

Comparison of Dilute Organic and Sulfuric Acid Pretreatment for Enzymatic Hydrolysis of Bamboo

Zhiqiang Li, Benhua Fei, and Zehui Jiang*

Pretreating bamboo is essential to overcome the recalcitrance of lignocellulose for bioethanol production. In this study, the effectiveness of formic, acetic, and sulfuric acids in pretreating bamboo were compared. To measure pretreatment efficiency, the enzymatic digestibility of the pretreated bamboo substrates was determined. Monomeric glucose conversion yield was measured after enzymatic hydrolysis. Additionally, the sugar degradation products fermentation inhibitors were measured after pretreatment. After conducting many tests, it was determined that pretreatment with dilute formic acid at 180 °C and 30 min can be an acceptable alternative to dilute sulfuric acid pretreatment.

Keywords: Bamboo; Pretreatment; Dilute acid; Formic acid; Acetic acid; Oxalic acid; Enzymatic hydrolysis

Contact information: International Centre for Bamboo and Rattan, Beijing 100102, China;

** Corresponding author: jiangzehui@icbr.ac.cn*

INTRODUCTION

Lignocellulosic biomass, such as agricultural by-products and forestry residues, is relatively cheap, abundant, and renewable. Therefore, it is the chief potential feedstock for second-generation bioethanol production. According to the China Forestry Development Report for the year 2013 issued by the State Forestry Administration of China, there is an annual production of 1.644 billion culms of bamboo in China, making bamboo a likely candidate for use in second-generation bioethanol production. Like other lignocellulosic biomass, bamboo requires pretreatment to improve cellulose accessibility to cellulolytic enzymes. Some researchers have focused on bamboo pretreatment for bioethanol production. The cellulose conversion to glucose yield of bamboo is lower than that of both wood and agricultural waste (Shimokawa *et al.* 2009; Leenakul and Tippayawong 2010; García-Aparicio *et al.* 2011; Li *et al.* 2014).

Dilute acid pretreatment is widely considered a promising method for industrial purposes. Dilute acid pretreatment is mainly used in hemicellulose removal from biomass without delignification (Mosier *et al.* 2005; Garlock *et al.* 2011). Dilute sulfuric acid applied in bamboo pretreatment has also been studied (Leenakul and Tippayawong 2010; Li *et al.* 2012c). Presently, sulfuric acid is used in the ethanol organosolv pretreatment of bamboo. In samples pretreated with sulfuric acid, it has been reported that the cellulose-to-glucose conversion yield of the pretreated bamboo substrates can be as high as 83.4%. Finally, the cellulose-to-glucose conversion yield for organosolv-alkali pretreatment was 95.5% after enzymatic hydrolysis (Li *et al.* 2012a, b). Although dilute sulfuric acid pretreatment is an effective approach that results in a high sugar yield from bamboo, it has some disadvantages. For example, a large problem stemming from dilute sulfuric acid

pretreatment is corrosivity, which requires expensive materials, leads to the formation of inhibitory compounds, and requires acid neutralization.

An organosolv system is one in which organic acids are employed at a high concentration. Formic acid, acting as an active agent, is able to effectively penetrate into the interior space of cellulose molecules, thus collapsing the rigid crystalline structure and allowing hydrolysis to occur in both the amorphous and crystalline zones (Sun and Lin 2010). An organosolv system of formic acid (78.22%), water (17.78%), and additional hydrochloric acid (4%, w/w) could potentially hydrolyze bamboo fiber into glucose with 35% conversion yield (Sun and Lin 2010). Such organosolv treatments increase the dosages of acid during pretreatment, and therefore require more alkali for subsequent neutralization or water for washing.

Dilute organic acid pretreatment has received minimal attention, especially regarding bamboo pretreatment. Dilute organic acid pretreatment has some desirable characteristics in comparison with dilute inorganic acid, including effective hydrolysis, less degradation of products, and a higher yield of oligomeric sugars (Kootstra *et al.* 2009a, b; Qin *et al.* 2012). Meanwhile, using sulfuric acid in pretreatment not only renders large quantities of gypsum (which can negatively affect the downstream process), but also results in a low-value by-product stream (Yang and Wyman 2008). The quality of the by-product stream improves significantly with organic acid pretreatment, as the organic substances can be more easily burned in co-firing installations, used for fertilizing soil, or applied in animal feed (Partanen and Mroz 1999; Kootstra *et al.* 2009a).

