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Original Article

Evaluation of dilute acid pretreatment for bioethanol fermentation from sugarcane bagasse pith

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ABSTRACT

Sugarcane bagasse pith is the most abundant agricultural waste in Thailand and an attractive raw material for biosugar production using dilute acid pretreatment and enzymatic hydrolysis. In this study, the raw material was pretreated at 121 °C with different sulfuric acid concentrations (0%, 1%, 2%, 3% or 4% volume per volume, v/v) and pretreatment times (30, 60 or 90 min). The pretreated solid was hydrolyzed using a commercial enzyme (Celluclast® 1.5L). The maximum total sugars yield (53.7 g/100 g dry bagasse pith) was achieved at 1–2% v/v H_2SO_4 for 90 min, representing 67% of total sugars in the bagasse pith. For ethanol production, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) processes were employed using *Pichia stipitis* JCM 10742. The results indicated that both the ethanol concentration and productivity using SSF were higher than from the SHF process. The ethanol concentration and productivity using SSF were 3.70 g/L and 0.15 g/L/hr in 24 h fermentation, respectively, while for the SHF process the results were 2.58 g/L and 0.09 g/L/hr in 30 h fermentation, respectively.

Introduction

The following information was synthesized from the Department of Alternative Energy Development and Efficiency, 2012. The development of biofuel is an important objective in a country's development strategy to reduce fuel imports and increase energy self-dependence. Ethanol can be produced from sugar (sugarcane and molasses) and starch (cassava, rice and corn). However, these raw materials may become insufficient for future ethanol production because they are edible crops. Many countries have already developed ethanol production technology using alternative raw materials. Lignocellulosic materials such as agricultural, softwood and hardwood residues are potential sources of sugars that interest scientists for ethanol production. Ethanol produced from lignocellulose materials is an attractive alternative as they do not compete with the food supply and are less expensive. A major lignocellulosic material found in large quantities in Thailand is sugarcane bagasse pith.

Thailand is the third largest sugar producer in the world after Brazil and Australia (Laopaiboon et al., 2010). The Thai 2014/2015 sugarcane harvest was estimated at 106 million tonnes (Krungsri, 2016). Sugarcane bagasse is a byproduct of the sugar industry, and consists of fiber bundles and other structural elements such as vessels, parenchyma and epithelial cells which can be summarized under the technical term pith (Sanjuán et al., 2001). Sugarcane bagasse contains 35% pith and with 60% depithing efficiency, around 20% of the production tonnage is removed as pith, at either the sugar or paper mill premises (Dasgupta et al., 2013). Therefore, a sugar mill processing 31.8 million tonnes per year of bagasse will generate 19.08 million tonnes per year of bagasse pith. The bagasse pith is considered an undesirable raw material for pulping, and so large quantities remains unutilized, posing serious waste disposal problems (Dasgupta et al., 2013).

Because it has a high carbohydrate content (39.8% cellulose, 32.2% hemicellulose), bagasse pith can potentially be a resource (Wang et al., 2015). Bagasse pith may be an excellent source of biosugar, which can be produced as either bioethanol or as a biochemical product (Tyagi et al., 2014). Bioethanol production from lignocellulosic material can be divided into three steps (Bensah and Mensah, 2013): 1) pretreatment, to improve enzyme access to the cellulose, 2) enzymatic hydrolysis, to convert the

