylose (Xyl).

The concentration of acetyl group linked with carbohydrate was calculated as follows,

$$Acetyl\_Group\ (g/L) = \frac{Acetic\_Acid\_After\_Acid\_Hydrolysis\ (g/L) - Acetic\_Acid\_Before\_Acid\_Hydrolysis\ (g/L)}{1.394} \quad (6)$$

where 1.394 is the ratio of molecular weight of acetic acid to the acetyl group.

Detoxification of Prehydrolysate by Over-liming

Prehydrolysate (100 mL) was held at 60°C in an incubator shaker, into which anhydrous CaO (Fisher Scientific) was added until the pH reached 10.5. After 30 min of incubation, hydrolysate was cooled to room temperature, and pH was readjusted to 5.5 with 72% sulfuric acid. Approximately 80% of the treated hydrolysate was decanted and centrifuged at 5000 RPM for 5 minutes to remove suspended particles before being subjected to fermentation experiments.

Digestibility Test for Prehydrolysate

The enzymatic digestibility of the extracted liquor was determined according to NREL Chemical Analysis and Testing Standard Procedures (NREL 2008). To each 20 mL glass scintillation vial, 0.5 mL 1 M sodium citrate buffer (pH 4.5), 0.1 mL of a 2% sodium azide solution were added to prevent microbial growth during the enzymatic digestion. The extracted liquor was neutralized by calcium carbonate until pH reached 5.0. Each different enzyme was diluted with DI water and added into the vial so that the enzyme loading was 25 mg protein/g-mannose oligomer. Enzyme reaction was carried out in an incubator at 50°C and 150 RPM. The sugar digestibility values for respective sugar oligomers were calculated by the following equation:

$$Sugar\_Digestibility\ (\%) = \frac{Sugar\_released\ (g)}{Total\_Sugar\_added\ (g)} \times 100 \quad (7)$$

Reported digestibility values are the average of duplicate tests, in which the standard deviation (SD) never exceeded 1% in all occasions.

Hydrolysis and Fermentation of Prehydrolysate

The test was performed according to NREL Chemical Analysis and Testing Standard Procedures (NREL 2008). Fermentation of prehydrolysate was done in two different modes: separate hydrolysis and fermentation (SHF) and simultaneous hydrolysis and fermentation (SSF). In the SHF, the treated prehydrolysate was first subjected to enzymatic hydrolysis for 48 h at 50°C and then to fermentation at 32°C. A 20 mL serum bottle was used as the bioreactor. To each serum bottle, 9.3 mL treated or untreated extracted liquor was added. The samples were steam sterilized at 121°C for 15 min. The enzyme loading was kept at 25 mg protein Multifect Pectinase PE /g-mannose oligomer. Our previous study has shown that acid-soluble ash in the paper sludge, such as calcium carbonate, acts as a buffer to stabilize the pH during fermentation (Kang *et al*. 2010). Calcium carbonate was, therefore, used in the SSF of softwood prehydrolysate. Fermentation was carried out under two different conditions: 0.5 mL 1M sodium citrate buffer (pH 4.5) for SHF, and $CaCO_3$ at the level of 0.05 g/10mL for SSF. To each serum bottle, 0.1 mL of yeast solution was added to the hydrolysate to make the initial yeast concentration of each sample 50 mg dry cell/L. The serum bottles were then sealed, vented, placed in an incubator shaker at 36 °C with 150 RPM for bioreaction. Hydrolyzate samples were taken at 6, 12, 24, and 48 h and analyzed for sugars and ethanol. In all of the microbial experiments, a sample from each flask was taken at the end of the run and streaked on a yeast extract peptone dextrose (YPD) plate and under an

optical microscope to check for contamination. Colony Forming Units (CFU) tests were also done over YPD agar plates to check microorganism viability. The ethanol yield was calculated as follows:

$$\text{Ethanol yield [\% of theoretical maximum]} = \frac{\text{Ethanol produced (g) in reactor}}{\text{Initial Total Sugar (g) in reactor} \times 0.511} \times 100 \quad (8)$$

Sugar denotes hexoses in the reactor.

Reported digestibility values are the average of duplicate tests in which the relative standard deviation was less than 1%.

