EFFECT OF LIGNIN ON ENZYMATIC SACCHARIFICATION OF HARDWOOD AFTER GREEN LIQUOR AND SULFURIC ACID PRETREATMENTS

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Red maple, sweet gum, trembling aspen, red alder, and *Eucalyptus globulus* samples were pretreated with dilute sulfuric acid and green liquor before enzymatic saccharification. Substrates showed different levels of delignification and sugar recovery, depending on the applied pretreatments and the syringaldehyde/vanillin ratio (S/V). Three major conclusions were drawn in this research. First, lignin is the greatest contributor to recalcitrance of hardwood to enzymatic saccharification. Second, a high S/V ratio is a useful indicator of high delignification during a pretreatment process. Third, green liquor pretreatment is a promising pretreatment method because of a high delignification degree and sugar recovery. In addition, xylan also contributes to the recalcitrance of hardwoods toward enzymatic saccharification.

Keywords: Fermentable sugar; Hardwood; Green liquor; Pretreatment; Enzymatic hydrolysis

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INTRODUCTION

Due to concerns of environment protection and energy safety, efforts are being exerted regarding the conversion of lignocellulosic biomass to bio-ethanol. The bio-energy produced from renewable sources can minimize the demand for the fossil fuels. Also, the replacement of fossil fuels with bio-energy can decrease the net emission of greenhouse gases substantially (Wang *et al*. 2007). The feedstock of lignocellulosic biomass is renewable, sustainable, and abundant. It includes forest and mill residues, animal wastes, aquatic plants, fast-growing trees and plants, and municipal and industrial wastes. Today, bio-ethanol is the most important renewable fuel in terms of volume and market value (Licht 2006). Most of it is produced from sugar- and starch-based materials such as sugarcane and corn, which compete with the food supply. Thus, scientists and engineers are turning to second-generation ethanol, derived from lignocellulosic biomass. This is a promising alternative energy technology that is being tested in pilot plants (Taherzadeh and Karimi 2007a, b). This is the most abundant biomass in the world, and it can be converted into fuels by enzymatic hydrolysis and microbial fermentation.

Typically, lignocellulosic biomass is composed of cellulose, hemicelluloses, lignin, extractives, and several inorganic materials (Sjöström 1993). Cellulose is a linear polysaccharide polymer of glucose made of cellobiose units, linked by β-1-4-glucoside bond (Delmer and Amor 2008; Morohoshi 1991) which is the main origin of fermentable sugar. The hemicelluloses are hetero-polymers composed mainly of mannose in softwood

and xylose in hardwood (Persson *et al*. 2006; Lavarack *et al*. 2002; Emmel *et al*. 2003). Lignin is a very complex molecule with a three-dimensional structure constructed of phenylpropane units. Lignin is particularly resistant to biodegradation; thus it is believed to be the most recalcitrant component of the plant cell wall. The present work considers a hypothesis that the content of lignin to a large extent determines the extent of enzymatic hydrolysis. Besides, the matrix of lignin, cellulose, and hemicelluloses, the properties of cellulose such as crystallinity and degree of polymerization, pore size and volume of fiber, distribution of lignin before/after pretreatment on fiber, and even lignin carbohydrate complexes (called LCCs) all contribute to recalcitrance on enzymatic saccharification of lignocellulosic biomass (Taherzadeh 1999; Palmqvist and Hahn 2000).

Due to the inherent properties of lignocellulosic biomass, pretreatment is necessary to make the biomass accessible to enzymes. The methods can be classified into "physical pretreatment," "physico-chemical pretreatment," "chemical pretreatment," and "biological pretreatment" (Wyman 1996; Berlin *et al*. 2006; Karimi *et al*. 2006; Sanchez *et al*. 2004; Alizadeh *et al*. 2005; Sassner *et al*. 2005). Dilute acid pretreatment is the most widely studied of these, with proof-of-concept well established (pilot scale). However, it has some unavoidable limitations, such as equipment corrosion, the requirement of neutralization before fermentation, and the inhibiting effects of degradation products, *etc*. All of these hinder the wide application of the dilute acid system. Development of optimal pretreatment technologies are demanded which can make sugar purification, utilization, and/or recovery economically feasible.

In this article, enzymatic saccharification was performed on five hardwood species with two different pretreatments including sulfuric acid and green liquor (Wu *et al*. 2010; Jin *et al*. 2010). The relationship between lignin content of substrate and sugar recovery was investigated. The influence of lignin structure (S/V ratio) on delignification was explored as well. Compared to acid pretreatment, alkaline treatment is more efficient in terms of delignification.