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polysaccharides into monomer sugars such as hexose and/or pentose, and 3) fermentation of these released sugars by specialized microorganisms, to produce ethanol. The natural structure of lignocellulosic material is extremely recalcitrant to enzymatic hydrolysis, and therefore the pretreatment step is necessary for removing hemicellulose and lignin, reducing cellulose crystallinity and increasing the porosity of the biomass, thus enhancing the enzymatic hydrolysis of the cellulose (Benjamin et al., 2014). Several physical and chemical pretreatment methods using diluted acid, concentrated acid, alkaline, steam explosion, ammonium fiber explosion and organic solvents have been studied to determine the most efficient methods for bioethanol production (Alvira et al., 2010). The pretreatment method must be effective and economical and thus feasible for commercial processing (Zheng et al., 2007). Economical pretreatment methods use inexpensive chemicals and require simple equipment and procedures; consequently, among the various pretreatment methods, dilute acid has been widely used because it is generally inexpensive, convenient, and effective for a wide range of lignocellulosic materials such as softwood, hardwood and herbaceous plants (Wei et al., 2012). The dilute acid pretreatment weakens the hydrolyzed glycosidic bond in the hemicellulose and lignin-hemicellulose bond and the lignin bond that leads to the dissolution of the sugar in the hemicellulose and to an increased porosity of the plant cell wall (Cheng et al., 2011; Jiang et al., 2013). The disadvantage of dilute acid hydrolysis is the formation of different type of inhibitors such as furan derivatives, carboxylic acids and phenolic compounds (Palmqvist and Hahn-Hägerdal, 2000). All these compounds can have negative effects on the fermentation process (Cara et al., 2008). Compared with hydrochloric, phosphoric and nitric acid, high hydrolysis yields have been reported when pretreating lignocellulosic biomass with dilute sulfuric acid (Alvira et al., 2010). Several studies of sulfuric acid pretreatment have been shown to be effective pretreatment options for different biomass. For example, the maximum hemicellulose yield (70%) was obtained when sugarcane bagasse was pretreated with 10% volume per volume (v/v) H_2SO_4 at 100 °C for 40 min (Candido et al., 2012). Eucalyptus chips were pretreated with 0.75% H_2SO_4 at 160 °C for 10 min resulting in 76% of hydrolysis yield and 82% of total sugars yield in eucalyptus biomass (Wei et al., 2012).

Moreover, only a small number of studies have focused on the dilute acid pretreatment of bagasse pith (Jiang et al., 2013; Tyagi et al., 2014). Therefore, the objectives of this study were: (a) to analyze the chemical composition of bagasse pith, (b) to identify the optimal sulfuric acid concentration and pretreatment time of bagasse pith in terms of both maximizing the biosugar yield in the prehydrolysate and improving the enzymatic hydrolysis of pretreated solid and (c) to investigate ethanol production from the bagasse pith using separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

Materials and methods

Raw material

The bagasse pith was provided by Mitr Phu Khieo Sugar Mill Factory in Chaiyaphum province, Thailand. The raw material was dried in an oven at 60 °C for 3–4 d and sieved to obtain 20–80 mesh fractions. The dry material was stored in plastic bags at room temperature until required for use.

Dilute acid pretreatment

The bagasse pith was prepared for sulfuric acid pretreatment by presoaking the particles at 10% weight per volume (w/v) solid loading at room temperature in a sulfuric acid solution of 0, 1, 2, 3 or 4% v/v overnight. Deionized water was used as the pretreatment control. The mixture was then pretreated at 121 °C and 1.5 bar pressure in an autoclave for 30, 60 or 90 min. After the pretreatment, the pretreated solids were separated using filtration through a muslin cloth and washed with tap water until the pH was neutral and then dried in an oven at 60 °C for 3–4 d. The pretreated solids were stored in plastic bags at room temperature prior to enzymatic hydrolysis. The prehydrolysate was analyzed using high-performance liquid chromatography (HPLC) to determine the concentration of monomeric sugars (glucose xylose, galactose, arabinose and mannose), and inhibitors (acetic acid, furfural, and 5-hydroxymethylfurfural (HMF)).

Enzymatic hydrolysis of bagasse pith

Enzymatic hydrolysis was carried out at a 5% w/v solid loading in a laboratory bottle (50 mL). A sample of pretreated bagasse pith was soaked in 50 mM sodium citrate buffer (pH 4.8). The enzyme loading was 15 filter paper units (FPU)/g dry pretreated solid (Celluclast® 1.5L, Novozymes A/s; Bagsvaerd, Denmark), with a filter paper activity of 58 FPU/ml. The laboratory bottles were incubated in a shaking water bath at 50 °C and 200 rpm for 24 h. The samples were then placed into boiling water for 5 min and centrifuged. The monomeric sugar concentrations in the enzymatic hydrolysate were determined using HPLC.