Simultaneous Saccharification and Fermentation (SSF) of Primary Sludge with and without Prehydrolysate

The SSF test of the sludge was performed according to NREL Chemical Analysis and Testing Standard Procedures (NREL 2008). Screw-capped 250 mL Erlenmeyer flasks were used as the reactor. The sludge samples were mixed with DI water or prehydrolysate to reach total working volume of 100 mL. Sludge loading was adjusted such that the solid content in the reactor was 15% (w/v, dry basis). Samples were steam sterilized at 121ºC for 15 min. The SSF was operated in an incubator at 37ºC and 150 RPM. The SSF was carried out 37°C since it is a compromise between the optimal temperatures for the enzymes and the yeast (Olofsson *et al.* 2008). The growth medium in the SSF was YP medium. The SSF of the sludges was carried out without pH control. Cellulase and β-glucosidase loadings were 15 FPU and 30 CBU/g-glucan, respectively. For the SSF of primary sludge supplemented with prehydrolysate, Multifect Pectinase FE was also added at the level of 25 mg protein/g-mannose oligomer. For comparison, the same amount of Multifect Pectinase FE was added for the SSF of paper primary sludge without prehydrolysate. Optical density (OD) was measured by UV Spectrophotometer (BioTek Synergy HT Multidetection Microplate Readers) at 600 nm for measurement of cell concentration (yeast, NREL-D_5A). The initial cell concentration after inoculation was 50 mg/L.

Analytical Methods

The solid samples were analyzed for carbohydrates, acid insoluble lignin (AIL), and acid soluble lignin (ASL) following the NREL standard procedures (NREL 2008). Sugars were determined by HPLC using a BioRad Aminex HPX 87P column. Ethanol, HMF, levulinic acid, acetic acid, and furfural were determined by HPLC using a BioRad-HPX-87H column. A refractive index detector was used with the HPLC. The acid-insoluble ash was determined following the TAPPI test method (TAPPI, Test Method T 244 cm-99). Liquid sample analysis and ash determination were done in triplicates. The total phenolics in the prehydrolysate before and after treatment were determined using the Folin-Ciocalteu method as described by Kujala *et al.* (2000). Where applicable, statistical analysis including mean value and standard deviation was done using Microsoft Office Excel 2003.

RESULTS and DISCUSSION

Water Pre-extraction of Softwood

Southern pine wood chips were treated with hot water under the conditions reported to be effective for selective removal of hemicellulose from wood (160 to 180°C) (Springer and Harris 1982). The weight loss in biomass due to loss of hemicellulose and other extraneous components increased with reaction time from 4% under the mildest extraction condition (0 min, 115 H-factor hours) to about 15% under the most severe condition (80 min, 1356 H-factor hours) (Table 2). The amount of hemicellulose extracted increased with H-factor up to about 11%. The cellulose fraction, however, remained intact even at high H-factor of 1300 h. This proves that all of the sugars in the prehydrolysate came from hemicellulose. We note that the pre-extraction in this work was to be done under the condition that the quantity of the cellulose fiber is not reduced and the quality of the pulp coming afterwards is not affected. Data in Table 2 proves that hot water treatment indeed satisfies the first condition if we choose proper operating conditions: 66 minutes, 170°C, with corresponding H-factor of 1124. To increase the sugar content in the extracted liquor and to simulate a counter-current continuous extraction, second and third stage extraction was done feeding the output extractant liquid repeatedly to the digester containing fresh biomass. The reaction and operating conditions were identical for all stages.

Table 2. Sugar Content of First Stage Prehydrolysate at Different Time Periods

Water Extraction at 170 °C			Soluble Sugars (Present as Monomer + Oligomer) in Prehydrolysate (g/L)					Polysaccharides Loss from Pine Chips (% dry basis)	
Time of Water Extraction (min)	H Factor	Wt. Loss (%)	Glu	Xyl	Gal	Ara	Man	Cellulose	Hemicellulose
0	115	4.24	0.33	0.74	0.91	0.53	1.81	0.00	2.24
11	277	5.83	0.56	1.20	1.18	0.69	2.39	0.00	3.12
25	483	8.57	1.07	2.23	1.79	1.05	4.47	0.00	5.50
45	839	11.64	1.60	3.13	2.53	1.48	6.69	0.00	8.00
66	1124	14.00	1.79	3.55	2.81	1.57	9.60	0.00	10.03
80	1356	14.94	2.40	4.05	3.11	1.83	9.71	0.03	10.91
All data in table are the mean value of duplicate (n = 2; standard deviation < 0.5).									

Composition of Softwood Prehydrolysate

During the pre-extraction process, the hydronium ion from water ionization causes cleavage of the acetyl group, which in turn forms acetic acid that acts as a catalyst for the hydrolysis of glycosidic bonds in hemicellulose (autocatalysis). The hemicellulose is first partially hydrolyzed to oligosaccharides and then into monomers. Hemicellulose sugars can degrade further into furfural, hydroxymethylfurfural (HMF), and organic acids including levulinic acid.

