Carbohydrate Polymers 79 (2010) 914–920

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Alkaline peroxide pretreatment for efficient enzymatic saccharification of bamboo

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ARTICLE INFO

Article history:
Received 19 July 2009
Received in revised form 9 October 2009
Accepted 9 October 2009
Available online 18 November 2009

Keywords:
Alkaline peroxide
Steam explosion
Sodium hydroxide
Hydrogen peroxide
Enzymatic saccharification
Bamboo

ABSTRACT

Bamboo is an alternative feedstock for the production of fine chemicals, such as fuel ethanol and lactic acid, because bamboo has a large amount of sugars. An effective pretreatment method for enzyme saccharification is required for the efficient production of these materials. Enzyme 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. In addition, pretreatments using 20 atm and 5 min steam explosion or mechanical milling for 5 min followed by 10 wt.% sodium hydroxide treatment at 121 °C for 60 min were attempted in order to enhance the digestibility of the holocellulose component. Both of these pretreatment methods had a large positive effect on the production of sugars by subsequent enzymatic hydrolysis. In particular, the maximum value of glucose production was obtained by the 20 atm steam explosion and 10 wt.% sodium hydroxide treatment. This produced 456 mg/(g initial dry sample) of glucose and 460 mg/(g initial dry sample) of reducing sugar. In comparison, the mechanical milling and 10 wt.% sodium hydroxide treatment produced 383 and 485 mg/(g initial dry sample) of glucose and reducing sugar, respectively. From these results, it was concluded that the pretreatment with 20 atm steam explosion and 10 wt.% sodium hydroxide treatment was the most effective pretreatment method for the production of glucose from bamboo by enzyme saccharification. However, since this pretreatment method requires the severe conditions of both high pressure and temperature steam explosion and high concentration sodium hydroxide, an alkaline peroxide pretreatment without a steam explosion and high concentration sodium hydroxide was also attempted. A comparatively large amounts of glucose and reducing sugar production, i.e. 399 and 568 mg/(g initial dry sample), respectively, were obtained in 1%(v/v) hydrogen peroxide and 1 wt.% sodium hydroxide treatment at 90 °C for 60 min. Therefore, it was concluded that alkaline peroxide pretreatment is an effective and environmentally friendly method for the enzyme saccharification of bamboo.

1. Introduction

Plant biomass is a natural renewable resource that can be converted into useful materials and energy (Klass, 1998; Saddler, 1993). Recently, lignocelluloses are the largest sources of hexose and pentose sugars with potential use for the production of fuel alcohol and various chemicals (Herrera, 2004; Kuhad & Singh, 1993; Kuhad, Singh, & Eriksson, 1997). Bamboo, which belongs to the Gramineae family, has been widely used as a feedstock for paper, textiles, food, construction and reinforcing fibers (Zhang, Yu, Huang, & Liu, 2007). In addition, bamboo has been investigated as a raw material for fine chemicals, such as ethanol (Ram & Seenayya, 1991), methane (Kobayashi, Take, Asada, & Nakamura, 2004), and lactic acid (Asada, Nakamura, & Kobayashi, 2005) because of the large amount of bamboo grown in Asia. In year bamboo growth reached 3300 million kg in Japan, but this was largely wasted (Saka, 2001). The most important advantages of bamboo are that it needs a short time (3–5 years) to mature compared to other plants (Krzesinska, Zachariasz, & Lachowski, 2009) and it contains a large amount of holocellulose components; 40–48% cellulose and 24–28% hemicellulose (Scurlock, Dayton, & Hames, 2000). However, holocellulose components are generally covered with the rigid lignin in bamboo. Therefore, it is difficult to convert from bamboo to fine chemicals efficiently due to the poor accessibility of enzyme and digestibility of holocellulose components. Therefore, the pretreatment of bamboo is necessary to degrade or remove the rigid lignin, in order to promote the production of sugars by enzymatic saccharification.

The steam explosion pretreatment is physicochemical; combining the destructing physical effect with chemical hydrolysis. The raw material is exposed to saturated steam at elevated temperature and pressure followed by the rapid reduction to atmospheric pressure, which causes lignocellulosic material to be broken down. Therefore, the accessibility of enzyme to cellulose component was enhanced and the digestibility was increased (Cara et al., 2008; Zimbardi et al., 2007).

