ETHANOL ORGANOSOLV PRETREATMENT OF BAMBOO FOR EFFICIENT ENZYMATIC SACCHARIFICATION

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Bamboo is a potential lignocellulosic biomass for the production of bioethanol because of its high cellulose and hemicelluloses content. In this research, ethanol organosolv pretreatment with dilute sulfuric acid as the catalyst was studied in order to enhance enzymatic saccharification of moso bamboo. The addition of 2% (w/w bamboo) dilute sulfuric acid in 75% ethanol had a particularly strong effect on fractionation of bamboo. It yielded a solids fraction containing 83.4% cellulose in the treated substrate. The cellulose conversion to glucose yield reached 77.1 to 83.4% after enzymatic hydrolysis of the solids fraction for 48 h at an enzyme loading of 15 FPU cellulase/g cellulose and 30 IU β-glucosidase/g cellulose. The enzymatic hydrolysis rate was significantly accelerated as the ethanol organosolv pretreatment time increased, reaching the highest enzymatic glucose yield of 83.4% after 48 h at 50 ^{o}C. The concentrations of fermentation inhibitors such as HMF (5-hydroxy-2-methyl furfural) and furfural were 0.96 g/L and 4.38 g/L in the spent liquor after the ethanol organosolv pretreatment, which were slightly lower than the concentrations quantified during H_2SO_4-water treatment. Spent liquor was diluted with water, and more than 87.2% of lignin in raw bamboo was recovered as ethanol organosolv lignin through the filtration process.

Keywords: Bamboo; Bioethanol; Ethanol organosolv pretreatment; Dilute acid

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INTRODUCTION

First-generation bioethanol, which is based on corn, has reached its limit due to limitations in feedstock supply and competition with the food and livestock feed market (Gray *et al.* 2006; Leibtag 2008; Elobeid *et al.* 2006). The exploitation and utilization of second-generation bioethanol from lignocellulose have attracted much interest from researchers and governments around the world. However, conversion of lignocellulosic biomass to ethanol is much more difficult than sugar and corn-to-ethanol production due to the recalcitrant nature of the lignocellulosic biomass (Sun and Cheng 2002). Thus, a pretreatment process is required to enhance the enzymatic digestibility of cellulosic materials. Many pretreatment methods have been proposed to increase cellulose-to-glucose conversion yield in the bioethanol production process.

Organosolv pretreatment has been evaluated as an effective pretreatment method for high-lignin lignocellulosic biomass (Chum *et al.* 1990; Pan *et al.* 2006). Organosolv pretreatment can break down internal lignin and hemicellulose bonds and thus remove almost all of the lignin from biomass (Holtzapple and Humphrey 1984). Through the removal of the lignin, the pore-volume and surface area increase so as to increase substrate enzymatic digestibility. A strong inorganic acid is usually applied in the organosolv pretreatment as a catalyst to hydrolyze the lignin-lignin and lignin-carbohydrate bonds in biomass (Holtzapple and Humphrey 1984). Inorganic acids including hydrochloric acid, sulfuric acid, and phosphoric acid have been frequently chosen. Generating high-quality lignin is one of the unique advantages of the organosolv pretreatment over alternative methods, such as steam explosion, dilute-acid pretreatment, and hot-water treatment, where the only proposed use for the lignin is as a boiler fuel (Pan *et al.* 2008). The organosolv spent liquor mixed with water could precipitate the dissolved lignin. In contrast to lignin produced by other technical processes, such as kraft pulping, ethanol organosolv lignin is a sulfur-free, low-molecular-weight product of high purity. High-quality lignin can be used as a substitute for polymeric materials, such as phenolic powder resins, polyurethane foams, and epoxy resins (Zhang 2008).

