Effect of Steam Explosion Pretreatment on Bamboo for Enzymatic Hydrolysis and Ethanol Fermentation

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Based on the steam explosion pretreatment that has been applied to other types of lignocellulosic biomass, the steam explosion pretreatment of bamboo, along with a study of the chemical compositions and enzymatic hydrolyzability of substrates, was conducted. The results show that steam explosion pretreatment can greatly enhance the cellulose-to-glucose conversion yield after enzymatic hydrolysis, which is sometimes affected by bamboo age and steam explosion conditions. When the steam explosion pretreatment conditions were 2.0 MPa (pressure) and 4 min (time), the cellulose-to-glucose conversion yield of 2-year-old bamboo substrate was 62.5%. However, the cellulose-to-glucose conversion yield of bamboo substrates after direct (without steam explosion pretreatment) sodium chlorite/acetic acid delignification was 93.1%. Fermentation of enzymatic hydrolyzates with *Saccharomyces cerevisiae* resulted in about 88.1% to 96.2% of the corresponding theoretical ethanol yield after 24 h.

Keywords: Bamboo; Steam explosion pretreatment; Enzymatic hydrolysis; Delignification; Fermentation

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INTRODUCTION

The world is gradually moving to greener sources of energy, but trapping that power is troublesome because energy is lost every time when it is moved or converted (Gammon 2014). This scenario is also applicable to biomass bioenergy. There should be an enormous amount of biomass feedstock for bioenergy production. For example, a sufficient amount of produced sugarcane can be used for sugarcane bioethanol production in Brazil; there is also vast farmland in the United States for corn planting and corn bioethanol production. Meanwhile, there are more than 500 species of bamboo distributed in South China. The annual production of bamboo is more than 1.644 billion culms in China (SFA 2013). Bamboo is an ideal renewable plant resource, with advantages such as fast growth, short renovation, and easy propagation. Bamboo also has a high content of cellulose and hemicellulose, which is a source of sugars for the production of bioethanol or other chemicals (Scurlock *et al*. 2000).

Similar to that for other types of biomass, bioethanol production from bamboo includes three steps: pretreatment, hydrolysis, and fermentation. Pretreatment is essential to enhance cellulase accessibility to cellulose for complete saccharification. Some pretreatment studies have been conducted on bamboo. Compared with other types of biomass, it is more difficult to obtain high enzymatic hydrolyzability from bamboo (Li *et al.* 2012a). Unlike wood trees, bamboo is fast growing and in a few days it can reach its mature height and culm diameter. It then grows only in density and inner structure and will

mature in three to five years. The growth age may therefore be an important factor affecting the efficiency of pretreatment (Shimokawa *et al*. 2009).

Steam explosion pretreatment has been studied for decades, and is considered to be an industrial pretreatment prospect for lignocellulosic bioethanol production (Jiang *et al*. 2011; Oliveira *et al*. 2013). Steam explosion pretreatment has also been used in some pilot bioethanol production lines, with agricultural residues as the dominant feedstocks. Research on steam explosion pretreatment of bamboo is currently in the laboratory research stage, and enzymatic hydrolyzability of pretreated bamboo substrates are very limited (Garcia-Aparicio *et al*. 2011). Some groups have reported that combining a second pretreatment step to enhance the digestibility of steam explosion pretreated bamboo substrates, like alkaline peroxide pretreatment and fungal pretreatment (Xing *et al*. 2013; Li and Chen 2014; Sun *et al*. 2014). While delignification by alkaline peroxide can enhance the enzymatic hydrolyzability of the substrate, it can also increase the cost of pretreatment.

In this study, we compared the steam explosion pretreatment of 2- and 5-year-old bamboo. The chemical changes of the cell-wall components were investigated, as well as the enzymatic digestibility of the pretreated bamboo substrates evaluated. Additionally, sodium chlorite/acetic acid delignification of the bamboo was also conducted.

EXPERIMENTAL

Materials

Bamboo specimens, including 2- and 5-year-old *Bambusa blumeana* (B. b.), and 2- and 5-year-old *Bambusa pervariabilis* McClure (B. p.), were harvested from a bamboo forest Guangxi, China. Air-dried bamboo was cut into small pieces with a relatively homogenous size of 50 mm × 5 mm for steam explosion pretreatment and milled to a screen opening size of 1 mm before delignification pretreatment.

