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Two-step liquid hot water pretreatment of *Eucalyptus grandis* to enhance sugar recovery and enzymatic digestibility of cellulose

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ABSTRACT

A two-step liquid hot water pretreatment (TSLHW) was developed with the objective of achieving complete saccharification of both hemicellulose and cellulose of *Eucalyptus grandis*, thereby avoiding the problems associated with the use of strong acid catalysts. The first step of the pretreatment was studied in the temperature range 180–200 °C, and the highest yield of total xylose achieved was 86.4% after 20 min at 180 °C. The second-step of the pretreatment was studied in the temperature range 180–240 °C and for lengths of time of 0–60 min. The conversion rate of glucan was more sensitive to temperature than time. The optimum reaction conditions for the second step of the pretreatment with minimal degradation of sugars were 200 °C for 20 min. the total sugar recovery from *E. grandis* with the optimized pretreatment and 72 h enzymatic digestion, reached 96.63%, which is superior to the recovery from a single-step pretreatment with hot water or dilute acid.

1. Introduction

Eucalyptus, poplar and pine are commercially important fast-growing trees. Eucalyptus provides raw material for papermaking and is widely used in the construction industries, where large amounts of wood residue, such as bark, cork residue, cross-cut ends, edgings, grinding dust, saw dust, and black liquor, have not been utilized efficiently. Fast-growing eucalyptus is one of the major promising cellulose feedstocks for ethanol production in the long term due to its high content of cellulose.

Nearly 90% of the dry weight of eucalyptus is in the form of cellulose, hemicelluloses, and lignin. Conversion of the cellulose and hemicellulose into sugars can provide the source material for the production of value-added chemicals, such as reducing sugars (Brito et al., 2008; Canettieri et al., 2007; Emmel et al., 2003; Zheng et al., 2009), and ethanol (Keffer et al., 2009; Sánchez and Cardona, 2008). Among the saccharification technologies available for the production of sugars from lignocellulosic biomass, a conversion process based on the use of cellulolytic enzymes appears to be the most promising for large-scale application. However, the efficiency of this technology is hindered by the complex chemical structure of the lignocellulosic starting material. Thus, pretreatment is necessary to alter the structure of the cellulosic biomass and make it more readily accessible to the enzyme. Any effective pretreatment needs to limit the formation of degradation products that can inhibit the growth of fermentative microorganism, minimize energy demands, and lower the cost of enzymes (Hamelinck et al., 2005; Hendriks and Zeeman, 2009; Mosier et al., 2005a,b; Wyman et al., 2005). The use of a liquid hot water (LHW) pretreatment using pressure to maintain the water in the liquid state at elevated temperatures, is an attractive approach because it does not require the addition of chemicals such as sulfuric acid, lime and ammonia, etc. An effective LHW pretreatment generates reactive cellulose fiber, allowing the recovery of most of the pentosan, and only a few of the degradation products that can hinder subsequent hydrolysis and fermentation (Rogalinski et al., 2008; Yun et al., 2007). There have been many studies of the pretreatment of lignocellulosic biomass with LHW to enhance the enzymatic digestibility, especially for herbaceous feed stocks such as corn fiber (Dien et al., 2006; Liu and Wyman, 2005; Mosier et al., 2005a,b), wheat straw (Garrote et al., 2007; Pérez et al., 2008), and sugarcane bagasse (Laser et al., 2002; Sasaki et al., 2003). However, there are few reports of the pretreatment of woody biomass with LHW, especially for the complex structure and composition of eucalyptus residues (He and Li, 2001).

We have investigated the use of a two-step liquid hot water (TSLHW) pretreatment of *Eucalyptus grandis* residue to recover hemicellulose-derived sugars and break down the cellulose structure to enhance enzyme digestibility, which could increase total sugar recovery using commercial cellulase preparations. We have

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optimized the parameters for the satisfactory pretreatment and fractionation of *E. grandis* residue.