Since 70% of liquor from the first stage was used in the second and third stage, the sugars and organic compounds accumulated with repeated extraction, raising their concentration (Table 3 and 4). However, the pH in the pre-hydrolyzate stayed relatively

constant at 3.50, 3.43, and 3.40 for the respective stages although the acetic acid concentration increased substantially. This was due to the fact that inorganic salts that dissolved out from pine wood acted as a buffer. In addition, the sugar oligomers released during pre-extraction of pine wood were acetylated.

Pre-extraction converted a large fraction of hemicellulose (10% based on dry wood or 48.5% of initial hemicellulose) to oligomers and monomers. Data in Table 3 indicate that most of the dissolved sugars were present as oligomers. The proportion of oligomers in the prehydrolysate varied greatly depending on the species of sugar. The oligomer/monomer ratio was higher for hexose than pentose, which is in line with a previous report of BeMiller (1967) that hexose is more recalcitrant to acid-catalyzed degradation. The arabinose substituent in the arabinoglucuronxylan, which is extremely labile to acid hydrolysis, was almost completely depolymerized during pre-extraction.

Table 3. Sugar Content in Three Stage Prehydrolysates

	Monomeric Sugars in Prehydrolysate (g/L)					Oligomeric Sugars in Prehydrolysate (g/L)					Total Sugars (g/L)
	Glu	Xyl	Gal	Ara	Man	Glu2	Xyl2	Gal2	Ara2	Man2	
First Stage	0.40	1.14	0.73	1.46	0.55	1.60	2.46	2.07	0.27	8.15	19.32
Second Stage	1.07	2.65	1.59	2.27	1.60	2.88	3.02	3.60	0.33	12.97	31.99
Third Stage	2.41	3.76	2.61	3.54	3.20	2.97	2.65	3.93	0.10	15.53	40.70
All data in table are the mean value of duplicate (n = 2; standard deviation < 0.5).											

Furfural and HMF are produced during the hemicellulose extraction and acid hydrolysis, under acidic and high temperature conditions (Nguyen *et al*. 1998). An observed increase of HMF and furfural (Table 4) indicates that hexose and pentose encounter significant degradation with additional stages of extraction. The degradation is prompted by increased hydroxyl ion at pH of 3.5, prevalent with hot water pre-hydrolysis. The hydroxyl ion is known to be a potent catalyst for sugar degradation. On the other hand, glycosidic linkages, such as those in the hemicellulose oligomer, are rather stable to hydroxyl ions, but were easily cleaved in the presence of hydrogen ions (BeMiller 1967). The proportion of oligomers for all sugars decreased with the pH in the prehydrolysate in all three stages. Obviously the rate of hydrolysis of the glycosidic linkages increases with the solution acidity.

Table 4. Organic Compounds Content of Three Stage Prehydrolysates

(g/L)	Acetic acid	Acetyl group	Levulinic acid	HMF	Furfural
First Stage	0.75	0.25	0.07	0.20	0.37
Second Stage	1.58	0.37	0.12	0.57	0.98
Third Stage	2.61	0.48	0.20	1.02	1.41
All data in table are the mean value of duplicate (n = 2; standard deviation < 0.5).					

Covalent linkages between lignin and hemicellulose exist in native wood (Erikkson and Lindgren 1977). These structures are commonly referred to as lignin-carbohydrate complexes (LCC). Lignin is covalently bound to hemicellulose, which in turn, is bound to cellulose through extensive hydrogen bonding. Lignin–carbohydrate bonds and some inter-unit lignin bonds, derived mainly from the benzyl alkyl ether type, may be cleaved during water pre-hydrolysis. We observed that part of the dissolved lignin precipitated as very fine particles during the cooling of the prehydrolysate. The precipitate was separated from the prehydrolysate by centrifugation and it is denoted as solid fraction in the composition table. The compositions of the solid fraction (Table 5) contained about 6% (w/w) carbohydrate and 85% lignin, which show that this part of lignin compounds was released in LCC form.

