Xylo-oligosaccharides and Ethanol Production from Liquid Hot Water Hydrolysate of Sugarcane Bagasse

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With the objective of maximizing the use of liquid hot water hydrolysate of sugarcane bagasse, xylo-oligosaccharides and ethanol were respectively produced by the methods of purification and microbial fermentation. The processes of purification with activated charcoal, overliming, solvent extraction, vacuum evaporation, and use of an ion exchange resin were evaluated, and the results indicated that anion exchange chromatography performed well in terms of by-product removal. The recovery and purity of xylo-oligosaccharides reached 92.0% and 90.4%, respectively, using column chromatography with the resin LS30 at a flow rate of 2 mL/min at 25 °C. The hydrolysate was used in ethanol fermentation with *Pichia stipitis* CBS6054 followed by the production of fermentable saccharides and detoxification. The highest ethanol concentration was 4.12 g/L with a theoretical yield of 47.9% for the hydrolysate after xylanase digestion and resin detoxification, similar to the data of the control experiment, which had an ethanol concentration of 4.64 g/L and a yield of 49.6%. However, the former had a higher ethanol productivity of 0.0860 g/(L·h), and the highest ethanol concentration appeared 12 to 24 h earlier compared to the control. This study suggests that combined generation of xylo-oligosaccharides and cellulosic ethanol could help maximize profits for a cane sugar factory.

Keywords: Liquid hot water; Xylo-oligosaccharides; Ethanol; Sugarcane bagasse

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INTRODUCTION

China is the third largest producer of sugarcane with 70 million tons, behind Brazil and India. The enormous sugar production in China generates huge amounts of bagasse, the fibrous waste product of the sugar refining industry. Take Guangxi province for example, more than 5.6 million tons of bagasse is produced every year. It is traditionally utilized for the animal feed, papermaking, or combustion. Recently, sugarcane bagasse has been persistently receiving attention as a raw material for production of ethanol because of its prominent content of cellulose and hemicellulose.

The polysaccharides hemicellulose and cellulose can be fermented into ethanol following the process of pretreatment and enzymatic hydrolysis (Chandel *et al.* 2012; Mussatto 2014). At present, research concerning cellulosic ethanol production focuses on the development of green and effective pretreatment technologies, the supply of cost-effective enzymes, and the isolation of microbial strains that maximize ethanol yields and tolerance. In essence, the common objective of these studies is to improve the economic efficiency of cellulosic ethanol. An effective pretreatment can reduce downstream pressure by making cellulose more accessible to the enzymes and minimizing the formation of

degradation products that inhibit the growth of fermentative microorganisms. Liquid hot water (LHW) pretreatment has become a promising method because of its environmentally friendly features, effective recovery of pentosans, and enhancement of cellulosic digestibility (Yu *et al.* 2013). The reaction condition optimization (Yu *et al.* 2010, 2011) and decomposition mechanism (Zhuang *et al.* 2012; Lv *et al.* 2013) of biomass in liquid hot water have been widely reported, but less has been written about the utilization of LHW hydrolysate.

Xylo-oligomers are typically used in the fields of pharmaceuticals, food ingredients, and feed formulations. In food-related applications, xylo-oligomers present advantages compared with other oligosaccharides such as fructo-oligosaccharide, galacto-oligosaccharide, and isomalto-oligosaccharide in terms of improvements to health and concentration thresholds (Vázquez *et al.* 2000). In general, xylan in lignocellulosic materials is first isolated with chemical methods and then further hydrolyzed enzymatically into xylo-oligomers (Rose *et al.* 2010; Mazzaferro *et al.* 2011). However, the comparatively high production costs from the use of xylanase are becoming an obstacle for its extensive use. Many studies have found that xylo-oligosaccharides can be extracted by LHW hydrolysis of xylan-rich materials (Samala *et al.* 2012). The composition of LHW hydrolysate mostly includes xylo-oligosaccharides (about 60% to 80%), xylose (10% to 15%), other sugars, and degradation products of sugar and lignin (10% to 25%) (Yu *et al.* 2012b; Zhuang *et al.* 2012). Electrospray ionization mass spectrometry (ESI-MS) of these xylose oligomers indicates that their DP range is lower than 5 (Yu *et al.* 2012a). The biggest challenge in the production of xylo-oligosaccharides from LHW hydrolysate is how to economically remove by-products like acetic acid and furfural.