2. Methods

2.1. Substrate

E. grandis residue that had been milled and screened to 40–80 mesh and dried at 105 °C to constant weight (~4 h) was obtained from a wood processing plant in Jiangmen, Guangdong Province, China. The chemical composition of the raw material (on a dry weight basis) was 44.9% glucan, 11.4% xylan, 26.2% acid-insoluble lignin, 3.5% extractives and 13.9% other.

2.2. Experimental setup and operation

The experimental system consisted of an autoclave reactor, a feeding system and a product collector. Details of the experimental apparatus are described elsewhere (Zhuang et al., 2009). About 35 g of the *E. grandis* residue (5% w/v in water) was put into the reactor, which was sealed and heated to the reaction temperature (180–200 °C) with the magnetic agitator operating at 500 rpm. Samples (5 ml) were collected every 10 min for 60 min and the conditions that gave the highest sugar recovery were determined as the optimum for the first step of the pretreatment. The hydrolyzate was collected and the feedstock was subjected to the second step of the pretreatment by adding water to maintain a 5% (w/v) dry solids slurry and heated at (180–240 °C). The hydrolyzate was collected and the reactor was cooled rapidly to <100 °C to terminate the reaction. The wet substrate was recovered from the reactor and divided into two portions; one was used for the measurement of weight loss and for composition analysis, and the other was used for the enzymatic digestibility assay.

2.3. Digestibility tests

After filtration, the water-insoluble solid was washed extensively with water, and tested for enzymatic digestibility using cellulase purchased from Imperial Jade Biotechnology Co., Ltd., China. The tests were done at 50 °C for 72 h in 250 ml Erlenmeyer flasks, each containing 100 ml of 0.05 M sodium citrate buffer (pH 4.8) and 5% (w/v) water-insoluble solid. The enzyme-loading amount was 40 FPU/g dry solid. The glucose content in the samples was measured by HPLC. All experiments were done in duplicate. The enzymatic digestibility was calculated as the ratio of glucose in the enzymatic hydrolysis per 100 g of potential glucose in the water-insoluble solid fraction.

2.4. Analytical methods

The raw material and the residue were analyzed according to the standard Laboratory Analytical Procedures (LAP) for biomass analysis provided by the National Renewable Energy Laboratory (NREL). Monomeric sugars, oligomeric sugars, and degradation products (5-hydroxymethyl-2-furaldehyde (HMF) and furfural) in the hydrolyzate were identified on the basis of NREL LAP. The oligomeric sugars in the liquid fraction were back-calculated after a secondary hydrolysis into monomerics with 4% sulfuric acid. The monomeric sugars in the hydrolyzate were determined by HPLC using a Shodex sugar SP-0810 column coupled with a refractive index detector, and degradation products were detected by an ultraviolet detector. The mobile phase was HPLC-grade water at a flow rate of 0.6 ml/min, with a column temperature of 80 °C. The total sugars in the liquid sample included the monomerics and oligomerics. The yield of sugars in the hydrolyzate was calculated on the basis of the amount of sugar polymers in the untreated solids, and the analysis of degradation products was based on the amount of untreated biomass material.

3. Results and discussion

3.1. Optimization of the first step of the pretreatment

E. grandis was pretreated under pressure (4.0 MPa) by liquid hot water at a temperature within the range 180–200 °C. Fig. 1a shows