Table 5. Composition of Solid Particles Fraction in Three Stage Prehydrolysates

	Total Solid Fraction Conc.(g/L)	Glu (Wt%)	Xyl (Wt%)	Gal (Wt%)	Ara (Wt%)	Man (Wt%)	AIL (Wt%)	ASL (Wt%)
First Stage	5.5	2.0	2.1	0.3	0.2	1.8	84.7	2.0
Second Stage	4.6	1.8	2.0	0.4	0.2	2.0	85.1	2.4
Third Stage	4.2	2.1	2.4	0.4	0.2	2.1	84.5	2.7
All data in table are the mean value of duplicate (n = 2; standard deviation < 0.5).								

When the bulk liquid is separated from the treated biomass, soluble lignin compounds are also removed. Pre-extraction could facilitate subsequent alkaline delignification because the partial hydrolytic removal of lignin and cleavage of alkali-stable carbohydrate–lignin bonds increase the accessibility of the cooking liquor (Sixta 2006).

Enzymatic Hydrolysis of Softwood Prehydrolysate

Secondary hydrolysis of softwood prehydrolysate to monosaccharides can be accomplished by enzymatic or diluted-acid hydrolysis. Dilute acid hydrolysis, however, produces toxins that negatively affect biocatalyst growth and metabolism (Klinke *et al.* 2004). Enzymatic hydrolysis was therefore adopted in this work. Various commercial enzymes were tested, applying uniform protein-based enzyme loading (25 mg protein/g mannose oligomer) to evaluate their abilities to hydrolyze the softwood prehydrolysates. The results are shown in Fig. 1.

The best overall performance was shown by Pectinase PE, as it gave the highest improvement in the yield of all sugars except xylose. Multifect xylanase has shown second best overall performance, and it gave the highest yield of xylose, which is not a surprise. Spezyme-CP and Novozyme-188 have shown low overall performance for the hemicellulose sugars in the prehydrolysates. This agrees with the findings of Berlin *et al.* that Multifect Pectinase FE has relatively high specific activity of mannanase (3.0 U/mg protein), compared to Multifect Xylanase, Spezyme CP, or Novozyme- 188 (Berlin *et al*. 2007). Data in Table 1 reaffirm that Multifect Pectinase FE contains relatively high specific activity of β-Glucosidase, Xylanase, β-Xylosidase, and α-Galactosidase. Similar to the cellulolytic enzymes, synergistic action also occurs with mannan structure. Due to the complexity of galactoglucomannan structure, both main and side-chain cleaving

enzymes are required to hydrolyze these types of oligomers (Filho 1998). For instance, β-Glucosidase, an exo-type enzyme, hydrolyzes 1,4-β-D-glucopyranose at the non-reducing end of the oligosaccharides released from glucomannan and galacto-glucomannan by β-mannanase. A side-chain cleaving enzyme, α-Galactosidase, hydrolyzes α-1, 6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan (Moreira and Filho 2008).

The highest galactose yield (above 70%) achieved by Multifect Pectinase FE is largely due to the synergistic action of various enzymes. Even though Novozyme-188 has the highest α-Galactosidase specific activity among four enzymes, only 50% galactose yield was attained. Clarke *et al.* also found that the galactose release from softwood pulp is enhanced by the presence of mannanase in combination with α-galactosidase (Clarke *et al.* 2000). Over 60% xylose yield was achieved, even though Multifect Pectinase FE has slightly lower specific activity of xylanase than that of Spezyme CP, and 30 times lower than that of Multifect Xylanase. However, Multifect Pectinase FE contains the highest β-xylosidase activity. Kumar and Wyman (2009) reported that supplementation of β-xylosidase improved glucose release during hydrolysis by decreasing the accumulation of xylose oligomers, which inhibit cellulase activity.