All chemicals were purchased from Sinopharm Chemical Reagent (Beijing) Co., Ltd. (China), and were of analytical grade. Commercial enzymes, Celluclast 1.5 L (cellulase) and Novozyme 188 (*β*-glucosidase) produced by Novozymes. *Saccharomyces cerevisiae* yeast was purchased from Angel Yeast Co., Ltd. (Hubei, China).

Methods

Pretreatments

Steam explosion pretreatment of bamboo was carried out in a batch pilot unit equipped with a 7.5-L reaction vessel; bamboo was exposed to saturated steam at 2.0 MPa for 4 min. After exposure to the saturated steam, a ball valve at the bottom of the reactor was quickly opened to bring the reactor rapidly to atmospheric pressure. The delignification was carried out by mixing bamboo powder 10 g and sodium chlorite/acetic acid solution 100 mL (0.6 g/g oven dry bamboo of sodium chlorite ($NaClO_2$) and 0.6 ml/g oven dry bamboo of acetic acid.) in a flask and heating the mixture to 80 °C in a water bath for 1 h. The bamboo was then carefully collected and mixed with fresh sodium chlorite/acetic acid solution and heated in a water bath for an additional hour. The sodium chlorite/acetic acid solution was used a total of 4 times. After the pretreatment, the substrate and liquor were separated by vacuum filtration. The solid substrate was washed with water until the pH of the wash was near neutral, and then stored at 4 °C for composition analysis

and enzymatic hydrolysis. Each pretreatment was conducted in duplicate, with the data reported as the average ± standard deviation (SD).

Enzymatic hydrolysis

All enzymatic hydrolysis experiments were carried out using cellulase (15 FPU/g glucan) and β-glucosidase (30 IU/g glucan) at 2% (w/v) solids loading in 0.05 M sodium acetate buffer (pH 4.8) in 100-mL plastic flasks on a shaking incubator (KYC-100C, Shanghai Fuma Laboratory Instrument Co., Ltd.; China) at 50 °C and 200 rpm. In order to control the growth of microorganisms and prevent consumption of liberated sugars, approximately 1.5 mg of tetracycline chloride was added in the hydrolysis solution. The enzymatic hydrolysate was sampled at 1, 3, 6, 12, 24, and 48 h to analyze glucose concentration. The data are presented as the average ± SD of duplicates from each experiment substrate.

Ethanol fermentation

The ethanol fermentation of the enzymatic hydrolyzates was performed as previously described (Li *et al*. 2012b). Briefly, for the preculture preparation of *Saccharomyces cerevisiae*, the yeast powder and 40 mL of 2% glucose solution were added to a 100-mL glass flask and placed in a 38 °C water bath. After 15 to 20 min, mixture was moved to a 33 °C water bath for 1.5 h. The enzymatic hydrolyzates solutions were prepared for fermentation by supplementing with the nutrients peptone (5.0 g/L), KH_2PO_4 (2.0 g/L), $MgSO_4$ (1.0 g/L), and $CaCl_2$ (0.25 g/L). The solutions were sterilized in an autoclave at 121 °C for 20 min and the pH was adjusted to 5.5 ± 0.1 using 0.6 M sodium hydroxide or 6% sulfuric acid. The precultured *S. cerevisiae* yeast solution was then inoculated into the prepared hydrolyzates for continuous ethanol fermentation and placed in a shaker at 150 rpm and 37 °C for 24 h. The flasks were sealed with plastic wrap to provide the anaerobic conditions. The fermentation solution were sampled and stored at 4 °C for ethanol determination.

Analytical methods

The component sugars (*i.e.*, glucose and xylose) and lignin (*i.e.*, acid-soluble and acid-insoluble) of the untreated and pretreated bamboo substrates were analyzed, with modifications, according to National Energy Laboratory (NREL) analytical procedures (Sluiter *et al*. 2008). The bamboo and substrates were prehydrolyzed in 75% sulfuric acid for 2 h at room temperature, and then diluted to 3% sulfuric acid for 1 h at 121 °C. The acid-soluble lignin was measured at 205 nm on a UV-Visible spectrophotometer using 3% sulfuric acid as the control blank. The acid-insoluble lignin contents were determined by drying the acid-treated samples in a vacuum oven (~5×10^3 Pa) at 60 °C for 10 h.

The liquid samples were analyzed by ion chromatography using an amperometric detector (Metrohm Corporation; Herisau, Switzerland). Detection of the sugars (*i.e.*, glucose, xylose, mannose, galactose, and arabinose) in the hydrolyzates and the carbohydrate analyses were performed at 32 °C with a Hamilton RCX-30 column and Metrosep RP2 guard column.