EXPERIMENTAL

Raw Materials

Red maple, sweet gum, trembling aspen, red alder and *Eucalyptus globulus* wood chips received from a pulp mill were ground and passed through a 40 mesh screen with a Wiley mill. The sawdust between 40 and 60 mesh was collected as raw material. All of substrates were extracted with a mixture of benzene and ethanol (2:1 v/v) for 8 hours to remove extractives. Then the extracted substrate was air-dried, homogenized in a single lot to keep the same composition, and stored for further use. The moisture content of the sawdust was measured by oven at 105 ˚C. Dilute sulfuric acid (0.1% wt.) and green liquor were prepared in the bio-energy group in Department of Forest Biomaterials of NC State University.

Green liquor 6 and green liquor 12 were prepared using Na_2S and Na_2CO_3. For green liquor 6, the TTA was 6% and composed of 25% Na_2S and 75% Na_2CO_3. For green

liquor 12, the TTA was 12%, and the ratio of Na_2S and Na_2CO_3 was the same as for green liquor 6.

Cocktail of Enzymes

Enzymatic saccharification was carried out with a complete *Trichoderma reesei* cellulase (C-TecI NS50013) treatment with β-glucosidase (C-TecI NS50010) and hemicellulase (C-TecI NS500014) (Novozymes, Denmark). The mass ratio of enzymes was 0.3:1:0.3. Activity of β-glucosidase was 280 CBU (Novozemes given data), cellulase was 84.1 FPU (measured), and hemicellulose was 330 FXU (data by Novozymes).

Pretreatments of Sawdust

Dried sawdust (about 0.75 g) was reacted with 3 mL of sulfuric acid (0.1% wt.) or green liquor at 185 ˚C for 30 min sealed in a 20 mL stainless steel tube. After reaction, the steel tube was cooled down immediately with an ice-water mixture. Then the slurry was filtered through a glass medium coarseness crucible by vacuum. The pretreated residue was washed with 500 mL of de-ionized water and air dried. Four batches of each sample were processed in order to obtain around 2 g of pretreated sample. Part of the pretreated sample was used to determine the moisture content by oven under 105 ˚C for 8 h. The oven-dried sample went to chemical composition analysis. The other portion was applied to enzymatic saccharification. In the case of acid pretreatment, hydrolyzed sugars and acid soluble lignin in filtrated were quantified by Dionex-IC and UV-VIS, respectively. The filtrate of green liquor pretreatment was discarded.

Composition of Substrates

Chemical composition of the substrates including the extractive-free and the pretreated substrates was determined by a modified version of TAPPI Standard Method T222 om-98. Briefly, the dried sawdust (around 0.1 g) reacted with 1.5 mL H_2SO_4 (72% wt.) at 25 ˚C for 2 h with stirring every 15 min. Then the slurry was diluted with 56 mL of de-ionized water (decreased acid concentration to 3% wt.) and transferred to a serum bottle. The slurry was subjected to autoclaving at 122 ˚C for 1.5 h then filtered through a fine coarseness crucible for gravimetric determination of acid-insoluble lignin. The sugar content (arabinose, rhaminose, galactose, glucose, xylose, and mannose) in the filtrate was quantified by Dionex-IC. The acid-soluble lignin was quantified by UV-VIS at a wavelength of 205 nm (HP8453E UV-VIS spectrophotometer). The IC system (Dionex IC-3000; Dionex) was equipped with guard column (carboPac PA1 4×50 mm) and an ion-exchange column (carboPac PA1 4×250 mm), a pulsed amperometric detector with a gold electrode, and a Spectra AS 300 autosampler. Prior to injection, samples were filtered through 0.2 µm Nylon filters (Millipore), and a volume of 25 µL was loaded. The column was pre-equilibrated with 250 mM NaOH and eluted with Milli-Q water at a flow rate of 1.1 mL/min.

Ratio of Syringaldehye and Vanillin

Lignin structure (S/V ratio) was characterized according to the method developed by Chen (1992). 200 mg OD of wood meal (40 to 60 meshes) was reacted with 7 mL 2N NaOH and 0.4 mL nitrobenzene in a stainless bomb at 170 ˚C for 2.5 h. Then the hot

stainless bomb was cooled down immediately with cool water, and 1 mL of 5-iodovanillin (80 mg dissolved in 5 mL acetone) was added as an internal standard. The mixture was extracted with CH_2Cl_2 (20 mL) three times, and the organic phase was discarded. The remaining water phase (alkali solution) was acidified with 2N HCl to pH 3 to 4. The acidified solution was further extracted with CH_2Cl_2 three times, and the organic phase was collected and dried with Na_2SO_4(s) over night. 1 mL of solution was dried by rotavaporater at 30˚C, and then 50 µL of pyridine and 60 µL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were added. The derivatized solution was directly injected into the GC device. Quantitative GC analysis was carried out on a HP 6890 GC equipped with a flame ionization detector and HP-1 column (30 m × 0.32 mm × 0.25 µm). The injection temperature was 200˚C, the detector temperature was 270˚C, and the column flow rate was 2 mL helium/min. The column was held for 3 min at 120˚C, raised at 5˚C/min to 200˚C, followed by 10˚C/min to 260˚C for 5 min.