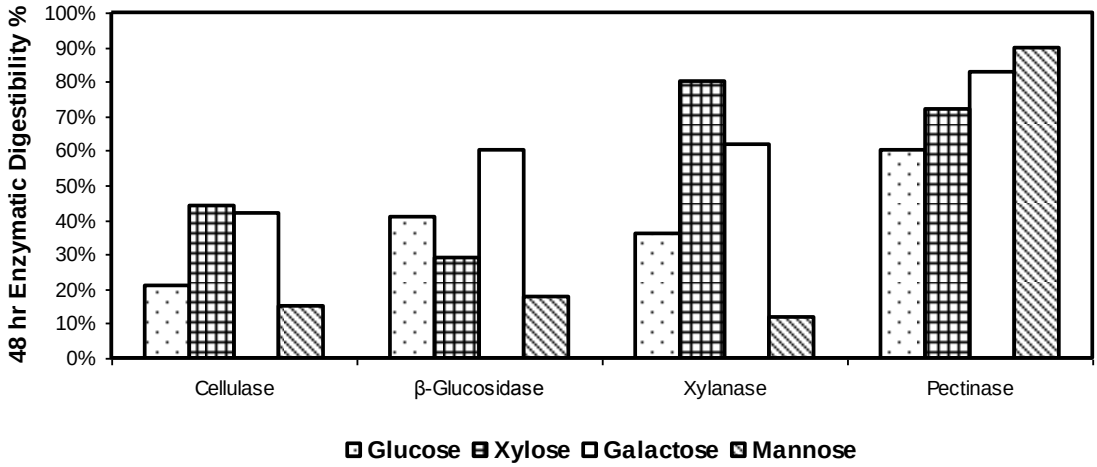

Fig. 1. Different commercial enzyme preparation undergo hydrolysis of softwood prehydrolysate The data represent the average of duplicate runs. The enzyme loading is 25 mg protein/g-mannose oligomer.

Detoxification and SHF of Softwood Prehydrolysate

Detoxification of lignocellulosic hydrolysates by treatment with CaO or $Ca(OH)_2$ to increase pH to 10 (over-liming) followed by pH readjustment to 5.5 with acid, was reported to remove certain inhibitory compounds generated in the hydrolysis process (Larsson *et al*. 1999). A similar procedure was applied in this study; addition of CaO at the level of 10 g /L followed by pH readjustment to 5.5 with 72% sulfuric acid at 60 °C for 30 minutes. Concentration of organic acids was not affected by over-liming. Phenolic compounds, HMF and furfural were partially removed. Slight decreases in concentrations

of total sugars (8% to 12%) and fermentable sugars (glucose and mannose, 6% to 9%) were observed, perhaps due to sugar degradation under high alkaline condition.

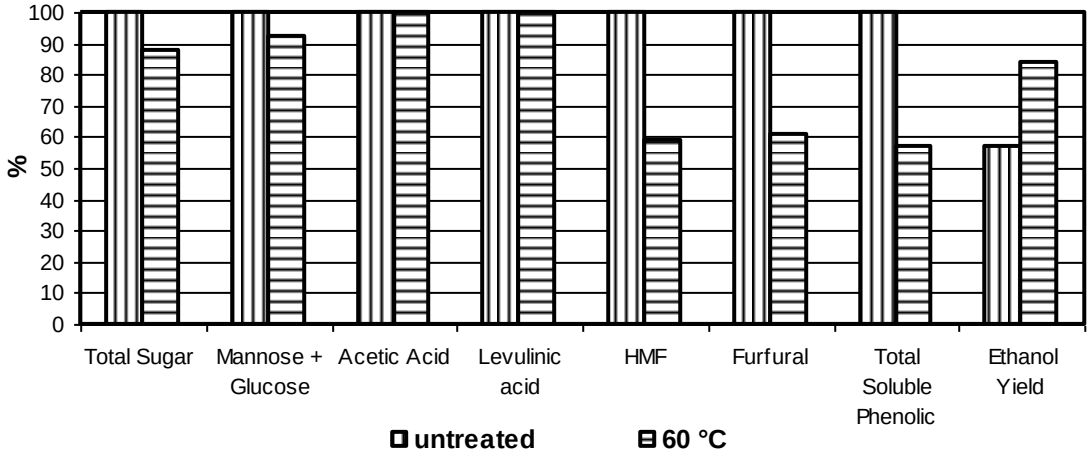

Fig. 2. Composition change after detoxification. The data represent the average of duplicate runs and are expressed as percentage of the concentration of compounds after detoxification.

The treated prehydrolysate was first subjected to enzymatic hydrolysis using Multifect Pectinase FE for 48 h at 50°C, then to fermentation at 32°C. The overall performance and the time-course data of the SHF are presented in Fig. 3. The concentration of xylose, galactose, and arabinose remained unchanged during the fermentation (data not shown). Although galactose is also a hexose, it remained unchanged during the fermentation. It has been reported that the flux through the galactose utilization pathway in *Saccharomyces cerevisiae* is only one-third the rate of glucose utilization (Ostergaard *et al.* 2000). Therefore only the uptake of glucose and mannose was accounted for in calculation of the ethanol yield.

