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Ethanol production from disposable aspen chopsticks using delignification pretreatments

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ABSTRACT

To convert waste disposable wooden chopsticks, cellulosic waste biomass, into ethanol efficiently, the effectiveness of pretreatments, i.e. ball-milling and steam explosion, for the enzymatic hydrolysis of aspen chopsticks and the dependence of substrate concentration using pretreated aspen chopsticks for the ethanol production were studied. In the enzymatic saccharification of ball-milled chopsticks, the maximum amount of glucose, 520 mg-glucoce/g-dry aspen chopsticks, was obtained from the chopsticks ball-milled for 60 min. On the other hand, the maximum amount of glucose, 598 mg-glucoce/g-dry aspen chopsticks, was obtained using steam-exploded aspen chopsticks treated at a steam pressure of 25 atm and a steaming time of 5 min. Simultaneous saccharification and fermentation (SSF) of the steam-exploded chopsticks by Meicelase and *Saccahromyces cerevisiae* AM12 provided 241 mg-ethanol/g-dry aspen chopsticks, corresponding to 79% of theoretical ethanol yield.

1. Introduction

Recently, bioethanol has been attracted as alternative energy of fossil materials, i.e. oil, coal, and natural gas (Farrell et al., 2006; Sukumaran, Singhania, Mathew, & Pandey, 2009). Since many popular biofuel sources, i.e. cane sugars and corn, are also generally used as staple foods, it is desired to develop for the efficient bioethanol production from cellulosic biomass, i.e. wood, bamboo, wheat straw, and bagasse, that does not compete with food (Boluda-Aguilar, Garcia-Vidal, Gonzalez-Castaneda, & Lopez-Gomez, 2010; Chen, Pen, Yu, & Hwang, 2011; Ewanick, Bura, & Saddler, 2007). However, as cellulose component, i.e. substrate for bioethanol, are generally covered with lignin in the cellulosic biomass, it is necessary to degrade and remove lignin in the cellulosic biomass. The ball milling and steam explosion are effective pretreatments for the delignification of cellulosic biomass (Adapa, Tabil, & Schoenau, 2011; Cara et al., 2008; Ohgren, Rudolf, Galbe, & Zacchi, 2006; Yamashita, Sasaki, & Nakamura, 2010; Zimbardi et al., 2007). In steam explosion, the cellulosic biomass is treated with high temperature and pressure saturated steam and then the pressure is reduced rapidly, which makes the cellulosic biomass undergoes an explosive decomposition. On the other hand, disposable wooden chopsticks are used in restaurants, school cafeterias, and supermarkets in Japan, and are generally made of aspen or white birch wood. The annual average amount of waste wooden chopsticks is about 90,000 *t* in Japan. Therefore, disposable wooden chopsticks can be used as source for bioethanol production.

In this work, the utilization of disposable wooden chopsticks in the bioethanol production was studied using ball milling, steam explosion, enzymatic saccharification, and simultaneous saccharification and fermentation (SSF). The effects of ball milling and steam explosion on enzymatic saccharification of disposable wooden chopsticks were clarified and the optimal condition of SSF was determined for the effective conversion of steam-exploded chopsticks into ethanol.

2. Methods

2.1. Samples

Aspen (*Popoulus termuloides*) chopsticks were cut into the half whose length is about 10 cm and then treated by various ball milling and steam explosional conditions as described bellow.

2.2. Mechanical grinding by a ball mill

100 g of chopsticks samples were ground by a vibrating sample mill (VIBRATING SAMPLE MILL CMT-TI-300, C. M. T Co. Ltd., working volume 250 ml × 2) at 60 cycles/s for 1–60 min. The particle size of ball-milled samples was measured by sieve analysis.