Ethanol in the fermentation solutions was determined with a Biosensor Analyzer (SBA-40E, Shandong Academy of Science; Shandong, China). The data are presented as the average ± SD of two measurements.

Degree of polymerization of the cellulose

The degree of polymerization of cellulose was indirectly determined by a viscometric method. The cellulose samples were obtained through the sodium chlorite/acetic acid delignification method from the pretreated bamboo substrates and original bamboo. The viscosity of cellulose in cupriethylenediamine solution was measured using a SYD-265C kinematic viscosity bath (Shanghai Changji Geological Instrument Co. Ltd., China), with an Ubbelohde type viscometer, according to the Chinese National Standard Method GB/T 1548 (2004). The viscosity average degree of polymerization of the cellulose samples was calculated from their intrinsic viscosity [η] in cupriethylenediamine solution. The viscosity determination was conducted in duplicate for each substrate; the averages ± SD are reported.

RESULTS AND DISCUSSION

Raw Bamboo Composition

The chemical compositions of the original *B. blumeana* (B. b.) and *B. pervariabilis* McClure (B. p.) are listed in Table 1. The cellulose content was calculated using the glucose concentration in the two-stage sulfuric acid hydrolysis solution. The concentration of hemicelluloses was based on the total concentrations of xylose, mannose, galactose, and arabinose in the two-stage sulfuric acid hydrolysis solution. The lignin content included both acid-insoluble lignin and acid-soluble lignin. The data indicated that the cellulose and lignin contents of 5-year-old bamboo were higher than those of 2-year-old bamboo. The amount of extractives depends on the bamboo species. The different analytical methods may have resulted in a difference in cellulose content.

Table 1. Chemical Composition of Three Raw Bamboos

Bamboo	Age of bamboo (years)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)
B. b.	2	37.7±1.5	27.5±1.7	25.7±0.0	7.6±0.0
	5	41.9±1.0	22.9±2.1	27.1±0.3	8.0±0.0
B. p.	2	38.8±1.8	28.8±2.4	24.9±0.1	5.8±0.1
	5	39.5±0.2	24.6±1.1	28.7±0.1	5.6±0.2

Composition of Pretreated Bamboo Substrates

Pretreatment is a key and high-cost step in the bamboo bioconversion process for bioethanol production. Because of its high density and lignin content, bamboo is more resistant to pretreatment than other forms of biomass. Xing *et al*. (2013) reported that the combined steam and alkaline peroxide pretreatment of bamboo resulted in a maximum glucose yield of 90.5%. In this study, alkaline peroxide pretreatment was effective in delignification to increase glucose yield of the steam explosion pretreated bamboo substrate. However, two steps of pretreatment increased the cost of the pretreatment. In this study, the original raw bamboo was delignified by sodium chlorite/acetic acid directly to measure the effect of lignin on bamboo's enzymatic hydrolyability. This sodium chlorite/acetic acid treatment is used to produce holocellulose, and lignin removal is selective when up to 90% of this component is removed (Siqueira *et al*. 2013; Kumar *et al.*

2013). For bamboo, much more lignin was removed, and only about 1% lignin remained in the delignified substrate.

Table 2. Chemical Composition of Bamboo Substrates after Pretreatment

Bamboo	Pressure (MPa)	Cellulose (%)	Lignin (%)	Hemicellulose (%)
B. b. (2 years)	2.25	59.6±0.8	34.9±0.3	11.4±2.7
	2.00	59.0±0.9	32.1±0.6	14.8±1.5
	1.75	56.9±3.1	27.6±0.4	21.2±0.6
B. b. (5 years)	2.00	55.3±2.6	34.8±0.5	15.4±1.8
B. p. (2 years)	2.00	63.8±3.1	25.8±0.8	16.8±1.5
B. p. (5 years)	2.00	57.9±2.2	34.3±0.9	13.6±0.5
Delignified substrate (B. p. 2 years)		61.6±2.0	1.0±0.3	37.4±1.3

Table 2 shows the chemical analysis of pretreated bamboo substrates after steam explosion pretreatment and delignification. During the steam explosion pretreatment, essentially no delignification occurred, so the proportion of lignin increased in the substrate. Steam treatment is more effective for removing pentosans compared with hot water extraction (Luo *et al.* 2013) and xylan is the main five-carbon sugar component of bamboo hemicelluloses. In contrast, steam explosion pretreatment hydrolyzed more hemicellulose because of hydrogen ions generated by water at high temperature. The lignin content was only 1% in the delignified bamboo substrate. The proportion of cellulose and hemicelluloses, which are the feedstocks for bioethanol production, increased in the bamboo substrate.