Bamboo is the vernacular term for members of a particular taxonomic group of large woody grasses (subfamily *Bambusoideae*, family *Andropogoneae/Poaceae*) (Scurlock *et al.* 2000). Bamboo has some advantages, such as fast growth, high cellulose content, and abundant availability, especially in China. So it has the potential to become one of most widely used bio-energy resources in the future. Some pretreatments have been investigated with bamboo for bioethanol production (Sathitsuksanoh *et al.* 2010; Li *et al.* 2010; Shimokawa *et al.* 2009). Enzymatic saccharification (48 h) using a 35 atm 5 min steam exploded bamboo produced 426 and 488 mg/(g initial dry sample) of glucose and reducing sugar, respectively (Yamashita *et al.* 2010). Otherwise, enzymatic saccharification (72 h) using dilute sulfuric acid pretreatment (0.6 to 1.2% (w/w) acid loading, 120 to 140 ^{o}C and 30 to 90 min) bamboo substrates produced only 43 to 85 mg/(g initial dry sample) reducing sugar (w/w) (Leenakul and Tippayawong 2010). Literature on organosolv pretreatment of bamboo is limited (Dang and Nguyen 2006; Li *et al.* 2012a, 2012b). Li *et al.* (2012a, 2012b) used formic acid/acetic acid/water organosolv treatment and focused on delignification of bamboo for pure and high-quality lignin utilizations. The substrates compositions and enzymatic digestibility were not discussed. However, to our knowledge, the combination of ethanol/water organosolv treatment aimed at enzymatic hydrolysis for bioethanol from bamboo has not been reported. In this investigation, the feasibility of an organosolv pretreatment using inorganic catalyst was evaluated. Dilute sulfuric acid was applied as the catalyst for organosolv pretreatment of moso bamboo, and the enzymatic hydrolysis was carried out after the pretreatment.

EXPERIMENTAL

Materials

Moso bamboo (*Phyllostachys heterocycla*) was acquired from the central area of Louisiana, USA in the fall of 2009. After being air-dried, the culms of mature (4 years old) bamboo were milled using a hammer mill with a screen opening size of 2.0 mm before pretreatment. The average moisture content of ground bamboo was 6.93% (wt). The moisture content of the ground samples was measured relative to samples that had been oven-dried (OD) for 24 h at 105±2 °C. The initial chemical composition of moso bamboo was determined as 41.3% of glucose, 22.0% of xylose, 1.1% of arabinose, 0.6% of mannose, 0.3% of galactose, 22.8% of Klason lignin, 1.5% of acid soluble lignin, 1.4% of ash, and 13.0% of water/ethanol extractives.

Commercial enzymes, cellulase and β-glucosidase produced by Novozymes, were purchased from Sigma-Aldrich (St. Louis, MO). All the chemical reagents used in this study were purchased from Fisher Scientific (Pittsburgh, PA).

Pretreatments

Bamboo samples were pretreated in a microwave accelerated reaction system made by CEM Corporation (Model MARS, CEM Corporation, Matthews, North Carolina, and USA). This apparatus provided microwave radiation at 3 variable power levels of 400 W, 800 W, and 1600 W. Each pretreatment was carried out in duplicate; the average results of the two runs were reported. Aqueous ethanol of 75% (w/w) and 2% of sulfuric acid (w/w bamboo) was used for the organosolv pretreatment, and 8 g of raw bamboo were mixed with the solvent in a 100 mL vessel. The liquor-to-bamboo ratio was 5:1. The vessel was positioned at the centre of a rotating circular ceramic plate in the microwave oven for treatment at the power level of 400 W. The temperature was raised to the target temperature (160 ^{o}C and 180 ^{o}C) in about 10 min and maintained for an additional 30 or 60 min. After the pretreatment, a few minutes were allowed for the temperature to drop down below 60 ^{o}C, and then the spent liquor was sampled immediately for determination of fermentation inhibitors. The pretreated substrate and spent liquor were then separated by filtration. The substrate was washed three times with 50 mL aqueous ethanol with the same concentration (75%) of pretreatment liquor at 60 ^{o}C, and the ethanol washes were combined with the spent liquor. The substrate was then washed three more times with water at 60 ^{o}C, and the water washes were discarded. The solid substrate then was stored at 4 ^{o}C for enzymatic hydrolysis.