Ethanol fermentation

The microorganism *Pichia stipitis* JCM 10742, (the Japan Collection of Microorganisms, Riken Bioresource Center; Ibaraki, Japan) was used for fermentation in the SSF and SHF experiments. The inoculum was prepared by growing the yeast on a rotary shaker at 150 rpm for 24 h at 30 °C in a yeast extract and malt extract medium. An inoculum size of 5% v/v was used to initiate the experiments.

Simultaneous saccharification and fermentation

The SSF experiment was performed in a 250 mL Erlenmeyer flask with a working volume of 150 mL. The solid loading of pretreated bagasse pith was 5% w/v. The culture medium contained 1.50 g/L yeast extract, 3 g/L peptone, 2 g/L KH_2PO_4, 1 g/L $(NH_4)_2SO_4$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$. The medium was sterilized by autoclaving at 121 °C for 15 min. The enzyme loadings of cellulase (Celluclast® 1.5L, Novozymes A/s, Bagsvaerd, Denmark) and beta-glucosidase (Novozym 188, Novozymes A/s, Bagsvaerd, Denmark) were 15 FPU/g dry bagasse pith and 7.50 IU/g dry bagasse pith, respectively. The SSF experiment was carried out using *P. stipitis* JCM 10742 at 30 °C and 150 rpm. Samples were taken periodically over 72 h and analyzed for glucose, xylose and ethanol concentrations using HPLC. Viable cells were determined by the standard method (plate count agar; Cappuccino and Sherman, 2008).

Separate hydrolysis and fermentation

Enzymatic hydrolysis of the pretreated bagasse pith was performed in a 250 mL Erlenmeyer flask using 5% w/v solid loading and 50 mM sodium citrate buffer (pH 4.8). The enzyme loadings of cellulase (Celluclast® 1.5L) and beta-glucosidase (Novozyme 188) were 15 FPU/g dry bagasse pith and 7.50 IU/g dry bagasse pith, respectively. The mixture was incubated at 50 °C for 12 h in a shaking water bath at 200 rpm. The hydrolysate obtained from the enzymatic hydrolysis supplement with 1.50 g/L yeast extract, 3 g/L peptone, 2 g/L KH_2PO_4, 1 /L $(NH_4)_2SO_4$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$ was

used as a fermentation medium. The same fermentation conditions as for the SSF process were used. Samples were taken periodically over 72 h and analyzed for glucose, xylose and ethanol concentrations using HPLC.

Analytical methods

The carbohydrate compositions of the raw material and the pretreated solid residue were determined by the National Renewable Energies Laboratory (NREL) according to Sluiter et al. (2012). The chemical composition of the raw material and the pretreated solid residue after pretreatment were determined, according to Technical Association of the Pulp and Paper Industry (1996). The concentration of glucose, xylose, galactose, arabinose and mannose were determined using HPLC. The HPLC system was equipped with an Aminex HPX-87P (Bio-Rad Labs; Hercules, CA, USA) and a refractive index detector (Waters; Mildford, MA, USA). The column was operated at 80 °C and 0.60 mL/min using a mobile phase of filtered deionized water (Milli-Q, Millipore Corp; Bedford, MA, USA). Furfural and HMF were measured using HPLC with an ultra violet/visible detector (Waters; Mildford, MA, USA). The Aminex HPX-87H operating at 60 °C with 5 mM H_2SO_4 as a mobile phase (0.6 mL/min), was used for separation. Detection was performed at 280 nm. Ethanol and acetic acid were analyzed using HPLC with a refractive index detector and the Aminex HPX-87H column maintained at 60 °C with a flow rate of 0.6 mL/min and H_2SO_4 as the mobile phase.