It is well known that alkaline pretreatment provides the effective delignification and chemical swelling of the fibrous cellulose (Zhao, Peng, Cheng, & Liu, 2009). However, Hsu reported that

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doi:10.1016/j.carbpol.2009.10.017

alkaline pretreatment is generally more effective on agricultural residues and herbaceous crops than on wood materials (Hsu, 1996). These effects enhance the accessibility of enzymes and the digestibility of holocellulose components because of the solvation and the saphonication during alkaline pretreatment. At the same time, the alkaline pretreatment can also cause condensation of lignin and modification of the crystal structure, which can introduce unwanted effects for lignin removal and cellulose degradation (Gregg & Saddler, 1996). Additionally, a lot of researchers have reported on alkaline peroxide treatment for various kind of lignocellulose biomass claiming that it improved enzymatic saccharification significantly (Chen, Han, & Xu, 2008; Sun, Fang, & Tomkinson, 2000; Yang, Boussaid, Mansfield, Gregg, & Saddler, 2002).

In this work, pretreatment methods for enzyme saccharification of Gramineae family bamboo were investigated to enable the efficient conversion to fine chemicals. The steam explosion, the consecutive pretreatment with steam explosion or mechanical milling followed by various concentrations of sodium hydroxide treatment, and alkaline peroxide pretreatment combing with hydrogen peroxide and sodium hydroxide were attempted for increasing the enzymatic saccharification of bamboo.

2. Experimental

2.1. Samples

Moso bamboo, *Phyllostachys pubescens* Mazel, (about 3 years old with 10–20 m of height and 10–20 cm of diameter) was collected in Miyoshi mountainous district in Tokushima prefecture in Japan and then chopped into small fragments whose length and width were about 5–8 and 3–5 cm, respectively, before undergoing the various pretreatments, as described bellow.

2.2. Steam explosion pretreatment

Steam explosion as a physicochemical pretreatment was carried out in a batch pilot unit equipped with a 1 L reaction vessel. Bamboo chips (100 g) were introduced into the reaction vessel and exposed to saturated steam with 20 atm (214 °C) and 35 atm (243 °C) for 5 min. After exposure to the saturated steam, a ball valve at the bottom of the reactor was suddenly opened to bring the reactor rapidly to atmospheric pressure. A product containing liquid and solid fractions was obtained as the steam exploded samples (Take et al., 2006).

2.3. Mechanical grounding by a ball mill

Twenty grams of bamboo chips were grounded by a vibrating sample mill (VIBRATING SAMPLE MILL CMT-TI-300, C.M.T. Co. Ltd.) at 60 cycles/s for 5 min. The milling samples obtained the particle size of approximately 0.1 mm.

2.4. Sodium hydroxide pretreatment

One gram of dry weight of solid material and 100 mL of aqueous sodium hydroxide (0.5 wt.%) were added to 200 mL Erlenmeyer flasks, which was equivalent to an initial dry solid material concentration of 1 wt.%. Sodium hydroxide pretreatment was performed in an autoclave at 121 °C for 60 min. After the reaction ended, the solid residue was washed with distilled water several times, and the residual sodium hydroxide was neutralized with acetic acid. Then the excess acetic acid was removed by washing with distilled water. The sodium hydroxide pretreated samples were stored at 4 °C for enzymatic hydrolysis (Chang & Holtzapple, 2000). The effect of sodium hydroxide concentration on enzyme saccharification was investigated from 0.5 to 10 wt.%.

2.5. Alkaline peroxide pretreatment

One gram of dry weight of solid material was added into 100 mL of 1 wt.% sodium hydroxide solution containing of 0.5–3%(v/v) hydrogen peroxide. Alkaline peroxide pretreatment was performed in a water bath at 90 °C for 60 min. After the reaction ended, the solid residue was washed with a distilled water several times and the sample was stored at 4 °C for enzymatic hydrolysis.

2.6. Enzyme saccharification

The solid bamboo samples were hydrolyzed by a cellulolytic enzyme, in 110 mL sample tubes at an initial sample concentration of 3%(w/v) in 10 mL of 100 mM sodium acetate buffer pH 5.0, and using an enzyme (Meicelase, Meiji Seika Co. Ltd.) loading of 20 FPU/g substrate. The enzyme reaction was carried out in a reciprocating water bath shaker at 140 strokes/min for 48 h at 45 °C. The supernatant was centrifuged and removed for sugar content testing. The cellulose hydrolysis and the enzyme saccharification were calculated using following equations (Sharma, Kalra, & Kocher, 2004).