Most of the published literature has focused on enzymatic saccharification and ethanol fermentation of LHW-treated solid residues (Lu *et al.* 2012; Wang *et al.* 2012). However, the absence of hexose, low monosaccharide content, high oligosaccharides content, and complex by-products of the LHW hydrolysate make it difficult for LHW to be utilized by microorganisms (Wierckx *et al.* 2010; Shi *et al.* 2014). With the objective of maximizing the use of the hydrolysate, the production of xylo-oligosaccharides and ethanol were investigated in this paper by the methods of purification and microbial fermentation, respectively.

EXPERIMENTAL

Materials

Preparation of LHW hydrolysate of sugarcane bagasse

Sugarcane bagasse (SB) was provided by the National Engineering Research Centre for Non-food Biorefinery, Guangxi Academy of Sciences, Guangxi province, China. This material is the solid residue remaining after juice extraction, milling, screening (through 8 to 18 mesh), and drying (80 °C for 24 h, to a constant weight). According to the laboratory analytical procedure (LAP) for biomass analysis used by the US National Renewable Energy Laboratory (NREL) (Sluiter *et al.* 2008) , the chemical composition of SB is 45.2% glucan, 23.6% xylan, 2.1% arabinan, 21.3% acid-insoluble lignin, 3.9% extractives, and 3.4% ash. The SB (containing 5% water w/v) was then treated with an experimental system that was composed of an autoclave reactor, a feeding system, and a product collector. The details of experimental apparatus were described in a previous article by the co-authors (Yu *et al.* 2010). As described previously (Yu *et al.* 2013),

nitrogen flowed into the reactor to maintain pressure (4 MPa), and the reactor was heated at 180 °C for 20 min. Hydrolysate was collected and centrifuged at 4024.8 xg for 5 min, and the precipitate was discarded.

Methods

Purification of xylo-oligosaccharides

Several purification methods were compared, as follows: The hydrolysate byproducts were adsorbed using powder activated charcoal (Kamal *et al.* 2011) with a liquid to solid ratio of 5:1 and incubated at 30 °C in a rotary shaker at 150 rpm for 1 h. The acidic products in the hydrolysate were precipitated when the pH was adjusted to 9.0 with calcium hydroxide or calcium carbonate. The organic phase in the hydrolysate was extracted by an equal volume of cyclohexanone using a separatory funnel. Vacuum evaporation was used at 75 °C with concentration times of 10 to eliminate substances with low boiling points, such as acetic acid and furfural, from the sugars (Vázquez *et al.* 2005). In addition, the hydrolysate was treated with ion exchange resins at 25% w/v loading in a 50-mL flask and incubated at 50 °C in a rotary shaker at 150 rpm for 6 h. The resins of ZGA451FD, ZGC151FD, 717, 750 were purchased from Ningbo Zhengguang Resin Co., Ltd, China, and LS30, LS106, LS600 from Shanxi Lanshen Resin Co.,Ltd, China. Chromatography experiments were performed with a 20 cm × 1 cm column packed with each type of resin and balanced with distilled water at 2 mL/min. Then, 10 mL of hydrolysate was passed through the column and washed by 40 mL of distilled water. The elution solution was collected, followed by the analysis of the concentration of xylo-oligosaccharides and by-products. All procedure was conducted in triplicate, except for the resins adsorption experiments.

Saccharification and detoxification of hydrolysate

The hydrolysate was concentrated to approximately 33% of its original volume using a rotatory evaporator at 57 °C, and xylo-oligosaccharides were hydrolyzed by 4% H_2SO_4 at 121 °C for 1 h or with a xylanase loading of 54 U/mL (produced in the authors' laboratory, with an activity of 900 U/mL) at 50 °C for 24 h. After saccharification, the hydrolysate was detoxified with the anion exchange resin ZGA451FD (12.5% w/v loading) at 50 °C, 150 rpm for 1 h.

Fermentation media

A solution of 20 g/L xylose was prepared as a control. The sugar solution described in "Saccharification and detoxification of hydrolysate" section was supplemented with 5 g/L peptone, 5 g/L yeast extract, 5 g/L KH_2PO_4, 0.2 g/L $(NH_4)_2SO_4$, and 0.4 g/L $MgSO_4.7H_2O$, adjusted to pH 5.0, and sterilized at 115 °C for 30 min. This was the fermentation medium.