Statistical analysis

These experiments were tested in duplicate. The experiment data were determined using a factorial test. The effects of acid concentration and pretreatment time were determined.

Results and discussion

Chemical composition of bagasse pith

Cellulose and hemicellulose accounted for 75.3% w/w of dry weight, consisting of cellulose (38.9%) and hemicellulose (36.4%). In a biorefinery context, these results are interesting because bagasse pith with high cellulose and hemicellulose contents is preferable for use as raw material for biosugar production. The cellulose and hemicellulose contents agreed with Wang et al. (2015) who reported the composition of sugarcane bagasse pith as 39.8% cellulose and 32.2% hemicellulose. Klason lignin at 23.2% w/w of dry weight tallied with results reported in the literature (Jain et al., 2011). The bagasse pith had a lower lignin content compared to other lignocellulosic biomasses such as peanut shells at 35.2%, *Prosopis juliflora* at 29.1% and eucalypt chips at 28.3% (Gupta et al., 2011; Baboukani et al., 2012; Wei et al., 2012). Extractives at 4.9% w/w of dry weight were lower than reported by Saad et al. (2008), and Canilha et al. (2011). The ash content at 5.2% was in agreement with Sanjuán et al. (2001), but lower when compared to other lignocellulosic materials such as rice and wheat straw at 14.5% and 7.1%, respectively (Hsu et al., 2010).

Substrate character after pretreatment

Table 1 summarizes the cellulose, hemicellulose and lignin contents in the pretreated solid residue after different pretreatment conditions. The main components in the pretreated solid residue, cellulose and lignin were in general well preserved. Cellulose recovery in the acid-pretreated solid was in the range 52.1–56.6% w/w of dry weight, but for the pretreatment performed under more severe conditions (4% v/v H_2SO_4, 90 min) this dropped to 52.1% w/w of dry weight, with the partial solubilization of the easily hydrolysable fraction. The acid-insoluble lignin recovered in the acid-pretreated solids increased with increasing acid concentration. The maximum acid-insoluble lignin content was 39.8% w/w of dry weight at 4% v/v H_2SO_4 and 30 min pretreatment time. Compared with the pretreatment without acid (control), the higher cellulose and acid-insoluble lignin in the acid-pretreated solid residue may have been due to the removal of the hemicellulose fraction. Recovery of hemicellulose in the acid-pretreated solid was low (12.0–14.4% w/w of dry weight), as it was mostly hydrolyzed during the acid pretreatment.

Table 1
Chemical analysis (mean ± SD) of pretreated bagasse pith after different dilute acid pretreatment conditions.

Time (min)	H_2SO_4 (% v/v)	Solid composition (% weight/weight on dry weight basis)		
		Cellulose	Hemicellulose [a]	Acid-insoluble lignin
30	Control	37.6 ± 0.9	36.5 ± 0.6	24.4 ± 0.6
	1	53.5 ± 0.6	14.4 ± 0.3	34.8 ± 0.5
	2	54.5 ± 0.2	13.5 ± 0.2	36.3 ± 1.3
	3	54.4 ± 0.4	13.5 ± 0.5	37.5 ± 2.9
	4	54.8 ± 1.3	13.0 ± 1.0	39.8 ± 2.9
60	Control	36.8 ± 1.7	36.8 ± 1.7	24.5 ± 0.1
	1	56.6 ± 0.4	12.7 ± 0.2	34.2 ± 0.7
	2	56.3 ± 0.6	12.5 ± 0.7	35.7 ± 0.1
	3	56.1 ± 0.6	12.0 ± 0.8	35.8 ± 0.8
	4	55.2 ± 0.8	12.5 ± 0.5	38.0 ± 0.3
90	Control	35.1 ± 0.5	35.1 ± 0.5	24.7 ± 0.6
	1	55.5 ± 0.5	12.6 ± 0.4	34.7 ± 0.2
	2	55.3 ± 0.8	12.1 ± 0.6	36.1 ± 0.5
	3	55.1 ± 0.1	12.1 ± 0.2	36.5 ± 0.1
	4	52.1 ± 1.2	14.3 ± 0.7	38.1 ± 0.1

% v/v = percent volume per volume.
[a] Hemicellulose = holocellulose-cellulose.