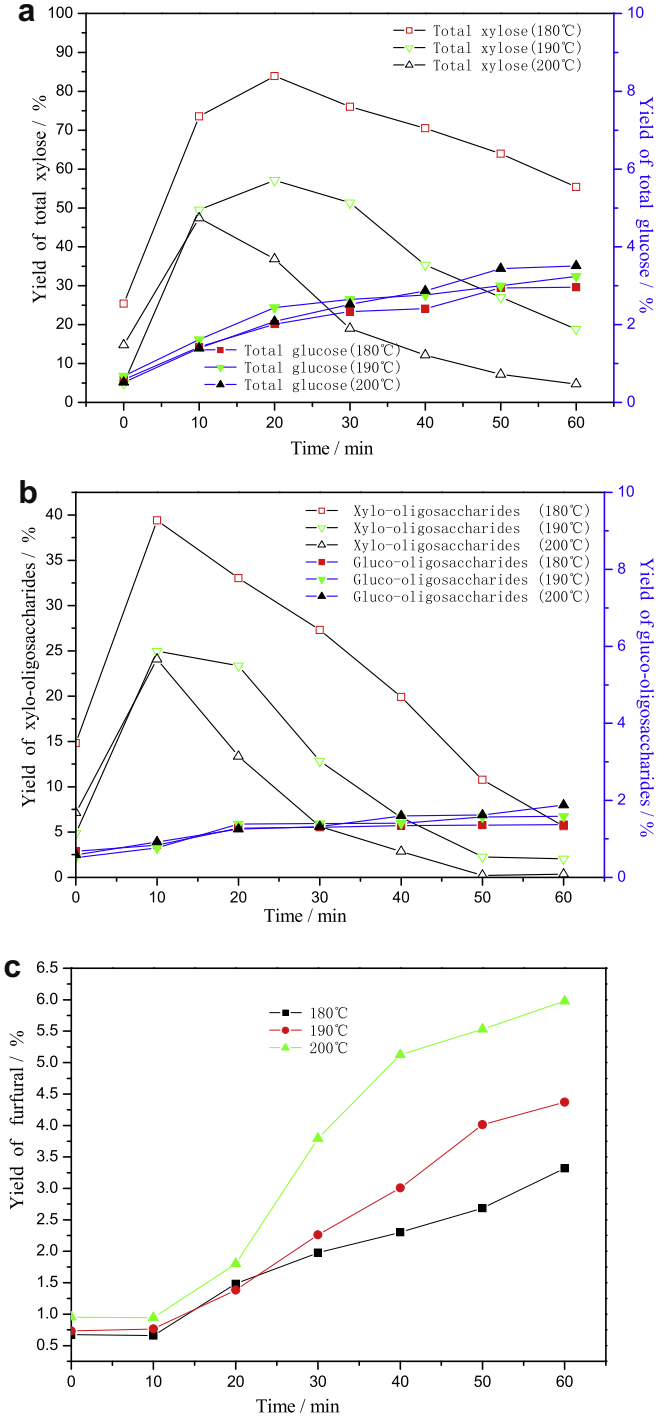

Fig. 1. Effect of reaction temperatures on the yield of total xylose and glucose (a), xylo-oligosaccharides and gluco-oligosaccharides (b), and furfural (c) in the liquid fractions.

the yield of total sugars in the liquid fractions as a function of time. As expected, the yield of total xylose increased significantly during the first 10 min, especially at 180 °C, and then decreased after about 10 min, which suggested that xylose is degraded more rapidly when the substrate is pretreated for a longer time or under more severe conditions. By contrast, the total glucose recovery was increased slightly from 0.6% to 3.5% with increasing time and temperature. Overall, these results support the observation reported by others that the decomposition of hemicellulose starts at a lower temperature (180 °C) than that of cellulose in the liquid hot water. It is necessary to recover their hydrolyzate separately to avoid sugar degradation (Ando et al., 2000).

Fig. 1b shows that more xylose is oligomeric at a relatively low temperature, and oligosaccharides are hydrolyzed into monomeric sugars over time. For example, the yield of xylo-oligosaccharides at 180 °C was 39.4% at 20 min, which is slightly less than half of the total xylose recovery (86.4%) and the yield of gluco-oligosaccharides in the liquid fractions was increased only slightly, from 0.5% to 1.8%, which indicated that *E. grandis* cellulose is recalcitrant to solubilization at these temperatures.

The hydrolysis of hemicelluloses is accompanied by the conversion of xylose to furfural. The amount of furfural produced is nearly linear because of the severity of the pretreatment conditions (Fig. 1c); a relatively low yield (1.5% of the dry mass) was detected after 20 min at 180 °C. The optimum conditions for the first step of the pretreatment were determined as: substrate, 5% w/v in water; temperature, 180 °C; time, 20 min; pressure, 4.0 MPa; agitation, 500 rpm.