Microorganism and fermentation of ethanol

Pichia stipitis (No. CBS6054) was purchased from the China Center of Industrial Culture Collection (CICC) and kept with 25% glycerinum at -20 °C. The inoculum was prepared in yeast extract peptone dextrose (YPD) media containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract at pH 5.0 and incubated at 30 °C in a rotary shaker at 150 rpm for 48 h. Seed culture was harvested by centrifugation at 4024.8 xg for 5 min and washed twice with sterile water. The seed culture was inoculated into the fermentation media at 1 g/L. Fermentation was conducted at 30 °C in a rotatory shaker at 70 rpm for 96

h. Samples were taken every 12 h to determine the concentrations of ethanol and xylose. This procedure was conducted in triplicate.

Analytical methods

Ethanol was analyzed using an Agilent 7890 GC gas chromatograph (Agilent Technologies, USA) equipped with an FFAP column, at 220 °C, with a hydrogen flow of 30 mL/min. Monosaccharide and by-product acids and aldehydes in the hydrolysate were detected by a Waters 2498 high performance liquid chromatograph (HPLC) (Waters Corp., USA) equipped with a Shodex SH1011 column (Shodex, Japan) coupled with a refractive index and ultraviolet detector. The mobile phase was 0.005 M H_2SO_4 at a flow rate of 0.5 mL/min, with a column temperature of 50 °C. The analysis of the xylo-oligosaccharides was calculated after a secondary hydrolysis into monosaccharide with 4% sulfuric acid at 121 °C. The concentration of xylo-oligosaccharides was given by the following expression: [Total xylose concentration after a secondary hydrolysis – initial xylose concentration] × 0.88.

RESULTS AND DISCUSSION

Comparison of Different Purification Methods

The composition of SB LHW hydrolysate was determined by HPLC. The concentration of xylo-oligosaccharides was 7.50 g/L, xylose 2.00 g/L, arabinose 0.300 g/L, glucuronic acid 0.800 g/L, furfural 0.600 g/L, glycolic acid 0.200 g/L, formic acid 0.150 g/L, and acetic acid 1 g/L. Figure 1 illustrates the effects of different purification methods on the removal of by-products and the yield of xylo-oligosaccharides.

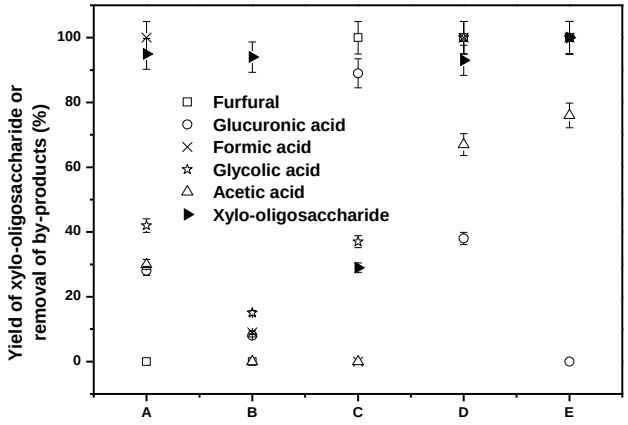

Fig. 1. Effect of purification methods on the yield of xylo-oligosaccharides and removal of by-products. (A) Calcium hydroxide; (B) Calcium carbonate; (C) Activated charcoal; (D) Cyclohexanone extraction; (E) Vacuum evaporation. Data provided as the mean ± standard deviation

As shown, the most effective method was vacuum evaporation, which removed 100% of formic acid, glycolic acid, and furfural, and 76.0% of acetic acid. Only glucuronic acid, which has a high boiling point, still was present in the hydrolysate. Although cyclohexanone extraction removed 100% of formic acid, glycolic acid, and furfural, 65.0% of acetic acid, and 37.0% of glucuronic acid, the large amount of solvent consumption and

high recycling cost limit its application in the future. The widely used activated charcoal adsorption was found to remove as much as 88.0% of glucoronic acid in the hydrolysate, but the yield of xylo-oligosaccharides was only 27.0%. Calcium carbonate removed less than 20.0% by-product, so it was an unsatisfactory method. Calcium hydroxide performed poorly in terms of furfural removal.