$$\text{Cellulose hydrolysis}\ (\%) = \frac{\text{The amount of glucose produced} \times 0.9}{\text{The amount of cellulose in raw bamboo}} \times 100$$

$$\text{Enzyme saccharification}\ (\%) = \frac{\text{The amount of reducing sugar produced} \times 0.9}{\text{The amount of holocellulose(cellulose and hemicellulose)in raw bamboo}} \times 100$$

2.7. Analytical methods

The cellulose and hemicellulose in raw and various pretreated samples were determined using an extraction and separation procedure based on Wayman's method (Chua & Wayman, 1979). One gram of dry weight of material was added to 100 mL of distilled water and extracted for 24 h at ambient temperature. After this time, the solid–liquid mixture was separated by filtration. After the water soluble polysaccharide was converted to the monomer by 4%(w/w) H_2SO_4 at 121 °C for 30 min, the reaction mixture determined by high performance liquid chromatography (HPLC) using an aminex column HPX-87H (300 × 7.8 mm, Bio-rad, Richmond, CA) (Davis, Rogers, Pearce, & Peiris, 2006). The solid fraction from water extraction was hydrolyzed with 10 mL of 72%(w/w) H_2SO_4 at 30 °C for 60 min. after then, the reaction mixture was diluted to 4%(w/w) H_2SO_4 and autoclaved at 121 °C for 60 min. This hydrolyzed solution was measured by HPLC as above condition. Cellulose and hemicellulose content was established based on monomer content both water and acid extractions. Sample recoveries after various pretreatments were determined based on the dry weight. For the enzymatic hydrolysis, the glucose concentration was measured by the mutarotase GOD method (Glucose C-Test; Wako Pure Chemical, Osaka, Japan) and the reducing sugar concentration was determined according to the Somogyi–Nelson method (Somogyi, 1952).

3. Results and discussions

3.1. Sugar composition

Fig. 1 shows the hemicellulose and cellulose contents in raw and steam exploded bamboo. The ratio of holocellulose content

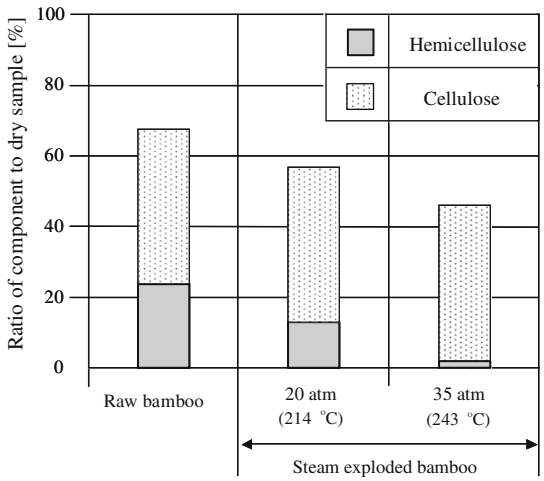

Fig. 1. Hemicellulose and cellulose contents in raw and steam exploded bamboo.

(defined as the sum of cellulose and hemicellulose contents) in raw bamboo was 68.3%, corresponding to 45.5% cellulose and 22.8% hemicellulose. This result is similar to that reported previously (Scurlock et al., 2000). While the cellulose content was not changed at all by the 35 atm steam explosion for 5 min, the hemicellulose content was significantly reduced from 22.8% to 12.4% at 20 atm and 1.0% at 35 atm. The observation of the large amount of hemicellulose reduction at 35 atm depended on the lignin structure of bamboo composes of a typical Gramineae lignin (Wang & Ren, 2008), which characterize the flexible structure (Sakakibara, 1982).

3.2. Enzyme saccharification of bamboo samples at various steam explosion conditions

For increasing the digestibility of holocellulose components, bamboo chips were pretreated by milling or steam explosion at 20 atm (214 °C) and 35 atm (243 °C) for 5 min. Fig. 2 shows the time courses of glucose and reducing sugar production in the enzyme saccharification of raw, milling, and steam exploded bamboo. There is no observation of glucose and reducing sugar production in the raw bamboo through enzyme saccharification. For the milled sample, the amounts of glucose and reducing sugar produced were 63 and 88 mg/(g initial dry sample), respectively. The steam explosion greatly enhanced the digestibility of holocellulose components compared to the raw and milled samples. In particular, 35 atm steam explosion provided maximum amounts of glucose and reducing sugar, i.e. 426 and 488 mg/(g initial dry sample), respectively.