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2.3. Steam explosion pretreatment

Steam explosion pretreatment, which was used as a physicochemical pretreatment, was carried out in a batch pilot unit equipped with a 1 l of reactor (Steam explosion apparatus NK-1L, Japan Chemical Engineering and Machinery Co. Ltd., Osaka, Japan) (Take et al., 2006). 100 g of chopsticks sample was introduced into the reactor and exposed to the saturated steam of 20 atm (214 °C), 25 atm (225 °C), and 30 atm (235 °C) for a steaming time of 5 min. After the 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. The product containing liquid–solid materials was obtained as the steam-exploded sample in the receiver.

2.4. Extraction and separation of components

The amounts of the components, i.e. water soluble material, methanol soluble material, holocellulose (cellolose and hemicellulose), and Klason lignin, in the chopsticks treated by various pretreatment conditions were measured by the procedure according to Wayman's extraction method (Chua and Wayman, 1979) as shown in Fig. 1. 5 g of dry steam-exploded sample was added to 100 ml of distilled water and extracted for 24 h at room temperature. The solid (Residue 1) and liquid (Extract 1) materials were separated by filtration, and the filtrate (water soluble material) was recovered from the liquid, then concentrated, dried, and weighed. Residue 1 was extracted at 80 °C for 24 h in a Soxhlet extractor with 150 ml methanol to dissolve the methanol soluble lignin, a low molecular weight lignin. After concentration and drying of the extract (Extract 2), the methanol soluble lignin was weighed. Residue 2 from the methanol extraction consisted of holocellulose and Klason lignin, a high molecular weight lignin. This residue (1 g) was added to 15 ml of 72% (w/w) sulfuric acid and left at room temperature for 4 h. This was placed in a 100 ml conical flask, washed with 560 ml distilled water, and then boiled for 4 h with reflux-cooling. After the insoluble matter (Residue 3) was washed with hot water, it was dried at about 105 °C by a heater to a constant weight, and weighed. This substance was Klason lignin. The weight of hollocelulose was calculated by subtracting the weight of Klason lignin from 1 g of Residue 2.

2.5. Enzyme saccharification

The ball-milled chopsticks and the steam-exploded chopsticks residue after water extraction were carried out using a cellulolytic enzyme, which was performed in 110 ml sample tubes at an initial sample concentration of 2% (w/v) in 10 ml of 100 mM sodium acetate buffer pH 5.0 and using enzyme (Meicelase, Meiji Seika Co. Ltd.) loading of 20 FPU/g sample. The enzymatic 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 the solid residue for sugar content.

2.6. Simultaneous saccharification and fermentation (SSF)

Saccharomyces cerevisiae AM 12 was obtained from *Bio Academia* Co. Ltd., Japan, and used for ethanol production. It was incubated on potato dextrose agar plates at 30 °C and then stored in the refrigerator at 4 °C. Pure yeast culture from an agar plate was added to 10 ml L-tubes containing 5 ml of sterile medium. The medium compositions were as follows: 10 g/l glucose, 1 g/l yeast extract, 0.1 g/l KH_2PO_4, 0.1 g/l $MgSO_4{\cdot}7H_2O$ and 0.1 g/l $(NH_4)_2SO_4$ (Itoh, Wada, Honda, Kuwahara, & Watanabe, 2003). This preculture was incubated at 30 °C for 24 h using a seesaw incubator at 60 rpm. The ball-milled chopsticks and the steam-exploded chopsticks residue after water extraction at various initial concentrations were put into 200 ml Erlenmeyer flasks, and then autoclaved for 20 min at 121 °C. Then, the sterilized nutrient solution, the enzyme and the sodium acetate buffer were added. The composition of the nutrient solution and enzyme loading in the fermentation medium were adjusted as follows: 2 g/l yeast extract, 0.05 g/l $MgSO_4{\cdot}7H_2O$, 1 g/l $(NH_4)_2HPO_4$, 20 FPU/g sample and 100 mM of sodium acetate buffer at pH 5.0. After the previously precultured yeast suspension was centrifuged and the supernatant was removed, the suspended yeast by sterilized water was inoculated and the mixture was incubated in a rotary shaker at 40 °C with gentle agitation at 100 rpm.