Resin Purification Process

Table 1 lists the xylo-oligosaccharides recovery and by-product removal data after adsorption by different resins. The anion exchange resin ZGA451FD had the highest yield of xylo-oligosaccharides at 92.3%, but the efficacy of by-product removal was relatively low. Moreover, although 87.5% and 95.5% furfural were removed by the macroporous adsorptive resins LS600 and LS106, respectively, the low xylo-oligosaccharides recoveries of 17.8% and 42.4%, respectively, made them unacceptable. In contrast, the anion exchange resins 717, D750, ZGA451FD, and LS30 not only had a good yield of xylo-oligosaccharides at more than 80%, but also had an acceptable level of by-products removal. Specifically, resin 717 had the greatest glycolic acid and acetic acid removal rates, at 100% and 86.9%, respectively. A comprehensive comparison of xylo-oligosaccharides recovery and by-product removal for the seven resins indicated that ZGA451FD was the most suitable, with a xylo-oligosaccharides recovery rate of 88%, furfural removal rate of 46.9%, glucuronic acid removal rate of 9.20%, glycolic acid removal rate of 58.5%, formic acid removal rate of 100%, and acetic acid removal rate of 71.7%.

Table 1. Yield of Xylo-oligosaccharides and Removal of By-products After Adsorption by Different Resins (%)

Resin	Xylo-oligosaccharides	Furfural	Glucuronic acid	Glycolic acid	Formic acid	Acetic acid
717	81.0	76.6	0	100	100	86.9
D750	83.8	40.9	2.80	69.5	100	80.9
ZGA451FD	88.0	46.9	9.20	58.5	100	71.7
LS30	81.3	52.6	7.80	62.8	100	64.6
ZGC151FD	92.3	28.4	6.90	15.2	14.0	11.3
LS600	42.4	87.5	13.2	14.0	14.7	22.2
LS106	17.8	95.5	15.2	15.2	14.0	35.1

Column chromatography experiments were conducted to further evaluate the purifying effects of different resins. Similar to the results of resin adsorption, ZGA451FD anion exchange resin had the highest yield of xylo-oligosaccharides at 96.2%, but the removal rates of furfural and acetic acid, the primary by-products in the LHW hydrolysate, reached only 59.0% and 19.0%, respectively (Fig. 2). In contrast, LS30 could remove all the formic acid, acetic acid, glycolic acid, and furfural, with a 92.0% xylo-oligosaccharides recovery. Cara *et al.* (2012) reported that about 80% oligosaccharides yields were obtained after purification by preparative gel filtration chromatography of olive tree pruning hydrolysate. The low glucuronic acid removal rate of 8% may have resulted from the formation of glucuronolactone at the pH value of 4, from which it is difficult to obtain a reaction with an anion exchange resin (Canilha *et al.* 2004). In spite of this, a good oligosaccharides purity of 90.4% was obtained due to the low initial concentration of glucuronic acid (0.800 g/L). The LS106 and LS600 are macroporous adsorptive resins and had lower selectivity between sugar and by-products. The 717 and D750 are strongly basic anion exchange resins, and nearly all acidic by-products could be removed by increasing

the resin loading to 5 times that of the adsorption experiment. Although ZGA451FD is a weakly basic anion exchange resin, about 10.0% of glucuronic acid was removed. In addition, an increase in resin loading led to the reduction of sugar recovery for 717, D705, and ZGA451FD in column chromatography.

The compositions of commercially available (enzymatically produced from corn cob by Qingdao Century Longlive International Trade Co., Ltd., China) and lab-made xylo-oligosaccharides were compared when 1 g/L solution was prepared. The 70% syrup contained 4.31 g/L xylose, 26.2 g/L xylo-oligosaccharides, 3.13 g/L arabinose, 4.58 g/L glucose, 12.6 g/L glucuronic acid, 0.776 g/L formic acid, and 0.071 g/L acetic acid, while 95% powdered sugar contained 0.088 g/L xylose, 12 g/L xylo-oligosaccharides, 0.415 g/L arabinose, 1.31 g/L glucose, and 5.54 g/L glucuronic acid. In contrast, the concentration of xylo-oligosaccharides was 5.92 g/L with 0.905 g/L xylose and 0.512 g/L glucuronic acid.