The spent liquor and ethanol washes were combined and mixed with three volumes of water to precipitate the dissolved lignin. The precipitated lignin, henceforth described as ethanol organosolv lignin (EOL), was collected on Whatman No. 1 filter paper through vacuum filtration and then washed thoroughly with water and air-dried. The filtrate and water washes were combined to obtain a water-soluble fraction containing monomeric and oligomeric hemicellulosic sugars, depolymerized lignin, and other unidentified components. It was stored in 4 ^{o}C until the sugar and fermentation inhibitors contents analysis was carried out by high performance liquid chromatography (HPLC).

Enzymatic Hydrolysis

Enzymatic hydrolysis of the pretreated bamboo substrates and original raw bamboo was conducted as described previously (Pan *et al.* 2008). Commercialized microcrystalline cellulose (MCC) was used as a comparison for the enzymatic digestibility. Enzymatic hydrolysis was carried out in 150 mL plastic jars at 50 ^{o}C on a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 220 rev/min. Based on cellulose content in the substrate, bamboo substrate equivalent to 0.8 g glucan was loaded with 40 mL of 0.05 M sodium acetate buffer (pH 4.8). Approximately 1.5 mg of tetracycline chloride was added to inhibit the growth of microorganisms and prevent consumption of liberated sugars. Two enzymes, cellulase (15 FPU, Filter Paper Units, per gram glucan) and β-glucosidase (30 IU, international Units, per gram glucan), were loaded into the plastic jars. Hydrolysates were sampled periodically at 1, 3, 6, 12, 24, and 48 hours to analyze glucose concentration. The hydrolysis was conducted in duplicate for each substrate; the average is reported here.

Analytical Methods

Insoluble lignin of bamboo and pretreated bamboo substrates was determined according to National Energy Laboratory (NREL) Analytical Procedure: Determination of Structural Carbohydrates and Lignin in Biomass (with modifications) (Sluiter *et al*. 2008). Acid-soluble lignin was measured at 205 nm on a UV-Visible spectrophotometer (Dence 1992).

Carbohydrate compositions of the original bamboo, pretreated bamboo substrates, and spent liquors were conducted using an improved high-performance anion exchange chromatography (Dionex HPLC system ICS-3000) device equipped with integrated amperometric detector and Carbopac™ PA1 guard and analytical columns at 20 ^{o}C. Eluent was provided at a rate of 0.7 mL/min, according to the following gradient: 0 to 25 min, 100% water; 25.1 to 35 min, 20% water and 80% 0.1 M NaOH; 35.1 to 40 min, 100% water. In addition, 0.5 M NaOH at a rate of 0.3 mL/min was used as post-column eluent to provide a stable baseline and detector sensitivity.

Fermentation inhibitors including acetic acid, formic acid, furfural, levulinic acid, and 5-hydroxylmethylfurural (HMF) were analyzed using the Dionex ICS-3000 system equipped with a Supelcogel C-610H column at temperature 30 ^{o}C and UV detector at 210 nm. Eluent was 0.1% phosphoric acid at a rate of 0.7mL/min for 0 to 85 min.

According to the filter paper assay recommended by the International Union of Pure and Applied Chemists (Ghose 1987), the cellulase activity was determined, and its expression will be given in filter paper units (FPUs). β-glucosidase activity was determined through p-nitrophenyl-b-D-glucoside as the substrate (Wood and Bhat 1988), and its expression is given in International Units (IUs).