Degree of Polymerization of Cellulose from Pretreated Bamboo

The degree of polymerization of cellulose from untreated and pretreated bamboo is listed in Table 3. Steam explosion pretreatment led to a decrease in the average degree of polymerization of cellulose, and also a decrease in the molecular weight of the bamboo substrates. This is one of the reasons why the steam explosion pretreated bamboo substrates had better enzymatic hydrolyzability than the untreated bamboo. Steam explosion pretreatment can be considered to be an acid-catalyzed hydrolysis process, and it results in auto-hydrolysis reactions that degrade cellulose macromolecules. The degree of polymerization of cellulose from high-pressure steam explosion pretreatment was lower than that of low-pressure steam explosion pretreatment. Therefore, higher steam pressure caused severe auto hydrolysis of cellulose and hemicelluloses from large molecules to small molecules, which was consistent with a decrease of the hemicelluloses content of the steam explosion pretreated bamboo substrate as the steam pressure increased. Cellulose had a high degree of polymerization and molecular weight in the delignified bamboo substrate, showing that the sodium chlorite/acetic acid delignification process did not seriously damage the cellulose. Bamboo cellulose from steam explosion with the 2.25 MPa pretreated substrate had the lowest degree of polymerization. For both species of bamboo, 2-year-old bamboo had a lower degree of polymerization than 5-year-old bamboo.

Table 3. Degree of Polymerization of Cellulose from Untreated and Pretreated Bamboo

Bamboo	Pressure (MPa)	DP of cellulose	Molecular weight of cellulose ($\times 10^4$)
B. b. (2 years)	2.25	458.7	7.43
	2	834.6	13.52
	1.75	1286.1	20.83
B. b. (5 years)	2	876.4	14.19
B. p. (2 years)	2	512.9	8.31
B. p. (5 years)	2	661.3	10.71
Delignification substrate (B. p. 2 years)		908.5	14.72
B. b. (2 years)	Untreated	1392.6	22.56
B. b. (5 years)		1411.6	22.87
B. p. (2 years)		939.1	15.21
B. p. (5 years)		1336.2	21.65

Enzymatic Hydrolyzability of Pretreated Bamboo Substrates

The enzymatic hydrolyzability of untreated and pretreated bamboo substrates is compared in Fig. 1. A comparison of bamboo (B. b., 2 years) substrates pretreated at various pressures is shown in Fig. 1a, and a comparison of bamboo at various ages, as well as delignified bamboo, is shown in Fig. 1b. At enzyme loadings of 15 filter paper units (FPU) cellulase and 30 international units (IU) β-glucosidase *per* gram cellulose, the steam explosion pretreated bamboo displayed much greater hydrolysis yield than did the untreated bamboo for every test bamboo species and age. For example, only about 2.5% of the cellulose in untreated bamboo (B. b., 2 years) was hydrolyzed to glucose after 48 h of hydrolysis, whereas 45.1% of the cellulose in steam explosion (2.25 MPa) pretreated substrates was saccharified in the same time. When the steam pressure of the pretreatment was reduced, the cellulose-to-glucose conversion yield (CGCY) after 48 h of hydrolysis of the steam explosion pretreated substrate was still substantially higher (23.3% for 2.0 MPa and 8.7% for 1.75 MPa) than those of the untreated bamboo. The steam explosion pretreated substrate contained lower hemicelluloses, had a slightly lower cellulose degree of polymerization, and might be assumed to have a better enzymatic hydrolyzability. However, based on other factors such as lignin content and distribution, the resulting situation was opposite to what was expected. The steam pretreatment method still suffers from incomplete disruption of the lignin-carbohydrate matrix (Chiaramonti *et al*. 2012), which caused incomplete hydrolysis of cellulose. The results also clearly indicated that the delignified bamboo substrate had better enzymatic digestibility than did the steam explosion substrate under the same hydrolysis conditions. As discussed above, the delignified substrate contained very little lignin compared to the steam explosion substrate. However, the delignified substrate had a higher cellulose degree of polymerization (molecular weight) than the steam explosion substrate. The results suggest that lignin plays a very important role in the interaction with enzymes, which affects the enzymatic hydrolyzability of bamboo substrates.