3.3. Effect of pretreatment with 20 atm steam explosion and sodium hydroxide treatment on enzyme saccharification of bamboo

Fig. 3 shows the glucose and reducing sugar production in 48 h enzyme saccharification of bamboo treated by 20 atm steam explosion and then hot water or 0.5 wt.% sodium hydroxide treatment. Significant differences in glucose and reducing sugar production between samples treated with steam explosion only and hot water were not observed. The hot water treatment produced 214 and 219 mg/(g initial dry sample) of glucose and reducing sugar, respectively, with a small decrease in sample recovery (95.4%). These observations suggest that low-pressure steam explosion degrades only a small part of lignin, and similar to that extracted by hot water treatment, these do not influence the enhancement of enzymatic digestibility. In contrast, a large increase in the production of glucose and reducing sugar were observed in 0.5 wt.% sodium hydroxide treatment of 20 atm steam exploded bamboo. This pretreatment resulted in 347 and 424 mg/(g initial dry sample) of glucose and reducing sugar, respectively. About 50% of recovery was obtained after treating 0.5 wt.% sodium hydroxide. Chen et al. reported that a large amount of lignin removal was observed with sodium hydroxide concentrations of between 0.5 and 2 wt.% while using raw herbaceous feedstock (Chen, Sharma-Shivappa, Keshwani, & Chen, 2007). In this work, the consecutive treatments of 20 atm steam explosion and 0.5 wt.% sodium hydroxide provided a large amount of lignin degradation and enhanced the accessibility of enzymes to the holocellulose components. This result demonstrates that pretreatment with steam explosion and alkaline treatment promotes effective enzyme saccharification of bamboo.

3.4. Effect of sodium hydroxide concentration on enzyme saccharification of bamboo treated with 20 atm steam explosion and sodium hydroxide

Fig. 4 shows the effect of sodium hydroxide concentration on glucose and reducing sugar and recovery ratio of bamboo treated with 20 atm steam explosion followed by sodium hydroxide. Sodium hydroxide (0.5 wt.%) treatment provided an effective enzymatic digestibility of holocellulose components as shown in

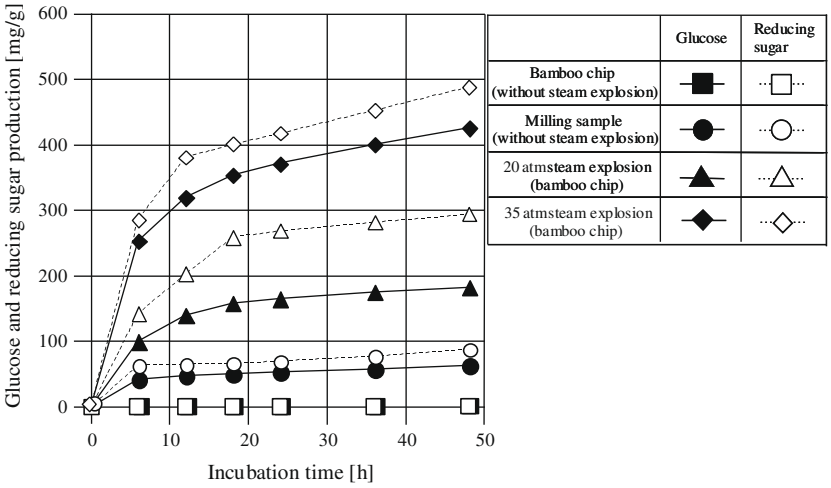

Fig. 2. Time courses of glucose and reducing sugar production in enzyme saccharification of raw, milling, and steam exploded bamboo.

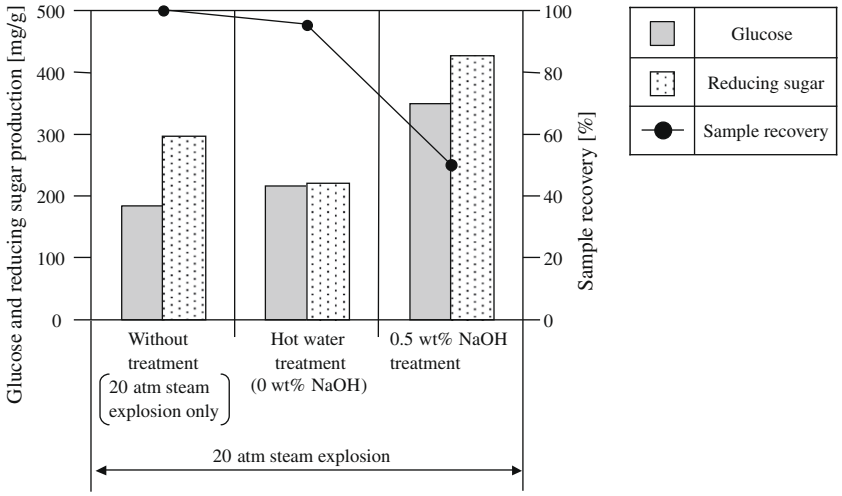

Fig. 3. Glucose and reducing sugar production in 48 h enzyme saccharification of bamboo treated with 20 atm steam explosion followed by hot water or 0.5 wt.% sodium hydroxide.