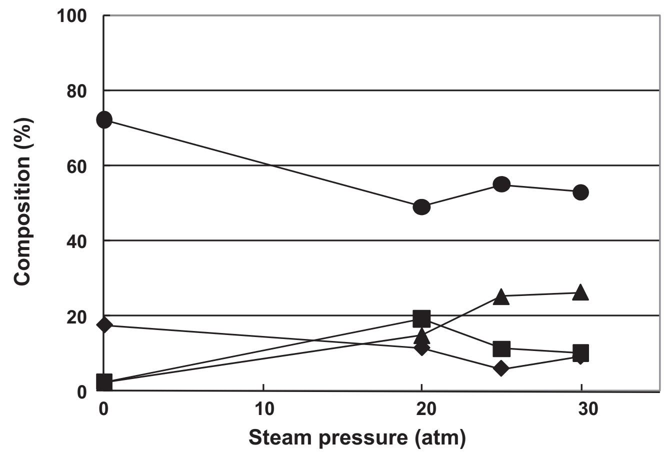

Fig. 2. Composition of ball-milled (zero of steam pressure) and steam-expolded aspen chopsticks under various steam pressures for a steaming time of 5 min. Symbols: (●): holocellulose (Extract 3); (■): water soluble material (Extract 1); (▲): methanol soluble lignin (Extract 2); (♦): klason lignin (Residue 3).

2.7. Analyses

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). Ethanol and monomeric sugars concentrations from the fermentation broth were analyzed by HPLC equipped with an Aminex column HPX-87H (300 mm × 7.8 mm) (Bio-rad, Richmond, CA).

3. Results and discussions

3.1. Effects of pretreatments

To increase the accessibility of enzyme to cellulose, the chopsticks were treated by ball milling and steam explosion. Fig. 2 shows the composition of ball-milled (zero of steam pressure) and steam-exploded aspen chopsticks under various steam pressures. In the case of ball-milled chopsticks, the amounts of holocellulose (Extract 3), i.e. cellulose and hemicellulose, and Klason lignin (Residue 3), i.e. high-molecular weight lignin, are comparatively high, and little amounts of water soluble materials (Extract 1), i.e. a comparatively low-molecular weight monosaccharides, oligosaccharides, and etc., and methanol soluble lignin (Extract 2), i.e. low-molecular weight lignin, were observed. In the case of steam-exploded chopsticks, the amount of water soluble material increased by a steam pressure of 20 atm because some part of the polysaccharides was hydrolyzed and converted into monosaccharides and oligosaccharides. The reason why the amount of water soluble material decreased beyond a steam pressure of 20 atm depended on the fact that the monosaccharides and oligosaccharides combined with the lignin at a high steam pressure. The amount of holocellulose decreased by the steam

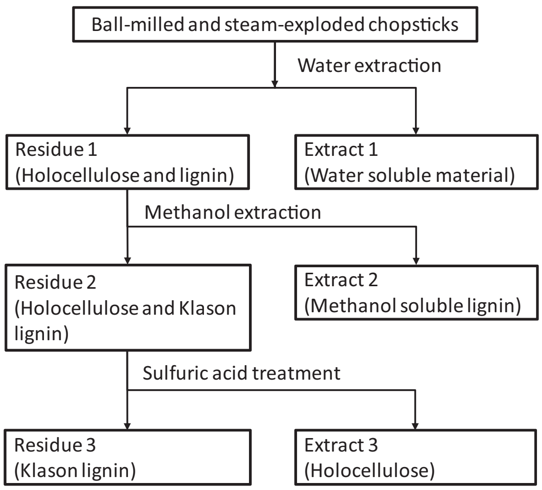

Fig. 1. Procedure for extraction and separation of each component of ball-milled and steam-exploded chopsticks.