RESULTS AND DISCUSSION

Comparison of Cell Wall Components of Pretreated Substrates

The cell wall chemical composition can provide some indications of the effect of chemical pretreatment on bamboo's chemical structure. Chemical components of

untreated bamboo and pretreated bamboo substrates are listed in Table 1. The untreated bamboo has glucose, xylose, and lignin contents of about 41.3%, 22.0%, and 24.3%, respectively. There are low contents of arabinose (1.1%), galactose (0.3%), and mannose (0.6%) in untreated bamboo. The organosolv pretreatment without acid catalyst slightly changed the chemical components composition of bamboo. The organosolv pretreatments with acid catalyst were very effective in removing hemicellulose and lignin, especially under the conditions of 180 ^{o}C and 60 min. As a result, cellulose was enriched in the pretreated substrates as high as 84.5% (180 ^{o}C, 30 min), 89.7% (180 ^{o}C, 60 min), and 70.4% (160 ^{o}C, 60 min), respectively. This suggested that the organosolv pretreatment with sulfuric acid could minimize the loss of cellulose, which serves as the main resource of glucose for bioethanol production. The addition of acid to the liquid mixture played a very important role in catalyzing the removal of hemicellulose and lignin. An increase in pretreatment duration and temperature increased lignin and hemicellulose removal.

Table 1. Chemical Analyses of Untreated and Pretreated Bamboo Substrates

Acid charge on OD bamboo (%)	Pretreatment temperature (°C)	Pretreatment time (min)	Component weight (%)						
			Arabinose	Galactose	Glucose	Xylose	Mannose	Acid-insoluble lignin	Acid-soluble lignin
Untreated bamboo			1.1±0.1	0.3±0.0	41.3±0.4	22.0±1.0	0.6±0.0	22.8±0.2	1.5±0.0
0	180	30	1.0±0.1	0.3±0.0	43.7±1.5	21.8±0.9	ND	24.9±0.5	2.2±0.0
2	180	30	0.1±0.0	ND	84.5±3.1	8.2±0.3	0.8±0.1	5.5±0.1	2.3±0.1
2	180	60	0.0±0.0	ND	89.7±2.9.	5.0±0.3	1.4±0.2	3.3±0.2	2.9±0.2
2	160	60	0.2±0.0	ND	70.4±3.5.	17.1±0.7	ND	9.9±0.4	1.9±0.1

The organosolv spent liquor was mixed with three volume of water to precipitate the dissolved lignin. Base on the 24.3 g lignin in 100 g raw original bamboo, the amount of precipitated lignin was 80.1% (180 ^{o}C, 30 min), 87.2% (180 ^{o}C, 60 min), and 67.9% (160 ^{o}C, 60 min), respectively. The sugars and soluble lignin in the combined liquor of filtrate and water washes are listed in Table 2. The concentration was calculated based on original volume of the spent liquors after pretreatments. As discussed above, an increase in pretreatment duration and temperature increased hemicellulose removal, and this was confirmed by the concentrations of sugars in spent liquors. More glucose and xylose were in 180 ^{o}C, 60 min pretreatment spent liquors than that in 180 ^{o}C, 30 min and 160 ^{o}C, 60 min spent liquors. But the content of arabinose and mannose in spent liquors were not the same. It is notable that arabinose and mannose are minor sugars in bamboo and easily degraded in acidic pretreatment.

Table 2. Chemical Analyses of Pretreated Spent Liquors

Acid charge on OD bamboo (%)	Pretreatment temperature (°C)	Pretreatment time (min)	Component in spent liquors (g/L)					
			Arabinose	Galactose	Glucose	Xylose	Mannose	Acid-soluble lignin
0	180	30	0.0±0.0	0.0±0.0	0.8±0.1	0.0±0.0	0.8±0.2	5.5±0.9
2	180	30	1.6±0.2	0.5±0.0	6.9±0.9	20.5±2.3	0.7±0.1	5.0±0.3
2	180	60	1.4±0.2	0.7±0.0	15.0±1.2	21.8±3.0.	0.3±0.0	5.3±0.3
2	160	60	2.0±0.5	0.1±0.0	1.0±0.3	10.6±0.7	0.0±0.	5.1±0.5