3.2. Optimization of the second-step of the pretreatment

In order to improve the reactivity of the cellulose-rich solid fraction with respect to enzymatic hydrolysis, the feedstock was subjected to the second step of the pretreatment, with temperature increasing stepwise from 180 °C to 240 °C by 20 °C at 20 min intervals. The yield of total glucose and gluco-oligosaccharides was nearly linear, depending on the severity of the pretreatment conditions, as illustrated in Fig. 2a. For example, the yield of total glucose is 2.3% after the first step of the pretreatment (180 °C, 20 min), which increased to 10.8% when the *E. grandis* residue was subjected to the second step of the pretreatment (240 °C, 20 min). In addition, Fig. 2a shows that the total glucose recovery increased significantly with time within the first 20 min at 200 °C, and was nearly constant during the next 40 min. This is the result of the combined effects of reduced cellulose solubilization and increased depolymerization of gluco-oligosaccharides. The data suggest that the formation of cello-oligosaccharides is a critical step for the hydrolysis of cellulose. A possible explanation for this observation is discussed elsewhere (Qiang et al., 2009).

The effects of reaction temperature on the yield of furfural and HMF are shown in Fig. 2b. As expected, glucose degradation was increased with temperature, which led to an increase in the yield of HMF. The yield of furfural increased when the temperature was increased from180 to 200 °C in the second step of the pretreatment, but it began to decrease above 200 °C because of further degradation into aldehyde, acid components (Xiao, 2006). Fig. 2b shows that the amount of furfural and HMF increased with reaction time at 200 °C, indicating that prolonged treatment is detrimental to the recovery of both xylose and glucose. Taken together, the results suggest that temperature has a more crucial role than time in accelerating the hydrolysis of cellulose.

3.3. Enzymatic digestibility test

All of the residual solids were tested for enzymatic digestibility to assess the effects of the TSLHW pretreatment. Pretreatment of

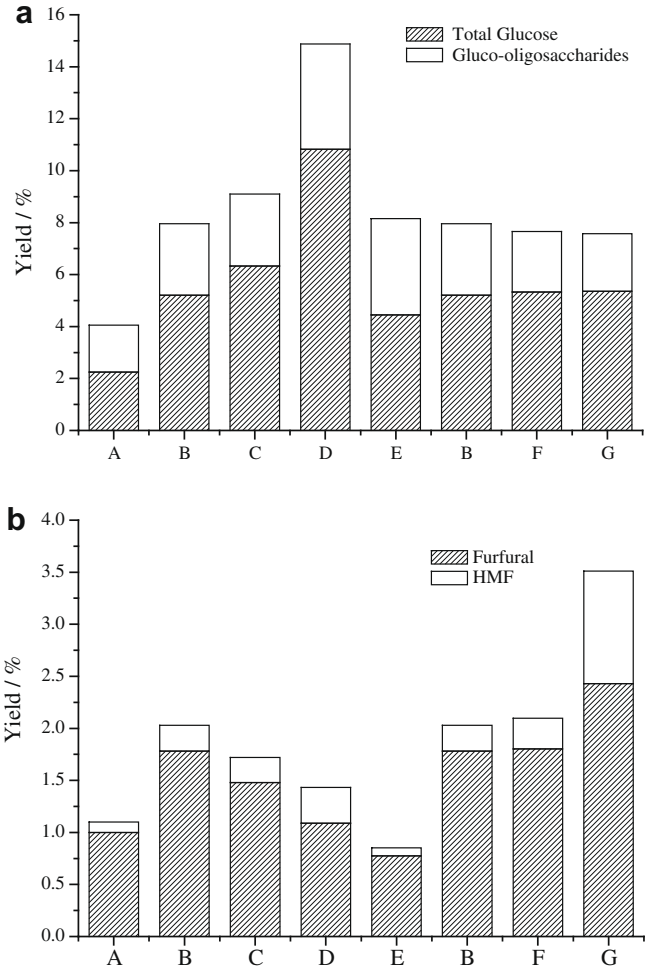

Fig. 2. Effect of reaction temperature and time on the yield of total glucose and gluco-oligosaccharides (a) and furfural and HMF (b) in the liquid fractions.

cellulose was more sensitive to reaction temperature than time. The 72 h digestibility of samples treated at 180 °C for 20 min was 72.8%, which was increased to 97.2% after the second step of the pretreatment at 240 °C (Fig. 3). A small increase in enzymatic digestibility from 81.2% to 86.6% occurred when the reaction time of the second step of the pretreatment was increased from 20 to 60 min at 200 °C.