Degradation Products during Acid Pretreatment (furfural content)

The filtrate of acid pretreatment was diluted to 250 mL with de-ionized water. The aliquot (5 mL) was reduced with $NaBH_4$ (30 mg) at 25 ˚C for 2 h, then neutralized with 0.5 N HCl. Furfural content was calculated from the difference of UV absorbance at 280 nm between the diluted filtrate and the reduced solution (Andrews 1980). The calibration curve was made with furfural standard solution. UV spectra were measured with a HP8453E UV-VIS spectrophotometer (Hewlett Packard Company, Palo Alto City, CA, USA).

Cellulase-Mediated Saccharification

The batch cellulase-mediated hydrolysis was carried out in a 40 mL sample flask containing 5% substrate (w/v) in 10 mL of 0.02 M acetate buffer (pH 4.8) supplemented with 40 µg/mL of tetracycline. Three enzyme charges were used including 10, 20, and 30 FPU/g based on the extracted sample. The slurry was incubated at 50˚C in a water bath shaker maintained at 150 rpm for 48 h. Then, the slurry was filtered and washed through medium coarseness crucible immediately. About 100 mL of the filtrate was collected and placed into boiled water for 10 min to denature the enzymes, then stored in a refrigerator. Sugar content in the filtrate was measured with a Dionex-IC *device*. The IC system (Dionex IC-3000; Dionex) was equipped with a guard column (carboPac PA1 4×50 mm) and an ion-exchange column (carboPac PA1 4×250 mm), a pulsed amperometric detector with a gold electrode, and a Spectra AS 300 autosampler. Prior to injection, samples were filtered through 0.2 µm Nylon filters (Millipore), and a volume of 25 µL was loaded. The column was pre-equilibrated with 250 mM NaOH and eluted with Milli-Q water at a flow rate of 1.1 mL/min. The residue was washed with 500 mL de-ionized water, and the composition was analyzed again.

RESULTS AND DISCUSSION

Chemical Composition of the Extracted Substrate

The compositions of the five substrates are summarized in Table 1. Eucalyptus showed the highest lignin content, around 27.1%, followed by sweet gum 26.9%, red

alder 26.0%, maple 23.9%, and aspen 22.1% (in Table 1). By contrast, aspen had the highest sugar content of about 64.7%, followed by maple 64.3%, sweet gum 60.0%, red alder 59.3%, and *Eucalyptus* 56.3%. Sugar recovery is shown for comparison of enzymatic saccharification, since the substrates have various sugar contents. It is defined as the percentage of saccharified sugar based on the carbohydrate content of biomass.

Table 1. Composition of Extractive-free Substrates

Species	Glucan	Xylan	Others*	Sugar*	Lignin*	Balance	S/V ratio
Maple	45.3±0.65	14.8±0.12	3.1±0.05	64.3±0.85	23.9±0.24	88.2±1.01	1.9±0.05
Sweet Gum	43.2±0.32	13.4±0.20	3.4±0.08	60.0±0.73	26.9±0.02	86.9±0.98	1.7±0.02
Aspen	49.0±0.56	12.5±0.14	3.2±0.04	64.7±0.69	22.1±0.14	86.8±1.12	2.9±0.07
Red Alder	42.8±0.30	13.9±0.15	2.6±0.05	59.3±0.71	26.0±0.25	85.4±1.17	2.2±0.03
Eucalyptus	40.2±0.25	13.3±0.14	2.7±0.02	56.2±0.47	27.1±0.12	83.3±1.05	2.0±0.01

Note: All of these values were based on original wood; Lignin content included the ISL (acid insoluable lignin) and ASL (acid soluble lignin); others included arabinan, rhamnan, and mannan.