Mass Balance of Sugars During Pretreatments

An ideal pretreatment should provide readily enzymatically digestible substrates. But that is not enough; it should also lead to maximal recovery of all the components of the original biomass. As discussed above, the original sample contained 41.3 g glucose, 22.0 g xylose, 1.1 g arabinose, 0.3 g galactose, and 0.6 g mannose from 100 g oven-dry bamboo. The total sugars and lignin in 100 g raw bamboo is 89.6 g without water/ethanol extractives and ash. As shown in Table 3, sugars and lignin's recoveries of the two fractions, solid substrates and spent liquors, were calculated based on 100 g untreated raw bamboo. The total sugars and lignin recovery was 86.7 g without acid pretreatment, while pretreatment with acid addition led to lower recovery (80.1 to 81.7 g). Although total components recovery of solid substrates and spent liquors was high, only 41.5 g, 33.1g, and 54.9 g solid substrates were obtained from 180 ^{o}C 30 min, 180 ^{o}C 60 min, and 160 ^{o}C 60 min pretreatments, respectively. Low substrate yield from pretreatment was due in part to the dissolution of lignin, as well as from dissolution and degradation of more cellulose and xylose. The detected sugars in the spent liquors ranged from 18.8 g (180 ^{o}C 30 min), to 24.5 g (180 ^{o}C 60 min), and to 8.5 g (160 ^{o}C 60 min). The spent liquors with such high concentration sugars can be detoxified and then used for ethanol fermentation.

For certain components of the bamboo, the recovery was calculated on the certain content in the raw bamboo. For example, glucose in the substrate and spent liquor were 38.7 g and 0.5 g after the pretreatment of 100 g raw bamboo, and the raw bamboo has 41.3 g glucose before the pretreatment. So the glucose recovery was 94.9% ((38.7+0.5)/41.3). The calculations from these data indicate that the total sugar recovery was 90.0% (180 ^{o}C 30 min), 84.7% (180 ^{o}C 60 min), and 87.1% (160 ^{o}C 60 min), respectively. The glucose recovery was 94.2% (180 ^{o}C 30 min), 92.5% (180 ^{o}C 60 min), and 95.6% (160 ^{o}C 60 min). While the xylose recovery was quite low, it was 73.6% (180 ^{o}C 30 min), 69.1% (180 ^{o}C 60 min), and 72.7% (160 ^{o}C 60 min), respectively. These results indicate that the xylose underwent greater degradation at high temperature and longer duration time than the glucose. This is one of the reasons why the 180 ^{o}C, 60 min spent liquor had a higher concentration of furfural than the other spent liquors (Table 4).

Table 3. Mass Balance of 100 g Raw Oven-Dry Bamboo during Pretreatment

Acid charge on OD bamboo (%)	Pretreatment temperature (°C)	Pretreatment time (min)	Component recovery (g)								
				Arabinose	Galactose	Glucose	Xylose	Mannose	Lignin	Sum	Recovery
0	180	30	Substrate	0.9±0.1	0.3±0.0	38.7±1.5	19.3±0.9	ND	23.1±0.5	82.3	86.7
			Liquor	0.0±0.0	0.0±0.0	0.5±0.1	0.0±0.0	0.5±0.2	3.4±0.9	4.4	
2	180	30	Substrate	0.0±0.0	0.0±0.0	34.6±3.1	3.4±0.1	0.3±0.1	3.2±0.1	41.5	81.2
			Liquor	1.0±0.2	0.3±0.0	4.3±0.9	12.8±2.3	0.4±0.1	20.8±0.3	39.6	
2	180	60	Substrate	0.0±0.0	0.0±0.0	28.8±2.9	1.6±0.3	0.4±0.2	2.3±0.2	33.1	80.1
			Liquor	0.9±0.2	0.4±0.0	9.4±1.2	13.6±3.0	0.2±0.0	22.5±0.3	47.0	
2	160	60	Substrate	0.1±0.0	0.0±0.0	38.9±3.5	9.4±0.7	ND	6.6±0.4	54.9	81.7
			Liquor	1.2±0.5	0.1±0.0	0.6±0.2	6.6±0.7	0.0±0.0	18.3±0.5	26.8	