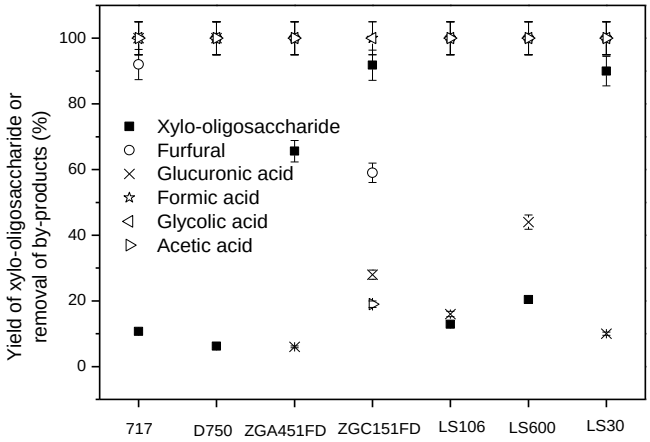

Fig. 2. Effect of column chromatography on removal of by-products and recovery of oligosaccharides. Data provided as the mean ± standard deviation

Production of Fermentable Sugar and Detoxification

About 75% of the total sugar in the sugarcane bagasse LHW hydrolysate was in the form of xylose oligomers. However, no reports indicate that *Pichia stipitis* CBS6054 can directly convert xylo-oligosaccharides into ethanol. Thus, hydrolysis of xylo-oligosaccharides might be a necessary step before fermentation.

The oligomers were decomposed with 4% sulfuric acid or xylanase, and Table 2 lists the chemical composition of different fermentation media after hydrolysis, detoxification, and sterilization treatment. Compared to untreated hydrolysate, resin adsorption was found to effectively remove most of the by-products, except glucuronic acid. For example, resin treatment of the unhydrolyzed sample removed 100% of furfural, 87.0% of glycolic acid, 81.0% of formic acid, and 45.0% of acetic acid. The enzymatic digestibility of xylo-oligosaccharides was 89.4% with the addition of 54 U xylanase *per* mL hydrolysate at 50 °C and 150 rpm for 24 h. In contrast, all oligomers were degraded after hydrolysis with 4% sulfuric acid at 50 °C for 1 h, but the amount of by-products increased due to the further degradation of the xylose monomer. Table 2 shows that acid-treated hydrolysate had the highest concentration of by-products, and the excess acid groups could not be totally exchanged with resin. Moreover, glucuronic acid was detected even in the control sample (a solution of 20 g/L xylose); this indicated that it was generated from the degradation of fermentation media supplements, such as peptone or yeast extract,

during the process of sterilization. Fortunately, glucuronic acid showed little toxicity to the microorganisms (Lawford and Rousseau 1997).

Table 2. Composition in the Fermentation Media (g/L)

Treatment	Xylose	Furfural	Glucuronic acid	Glycolic acid	Formic acid	Acetic acid
Unhydrolyzed	4.20	0.0230	2.14	0.582	0.622	1.80
Enzymolysis	16.8	0.0245	5.82	0.670	0.876	1.91
Acid hydrolysis	17.5	0.0830	2.89	0.921	1.06	4.67
Unhydrolyzed + Resin	4.14	<0.001	5.63	0.0740	0.120	0.991
Enzymolysis + Resin	15.5	<0.001	4.75	0.0890	0.387	1.53
Acid hydrolysis + Resin	16.9	0.351	2.31	0.452	0.435	3.38
Control	18.3	<0.001	3.52	0.110	0.250	<0.001

Ethanol Fermentation

As shown in Table 3, a detoxification step was necessary for all hydrolysates. However, hydrolysate treated with 4% sulfuric acid followed by resin detoxification could not be fermented, which resulted from the high concentration of by-products. As has been reported elsewhere, when the concentration of acetic acid is higher than 2 g/L (Canilha *et al.* 2010), or that of furfural is higher than 1 g/L (Roberto *et al.* 1991), the growth of *Pichia stipitis* can be inhibited. The ethanol concentration, yield, and productivity of the control experiment were 4.64 g/L, 0.253 g/g xylose, and 0.0770 g/(L· h), respectively.