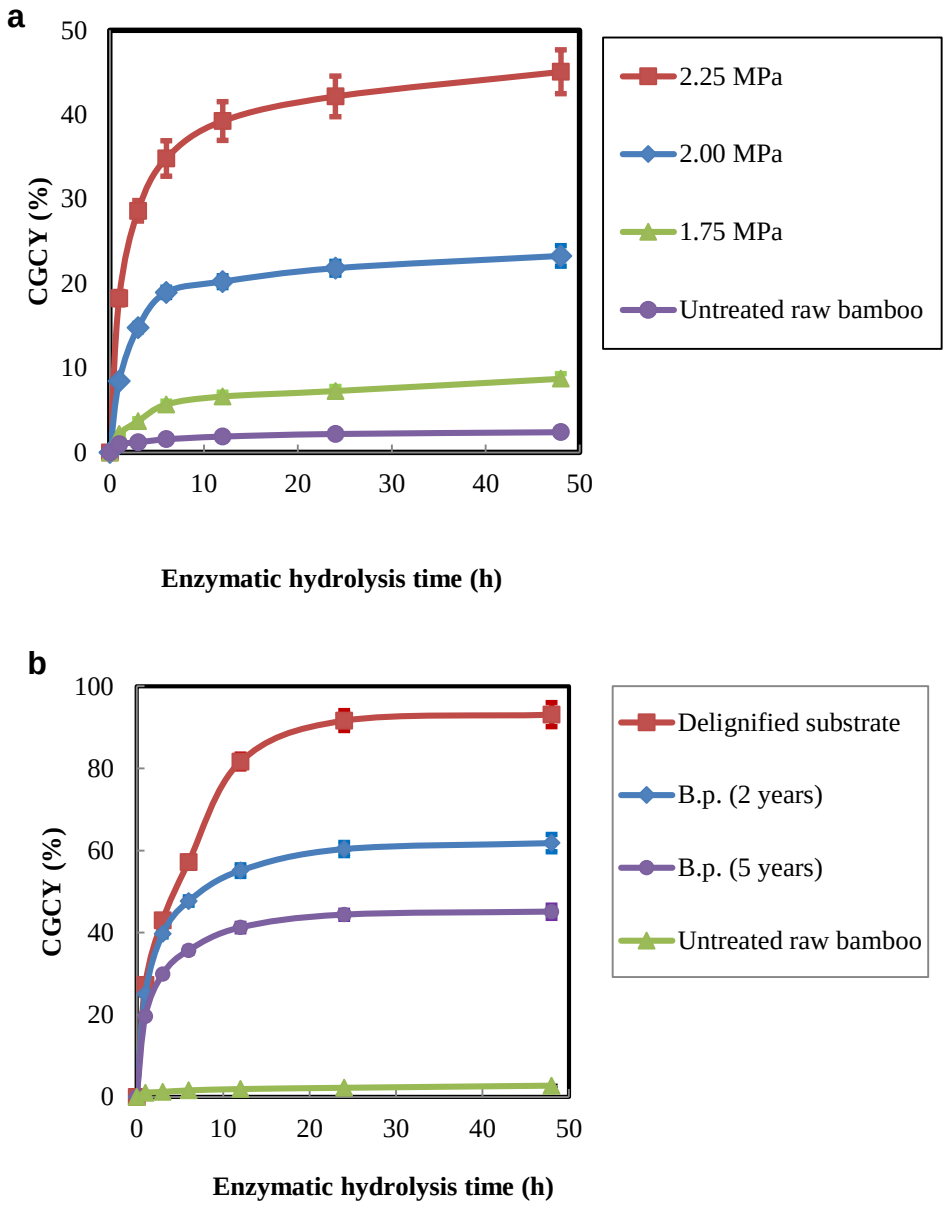

Fig. 1. Comparison of enzymatic hydrolyzability of untreated and pretreated bamboo substrate with an enzyme loading of 15 FPU cellulase and 30 IU β-glucosidase *per* gram of cellulose, at 50 °C, pH 4.8, on a 220 rpm shaker. CGCY: cellulose-to-glucose conversion yield. (a) Bamboo (B. b., 2 years) substrates pretreated at various pressures; (b) Bamboo substrates at various ages, and delignified bamboo