Pretreatment of Substrates

After acid pretreatment, the slurry was filtered by vacuum to separate the filtrate and residue. Sugar content in the filtrate was determined by Dionex-IC (Table 2). The composition of residues (after acid, green liquor 6 and green liquor 12 pretreatment) was also analyzed (Table 3). Around 50% of xylan, the main component of hemicelluloses in hardwood, was hydrolyzed; however, only 1 to 2% lignin based on the extracted substrate was removed by acid pretreatment (Table 2 and 3). Although little lignin was removed by acid pretreatment, the removal of hemicelluloses still improved the sugar recovery. Such an effect can be attributed to an increase of relative surface area and/or pore volume and size (Brunecky *et al.* 2009). The considerable amount of sugar hydrolyzed during acid pretreatment must be recovered in order to maximize sugar production (Table 2). Due to the severity of acid pretreatment (185˚C and 30 min), a significant amount of pentose and even parts of hexose were degraded into furfural, hydroxymethylfurfural, and other organic acids. Furfural, from 0.8% to 1.8% depending on species, was quantified by UV-VIS spectrophotometer in the filtrates (Table 2). These degradation products inhibit the activity of enzymes and result in low sugar recovery or even inhibit the fermentation (Yang and Wyman 2004; Kabel *et al.* 2007). Therefore, it is essential to remove these derivatives before enzymatic saccharification; however it could increase the cost of final product bio-ethanol substantially.

Table 2. Composition of the Acid Pretreated Hydrolysate

Species	Others*	Glucan	Xylan	ASL*	Furfural	Sugar
Maple	2.5±0.08	3.1±0.04	8.5±0.21	2.4±0.07	2.1±0.03	14.1±0.31
Sweet Gum	1.3±0.05	3.3±0.08	7.1±0.15	2.1±0.02	1.2±0.01	11.7±0.21
Aspen	1.2±0.02	4.8±0.11	7.6±0.23	3.1±0.05	0.8±0.00	13.6±0.18
Red Alder	1.0±0.04	5.2±0.10	5.4±0.12	2.0±0.03	1.8±0.05	11.6±0.12
Eucalyptus	1.2±0.03	4.6±0.09	6.2±0.24	2.5±0.01	1.2±0.03	12.0±0.20

Note: all of these values were based on original wood; ASL: acid soluble lignin; others included arabinan, rhamnan, and mannan

In contrast to acid pretreatment, alkaline pretreatment resulted in more carbohydrates remaining (around 80%) and more lignin being removed (around 15% by green liquor 6 and 30% by green liquor 12). The removal of lignin may have facilitated carbohydrates accessibility to enzymes because of the increase of pore volume and size, thus increasing the available surface area. It has been shown that enzymes can be non-productively adsorbed by lignin (Berlin *et al*. 2006; Kumar and Wyman 2009). Thus, this negative effect on enzymatic saccharification was minimized when substrates had less lignin. These two advantages led to better sugar recovery of the substrates pretreated by green liquor. Additionally, structure change of the pretreated substrates also is believed to affect the enzyme saccharification significantly. The alkaline pretreatment could decrease the crystallinity of cellulose by the effect of swelling, which facilitates digestion of carbohydrates (Parveen *et al*. 2009; Taherzadeh and Karimi 2008; Zhu *et al*. 2009).

Table 3. Composition of Residues

Species	PT method	Yield	Glucan	Xylan	Sugars*	Lignin*
Maple	Original	100.0	45.3±0.65	14.8±0.12	64.3±0.85	23.9±0.24
	Acid	67.8±1.01	41.0±0.45	2.0±0.01	43.1±0.42	22.1±0.01
	GL6	76.0±1.11	44.2±0.52	10.5±0.07	55.5±0.62	20.8±0.12
	GL12	70.0±0.89	40.9±0.32	10.8±0.10	52.5±0.33	17.5±0.15
Sweet Gum	Original	100.0	43.2±0.32	13.4±0.20	60.0±0.73	26.9±0.02
	Acid	69.2±0.95	40.1±0.34	1.9±0.03	42.8±0.41	24.9±0.21
	GL6	75.6±1.14	42.0±0.21	11.0±0.09	53.6±0.38	23.8±0.17
	GL12	65.7±1.02	38.3±0.22	9.7±0.10	48.7±0.32	20.3±0.12
Aspen	Original	100.0	49.0±0.56	12.5±0.14	64.7±0.69	22.1±0.14
	Acid	70.5±0.69	45.7±0.36	1.7±0.00	47.9±0.45	19.3±0.22
	GL6	78.0±1.32	48.1±0.65	9.4±0.11	57.8±0.75	17.8±0.07
	GL12	68.6±0.97	44.9±0.44	8.7±0.07	53.6±0.52	14.7±0.19
Red Alder	Original	100.0	42.8±0.30	13.9±0.15	59.3±0.71	26.0±0.25
	Acid	71.8±1.00	37.7±0.32	2.1±0.01	41.2±0.49	23.6±0.21
	GL6	76.0±1.35	42.0±0.47	10.5±0.14	53.0±0.51	22.1±0.16
	GL12	69.5±0.91	39.8±0.41	9.0±0.08	49.3±0.50	18.3±0.05
Eucalyptus	Original	100.0	40.2±0.25	13.3±0.14	56.2±0.47	27.1±0.12
	Acid	65.9±0.78	34.3±0.31	1.7±0.02	37.0±0.44	25.0±0.23
	GL6	72.7±1.17	39.6±0.43	10.4±0.06	50.6±0.56	22.2±0.11
	GL12	66.3±0.83	37.5±0.28	9.8±0.02	47.3±0.37	19.5±0.24