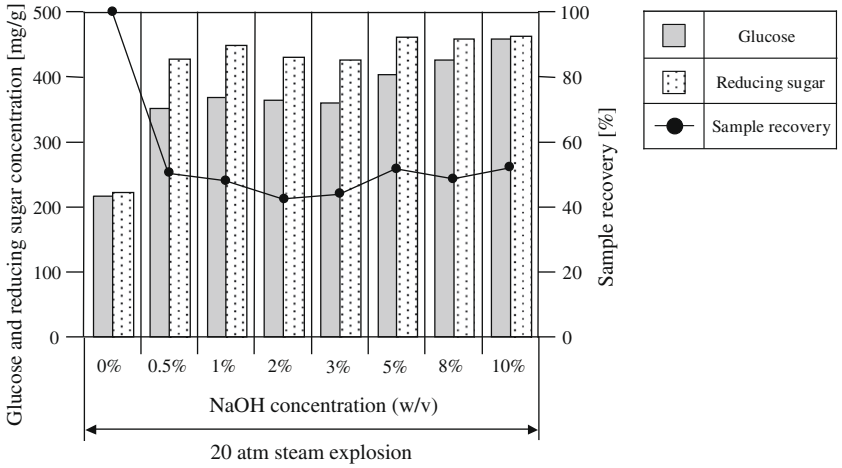

Fig. 4. Glucose and reducing sugar production in 48 h enzymatic saccharification of bamboo treated with 20 atm steam explosion followed by various concentration sodium hydroxide.

Fig. 3. Little difference in the production of glucose and reducing sugar for 0.5–3 wt.% sodium hydroxide treatment was observed: about 350 and 420 mg/(g initial dry sample) of glucose and reducing sugar were produced, respectively, within this range. However, at sodium hydroxide concentrations greater than 5 wt.%, glucose and reducing sugar production increased and then reached maxi-

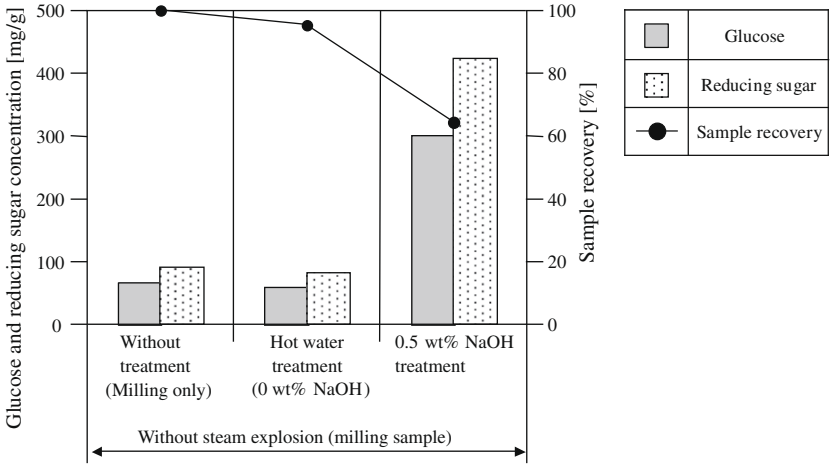

Fig. 5. Glucose and reducing sugar production in 48 h enzyme saccharification of bamboo treated with milling followed by hot water or 0.5 wt.% sodium hydroxide.

mum values of 456 and 460 mg/(g initial dry sample), respectively, at 10 wt.% sodium hydroxide. This indicates that sodium hydroxide removed a large amount of lignin regardless of the concentration; however, the digestibility of holocellulose components was only enhanced at concentrations above 3 wt.%. At the higher concentrations, sodium hydroxide modified the crystalline structure of cellulose and increased the production of sugars. However, lignin condensation occurred at the same time, but had no effect on the enzymatic digestibility. He et al. reported that the cellulose crystal was not obviously changed in 6 wt.% sodium hydroxide treatment in using rice straw (He, Pang, Liu, Li, & Wang, 2008). In addition, Mansour et al. reported that the crystallinity is reduced most at concentrations of 9–12 wt.% sodium hydroxide during the alkaline treatment (Mansour, Ssaady, & Mottaleb, 1972). In this work, 20 atm steam explosion may have enhanced the reduction of cellulose crystallinity by even 5 wt.% sodium hydroxide treatment. Furthermore, these results indicate that consecutive treatment with 20 atm steam explosion and 10 wt.% sodium hydroxide produce a large amount of sugars from bamboo.