explosion. The reason why the amount of holocellulose decreased seems that almost part of the hemicellulose was converted into a water soluble material by the hydrolysis of steam explosion. The amount of methanol soluble lignin increased by the increase of steam pressure. The amount of Klason lignin decreased by a steam pressure of 25 atm and then increased. Since in the steam explosion with a comparatively low steam pressure the high-molecular weight lignin was hydrolyzed into low-molecular weight lignin, the amount of Klason lignin decreased by a steam pressure of 25 atm. The increase of Klason lignin at a comparatively high steam pressure depended on the recondensation reaction of the low-molecular lignin and the combination reaction of the water soluble material and the high-molecular lignin (Kobayashi, Take, Asada, & Nakamura, 2004). The steam-exploded chopsticks treated at a comparatively low steam pressure, i.e. 25 atm, with a small amount of high-molecular lignin seems to be suitable for the microbial conversion into sugars because the high-molecular lignin inhibited the hydrolysis reaction of the cellulose by the amylolytic microorganism. Furthermore, since the water soluble material (Extract 1) in the steam-exploded chopsticks contained not only sugars and polyphenols but also furfural and 5-HMF derived from aspen chopsticks by steam explosion (data not shown), and the furfural and 5-hydroxymethylfurfural (5-HMF) are well known as inhibitors for alcohol fermentation (Palmqvist and Hahn-Hagerdal, 2000; Sanchez and Bautista, 1988), Residue 1, i.e. steam-exploded chopsticks after water extraction, was used as a substrate in the next enzymatic saccharification and SSF experiments of steam-exploded chopsticks.

3.2. *Enzymatic saccharification of ball-milled aspen chopsticks*

For increasing the digestibility of holocellulose components, chopsticks were pretreated by ball-milling for 1–60 min. Fig. 3 shows the time courses of reducing sugar and glucose production in the enzyme saccharification of 20 g/l ball-milled aspen chopsticks. There is no observation of reducing sugar and glucose production in the raw chopsticks through enzyme saccharification. For the ball-milled chopsticks, the concentrations of reducing sugar and glucose increased significantly with the increase of ball-milling time and reached their maximum values of 13.2 and 10.4 g/l, respectively, at 60 min of ball-milling. These values corresponded to 660 mg-reducing sugar/g-chopsticks and 520 mg-glucose/g-chopsticks, respectively. Furthermore, no increase of sugar production was observed beyond 60 min of ball milling time. This observation suggests that a long time ball-milling

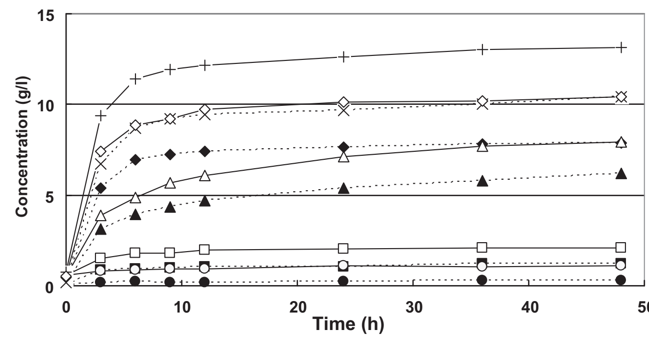

Fig. 3. Production of reducing sugar and glucose in enzymatic saccharification of 20 g/l ball-milled aspen chopsticks under various ball-milling times. Symbols: reducing sugar, (◯), 1 min; (□), 5 min; (□), 10 min; (□), 30 min; (+) 60 min; glucose, (●), 1 min; (■), 5 min; (▲), 10 min; (□), 30 min; (×) 60 min.

such as 60 min is an effective pretreatment for enzymatic saccharification of chopsticks even if it needs a high energy cost.