The crystallinity of lignocelluloses has often been suggested as a major obstacle to their enzymatic digestibility. The X-ray diffraction (X'Pert PRO, PANalytical B.V. Co., Netherlands) patterns of treated and untreated *E. grandis* were determined and the intensity of the amorphous region at $2\theta = 18°$ and the crystalline region at $2\theta = 22°$ were used to calculate the crystallinity index (CrI):

$$\mathrm{CrI} = [\text{intensity at } 2\theta = 22\,\text{deg.} - \text{intensity at } 2\theta = 18\,\text{deg.}]/[\text{intensity at } 2\theta = 22\,\text{deg.}]$$

The CrI value of pretreated *E. grandis* was greater than that of the untreated material (52.9%), reflecting the removal of hemicellulose and lignin. Fig. 3 and Table 1 show that the CrI value was increased with increased amount of hemicellulose and lignin removed. Generally, the removal of hemicellulose and lignin enlarges the area of contact between the cellulose and the enzyme, which enhances the enzymatic digestibility. Fig. 3 shows that the CrI value of pretreated *E. grandis* was increased slightly from 72.7% to 76.8% after the second step of the pretreatment at 200 °C for 0–60 min. In addition, the CrI value was increased at

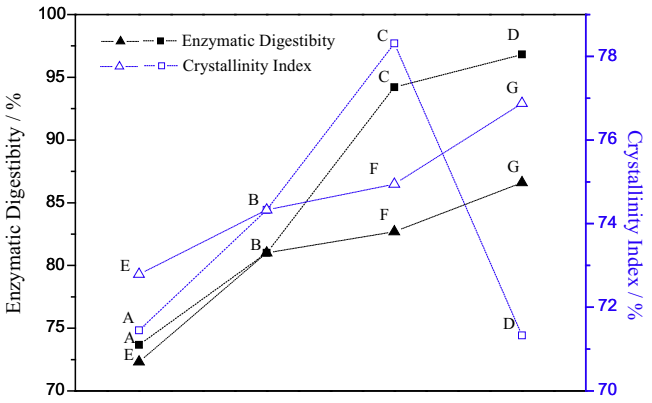

Fig. 3. Enzymatic digestibility and crystallinity index of pretreated *E. grandis*.

reaction temperature <240 °C in the second step of the pretreatment, but was decreased at 240 °C, reflecting the greater degradation of cellulose. These results support the suggestion that there is no direct relationship between enzymatic digestibility and CrI value, but there is a close relationship with the removal of hemicellulose and lignin (Kim and Lee, 2005, 2006).

In order to clarify the influence of TSLHW pretreatment on enzymatic digestibility, treated and untreated *E. grandis* material was examined in a scanning electron microscope (JSM-6360LV, *JEOL Ltd.*, Tokyo, Japan). The results show that the untreated *E. grandis* material consists of rigid and highly ordered fibrils, while the treated material does not. The fibers of the treated samples were separated from the initial connected structure and fully exposed, especially after the TSLHW pretreatment because of the removal of hemicellulose and lignin, which improved the accessibility of the substrate to the enzyme.