Although steam explosion pretreatment is a promising technology for many kinds of lignocelluloses, it did not exhibit significant effects on bamboo enzymatic hydrolysis in this research. Besides, some other disadvantages, such as only partial degradation and removal of hemicelluloses and lignin, as well as incomplete disruption of lignin-hemicelluloses matrix will impede the enzymatic digestibility of pretreated substrate. Therefore, a further post-treatment is required to remove lignin and degraded hemicellulosic products from the steam-exploded substrates so as to improve enzymatic digestibility of the pretreated substrates (Sun *et al*. 2014). Moreover, an important step which influences the effect of steam explosion pretreatment is whether to presoak

lignocelluloses before steam explosion to soften fiber, which makes the fiber more free from mechanical damage. Steam explosion associated with NH_4Cl preimpregnation with the glucose yield of 62.64%, which was 0.69 times higher than that of steam explosion pretreated samples (Chen *et al.* 2014). Therefore, for bamboo steam pretreatment, it also should be divided into two steps – preimpregnation-steam explosion or steam explosion-post pretreatment. Two-step pretreatment may need more energy consumption than one step pretreatment such as sodium chlorite/acetic acid delignification.

Separate Hydrolysis and Fermentation of Pretreated Bamboo Substrates

Separate hydrolysis and fermentation (SHF) includes two steps. First, the cellulose in bamboo substrates was enzymatically hydrolyzed to glucose. Second, the glucose in the hydrolyzate was converted to ethanol by fermentation. Both steps were conducted under their optimal experimental conditions (*i.e.*, temperature 50 °C, pH 4.8 for hydrolysis, and temperature 37 °C, pH 5.5 for fermentation). Simultaneous saccharification and fermentation (SSF) is the production of ethanol in one step. The ethanol yield trend line was very similar as the time-dependent enzymatic hydrolyzability (glucose yield) of bamboo substrates. This indicated that the generation rate of ethanol was consistent with the generation rate of glucose. The ethanol yields of all bamboo substrates were lower in SSF than in SHF (Li *et al*. 2014). Therefore, in this study, SHF was used for the fermentation of steam explosion pretreated bamboo substrate.

The CGCY, reducing sugars yield (48 h), and glucose-to-ethanol conversion yield of hydrolyzate fermentation (24 h) are shown in Table 4. At enzyme loadings of 15 FPU cellulase and 30 IU *β*-glucosidase *per* gram cellulose, the steam explosion pretreated bamboo with lower steam pressure displayed a lower yield than did the high-pressure pretreated bamboo. After enzymatic hydrolysis, the hydrolyzate was autoclaved at 121 °C for 20 min for sterilization. Then, hydrolyzate fermentation was conducted. The glucose-to-ethanol conversion yield (GECY) is also shown in Table 4. The GECY of pure glucose was 88.4%, and the GECY of bamboo substrate hydrolyzate ranged from 88.1% to 96.2%. No glucose was detected in the solution after 24 h of fermentation. This indicates that all glucose was consumed during fermentation. Most of the glucose was converted to ethanol; however, some glucose was consumed as nutrient for yeast (Fonseca *et al.* 2007).

Table 4. Glucose and Ethanol Yield of SHF of Bamboo Substrates

Bamboo	Pressure (MPa)	CGCY (%)	Reducing sugars Yield (%)	GECY (%)
B. b. (2 years)	2.25	45.09	34.60	96.2
	2.00	23.27	47.34	94.5
	1.75	8.73	46.58	88.1
B. b. (5 years)	2.00	40.00	47.56	93.8
B. p. (2 years)	2.00	62.55	62.82	90.6
B. p. (5 years)	2.00	45.09	56.14	93.5
Delignified substrate (B. p. 2 years)		93.09	98.79	93.2

CONCLUSIONS

1. The bamboo growth ages are the primary factors affecting the hydrolyzabilities of steam explosion pretreated bamboo substrates.
2. Bamboo delignified by sodium chlorite/acetic acid has higher enzymatic hydrolyzability than steam explosion pretreated bamboo. The cellulose-to-glucose conversion yield of steam explosion (2.0 MPa and 4 min) pretreated bamboo (B. p., 2 years old) substrate reached 62.55% with a cellulase loading of 15 FPU/g glucan.
3. Ethanol fermentation of enzymatic hydrolyzates of steam explosion pretreated bamboo substrate with *Saccharomyces cerevisiae* yeast resulted in about 88.1% to 96.2% of the corresponding theoretical ethanol yield after 24 h.

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