Organic acids such as formic acid and acetic acid have been used for pulping of woody and herbaceous plants (Muurinen 2000). Pretreatments of biomass by formic acid and acetic acid have been reported by several authors. (Sun *et al.* 2007; Sun *et al.* 2008; Xu *et al.* 2009). Organic acid pretreatment increase accessible surface area of substrates, solubilization of hemicelluloses and lignin, and alteration of lignin structure (Zhao *et al.* 2009). Dicarboxylic organic acids such as oxalic acid can hydrolyze β -(1, 4)-bonds more selectively than sulfuric acid (Mosier *et al.* 2002; Lu and Mosier 2007). In this study, we compared the effectiveness of formic acid, acetic acid, and oxalic acid to that of sulfuric acid in the pretreatment of bamboo. Furthermore, this study investigated the effect of inorganic acid combination (boric acid and sulfuric acid) on the efficiency of the pretreatment, as well as the formation of sugar degradation products. To measure pretreatment efficiency, the enzymatic digestibility of pretreated bamboo substrates was determined by calculating the glucose yields from cellulose conversion.

EXPERIMENTAL

Materials

Moso bamboo (*Phyllostachys heterocycla*) was acquired from Guangxi, China, in the winter of 2012. Air-dried bamboo was milled using a hammer mill with a screen opening size of 2.0 mm before chemical pretreatment. Milled bamboo powder was kept in a sealed plastic barrel at room temperature until it was used. The moisture content of the ground powder was measured in an oven at 103 ± 2 °C for 24 h. The ash, water-ethanol extractives, and chemical composition of the bamboo were also determined.

Two commercial enzymes, Celluclast 1.5 L (cellulase) and Novozyme 188 (β -glucosidase), were purchased from Sigma-Aldrich (St. Louis, MO). All chemical reagents used in this study were purchased from Sinopharm Chemical Reagent Beijing Co., Ltd., China.

Methods

Pretreatments

All the acids acquired were of research grade and used shortly after being received. The acid pretreatment was carried out in a microwave accelerated reaction system with the microwave frequency 2450 MHz and power 400 W, made by CEM Corporation (Model MARS, USA). A bamboo powder sample, weighing 8 g on an oven-dry (OD) basis, was used for each pretreatment experiment. Each sample was immersed in 50-mL water solutions of the particular acid being studied. The mixture was placed in a 100-mL vessel and positioned at the center of a rotating circular ceramic plate in the microwave oven for treatment. The temperature was raised from room temperature to 180 °C over the span of approximately 10 min and maintained at 180 °C for an additional 30 min. After pretreatment, the mixture was left to sit for about 30 minutes to allow the temperature to drop below 80 °C. Then, the bamboo substrate and spent liquor were separated by vacuum filtration. The liquor was sampled and stored at 4 °C, ideal for the sugar and fermentation inhibitors, and an analysis by high-performance liquid chromatography (HPLC) was performed. The solid bamboo substrate was washed with water until the pH of the washings was near neutral. Then, the substrate was kept in a sealed plastic bag and stored at 4 °C for chemical composition analysis and enzymatic hydrolysis. Each pretreatment was carried out in duplicate, and the average result was recorded.

Enzymatic hydrolysis

Enzymatic hydrolysis was carried out in 100-mL plastic jars at 50 °C on a shaking incubator (KYC-100C; Shanghai Fuma Laboratory Equipment Co. Ltd., China) at 220 rpm. Bamboo substrates, equivalent to 0.8 g of glucan, were loaded with 40 mL of 0.05 M acetic acid/sodium acetate buffer (pH 4.8). Approximately 1.5 mg of tetracycline chloride, dissolved in ethanol solution, was added to prevent the consumption of liberated sugars and to control the growth of microorganisms. Two enzyme solutions, cellulase (15 filter paper units (FPU) *per* gram glucan) and β -glucosidase (30 international units (IU) *per* gram glucan), were loaded. Enzymatic hydrolyzates were sampled periodically, at 1, 3, 6, 12, 24, and 48 h, to analyze the glucose concentration. Enzymatic hydrolysis was conducted twice for each pretreated bamboo substrate, and the average was recorded.