3.5. Effect of pretreatment with milling and sodium hydroxide treatment on enzyme saccharification of bamboo

Fig. 5 shows the glucose and reducing sugar production in 48 h enzyme saccharification of bamboo treated by milling and then hot water or 0.5 wt.% sodium hydroxide treatment. The hot water treatment had no effect on the production of sugar and the sample recovery compared to milling only. However, 0.5 wt.% sodium hydroxide treatment of milled bamboo facilitated a large amount of glucose and reducing sugar production, 298 and 420 mg/(g initial dry sample), but decreased the sample recovery to 64.5%. Though the pretreatment method was different from this research, Zhang et al. reported that 223 mg/(g initial dry sample) of reducing sugar was obtained from biologically pretreated bamboo with white rot fungi (Zhang et al., 2007). The greater production of reducing sugar in this work may be due to the large amount of lignin removal during alkaline extraction. Therefore, this result indicates that sodium hydroxide pretreatment improves the enzymatic digestibility of raw milled bamboo, in spite of the large amount of rigid lignin accumulation.

3.6. Effect of sodium hydroxide concentration on enzyme saccharification of bamboo treated with milling and sodium hydroxide

Fig. 6 shows the effect of sodium hydroxide concentration on glucose and reducing sugar and recovery ratio of bamboo treated with milling followed by sodium hydroxide. There was little difference in sugar production for sodium hydroxide concentrations between 0.5 and 1 wt.%. Higher sodium hydroxide concentrations led to increased glucose production. The maximum value of glucose production was 383 mg/(g initial dry sample) at 10 wt.% sodium hydroxide. However, the reducing sugar production did not change with sodium hydroxide concentrations between 0.5 and 5 wt.%, providing around 400 mg/(g initial dry sample). In contrast, approximately 480 mg/(g initial dry sample) of reducing sugar was produced at 8 and 10 wt.% sodium hydroxide. This result may be due to the modification of cellulose crystal structure by sodium hydroxide concentrations above 8 wt.%, as shown in Fig. 4. However, it seems that the lignin structure of milled bamboo is less susceptible to the alkaline treatment, and produced a smaller amount of glucose compared to the two-step 20 atm steam explosion and 10 wt.% sodium hydroxide treatment.

3.7. Enzyme saccharification of bamboo using various pretreatment methods

Table 1 shows the cellulose hydrolysis and the enzyme saccharification after 48 h incubation using the various pretreatment methods. The comparatively high values of cellulose hydrolysis and enzyme saccharification, i.e. 84.3% and 64.3%, were obtained using 35 atm steam explosion only. This result showed that almost all of holocellulose component included of 35 atm steam exploded bamboo was dissolved and a large amount of cellulose converted into glucose by enzymatic hydrolysis. However, the maximum glucose production, i.e. 90.1%, was obtained with 20 atm steam explosion and 10 wt.% sodium hydroxide treatment. In contrast to cellulose hydrolysis, the enzyme saccharification could not be improved due to the fact that a large amount of the hemicellulose component was degraded by the 10 wt.% sodium hydroxide treatment. The milling and 10 wt.% sodium hydroxide treatment also produced a large amount of glucose and reducing sugar; enhancing the cellulose hydrolysis and enzyme saccharification to 75.8% and 63.9%, respectively. However, since the crystal structure of cellulose was not changed, the values of cellulose hydrolysis and enzyme saccharification were low compared to the 20 atm steam explosion and 10 wt.% sodium hydroxide treatment. As a result, it was found that the 20 atm steam explosion and 10 wt.% sodium hydroxide treatment is the most effective pretreatment method for the cellulose hydrolysis of bamboo into glucose. However, since this method needs the high pressure of steam explosion and high concentration of sodium hydroxide, it is necessary to examine and develop the more feasible and environmentally friendly pretreatment method.