Comparisons of Fermentation Inhibitors Formation during Pretreatments

The cellulose and hemicellulose removed by organosolv pretreatments were partially hydrolyzed to fermentable sugars and even further degraded to potential fermentation inhibitors, such as formic acid, levulinic acid, furfural, and hydroxymethylfurfural (HMF). Furfural was derived from pentoses, HMF from degradation of hexoses, and levulinic and formic acids from successive decomposition of HMF. The acid-soluble lignin and other phenol type compounds are also inhibitors of the fermentation process. Acetic acid was released from acetyl groups on hemicelluloses.

All the potential fermentation inhibitors mentioned above are listed in Table 4. The data indicated that the acid-soluble lignin in the four spent liquors might be slightly different. The total of inhibitors (formic, acetic and levulinic acids, furfural, and HMF) were significantly different. There was no inhibitor detected in organosolv pretreatment liquors without acid addition. The total inhibitors increased with increases in the pretreatment temperature and time duration. The amounts of total inhibitors formed in case of 160 ^{o}C, 60 min and 180 ^{o}C, 30 min pretreatments were only 25.8% and 62.1% of those formed in the case of 180 ^{o}C, 60 min pretreatment (18.2 g/L), respectively. Meanwhile, the furfural concentrations in the spent liquors were much higher than HMF concentrations. Furfural was derived from pentoses, whereas HMF was from degradation of hexoses. The results also indicated that xylose was much more easily degraded than glucose during the pretreatments.

Table 4. Fermentation Inhibitors in Pretreated Spent Liquors

Acid charge on OD bamboo (%)	Pretreatment temperature (°C)	Pretreatment time (min)	Inhibitors (g/L)						
			Acid-soluble lignin	Formic acid	Acetic acid	Furfural	HMF	Levulinic acid	Total
0	180	30	5.5±0.9	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0
2	180	30	5.0±0.3	2.4±0.2	1.6±0.4	4.4±0.1	1.0±0.1	2.0±0.3	11.3
2	180	60	5.3±0.3	4.3±0.4.	1.8±0.2	9.4±0.2	1.1±0.1	1.6±0.4	18.2
2	160	60	5.1±0.5	1.0±0.1	0.9±0.1	1.4±0.1	0.4±0.1	1.0±0.2	4.7

Enzymatic Hydrolyzability of Pretreated Bamboo Substrates

There are many factors that affect enzymatic hydrolyzability of substrates, such as hemicellulose content, lignin structure, distribution and content, cellulose crystallinity, the degree of polymerization, and so on (Mansfield *et al.* 1999; Alvira *et al.* 2010). All these known and other unknown factors cause bamboo to be a unique biomass for efficient pretreatment and distinct relative to agricultural wastes and wood. One of the structural differences is that bamboo has high density and hardness, even for the outer part of bamboo (bamboo green) (Chand *et al.* 2006). The wax in the bamboo green also may affect the pretreatment effect.