Analytical methods

The carbohydrate and lignin (acid-soluble and -insoluble lignin) contents of both the untreated and pretreated bamboo substrates were analyzed according to the National Energy Laboratory (NREL) analytical procedure: Determination of Structural Carbohydrates and Lignin in Biomass (with some modifications) (Sluiter *et al.* 2008). The method was based on the degradation of carbohydrates into monomeric sugars by a two-step hydrolysis with sulfuric acid (72% for 2 h at 30 °C; 3% for 1 h at 121 °C). The sugars were determined using HPLC. Acid-soluble lignin was measured at a wavelength of 205 nm on a UV-visible spectrophotometer (Shanghai Sunny Hengping Scientific Instrument

Co. Ltd., China) (Dence 1992). The acid-insoluble lignin contents were determined by drying the acid-treated samples in a vacuum oven at 60 °C for 10 h.

The liquid samples were analyzed by HPLC equipped with a refractive index detector (Waters Corporation; Milford, MA). The detection of arabinose, galactose, glucose, mannose, and xylose sugars in the hydrolyzates as well as the carbohydrate analyses were performed using an anion exchange column (Aminex HPX-87P, Bio-Rad; USA) at 85 °C with 0.6 mL/min of water.

The enzymatic hydrolytic reaction of each pretreated substrate was monitored by measuring the time-dependent glucose concentrations in the enzymatic hydrolysis solutions. For expedited analysis, the glucose in the solutions was determined using a commercial biosensor analyzer (SBA-40E, Shandong Academy of Science; Shandong Province, China). The instrument's precision is within 2% of the actual value, based on the manufacturer's specifications. The average of duplicate runs was recorded.

RESULTS AND DISCUSSION

Comparison of Cell Wall Components of Pretreated Substrates

In general, in order to reach the range 150 to 180 °C, the acid pretreatment could be finished in an oil bath. However, the thermal treatment of lignocellulosic materials in an aqueous medium is known to release acetic acid, hence providing an acidic environment for auto-hydrolysis (Lora and Wayman 1978). Furthermore, microwave radiation supplies internal heat to the biomass and generates a continuously changing magnetic field, causing the polar bonds to vibrate as they align with the magnetic field. This disruption and shock to the polar bonds accelerates chemical, biological, and physical processes (Sridar 1998). Therefore, in this paper, we selected microwave oven instead of oil bath for pretreatment.

The cell wall chemical composition can provide some indication of the effect of chemical pretreatment on the bamboo chemical structure. The chemical compositions of both the original and pretreated bamboo substrates are listed in Table 1.

Table 1. Chemical Composition of Organic Acids in Pretreated Bamboo Substrates

Components (%)	Arabinose	Galactose	Glucose	Xylose	Mannose	Acid-insoluble lignin	Acid-soluble lignin
Untreated bamboo	1.10	0.40	44.50	22.03	0.61	23.82	1.50
Sulfuric acid	0.10	0.00	58.46	1.80	ND	34.97	1.78
Boric acid + sulfuric acid	0.11	0.00	55.01	1.64	ND	34.00	1.52
Oxalic acid	0.40	0.43	56.68	9.34	ND	33.15	4.03
Formic acid	0.07	0.01	61.64	6.15	ND	34.92	4.26
Acetic acid	0.13	0.03	59.31	7.63	ND	34.25	4.44
Acetic acid + sodium sulfite	0.28	0.12	62.06	16.35	ND	26.95	3.93

The data indicated that the dominant components in bamboo are glucan, lignin, and xylan. Cellulose (glucan) and hemicelluloses (arabinan, galactan, mannan, and xylan) accounted for more than 59% of the oven-dry weight of bamboo, making it a good potential feedstock for ethanol production. Xylose was the main sugar for the hemicellulose fraction, accounting for 91.3% of the total sugars. Water and ethanol extractives of the Moso bamboo are much higher (13.04%) than those of wood and other bamboos (Scurlock *et al.* 2000). However, the ash content (1.5%) was much lower than agricultural wastes (Sarkar *et al.* 2012). The content of cellulose, hemicellulose, ash, and lignin was calculated for the water and ethanol-extractive free bamboo material.