Table 3. Ethanol Fermentation of Hydrolysates after Various Treatments

Treatment	Highest ethanol concentration (g/L)	Yield g/g sugar	Productivity g/(L. h)
Unhydrolyzed	0	0	0
Unhydrolyzed + Resin	2.45	0.145	0.100
Enzymolysis	0	0	0
Enzymolysis + Resin	4.12	0.266	0.0860
Acid hydrolysis	0	0	0
Acid hydrolysis + Resin	0	0	0
Control	4.64	0.253	0.0770

Figure 3a presents the ethanol fermentation of detoxificated unhydrolysis samples and samples that underwent enzymolysis. The theoretical yield of ethanol from xylose is 0.511 g/g sugar, which is significantly higher than the values shown in Table 3. One of the possible reasons for the high yield from untreated hydrolysate was that the strain could convert part of the xylo-oligosaccharides into xylose, leading to the production of ethanol. Correspondingly, the utilization of xylose of the unhydrolyzed group reached 100% at 36 h, but it decreased a little afterwards due to the formation of new xylose (Fig. 3b). The concentration of ethanol of the unhydrolyzed group peaked at 24 h, and the productivity was 16.3% higher than that of the enzymolysis group, but little difference was observed in their 24-h ethanol concentrations (Fig. 3a). Comparing the ethanol produced from the enzymolysis and control experiments, the former had a higher ethanol yield and productivity. Moreover, the peak of ethanol concentration appeared around 12 to 24 h

earlier than the control. Similarly, more than 90% xylose in the enzymolysis group was utilized in the first 36 h, while 60 h was needed for the control experiment (Fig. 3b).

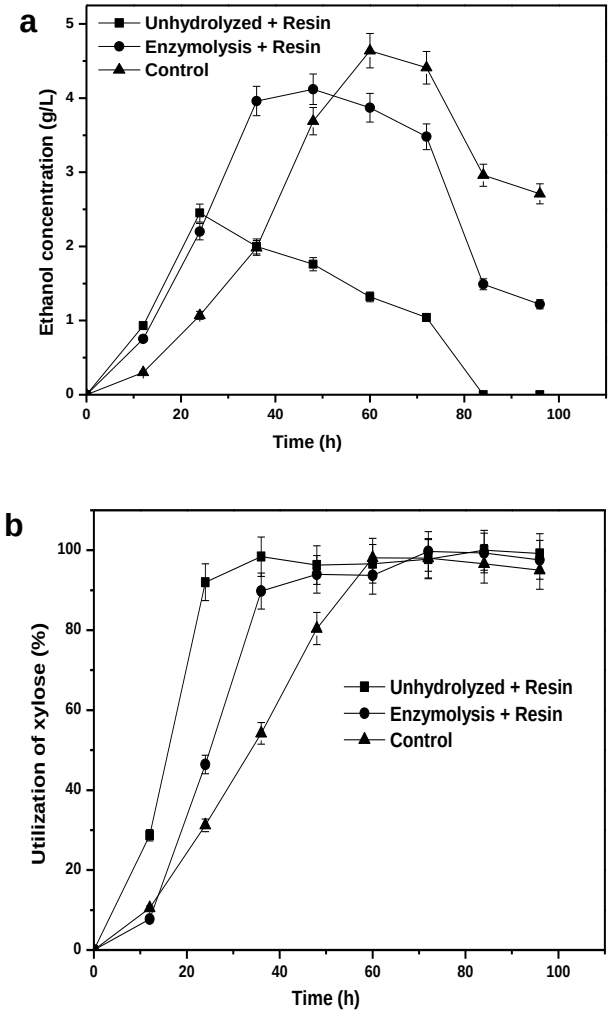

Fig. 3. (a) Ethanol fermentation and (b) xylose utilization for different treated hydrolysate. Data provided as the mean ± standard deviation