Note: All of these values were based on original wood; others include arabinan, rhamnan, galactan, and mannan; Lignin content included the ISL (acid insoluable lignin) and ASL (acid soluble lignin).

High delignification was observed when the S/V ratio was high (Fig. 1). Compared to acid pretreatment, alkaline pretreatment (green liquor 12 and green liquor 6) could remove more lignin from the substrates. Different behaviors have been observed for acid *vs*. alkaline pretreatment as a function of lignin removal (Gharpuray *et al*. 1983; Thompson *et al*. 1992).

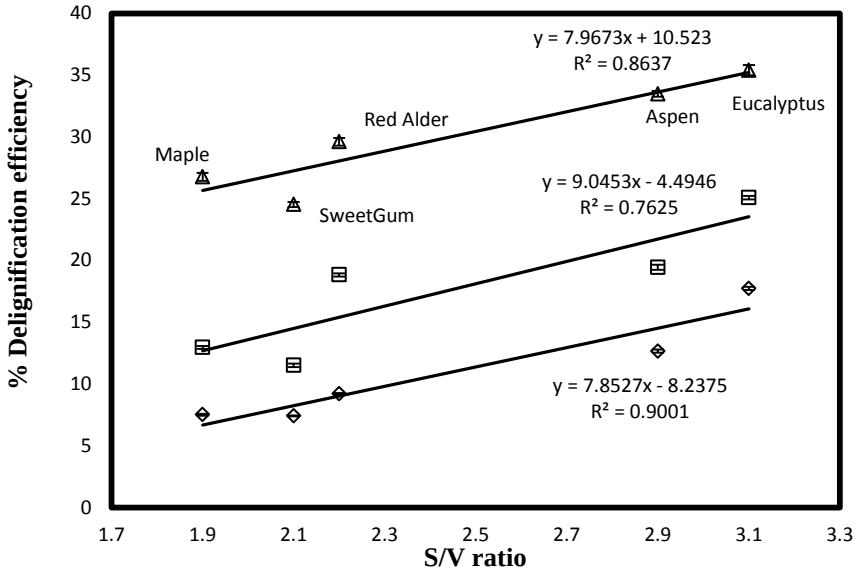


Fig. 1. Relationship between S/V ratio and delignification efficiency: △ acid, □ green liquor 6, and ◇ green liquor 12

Cellulase-Mediated Saccharification

Three charges, 10FPU/g, 20FPU/g, and 30FPU/g based on the pretreated substrates, were applied. Only 20 FPU/g was performed on green liquor 6 pretreated substrates and pretreated red alder. Sugar production from enzymatic saccharification after the pretreatments increased according to enzyme charges (Fig. 2). A significant increase of fermentable sugar was observed from 10 to 20FPU/g, and the amount leveled off from 20 to 30 FPU/g. Sugar production after the pretreatments was in the following order: green liquor 12, green liquor 6, and dilute acid (Fig. 2 (a), (b) and (d)), which was consistent with the amounts of lignin in the samples (see Table 2). For instance, after green liquor 12 pretreatment, there was only 17.5% (based on extracted substrate) lignin left, 20.8% lignin after green liquor 6 pretreatment, and little decrease of lignin (22.1% compared to original 23.9%) after acid pretreatment. These observations showed that lignin was the main contributor to recalcitrance of the wood towards enzymatic saccharification. Green liquor was more favorable than acid used for pretreatment because the substrates pretreated with green liquor had less lignin and gave higher sugar production.

There was a substantial amount of sugars hydrolyzed from hemicelluloses during acid pretreatment (Table 2). And the combined amount of sugars from acid pretreatment and enzymatic saccharification (Fig. 2 (c)) equaled to sugars produced from enzymatic saccharification after green liquor 12 pretreatment. However, neutralization was an essential step after acid pretreatment, because acid could inhibit and/or kill the yeast, or even corrode fermentation equipment. The additional step could increase the cost of sugar and/or bio-ethanol eventually.