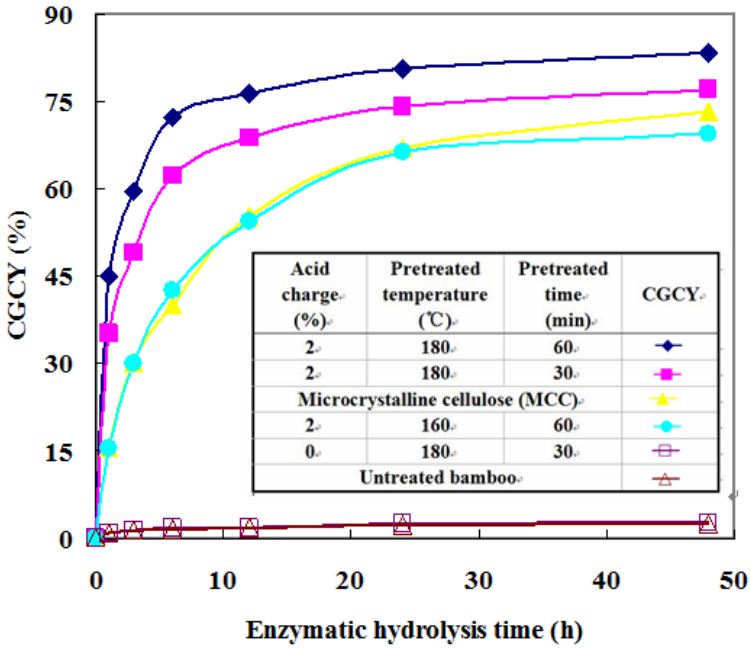

Fig. 1. Comparison of enzymatic hydrolysability of different pretreated bamboo substrates with a enzyme loading of 15 FPU cellulase and 30 IU β-glucosidase per gram of cellulose, 50 °C, pH 4.8 and on a 220 rpm shaker. CGCY: Cellulose-to-Glucose Conversion Yield

The enzymatic hydrolyzability of organosolv pretreated bamboo substrates are shown in Fig. 1. Commercialized microcrystalline cellulose (MCC) was used as a comparison for the enzymatic digestibility. The enzymes loading were 15 FPU (Filter Paper Units) cellulase and 30 IU (International Units) β-glucosidase per gram cellulose for all enzymatic hydrolyses. The cellulose-to-glucose conversion yield of untreated raw bamboo after 48 h of hydrolysis was only 2.4%. The cellulose-to-glucose conversion yield of organosolv pretreatment without acid addition was just 2.6%, not significantly increasing. Otherwise, the acid-catalyzed organosolv pretreatment significantly improved the enzymatic digestibility of bamboo. Meanwhile, with increasing pretreatment temperature and time duration, the cellulose-to-glucose conversion yield was increasing. When the temperature was 180 ^{o}C and the duration was 30 min or 60 min, the glucose yield reached 77.1% (180 ^{o}C, 30 min) or 83.4% (180 ^{o}C, 60 min), respectively, which were even higher than for the microcrystalline cellulose to glucose conversion yield (73.2%). Including glucose in the pretreated spent liquor and enzymatic hydrolysate, the total glucose yields from raw bamboo were 27.6% (160 ^{o}C, 60 min), 31.0% (180 ^{o}C, 30 min), and 33.4% (180 ^{o}C, 60 min), respectively. These results were calculated based on 41.3% cellulose content in raw bamboo.

Based on comparison among the organosolv pretreatments, the cellulose-to-glucose conversion yields were increasing as the contents of xylose and lignin were decreasing. The removal of hemicellulose and lignin increased the susceptibility of the cellulose to enzymes. The results indicated that the content of hemicellulose and lignin affected the enzymatic digestibility of the substrates.

CONCLUSIONS

Organosolv pretreatment (180 ^{o}C, 30 min) without acid addition had no observable influence on cell-wall change or enzymatic digestibility of bamboo. Organosolv pretreatment with sulfuric acid as a catalyst significantly accelerated hemicellulose and lignin removal and increased the enzymatic digestibility of bamboo substrates. Based on the same acid loading (2% on OD bamboo), when the pretreatment temperature and duration time were increased, much more hemicellulose and lignin were removed, and the cellulose-to-glucose conversion yield of enzymatic hydrolysis was increased, too. The total glucose yield (including pretreatment and hydrolysis) from raw bamboo were 27.6% (160 ^{o}C, 60 min), 31.0% (180 ^{o}C, 30min), and 33.4% (180 ^{o}C, 60 min), respectively.

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