Comparison of the Two Applications

Nowadays, the cost of fuel ethanol production in China is expensive and still needs government subsidies to be economically viable. A possible technology line for fuel ethanol production from sugarcane bagasse can be proposed based on the analysis of application of liquid hot water hydrolysate. According to the empirical data from the sugar factory in Guangxi province, China, about 2 tons of cane sugar was extracted from 16 tons of raw sugarcane, with 6 tons of bagasse remaining (Li 2010). As shown in Fig. 4, when sugarcane bagasse was treated with hot water at 180 °C, 5% w/v, for 20 min, about 80 tons of hydrolysate and 3.6 tons of solid residues were recovered according to the data of the authors' previous studies (Yu *et al.* 2013). Also, more than 80% of the hemicellulose was dissolved in the liquid fraction in the form of xylose oligomer (Yu *et al.* 2012b; Zhuang *et al.* 2012). After the purification and spray drying, about 0.35 tons of xylo-oligosaccharides were produced from the hydrolysate, with a recovery rate of 80%. If the hydrolysate is treated with xylanase and then fermented with an enzymatic digestibility of 90%, with a loss ratio in detoxification of 10%, and ethanol conversion ratio of 47.9%, about 0.12 tons

of hemicellulosic ethanol will be produced. Furthermore, about 1 ton of ethanol was obtained after the simultaneous saccharification and fermentation (SSF) of cellulose in the solid residues. About 1.12 tons of fuel ethanol (including cellulosic and hemicellulosic ethanol) or 0.35 tons of xylo-oligosaccharides with 1 ton of cellulosic ethanol could be generated, combined with the 2 tons of cane sugar. As a conservative estimate, the cost per ton of xylo-oligosaccharides should be estimated to be at least $10,000 (Sold as food ingredients by Qingdao Century Longlive International Trade Co., Ltd. for $20 per 1 kg, http://www.longlive.cn). Thus, the cost of producing fuel ethanol would be reduced when combined with the generation of xylo-oligosaccharides and cane sugar.

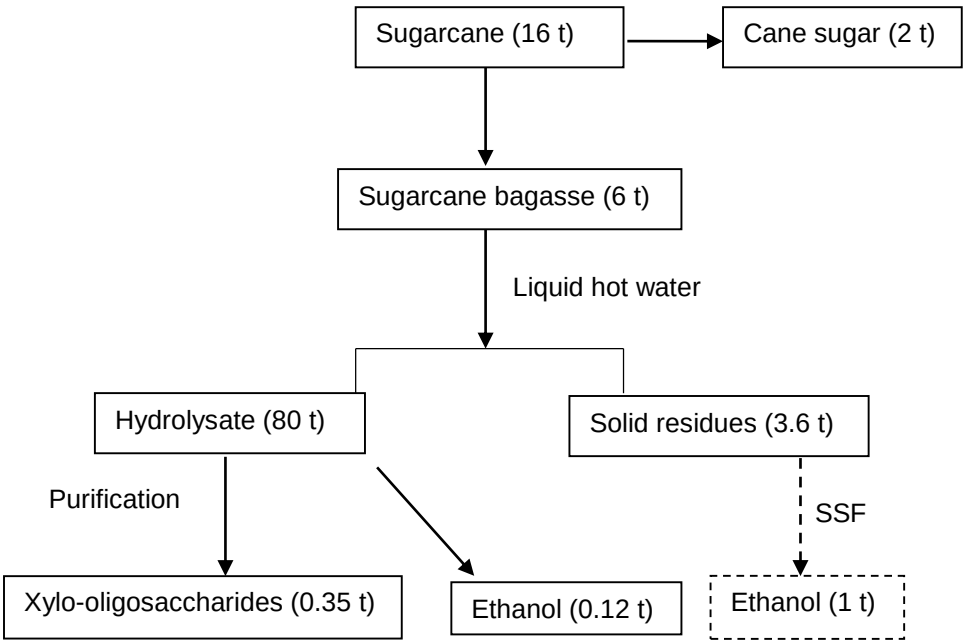

Fig. 4. Flow chart of xylo-oligosaccharides and ethanol production from sugarcane bagasse

CONCLUSIONS

1. Xylo-oligosaccharides can be purified and separated with column chromatography. The recovery and purity of xylo-oligosaccharides reached 92.0% and 90.4%, respectively, after column chromatography with the anion exchange resin LS30.
2. High ethanol productivity of 0.0860 g/(L·h) can be observed from the hydrolysate treated with xylanase and detoxification.
3. The production of xylo-oligosaccharides from hot water hydrolysate of sugarcane bagasse can maximize profits for cellulosic ethanol refining.

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