Ethanosolv Pretreatment of Bamboo with Dilute Acid for Efficient Enzymatic Saccharification

Zhiqiang Li, Zehui Jiang, Benhua Fei*
International Centre for Bamboo and Rattan, Beijing 100102, China.
** Corresponding author: jiangzehui@icbr.ac.cn*
Zhiyong Cai

USDA Forest Service, Forest Products Laboratory, 1 Gifford Pinchot Dr., Madison, WI 53726, United States
Xuejun Pan
Biological Systems Engineering, University of Wisconsin, 460 Henry Mall, Madison, WI 53706, United States

Abstract

Bamboo is a potential lignocellulosic biomass for the production of bioethanol because of its high cellulose and hemicelluloses content. In this research, ethanosolv pretreatment catalyzed by sulfuric acid was studied in order to enhance enzymatic saccharification of moso bamboo. The addition of 2% (w/w on bamboo) sulfuric acid in water or 75% (v/v) ethanol was demonstrated effective in pretreatment and fractionation of bamboo. The ethanosolv pretreatment yielded a solid fraction with 84.5% cellulose. The cellulose-to-glucose conversion yields were 77.1% (ethanosolv) and 57.3%(water) after enzymatic hydrolysis of the solid fraction at 50 °C for 48 h using a enzyme loading (15 filter paper units of cellulase/g cellulose and 30 IU β-glucosidase/g cellulose). The concentrations of fermentation inhibitors such as 5-hydroxy-2- methyl furfural (HMF) and furfural were 0.96 g/L and 4.38 g/L, respectively, in the spent liquor after the ethanosolv pretreatment, which were slightly lower than those in the spent liquor from H_2SO_4-water treatment. When the pretreatment spent liquor was diluted with water, dissolved lignin was precipitated and then recovered through filtration. More than 87.2% of the original lignin in untreated bamboo was recovered as ethanol organosolv lignin.

***Keywords*:** Bamboo, Bioethanol, Ethanosolv, Organosolv pretreatment, Dilute acid

Introduction

The first-generation bioethanol which based on corn reached the limit due to limitation in feedstock supply and competition with 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 of researchers and the 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 et al., 2002). Thus, a pretreatment process is required to enhance its enzymatic digestibility. 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). It can breaks down internal lignin and hemicellulose bonds and thus, removes the portion of lignin from biomass. Through the removal of the lignin, pore-volume and surface area increase so as to increase substrate enzymatic digestibility. A strong inorganic acid is usually carried out in the organosolv pretreatment as a catalyst which to hydrolyze the lignin-lignin and lignin-carbohydrate bond in biomass (Holtzapple et al., 1984).

Bamboo is the vernacular or common term for members of a particular taxonomic group of large woody grasses (subfamily *Bambusoideae*) (Scurlock et al., 2000). Bamboo has some advantages such as fast growth, high cellulose content and bamboo resource is very abundant in China. So it may be one of most potential bio-energy resources with Chinese characteristics in the future. Some pretreatments have been investigated on bamboo for bioethanol production. Enzymatic saccharification (48 h) using a 35 atm and 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, the sugar yield was only 43-85mg/(g initial dry sample) of dilute sulfuric acid pretreatment which carried out at 120-140°C for 30-90min with a sulfuric acid loading of 0.6-1.2% (w/w) (Leenakul et al., 2010). Literature on organosolv pretreatment of bamboo is scarce. In this investigation, the feasibility of an ethanosolv pretreatment using inorganic catalyst was evaluated. Dilute sulfuric acid was applied as the catalyst for ethanosolv pretreatment of moso bamboo and the enzymatic hydrolysis were carried out after the pretreatment.