The pretreatments utilizing an organic acid catalyst were very effective in changing cell wall components. As a result, cellulose increased in the pretreated substrates from 55% to 62.1%. The increased cellulose suggests that the pretreatment with organic acid minimizes the loss of cellulose, which serves as 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. In comparison to sulfuric acid pretreatment, organic acid pretreatment could potentially preserve more hemicelluloses. Discounting hemicelluloses, the lignin content in the substrates was almost the same for sulfuric acid and organic acid pretreatment. The lignin contents indicated that neither sulfuric nor organic acid had appreciable effect on delignification. For acetic acid pretreatment, the bamboo substrate was composed of more xylose than the sulfuric acid pretreated bamboo substrates.

To evaluate the effectiveness of dilute acid pretreatment on monomer sugar formations, a mild post-acid hydrolysis (3% H₂SO₄, 121 °C, 60 min) of the pretreated spent liquors fraction was carried out. The sugars and soluble lignin in the pretreated spent liquors proceeding post-acid hydrolysis are listed in Table 2. The concentration was calculated for the original spent liquors. As discussed above, the glucose content of organic acid pretreated spent liquors were lower than sulfuric acid pretreated spent liquor. However, the content of arabinose, galactose, and mannose in spent liquors pretreated with organic acid were much higher than sulfuric acid spent liquor, because there are minor sugars present in bamboo that are easily degraded in severe acidic pretreatment. Furthermore, the xylose content in formic and acetic acid pretreated spent liquors was lower than sulfuric acid pretreated spent liquor. Acetic acid and sodium sulfite pretreatment essentially had the same delignification effect as sulfite pretreatment in order to overcome the recalcitrance of lignocellulose (SPORL), which can remove lignin from the bamboo cell wall (Zhu *et al.* 2009).

Table 2. Chemical Composition of Organic Acids Pretreated Spent Liquors

Components (g/L)	Arabinose	Galactose	Glucose	Xylose	Mannose	Acid-soluble lignin
Sulfuric acid	0.04	0.74	3.28	8.36	0.00	5.73
Boric acid + sulfuric acid	0.03	0.59	2.52	8.31	0.00	4.09
Oxalic acid	0.88	0.26	1.77	10.93	0.30	5.51
Formic acid	0.16	0.15	0.90	3.98	0.28	4.87
Acetic acid	0.22	0.18	0.92	7.42	0.21	5.49
Acetic acid + sodium sulfite	0.43	0.06	0.85	2.08	0.52	11.91

Comparisons of Fermentation Inhibitors Formation during Pretreatments

The potential fermentation inhibitors formed during acid pretreatments are listed in Table 3. The inhibitors include acid-soluble lignin, furfural derived from pentoses, 5-hydroxymethyl-2-furaldehyde (HMF) from degradation of hexoses, levulinic, and formic acids from successive decomposition of HMF and acetic acid, which is released from acetyl groups on hemicelluloses. The data in Table 3 clearly indicate that the total amounts of inhibitors (formic, acetic and levulinic acid, furfural, and HMF) were slightly different when using organic acid and the combination of boric acid and sulfuric acid pretreated spent liquors. The sulfuric acid pretreated spent liquor was typically paired with the higher inhibitors (15.88 g/L). The total acid-soluble lignin in the four spent liquors were each slightly different. There were lower amounts of inhibitors detected in the acetic acid and sodium sulfite pretreatment spent liquors than in spent liquors pretreated with only acetic acid. The total number of inhibitors was also the same as the SPORL pretreatment, which had less inhibitors than the dilute sulfuric acid pretreatment (Li *et al.* 2012c). Due to the fact that furfural is easily degraded in the presence of sulfuric acid, the furfural concentration of sulfuric acid pretreated spent liquor was lower than in formic acid and acetic acid pretreated spent liquors.