Efficient ethanol production was indeed observed from fermentation of the prehydrolysate after detoxification. The ethanol yields based on initial fermentable sugars (glucose and mannose) were 85.0% at 48 h, a phenomenal improvement from 56.9% ethanol yield for untreated prehydrolysate. The ethanol concentration at 96 h increased from 6.0 to 10.6 g/L. It is noteworthy that the sugar concentration also increased after detoxification. The glucose concentrations at 48 h rose from 4.1 to 4.4 g/L after detoxification. The mannose concentration also rose from 10.8 to 11.5 g/L. The hydrolysis yields of sugars have also increased after detoxification for various enzyme systems including Multifect Xylanase, Multifect Pectinase FE, Spezyme CP, and Novozyme-188 (data not shown here). Apparently, the toxins in the prehydrolysate are also inhibitory to these enzymes.

During fermentation, glucose was rapidly consumed within 12 h even for untreated prehydrolysate. However, mannose consumption was much slower for untreated hydrolysate requiring 24 h for complete consumption. The sugar consumption was of diauxic pattern, glucose preferentially consumed over mannose, which agrees with Smith *et al.* (1997).

It is unlikely that the inhibition of ethanol fermentation is from furfural or HMF, because their concentration after detoxification was less than 1 g/L and 0.6 g/L, respectively, which are far below the reported toxicity threshold level in the fermentation of lignocellulosic hydrolysate (Tu *et al.* 2009; Taherzadeh *et al.* 1997). Furthermore, *Saccharomyces cerevisiae* can convert furfural and HMF to their corresponding alcohols via NADH-dependent alcohol dehydrogenase (Palmqvist *et al.* 1999). Although different conditions were used in this study, we also observed that after 24 h of fermentation, furfural and HMF were completely consumed by the yeast.

The organic acids existing in the prehydrolysates (acetic, levulinic, and formic acids) are a significant factor inhibiting the yeast fermentation and suppressing cell growth, as well as ethanol yields (Larsson *et al.* 1999). The concentration of organic acids, however, was not reduced after detoxification. Van Zyl *et al*. (1991) reported that the degree of inhibition caused by acetic acid depends not only on its concentration, but also on the pH of the medium. When the medium pH is low, acetic acid (pK_a = 4.75 at 25ºC), formic acid (pK_a = 3.75 at 20°C), and levulinic acid (pK_a = 4.66 at 25°C) exist in undissociated form; thus they can easily diffuse into the cell through the plasma membrane. Inside the cell, they dissociate due to higher intracellular pH, eventually decreasing the cytosolic pH (Pampulha *et al*. 1989). The reduced internal pH strongly inhibits cell activity, and even causes cell death. According to the Henderson–Hasselbalch equation, the concentration of the undissociated form of organic acid has an inverse relationship with pH value. The concentration of the undissociated form of organic acid decreases with increasing pH. Therefore, the inhibition of organic acids is less at higher pH. However, pH above 7 is unfavorable for ethanol production. From this reasoning, we have determined that pH of 5.5 is near the optimum for fermentation and SSF operation.

Since we eliminated furfural and HMF as inhibitors, phenolic compounds originated from lignin are believed to be the major inhibitors in the hydrolysate. Phenolic compounds are known to increase biological membrane fluidity and cause loss of cellular integrity, thereby affecting its role in selective barriers and enzyme matrices (Heipieper *et al.* 1991, 1994).

SSF of Softwood Prehydrolysate

SSF of prehydrolysate after detoxification was also investigated in this study. In the SSF, enzymatic hydrolysis and fermentation are carried out concurrently. The profile of ethanol production in the SSF is shown in Fig. 4. The overall trends for sugar consumption and ethanol production in the SSF of softwood prehydrolysate were similar to those of the SHF. In the SHF, ethanol was produced at a concentration of 10.5 g/L in 72 h of total processing time (48 h hydrolysis and 24 h fermentation) giving 84.6% of the yield. The similar ethanol yield of 82.6% and the ethanol concentration of 10.3 g/L were attained when the process was carried out under SSF mode, but it was achieved within 48 h, shorter than SHF by 24 h. SSF process can greatly reduce the product inhibition to the enzymes and enhance reaction rate. However, the temperature of the hydrolysis and fermentation is suboptimal in the combined process-SSF (Olofsson *et al.* 2008).