Materials and Methods

Materials. Moso bamboo (*Phyllostachys heterocycla*) was acquired from the central area of Louisiana, USA. 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 under oven-dried (OD) for 24 h at 105±2°C. 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). Each pretreatment was carried out in duplicate; the average results of the two runs were reported. In general, 8 g of raw oven-dry bamboo was loaded into a 100 mL vessel. Prepared pretreatment solution of 50 mL 0.32% (2% on OD bamboo) H_2SO_4 in water or 75% (w/w) ethanol was then poured into the vessel. The vessel positioned at the centre of rotating circular ceramic plate in the microwave oven for treatment at the power level of 400W. The temperature was raised to the 180°C in 10 min and maintained for an additional 30 min. After the pretreatment, the pretreatment spent liquor was separated from the pretreated substrate (solid) by vacuum filtration. The ethanosolv pretreated substrate was washed three times with 50 mL aqueous ethanol with the same concentration (75%) of pretreatment liquor at 60 °C and the washes combined with the spent liquor. The substrate was then washed three more times with water at 60 °C and the washes discarded. The solid substrate then stored at 4°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, described as ethanol organosolv lignin (EOL), was collected on 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°C for fermentation inhibitors and chemicals analysis 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°C on a shaking incubator (Thermo Fisher Scientific, Model 4450, Waltham, MA) at 220 rev/min. Bamboo substrate equivalent to 0.8 g glucan was loaded with 40 mL of 0.05M sodium acetate buffer (pH 4.8). Approximately 1.5 mg of tetracycline chloride was added to control 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 hour to analyze glucose concentration. The hydrolysis was conducted in duplicate for each substrate; the average is reported here.

Analytical methods. In-soluble 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). Acid-soluble lignin was measured at 205 nm on a UV-Visible spectrophotometer.

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) equipped with integrated amperometric detector and Carbopac™ PA1 guard and analytical columns at 20°C. Fermentation inhibitors including acetic acid, formic acid, furfural, levulinic acid and 5-hydroxylmethylfurural (HMF) were analyzed using the Dionex ICS-3000 equipped with a Supelcogel C-610H column at temperature 30°Cand UV detector at 210 nm.

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

Results and Discussions

Comparison of cell wall components of pretreated substates. The cell wall chemical composition can provide some indications of the effect of chemical pretreatment on bamboo chemical structure. Chemical components of untreated bamboo and pretreated bamboo substrates are listed in table1. The untreated bamboo has glucose, xylose and lignin contents of about 41.3%, 22.0% and 24.3%, respectively. There are low content of arabinose (1.1%), galactose (0.3%) and mannose（0.6%) in untreated bamboo. The ethanosolv pretreatment without acid catalyst slightly changed the chemical components composition of bamboo. The ethanosolv pretreatment with acid catalyst were very effective in removing hemicellulose and lignin. As a result, cellulose was enriched in the pretreated substrates as high as 84.5%. This suggested that the ethanosolv pretreatment with sulfuric acid could minimize the loss of cellulose, which served the main resource in bioethanol production. The addition of acid to the liquid mixture played a very important role in catalyzing the removal of hemicellulose and lignin.

Table 1. *Chemical analyses of untreated and pretreated bamboo substrates after 30 min at 180 °C*

Acid charge on OD bamboo (%)	Pretreated solutions	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	Ethanosolv	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	Ethanosolv	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	Water	0.1±0.0	ND	52.0±2.9	1.8±0.2	ND	35.0±0.4	1.8±0.4

The spent liquor and ethanol washes were combined and mixed with three volumes of water to precipitate the dissolved lignin. The precipitated lignin was collected on filter paper through vacuum filtration and then washed thoroughly with water and air-dried. 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. Generating high-quality lignin is one of the unique advantages of the ethanosolv 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). High-quality lignin can be used as a substitute for polymeric materials, such as phenolic powder resins, polyurethane foams, and epoxy resins.

The sugars and soluble lignin in the combined liquor of filtrate and water washes were listed in table 2. The concentration was calculated on original spent liquors. More glucose and xylose were detected in ethanosolv pretreatment spent liquors than that in water pretreated spent liquors.