3.3. *Enzymatic saccharification of steam-exploded aspen chopsticks*

Fig. 4 shows the time courses of reducing sugar and glucose production in the enzyme saccharification of 20 g/l Residue 1, i.e. steam-exploded aspen chopsticks residue after water extraction, under various steam pressures for a steaming time of 5 min. In case of untreated chopsticks no reducing sugar and glucose was observed even after an incubation time of 48 h (data not shown). On the other hand, in case of steam-exploded chopsticks the amounts of reducing sugar and glucose produced rapidly increased with the increase of incubation time and reached their maximum values at an incubation time of 48 h. When the chopsticks were treated at 25 atm, the amounts of reducing sugar and glucose were higher (14.8 and 13.5 g/l, respectively) compared to those at 20 and 30 atm. These values corresponded to 740 mg-reducing sugar/g-Residue 1 and 675 mg-glucose/g-Residue 1, respectively. Furthermore, they corresponded to 656 mg-reducing sugar/g-chopsticks and 598 mg-glucose/g-chopsticks, respectively, because of steam-exploded chopsticks at 25 atm contained Residue 1 (88.6%) and Extract 1 (11.4%) as shown in Fig. 1. Since 75% of Extract 1 was reducing sugar, future study will be focused on utilizing Extract 1. As a result, it is found that the steam explosion at 25 atm is more efficient in the pretreatment of chopsticks compared to ball milling or steam explosion at other steam pressures for the enhancement of enzymatic saccharification.

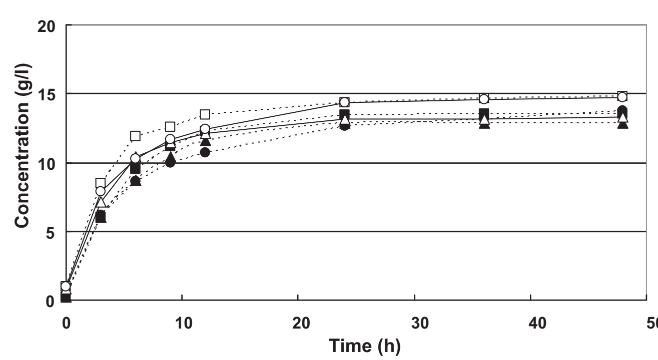

Fig. 4. Production of reducing sugar and glucose in enzymatic saccharification of 20 g/l steam-exploded aspen chopsticks after water extraction under various steam pressures for a steaming time of 5 min. Symbols: reducing sugar, (◯), 20 atm; (□), 25 atm; (□), 30 atm; glucose, (●), 20 atm; (■), 25 atm; (▲), 30 atm.

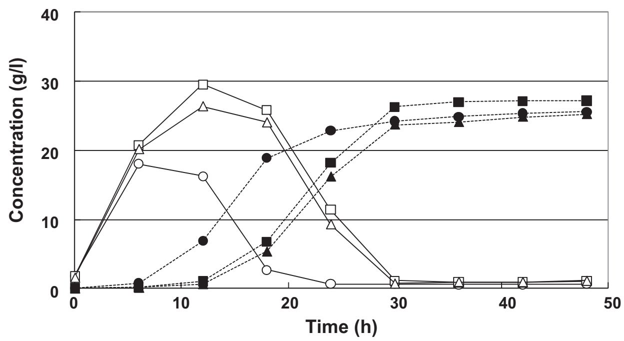

Fig. 5. Production of ethanol in simultaneous saccharification and fermentation of 100 g/l steam-exploded aspen chopsticks after water extraction under various steam pressures for a steaming time of 5 min. Symbols: glucose, (○), 20 atm; (□), 25 atm; (□), 30 atm; ethanol, (●), 20 atm; (■), 25 atm; (▲), 30 atm.

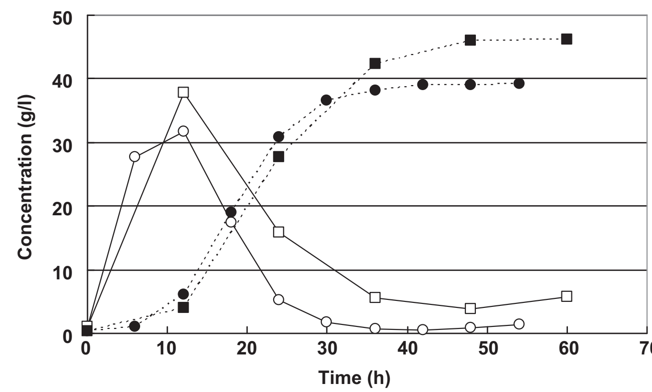

Fig. 6. Production of ethanol in simultaneous saccharification and fermentation of 150 and 200 g/l steam-exploded aspen chopsticks after water extraction at a steam pressure of 25 atm for a steaming time of 5 min. Symbols: glucose, (○), 150 g/l; (□), 200 g/l; Ethanol, (●), 150 g/l; (■), 200 g/l.