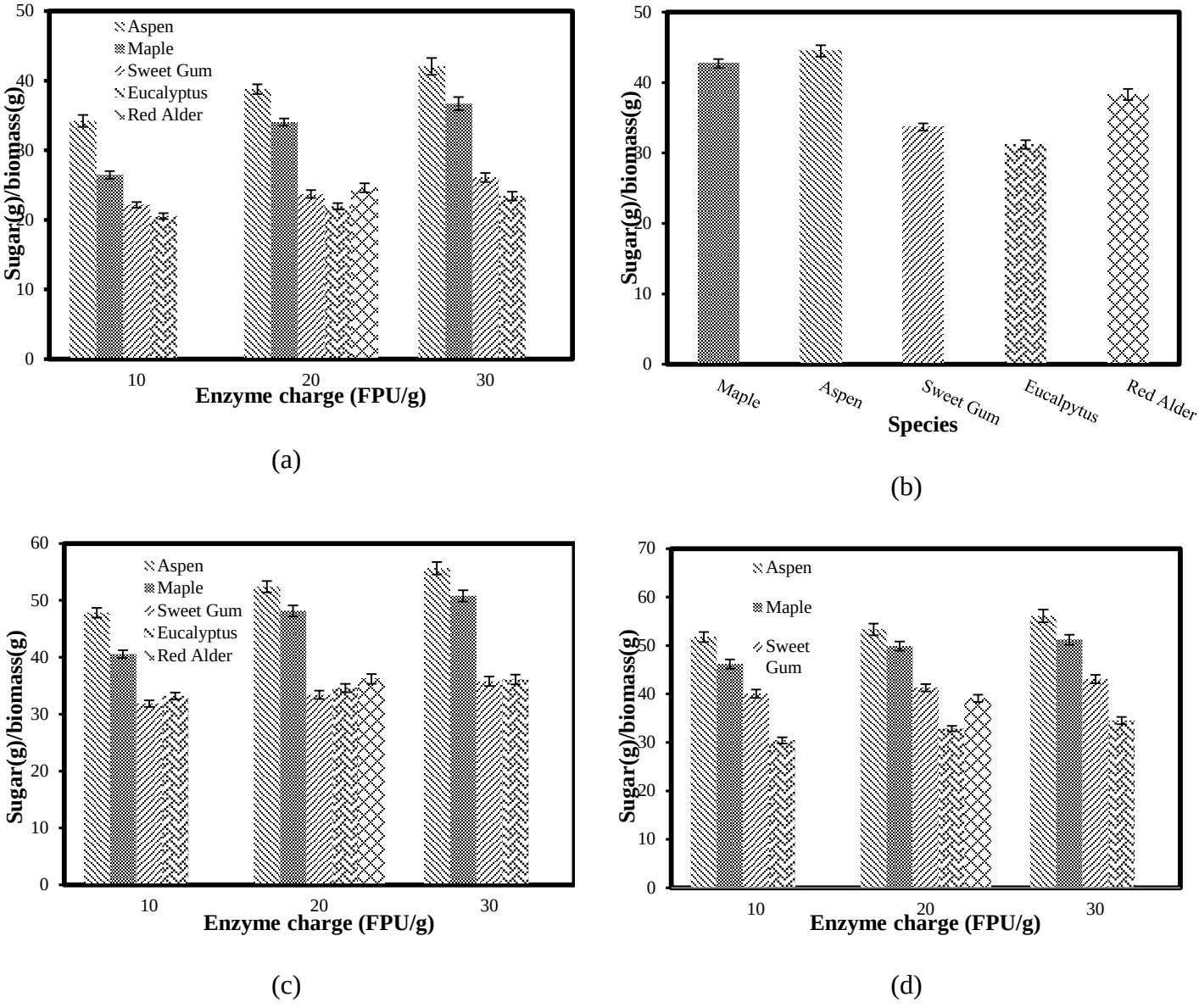
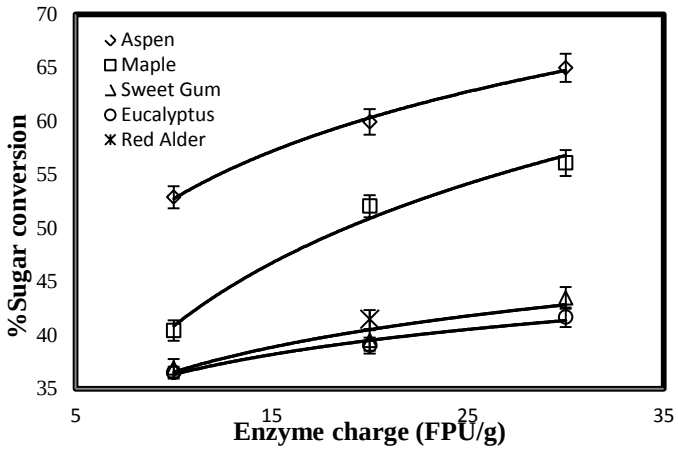