Table 2. *Chemical analyses of pretreated spent liquors*

Acid charge on OD bamboo (%)	Pretreated solutions	Component in spent liquors (g/L)					
		Arabinose	Galactose	Glucose	Xylose	Mannose	Acid-soluble lignin
0	Ethanosolv	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	Ethanosolv	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	Water	0.04±0.0	0.7±0.0	3.3±0.1	8.4±0.6	ND	5.7±0.3

Mass balance of sugars during pretreatments. For an ideal pretreatment, it should provide readily enzymatic digestible substrates. But it is not enough; it should also to maximally recover all the components of original biomass. As shown in table 3, sugars and lignin in two fractions, solid substrates and spent liquors, were calculated based on 100 g untreated raw bamboo. As discussed above in table1, it contained 41.3 g glucose, 22.0 g xylose, 1.1 g arabinos, 0.3 g galactose and 0.6 g mannose from 100 g oven-dry bamboo. The total sugars and lignin recovery was 86.7 g without acid pretreatment. Ethanosolv pretreatment with acid addition obtained lower recovery 81.2 g. Although total components recovery was high, only 41.5 g substrates were obtained. Low substrate yield from ethanosolv pretreatment was due in part to the dissolution of more hemicellulose and lignin. The detected sugars in the spent liquors were 18.8 g (ethanosolv). The spent liquors with so high concentration sugars can be detoxified and then used for ethanol fermentation.

The calculations from these data indicated that total sugar recovery was 87.4 % (ethanosolv) and 63.7 % (water). The glucose recovery was 94.2 % (ethanosolv) and 84.0% (water). While the xylose recovery was quite low, it was 73.6% (ethanosolv) and 28.6 % (water). The result indicated xylose was subject to greater degradation than glucose in acid pretreatment, especially in water pretreated solution. This is one of the reasons why water pretreated spent liquor had high concentration of furfural than ethanosolv pretreated spent liquor, which was listed in table 4.

Table 3. *Mass balance of 100 g oven-dry bamboo powder during pretreatments*

Acid charge on OD bamboo (%)	Pretreated solutions	Component recovery, g								
			Arabinose	Galactose	Glucose	Xylose	Mannose	Lignin	Sum	Recovery
0	Ethanosolv	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	Ethanosolv	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	Water	Substrate	0.1±0.0	0.0±0.0	32.6±2.9	1.1±0.2	0.0±0.0	23.0±0.8	56.6	68.0
		Liquor	0.0±0.0	0.5±0.0	2.1±0.2	5.2±0.8	0.0±0.0	3.6±0.4	11.4	

Comparisons of fermentation inhibitors formation during pretreatments. The cellulose and hemicellulose removed by ethanosolv 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 derived from pentoses, HMF from degradation of hexoses, and levulinic and formic acids from successive decomposition of HMF. The acid-soluble lignin was also a kind of fermentation inhibitors. Acetic acid released from acetyl groups on hemicelluloses, which could be detected in both acidic and basic pretreatment liquors.

All the potential fermentation inhibitors mentioned above were listed in table 4. The data clearly indicated that the acid-soluble lignin in the spent liquors were slightly different. The total of inhibitors (formic, acetic and levulinic acid, furfural and HMF) were significantly different. There was no inhibitor detected in ethanosolv pretreatment liquors without acid addition. The total inhibitors in organsolv pretreated spent liquor was less than that in water pretreated spent liquors.

Table 4. *Fermentation inhibitors in pretreated spent liquors after 30 min at 180 °C*

Acid charge on OD bamboo (%)	Pretreated solutions	Inhibitors (g/L)						
		Acid-soluble lignin	Formic acid	Acetic acid	Furfural	HMF	Levulinic acid	Total
0	Ethanosolv	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	Ethanosolv	5.0±0.3	2.4±0.2	1.6±0.4	4.4±0.1	1.0±0.1	2.0±0.3	11.4
2	Water	5.7±0.3	4.0±0.3	5.7±0.4	4.6±0.1	1.2±0.1	0.4±0.0	15.9

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 and degree of polymerization, and so on(Mansfield et at., 1999; Alvira et al., 2010). All these known and unknown factors caused bamboo to be a unique biomass for efficient pretreatment not likes 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).