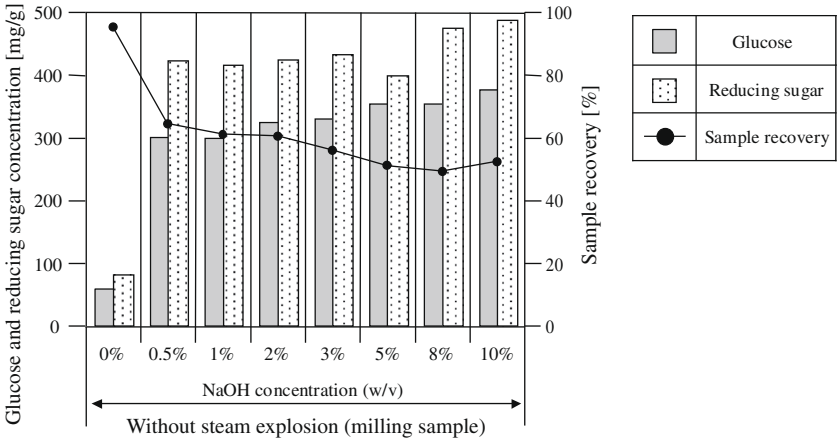

Fig. 6. Glucose and reducing sugar production in 48 h enzymatic saccharification of bamboo treated with milling followed by various concentration sodium hydroxide.

Table 1
Cellulose hydrolysis and enzyme saccharification of bamboo treated using various pretreatment methods.

Pretreatment method	Glucose (mg/g initial dry sample)	Reducing sugar (mg/g initial dry sample)	Cellulose hydrolysis (%)	Enzyme saccharification (%)
Steam explosion (35 atm) for 5 min	426	488	84.3	64.3
Steam explosion (20 atm) for 5 min followed by 10%NaOH treatment for 60 min	456	460	90.1	60.6
Milling for 5 min followed by 10%NaOH treatment for 60 min	383	485	75.8	63.9

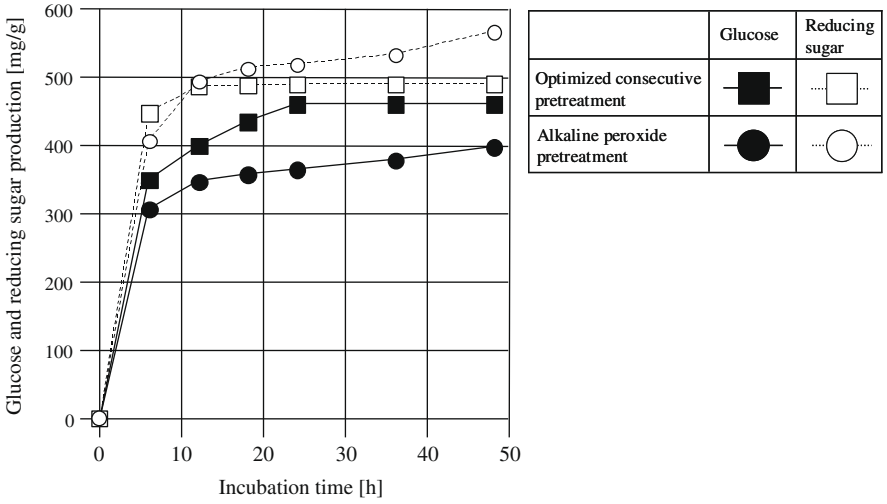

Fig. 7. Comparison of optimized consecutive pretreatment and alkaline peroxide pretreatment in the enzymatic saccharification of bamboo.

3.8. Effect of alkaline peroxide pretreatment on enzyme saccharification of milled bamboo

In the enzymatic saccharification of herbaceous lignocellulose, alkaline peroxide pretreatment was expected to be an effective and mild method for decreasing the sodium hydroxide concentration. In this work, the milled bamboo was treated with 1%(v/v) of hydrogen peroxide and 1 wt.% of sodium hydroxide. Fig. 7 shows the time courses of glucose and reducing sugar production in the enzymatic saccharification of bamboo treated by the optimized consecutive pretreatment (20 atm steam explosion and 10 wt.% sodium hydroxide treatment) and the alkaline peroxide pretreatment. The optimized consecutive pretreatment provided the large amount of glucose production, i.e. 456 mg/(g initial dry sample), with a shorter incubation time (24 h), due to the effects of both high delignification and the change of cellulose crystallinity. However, since little difference between glucose and reducing sugar production, i.e. 456 and 460 mg/(g initial dry sample), was not observed, it means that a large amount of the hemicellulose component was degraded. The glucose and reducing sugar production increased with the incubation time and reached the maximum values of 399 and 568 mg/(g initial dry sample), respectively, after 48 h incubation. The large difference between the amounts of glucose and reducing sugar indicates that the sugars derived from the