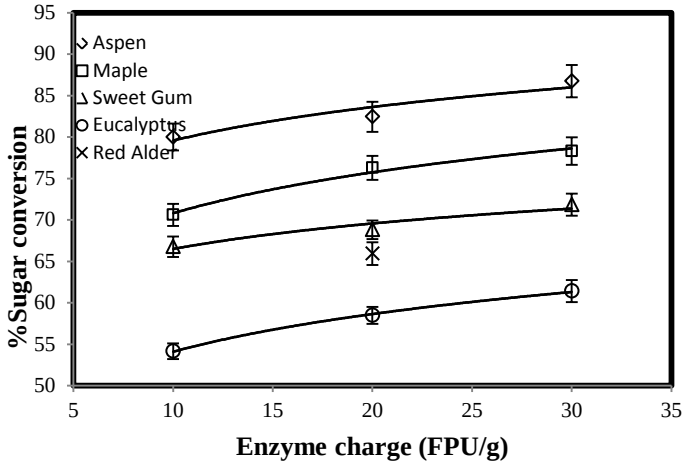
Fig. 2. Total fermentable sugar production in enzymatic hydrolysate: (a) total sugar production after the acid pretreatment, (b) total sugar production after the green liquor6 pretreatment, (c) combination of total sugar production from the enzymatic and the acid hydrolysates, and (d) total sugar production after the green liquor12 pretreatment

The substrates pretreated by green liquor 12 had the highest sugar recovery, followed by green liquor 6 and diluted sulfuric acid (Fig. 3 (a), (b) and (c)). These results roughly correspond to the levels of lignin in the samples, thus demonstrating a pronounced impact of lignin on enzymatic saccharification. The correlation between sugar recovery and lignin is shown in Fig. 4. The pretreated substrates with low lignin

had high sugar recovery. The behavior of acid and green liquor pretreatments (green liquor 6 and green liquor 12) on lignin removal was different (Fig. 1). It is worth keeping in mind that lignin is the main, but not only recalcitrant material affecting enzymatic saccharification. Other factors such as lignin structure, lignin distribution on the fiber surface, and crystallinity of cellulose are believed to influence enzymatic saccharification as well (Taherzadeh and Karimi 2008; Zhu *et al*. 2009; Gharpuray *et al*. 1983; Thompson *et al*. 1992).



(a)



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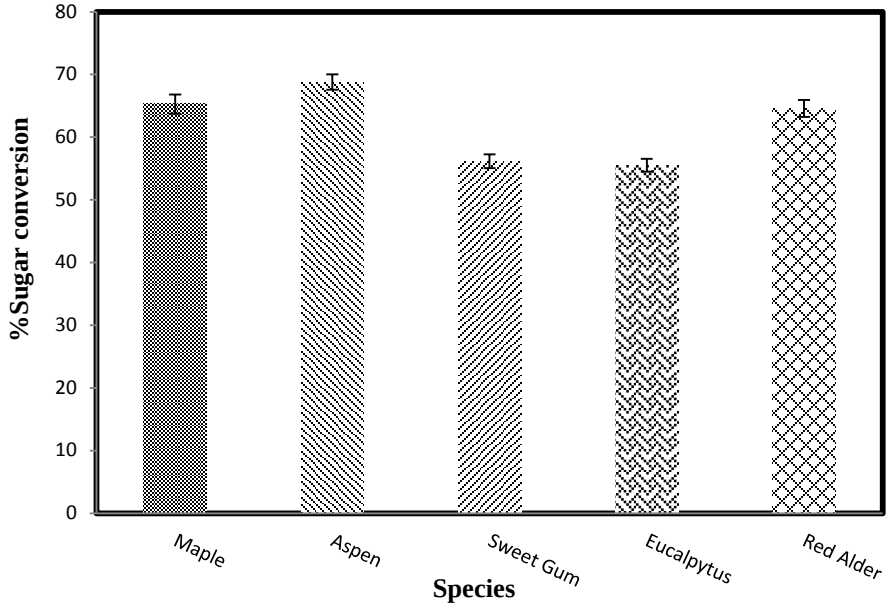

(c)

Fig. 3. Summary of sugar recovery: (a) sugar recovery after the acid pretreatment, (b) sugar recovery after green liquor 12 pretreatment and (c) sugar recovery after green liquor 6 pretreatment