Sugar in prehydrolysate

The effects of acid concentration and pretreatment time on the sugar content of the prehydrolysate were investigated (Table 2). The sugar yield in the prehydrolysate was reported in grams based on 100 g of dry bagasse pith. The prehydrolysate contained monomeric sugars as glucose, xylose, galactose and arabinose. No mannose was detected in the prehydrolysate. Xylose was the main sugar after hydrolysis of the bagasse pith. The xylose released was mainly soluble hemicellulose, because xylose represented the most abundant hemicellulose sugar monomer in bagasse. The xylose yield increased with increasing pretreatment time. The maximum xylose yield was in the range 80–83% of xylose in the bagasse pith for the bagasse pith pretreated at 1–4% v/v H_2SO_4 for 90 min. This indicated that a longer pretreatment time resulted in greater solubilization of hemicellulose. Generally, the xylose yield during the hydrolysis of sugarcane bagasse was reported in the range 71–80% of xylose in the raw material (Lavarack et al., 2002; Benjamin et al., 2014). The lowest xylose yield was observed when the hydrolysis was pretreated without the addition of acid (control). This result was similar to that obtained by Benjamin et al. (2014) who reported a lower xylose yield after pretreatment without the addition of acid, because most of the sugars found in the prehydrolysate remained as oligomers. This result confirmed the importance of acid during the pretreatment of bagasse pith to improve the xylose yield (Canilha et al., 2011). Arabinose is a sugar formed from the arabinoxylan in hemicellulose (Gámez et al., 2006). Arabinose existed in the furanose form; thus, it was hydrolyzed faster than

Table 2
Composition (mean ± SD) of the prehydrolysate after different dilute acid pretreatment conditions.

Pretreatment condition		Liquid composition						
Time (min)	H_2SO_4 (%v/v)	Sugar analysis (g/100 g dry bagasse pith)				Inhibitor analysis		
		Glucose	Xylose	Galactose	Arabinose	Acetic acid (g/L)	HMF (mg/L)	Furfural (mg/L)
30	Control	0.1 ± 0.0	ND	ND	0.04 ± 0.01	ND	ND	ND
	1	0.5 ± 0.5	11.5 ± 0.2	0.6 ± 0.0	2.2 ± 0.1	3.1 ± 0.1	14.8 ± 1.3	49.8 ± 3.5
	2	2.4 ± 0.2	16.7 ± 0.3	1.1 ± 0.0	3.2 ± 0.0	3.2 ± 0.1	28.2 ± 2.8	137 ± 15.4
	3	2.6 ± 0.2	17.2 ± 0.0	1.1 ± 0.1	3.2 ± 0.2	4.3 ± 0.6	35.0 ± 4.2	235 ± 79.7
	4	3.4 ± 0.4	17.8 ± 0.0	1.1 ± 0.2	3.3 ± 0.4	4.1 ± 0.6	40.9 ± 6.6	472 ± 65.3
60	Control	0.1 ± 0.0	ND	ND	0.1 ± 0.0	ND	ND	ND
	1	2.0 ± 0.5	14.4 ± 0.0	1.0 ± 0.2	2.8 ± 0.4	3.8 ± 0.6	39.1 ± 9.1	138 ± 40.5
	2	2.4 ± 0.3	14.3 ± 0.0	1.0 ± 0.0	2.9 ± 0.0	4.1 ± 0.4	44.1 ± 1.7	243 ± 9.4
	3	3.3 ± 0.9	17.5 ± 0.0	1.0 ± 0.2	3.5 ± 0.0	4.6 ± 0.2	50.8 ± 1.0	576 ± 148
	4	4.2 ± 0.8	17.6 ± 0.0	1.2 ± 0.1	3.6 ± 0.5	4.0 ± 0.2	39.3 ± 0.4	863 ± 89.9
90	Control	0.1 ± 0.0	0.1 ± 0.0	ND	0.2 ± 0.0	ND	ND	ND
	1	4.2 ± 0.2	22.5 ± 0.7	1.5 ± 0.0	4.3 ± 0.1	4.4 ± 0.6	59.8 ± 13.0	350 ± 139
	2	4.8 ± 0.5	22.4 ± 2.5	1.5 ± 0.2	4.4 ± 0.4	4.6 ± 0.3	57.5 ± 3.3	782 ± 96.0
	3	4.9 ± 0.8	21.6 ± 0.0	1.6 ± 0.0	4.3 ± 0.6	5.1 ± 2.0	55.0 ± 23.2	1041 ± 504
	4	5.9 ± 1.0	22.6 ± 1.3	1.7 ± 0.3	4.6 ± 0.2	10.5 ± 0.1	104 ± 13.2	1479 ± 338