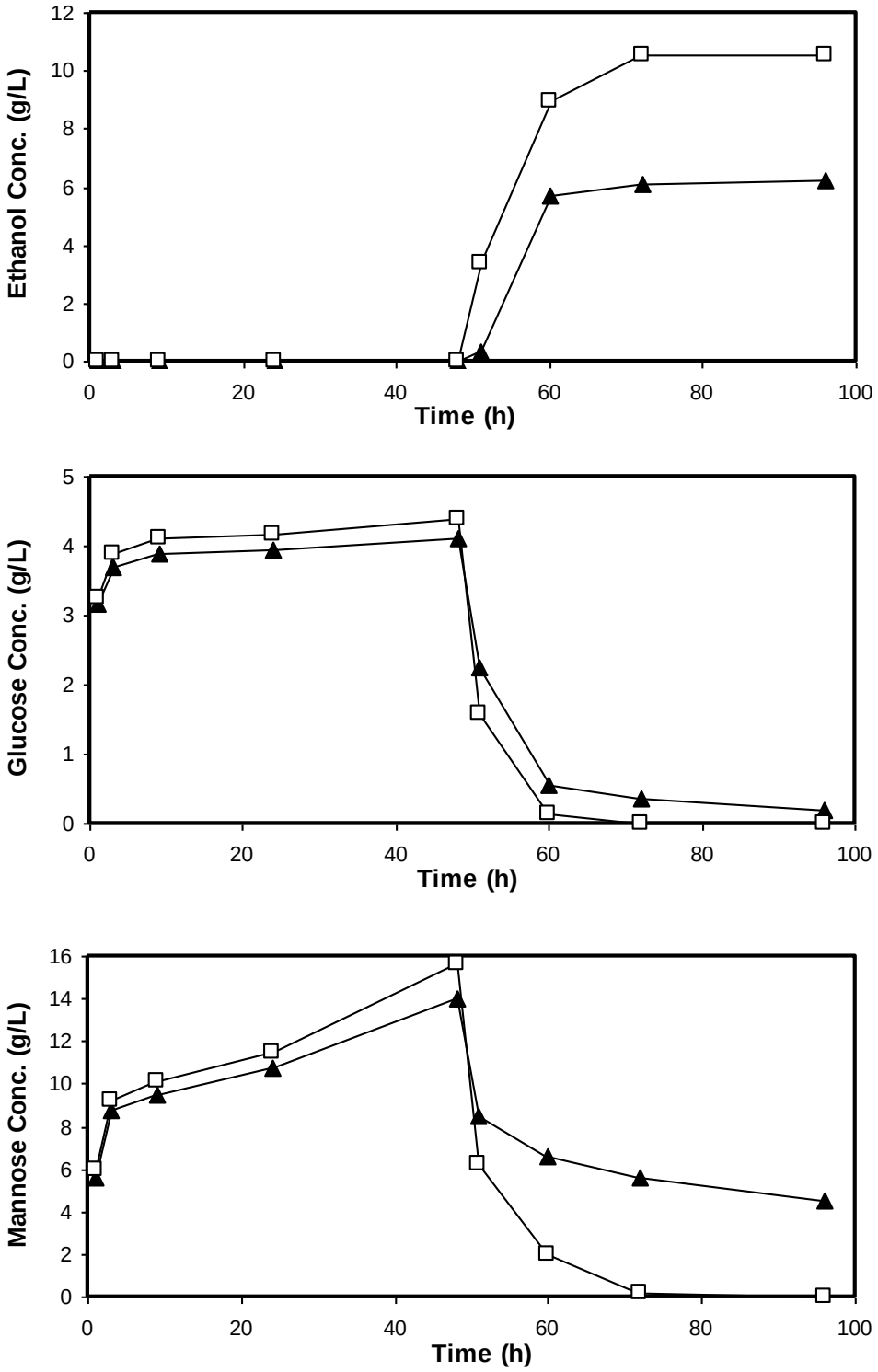

Fig. 3. Separate hydrolysis and fermentation (SHF) of prehydrolysate by multifect pectinase FE and *Saccharomyces cerevisiae* (ATCC-200062). Squares represent prehydrolysate after 60°C detoxification treatment. Filled triangles represent untreated prehydrolysate. The data points represent average of duplicate runs.