Table 3. Fermentation Inhibitors in Organic Acids Pretreated Spent Liquors

Inhibitors (g/L)	Formic acid	Acetic acid	Levulinic acid	HMF	Furfural	Total
Sulfuric acid	4.01	5.71	0.38	1.23	4.56	15.88
Boric acid + sulfuric acid	3.93	4.48	0.20	0.98	3.03	12.63
Oxalic acid	3.01	3.39	0.91	0.94	3.98	12.22
Formic acid	1.16	3.19	1.93	1.24	5.91	13.43
Acetic acid	1.28	2.58	1.71	1.11	6.02	12.70
Acetic acid + sodium sulfite	0.12	2.35	0.11	0.20	0.62	3.40

Mass Balance of Sugars during Pretreatments

Ideal pretreatment should not only provide readily available enzymatic digestible substrates, but also should maximize the recovery of all the components of the original biomass. As shown in Table 4, sugars and lignin were divided into two fractions, solid substrates and spent liquors, and they were calculated based on 100 g of untreated raw bamboo. As discussed in Table 1, the raw bamboo contained 44.5 g of glucose, 22.0 g of xylose, 1.1 g of arabinose, 0.4 g of galactose, and 0.6 g of mannose per 100 g of oven-dried bamboo. Pretreatment with acid yielded lower recovery, ranging from 72.5 to 83.0 g. The low substrate yield after pretreatment was due in part to the dissolution of cellulose and xylose. Spent liquors with a high concentration of sugars can be detoxified and condensed for use in ethanol fermentation.

The calculations from these data indicated that total glucose recovery was 87.4% (sulfuric acid), 80.2% (boric acid + sulfuric acid), 95.2% (formic acid), 92.6% (oxalic acid), 92.3% (acetic acid), and 97.2% (acetic acid + sodium sulfite), respectively. The total xylose recovery was 28.9% (sulfuric acid), 28.1% (boric acid + sulfuric acid), 30.2% (formic acid), 61.0% (oxalic acid), 44.7% (acetic acid), and 57.0% (acetic acid + sodium sulfite), respectively. The results indicate that xylose was subjected to greater degradation

at severe acid pretreatment than cellulose. This is one of the reasons why the sulfuric acid spent liquor had a higher concentration of inhibitors than other spent liquors, as seen in Table 3. It indicated that organic acid pretreatment could reserve large quantities cellulose and hemicellulose, which is beneficial for bioethanol production.

Table 4. Mass Balance of Organic Acids Pretreated Bamboo

Components (g)		Arabinose	Galactose	Glucose	Xylose	Mannose	Lignin	Sum	Recovery
Untreated bamboo		1.10	0.40	44.50	22.03	0.61	25.32	93.96	93.96
Sulfuric acid	Substrate	0.06	0.00	36.83	1.14	ND	23.15	61.18	72.52
	Liquor	0.03	0.46	2.05	5.22	ND	3.58	11.34	
Boric acid + sulfuric acid	Substrate	0.07	0.00	34.10	1.01	ND	22.03	57.21	66.92
	Liquor	0.02	0.37	1.57	5.19	ND	2.56	9.71	
Oxalic acid	Substrate	0.28	0.30	40.10	6.60	ND	23.45	70.74	83.02
	Liquor	0.55	0.16	1.10	6.83	0.19	3.44	12.28	
Formic acid	Substrate	0.05	0.01	41.82	4.17	ND	23.69	69.73	76.18
	Liquor	0.10	0.10	0.56	2.49	0.17	3.03	6.45	
Acetic acid	Substrate	0.09	0.02	40.50	5.21	ND	23.39	69.21	77.51
	Liquor	0.14	0.11	0.58	4.64	0.13	2.71	8.30	
Acetic acid + sodium sulfite	Substrate	0.19	0.08	42.74	11.26	ND	18.56	72.84	82.74
	Liquor	0.27	0.04	0.53	1.30	0.33	7.44	9.90	

Enzymatic Hydrolyzability of Pretreated Bamboo Substrates

The enzymatic hydrolyzability of acid pretreated bamboo substrates is shown in Fig. 1. Untreated raw bamboo was used as a comparison for the enzymatic digestibility. The enzymes that were loaded were composed of 15 FPU cellulase and 30 IU β -glucosidase *per* gram glucan for all enzymatic hydrolysis. The cellulose-to-glucose conversion yield (CGCY) of untreated raw bamboo after a 48-h hydrolysis period was only 2.5%. The CGCY of sulfuric acid pretreatment significantly improved the enzymatic digestibility of bamboo, at 49.4%. For the organic acid pretreatment, the CGCY was decreased significantly. The acetic and sodium sulfite pretreatment possessed a lower CGCY than acetic acid alone pretreatment, which was consistent with the bamboo SPORL pretreatment (Li *et al.* 2014). The sugar content in the hydrolyzates increased sharply in the first 12 h and gradually continued to increase until 48 h. The removal of hemicellulose increased cellulose susceptibility to enzymes.