3.4. Simultaneous saccharification and fermentation (SSF) of steam-exploded aspen chopsticks

The glucose, hydrolyzed from steam-exploded chopsticks by a cellulolytic enzyme, is converted into ethanol by *S. cerevisiae*. Fig. 5 shows the time courses of glucose and ethanol concentrations in SSF of 100 g/l Residue 1, i.e. steam-exploded aspen chopsticks residue after water extraction, under various steam pressures for a steaming time of 5 min. The glucose produced in SSF was completely consumed, because it was converted into ethanol by *S. cerevisiae*. The ethanol appeared after about 10 h of incubation time and increased with the increase of incubation time. In Residue 1 at 20 atm the ethanol appeared at earlier incubation time compared to those at 25 and 30 atm. This reason seems to be that Residue 1 at 20 atm contained lower amount of methanol soluble lignin, i.e. low-molecular weight lignin, which has inhibitory effect on alcohol fermentation, compared to those at 25 and 30 atm as shown in Fig. 2. On the other hand, in case of SSF using 100 g/l steam-exploded chopsticks without washing with water, though the significant amount of glucose was obtained, no production of ethanol was observed by 48 h of incubation time because 100 g/l steam-exploded chopsticks contained a large amount of furfural and 5-HMF that inhibited the growth of fermenting cells (Palmqvist and Hahn-Hagerdal, 2000). In SSF of Residue 1 under various steam pressures, the maximum ethanol concentration, i.e. 27.2 g/l, was obtained from Residue 1 of steam-exploded chopsticks at 25 atm. As a result, it was found that a high ethanol yield, i.e. 241 mg-ethanol/g-dry chopsticks, was obtained from the steam-exploded chopsticks at 25 atm, because of they contained 11.4% of water soluble material (Extract 1) as shown in Fig. 2.

Fig. 6 shows the time courses of glucose and ethanol concentrations in SSF using various initial substrate concentrations of Residue 1 of 25 atm steam-exploded aspen chopsticks. The increase of the amount of substrate concentration resulted in the elevated ethanol production. First, 39.2 g/l of ethanol was generated from 150 g/l of substrate at 48 h of incubation by SSF process. In addition, the increase of the amount of substrate concentration led to the enhancement of ethanol production, and 46.2 g/l of ethanol was obtained at 48 h of incubation from the initial substrate concentration of 200 g/l. The use of high concentration of substrate, 200 g/l, introduced the large amount of ethanol production, however, glucose uptake by fermentation cells was ceased at 36 h incubation and after that, the some amounts of glucose was accumulated in the fermentation broth with the incubation time. This result was caused that the ethanol production inhibition occurred in high concentration of substrate due to the accumulation of high concentration of lignin into fermentation broth. The ethanol yields obtained from Residue 1 at 150 and 200 g/l were 232 and 205 mg-ethanol/g-dry chopsticks, respectively.

From these results, it was concluded that the steam-exploded chopsticks after water extraction with 100 g/l of substrate concentration could obtain a high ethanol yield, i.e. 241 mg-ethanol/g-dry chopsticks. This value corresponds to 79% of theoretical ethanol yield. Furthermore, this value was almost consistent with the value, i.e. 200 mg-ethanol/g-sample (79% of theoretical ethanol yield), reported by Sassner, Martensson, Galbe, & Zacchi (2008) using the steam pretreatment of H_2SO_4-impregnated Salix (fast-growing willow).