SSF of Prehydrolysate Supplemented with Paper Sludge

In the SHF or SSF process of the prehydrolysate, the terminal ethanol concentration was only slightly above 1%, which is far below the level acceptable as a distillation feed. The concentration of ethanol in the bioreactor significantly affects the cost of the downstream separation process. Use of the prehydrolysate and pulp mill sludges mixture as the fermentation feed can increase the product concentration. In our previous study, we demonstrated that the ethanol production from SSF using Spezyme CP and *S. cerevisiae* (D_5A) can attain 75.1% ethanol yield and 25.5 g/L of concentration from untreated Kraft paper sludges (Kang *et al.* 2010). SSF of prehydrolysate supplemented with paper sludge was performed in this work. The results are shown in Fig. 4. The bioreaction was started with a total sludge loading of 150 g/L, which is equivalent to 66.75 g-glucan/L. With the use of the mixed feedstock, the ethanol concentration was significantly increased from the single feedstock runs. The improvements were: from 10.3 g/L (prehydrolysate only) or 24.9 g/L (paper sludge only) to 31.0 g/L (mixed feedstock). The ethanol concentration can be further increased if one applies multiple feeding of the sludge in the mixed-feed SSF (a process known as fed-batch operation). We noticed that the ethanol yield from the SSF of the mixture (71.5% of the theoretical maximum) was lower than that of the paper sludge (73.2%) or the prehydrolysate (82.6%). The main reason appears to be inhibition on the cellulase enzyme by ethanol (Wu and Lee 1997), phenolic compounds (Ximenes *et al.* 2010), furans (Hodge *et al.* 2008), sugar monomers, and oligomers (Xiao *et al.* 2004; Nigam and Prabhu 1991; Kumar and Wyman 2009).

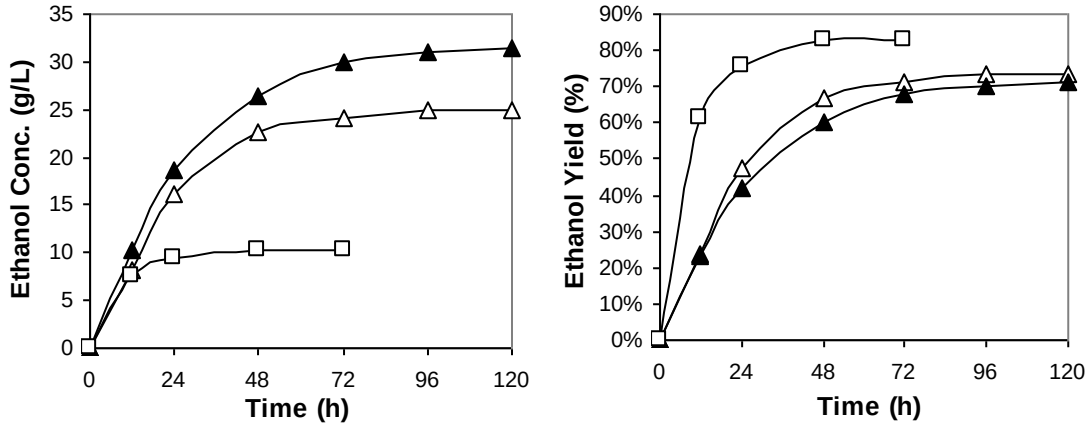

Fig. 4. Simultaneous saccharification and fermentation (SSF) of prehydrolysate, paper sludge, and the mixture of prehydrolysate and paper sludge by *Saccharomyces cerevisiae* (ATCC-200062), and Multifect Pectinase FE. Filled triangles represent paper sludge with prehydrolysate. Triangles represent paper sludge only. Squares represent prehydrolysate only. The data represent average of duplicate runs. The conditions of the SSF of paper sludge and paper sludge with prehydrolysate were: 15% (w/v, dry basis) solid loading, 15 FPU Spezyme CP + 30 CBU of Novozyme-188/g-glucan + 25 mg protein Multifect Pectinase PE /g-mannose oligomer. The conditions of the SSF of prehydrolysate were: 25 mg protein Multifect Pectinase PE /g-mannose oligomer.

CONCLUSIONS

1. With hot water treatment, a part of the hemicellulose sugars in southern pine can be selectively recovered without degrading its glucan content. The recovered hemicellulose sugar mixtures (prehydrolysate) can be converted to ethanol through sequential processing of detoxification, enzymatic hydrolysis, and fermentation.
2. The prehydrolysates contain a large amount of mannose oligomers. The Multifect Pectinase FE product was found to be most efficient in the conversion of the oligomers monomers.
3. Detoxification by over-liming improved the enzymatic hydrolysis as well as the microbial ethanol conversion of the prehydrolysates, attaining 85.0% yield of the theoretical maximum. In the SHF of the prehydrolysate, the ethanol concentration of 10.51 g/L and the yield of 84.6% were obtained after 72 h of total processing (48 h of hydrolysis plus 24 h of fermentation). The same bioprocessing under the SSF reduced the processing time to 48 h.
4. Use of mixed feed, prehydrolysate, and kraft pulp mill sludge in the SSF increased the ethanol concentration to 31.5 g/L. The ethanol yield from the mixed-feed SSF was 71.5%.

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