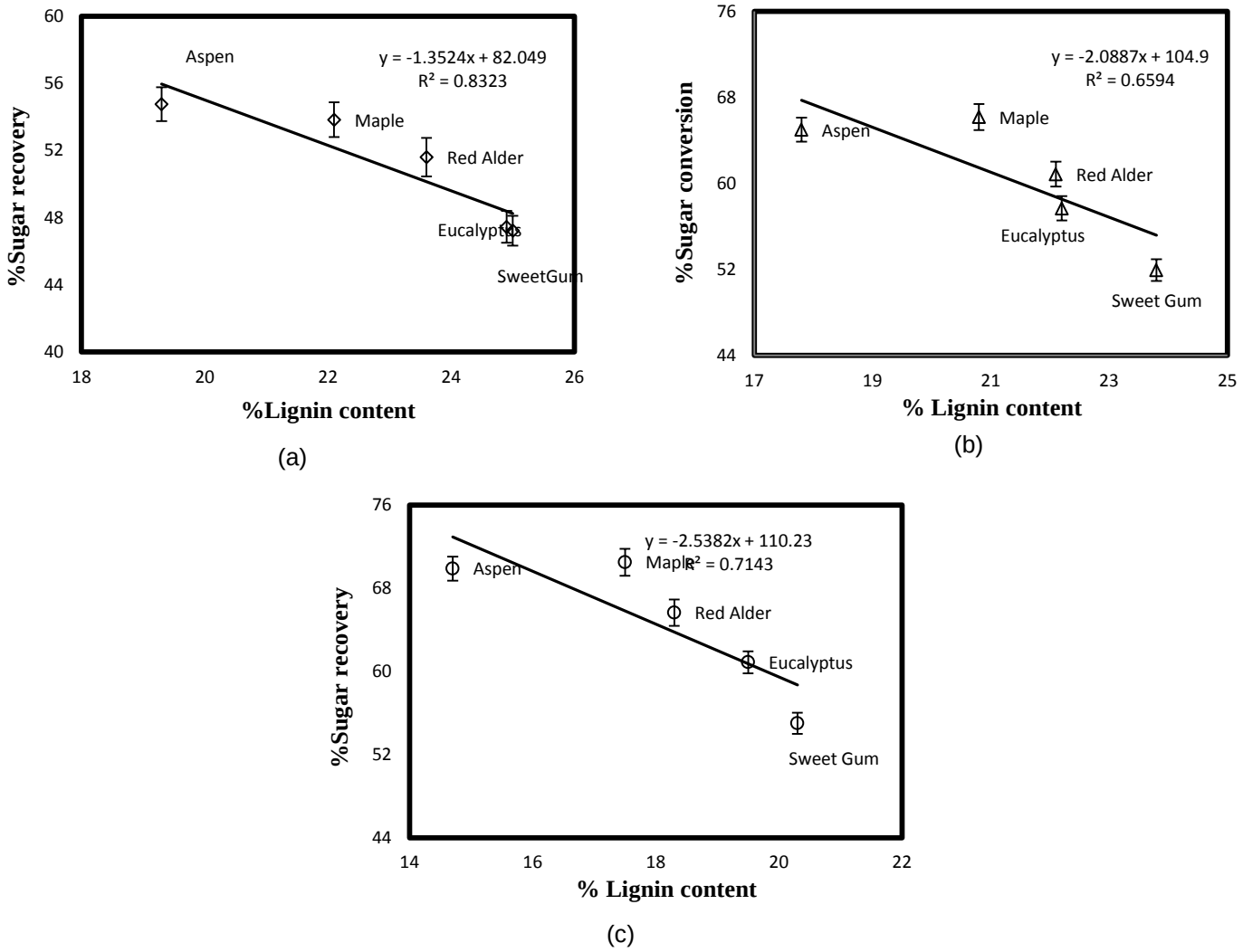

Fig. 4. Relationship between sugar recovery and lignin at 20 FPU/g: (a) acid pretreatment, (b) green liquor 6 pretreatment and (c) green liquor 12 pretreatment

CONCLUSIONS

First of all, lignin structure (S/V ratio) had a significant effect on delignification during the pretreatment. High S/V ratio indicated a high level of lignin removal from substrates. Secondly, the relationship between lignin and enzymatic saccharification confirmed that low lignin is predictive of high sugar production and high sugar recovery. Thirdly, the behavior of removal of lignin brought about by acid *vs.* alkaline (green liquor) pretreatments was different. Substrates pretreated by alkaline solution (green liquor) give higher sugar production and higher sugar conversion due to greater lignin removal. In addition, green liquor can be recovered easily from a kraft pulp mill, which makes the cost of bio-ethanol low compared to other pretreatments.

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