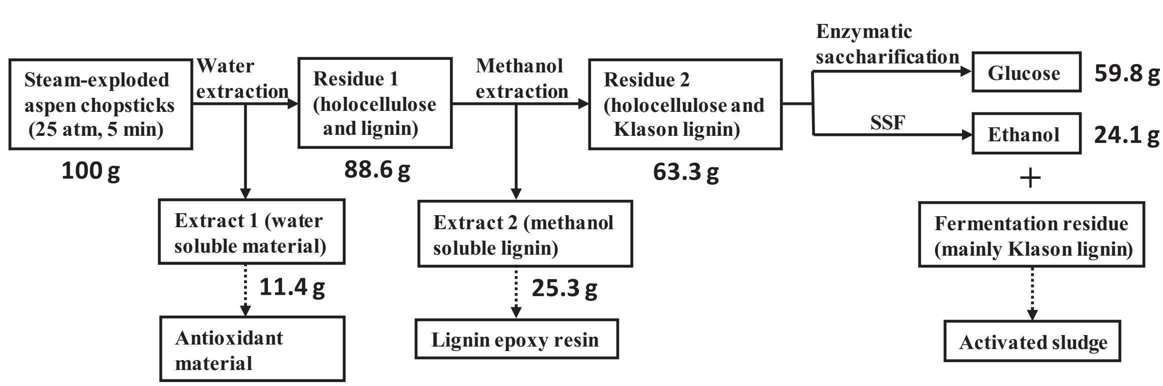

Fig. 7. Possible mass balance of useful materials produced from steam-exploded aspen chopsticks.

3.5. Possible conversion process of aspen chopsticks into useful materials

Considering above results the possible production process of fuel and materials from the structural components of aspen chopsticks is presented as shown in Fig. 7. 100 g of dry steam-exploded chopsticks is extracted and separated into 11.4 g of Extract 1 (water soluble material), 25.3 g of Extract 2 (methanol soluble lignin), and 88.6 g of Residue 1 (holocellulose and Klason lignin). Extract 1 may show an antioxidant activity because it contains water-soluble polyphenol materials derived from lignin (Kurosumi et al., 2007). Extract 2 can be used as a raw material for the synthesis of epoxy resin, and it has the heat-resisting property for solder demanded in the electronic material field (Asada, Nakamura, & Kobayashi, 2005). Residue 2 is converted into 59.8 g of glucose and 24.1 g of ethanol by enzymatic saccharification and SSF, respectively. In this figure the amount of ethanol obtained is estimated from the experiment result of SSF using Residue 1. Therefore, if Residue 2 is used as a substrate for the SSF experiment, the final ethanol production may be increased because Residue 2 contains little inhibitory materials such as Extract 2. This point is a future subject. Furthermore, the fermentation residue (mainly Klason lignin component) can be carbonized into activated carbon. This process seems to be a novel production process of energy and useful materials from woody biomass using the steam explosion, extraction and various conversion methods. Future study will be focused on the extraction and separation of antioxidant materials from Extract 1 and the synthesis of epoxy resin from Extract 2.

4. Conclusion

This study clarified the effect of steam explosion on enzymatic saccharification of disposable chopsticks and the optimal condition of simultaneous saccharification and fermentation (SSF) for the effective conversion of steam-exploded chopsticks into ethanol. The steam explosional condition at 25 atm and 5 min provided the most positive effects on enzyme saccharification due to breakdown the wood fragment following the enhancement of the accessibility of enzyme and the digestibility of cellulose. In addition, the ethanol production from steam-exploded chopsticks after water extraction was attempted using SSF and obtained the comparatively high theoretical yield and the ethanol production as same as the steam pretreatment of H_2SO_4-impregnated wood. Since the steam explosion uses no chemical reagents such as acid and alkali, it seems to be an environmentally friendly method. Therefore, it was confirmed that the steam explosion is an effective pretreatment method of disposable chopsticks not only for ethanol production but also for environmental protection. Furthermore, this process might be applied on an industrial scale to the effective conversion of not only disposable chopsticks but also waste wooden biomass, i.e. wood chips, bark, and etc.

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