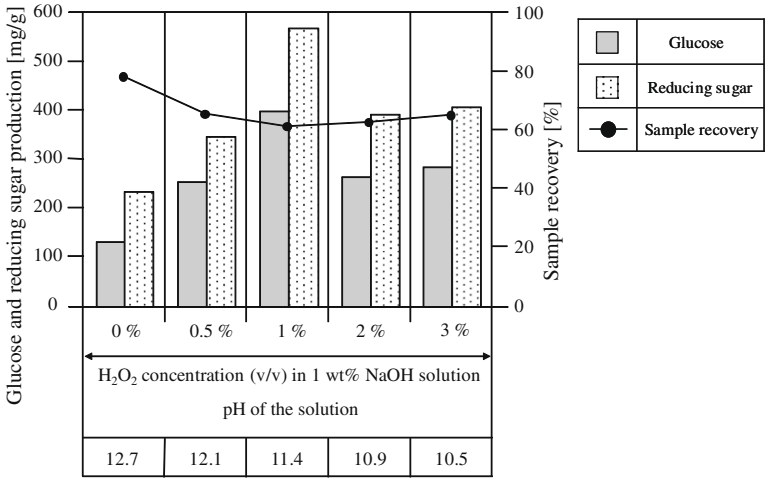

Fig. 8. Effect of hydrogen peroxide concentration with 1 wt.% sodium hydroxide on the production of glucose and reducing sugar by enzyme saccharification of bamboo.

hemicellulose component were produced by enzymatic saccharification without being degraded by the alkaline peroxide pretreatment. Therefore, this observation showed that the alkaline peroxide pretreatment is more effective for enzyme saccharification of bamboo.

Fig. 8 shows the effect of hydrogen peroxide concentration with 1 wt.% sodium hydroxide on the production of glucose and reducing sugar by enzyme saccharification. When the hydrogen peroxide concentration of 0%(v/v), which is equivalent to 1 wt.% sodium hydroxide treatment, was performed, the glucose and the reducing sugar production decreased to approximately half that compared to 1 wt.% sodium hydroxide pretreatment shown in Fig. 6. This is due to the difference of reaction temperatures, i.e. 121 °C for the sodium hydroxide pretreatment and 90 °C for the alkaline peroxide pretreatment. However, the addition of hydrogen peroxide increased the sugars production. The maximum production of glucose and reducing sugar, i.e. 399 and 568 mg/(g initial dry sample), were obtained in 1%(v/v) of hydrogen peroxide addition. Gould reported that the optimal pH is 11.5 for the alkaline peroxide treatment (Gould, 1984). In this work, the large amount of sugars production was obtained by the suitable pH, i.e. 11.4, of the reaction mixture in 1%(v/v) hydrogen peroxide and 1 wt.% of sodium hydroxide. Therefore, it is concluded that the alkaline peroxide pretreatment combined with 1%(v/v) hydrogen peroxide and 1 wt.% of sodium hydroxide was the most effective pretreatment method for enzyme saccharification of bamboo.

4. Conclusion

Pretreatment methods for enzyme saccharification using herbaceous lignocellulose, bamboo, were investigated for the purpose of producing fine chemicals. Steam explosion (35 atm) produced a large amount of glucose and reducing sugar because of the enhancement of enzyme accessibility due to the destruction of the bamboo structure. In addition, the consecutive pretreatment with 20 atm steam explosion or mechanical milling and various concentrations of sodium hydroxide treatment were attempted. The consecutive pretreatment with 20 atm steam explosion and 10% sodium hydroxide provided the maximum values for glucose production and cellulose hydrolysis by enzyme saccharification. An alkaline peroxide pretreatment combing with 1%(v/v) hydrogen peroxide and 1 wt.% sodium hydroxide, also produced a large amount of glucose and reducing sugar without severe conditions of high pressure steam explosion and high concentration sodium hydroxide treatment, and this method seems to be the most effective as an environmentally friendly pretreatment. Future study will be focused on the research for the further improvement of enzymatic saccharification by clarifying not only chemical modification of cellulose crystal structure but also delignification characteristics of bamboo with alkaline peroxide pretreatment.

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