3.4. Composition analysis

Table 1 gives the composition of liquid hydrolyzate and solid residue after various pretreatment steps. The conversion rate of xylan:

[initial xylan – residual xylan in solid]/[initial xylan]

reaches 100%, and about 87.5% xylan recovery:

[xylan recovered in liquid phase]/[initial xylan])

is obtained. The conversion rate of glucan:

[initial glucan – residual glucan in solid]/[initial glucan]

increased with increased pretreatment temperature. For example, the conversion rate of glucan was 6.68% when the second step of the pretreatment was done at 180 °C for 20 min, and increased to 61.44 % (9.1-fold increment) at 240 °C for 20 min. However, the increase of the glucan recovery:

[glucan recovered in liquid phase]/[initial glucan]

was not proportional to the conversion rate because the sugar degradation occurred at a higher temperature. The glucan recovery was 2.25% at 180 °C and only a 4.8-fold glucan recovery increment was obtained at 240 °C. In addition, increasing amounts of lignin were removed with increasing temperature and about 57.7% was removed when the TSLHW pretreatment was done first at 180 °C for 20 min and then at 240 °C for 20 min. Comparing the recovery of total sugars, the optimum operating conditions were 180 °C for 20 min in the first step, and 200 °C for 20 min in the second step of the pretreatment. The total sugars recovery was 96.63% after enzymatic hydrolysis with 40 FPU/g dry solid.

3.5. Comparison of TSLHW and other pretreatment methods

Owing to the lack of studies of the LHW pretreatment of *E. grandis* to enhance the enzymatic digestibility of cellulose, the TSLHW pretreatment described here was compared with a single-step hot water and dilute acid pretreatment of other hardwood species, such as hybrid poplar and olive. Table 2 gives sugar yields as a percentage of the total xylose and glucose available in the substrate material. The results show that there was no significant difference in the recovery of hemicellulosic-derived sugars among these three pretreatment methods.

However, there was a significant difference in the glucose recovery. The lowest recovery of glucose after the single-step hot water pretreatment and 72 h hydrolysis with 40 FPU cellulose/g glucan

Table 1
Composition of liquid hydrolysate and solid residues.

Methods	Pretreatment[a]						Glucan yield in the 72 h enzymatic hydrolysis[a] (%)	Total sugars recovery (glucan + xylan)[b] (%)
	Solid remaining (%)	Glucan (%)		Xylan (%)		Klason lignin (%)		
		Solid	Liquid	Solid	Liquid			
Untreated biomass	100	44.9		11.4		26.2		
Residues A	67.3	41.9	1.0	1.1	9.6	19.6	34.3	81.2
Residues B	61.1	41.1	2.3	0	10.3	17.6	41.9	96.6
Residues C	49.3	33.9	2.8	0	10.1	11.5	35.5	85.9
Residues D	29.8	17.3	4.9	0	10.0	11.1	18.6	59.4

Note: The reaction conditions for A–D are the same as those in Fig. 2.
[a] Based on the original oven-dry untreated biomass.
[b] Based on the initial amount of glucan and xylan in the untreated biomass.

Table 2
Comparison of two-step LHW and other pretreatment methods.

Materials	Methods	Hemicellulose-derived sugars recovery (%)	Glucose recovery (%)
E. grandis	Liquid hot water + enzymatic saccharification	86.4	98.4
Hybrid poplar (Kim et al., 2009)	Liquid hot water + enzymatic saccharification	82	61
Olive (Cara et al., 2008)	Dilute acid + enzymatic saccharification	80	75

was from the hybrid poplar, indicating that very little lignin was released into the liquid fractions and the reactivity of the cellulose-rich solid fraction needed to be improved (Kim et al., 2009). Comparing with (Cara et al., 2008), the TSLHW pretreatment shows a better performance than dilute acid pretreatment in terms of the greatest sugar recovery and without the need for chemical additives.

4. Conclusions

The results of this study indicate that the TSLHW pretreatment allows a greater recovery of total sugars and improves the enzymatic digestibility of a woody biomass compared with a single-step, combined hot water and dilute acid pretreatment. The structure of *E. grandis* is altered substantially after the TSLHW pretreatment; 81.5% enzymatic digestibility and 96.6% total sugars recovery were achieved after TSLHW pretreatment at 180 °C for 20 min and at 200 °C for 20 min. Selection of the appropriate temperature is crucial for TSLHW pretreatment, especially for the second step.

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