There are many factors that affect the enzymatic hydrolyzability of biomass substrates, such as the reaction temperature, reaction time, and dilute acid concentration. The three factors were combined through the combined severity parameter (CS). Glucan conversion after 96 h of enzymatic hydrolysis increased with CS, reaching a maximum of 68% at CS 2.76 (Scordia *et al.* 2011; Sun *et al.* 2007). Bamboo is much more difficult to efficiently pretreat than other grass. Further study should be conducted for the combined optimization of pretreatment temperature, time, and acid concentration.

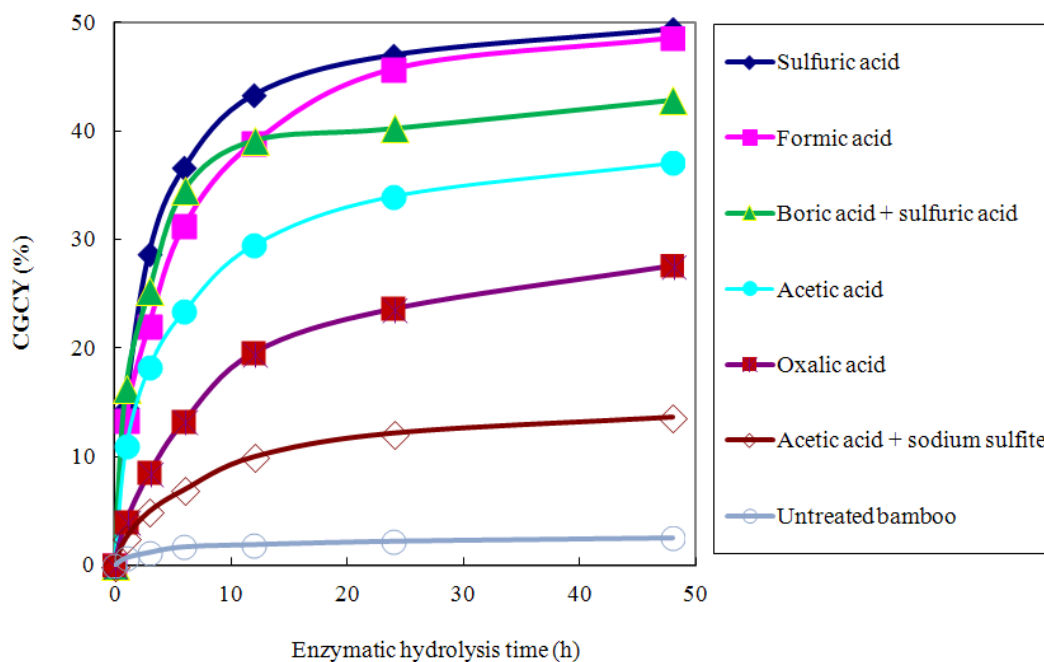


Fig. 1. Comparison of enzymatic hydrolysability of different pretreated bamboo substrates with a enzyme loading of 15 FPU cellulase and 30 IU β -glucosidase per gram of cellulose, at 50 °C, pH of 4.8, on a 220 rpm shaker. CGCY: Cellulose-to-Glucose Conversion Yield.

CONCLUSIONS

1. The study demonstrated that organic acid pretreatments can observably influence the cell-wall structure and enzymatic digestibility of bamboo.
2. Using an organic acid pretreatment not only results in formation of less fermentation inhibitor than a sulfuric acid pretreatment, but also it increased the recovery of cellulose and hemicelluloses.
3. This study showed that the application of dilute organic acids in the pretreatment of bamboo can be effective, therefore, making dilute organic acids (formic acid) a serious alternative for the dilute sulfuric acid pretreatment of bamboo.

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