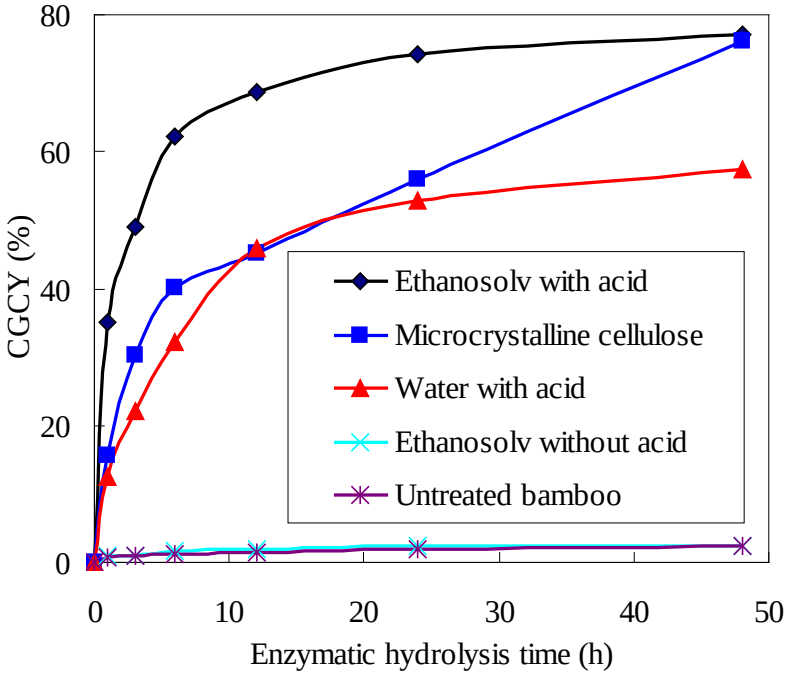

Figure 1. *Comparison of time-dependent enzymatic hydrolysability of different pretreated bamboo substrates with a enzyme loading of 15 FPU cellulase and 30 IU β-glucosidase per gram of cellulose, 50°C, pH 4.8 and on a 220 rpm shaker. CGCY: cellulose-to-glucose yield.*

The enzymatic hydrolyzability of ethanosolv pretreated bamboo substrates were shown in figure 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 hydrolysis. The cellulose-to-glucose conversion yield of untreated raw bamboo after a 48 h hydrolysis was only 2.4%. The cellulose-to-glucose conversion yield of ethanosolv pretreatment without acid addition was just 2.6%, no significantly increasing. Otherwise, the water and ethanosolv with acid pretreatment had significantly improved the enzymatic digestibility of bamboo. Meanwhile, the cellulose-to-glucose conversion yield of ethanosolv pretreatment (77.1%) was higher than that of water pretreatment (57.3%). Which were even higher than microcrystalline cellulose to glucose conversion yield (76.2%)? Including glucose in the spent liquor, the total glucose yield from raw bamboo were 31.0% (ethanosolv) and 20.8% (water), respectively. It was calculated based on 41.3% cellulose content in raw bamboo.

Comparison among the pretreatments, the cellulose-to-glucose conversion yields were increasing with the content of lignin decreasing. The removal of hemicellulose and lignin increased cellulose susceptibility to enzymes. Meanwhile, the cellulose-to-glucose conversion yield of MCC was 76.2%, not reach 100%. The results indicated that besides the content of hemicellulose and lignin, other factors like cellulose crystallinity and degree of polymerization affected the enzymatic digestibility of the substrates together.

Conclusion

Ethanosolv pretreatment without acid addition has no observably influence on cell-wall change or enzymatic digestibility of bamboo. Ethanosolv pretreatment with sulfuric acid as catalyst has significantly hemicellulose and lignin removal and enzymatic digestibility increasing of bamboo substrates. Based on the same acid loading (2% on OD bamboo), the ethanosolv pretreatment has much more hemicellose and lignin removal and the cellulose-to-glucose conversion yield was higher than water-pretreatment. The total glucose yield (including pretreatment and hydrolysis) from raw bamboo were 31.0% (ethanosolv) and 20.8% (water), respectively.

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