%v/v = percent volume per volume; HMF = 5-hydroxymethylfurfural; ND = not detected.

pyranose, xylose and glucose. The maximum arabinose yield of 91–99% of arabinose in the bagasse pith (4.3–4.6 g/100 dry bagasse pith) was achieved under the same pretreatment conditions as for xylose.

The glucose yield increased with increasing acid concentration and pretreatment time, with a maximum of 5.9 g/100 g dry bagasse pith (13% of the glucose in the bagasse pith) at 4% v/v H_2SO_4 for 90 min. The relatively low glucose yields implied that the glucan fraction was not altered during acid pretreatment; thus the glucose obtained was probably produced from the hemicellulose fraction. The low glucose in the prehydrolysate was considered as a positive result, since the objective of this study was the solubilization of only the hemicellulose fraction, while retaining pretreated solids with a high cellulose content. The maximum galactose yield was in the range 56–65% of the galactose in the bagasse pith for the bagasse pith pretreated at 1–4% v/v H_2SO_4 for 90 min.

All experimental results were analyzed using a factorial design. The statistical analysis showed that pretreatment time and acid concentration had a significant ($p < 0.05$) effect on xylose, galactose, glucose and arabinose yield.

Table 2 shows the concentration of fermentative inhibitors (acetic acid, furfural and HMF) under different pretreatment conditions. Acetic acid was produced from the hydrolysis of the acetyl group in hemicellulose, while furfural and HMF were generated from pentose (xylose and arabinose) and hexose sugar degradation. All these compounds not only caused a lower sugar yield, but also could potentially inhibit the growth of yeast and reduce ethanol production (Ertas et al., 2014). As seen in Tables 2 and 3.1–10.5 g/L acetic acid and 49.8–1479 mg/L furfural were generated in the prehydrolysate with differing acid concentrations and pretreatment times. Higher concentrations of acetic acid and furfural were generated under harsher pretreatment conditions. This indicated that greater amounts of hemicellulose and xylan were solubilized. Acetic acid and furfural had the highest concentration at 4% v/v for 90 min. This indicated that increasing the acid concentration and pretreatment time led to greater amounts of acetic acid and furfural being formed. However, low acetic acid and furfural concentrations were observed when the hydrolysis was pretreated without the addition of acid. The HMF concentration in the prehydrolysate was low (14.8–104 mg/L), which indicated limited degradation of glucose.

The yields of monomeric sugar in the prehydrolysate indicated the solubilization of hemicellulose after acid pretreatment. The total hemicellulosic sugars in the prehydrolysate included xylose, galactose and arabinose. The yield from the control was 0.1–0.9% of the total hemicellulosic sugars in the bagasse pith for all pretreatment times, and the addition of acid increased the values by 42–84% of the total hemicellulosic sugars in the bagasse pith, depending on the acid concentration and pretreatment time. This showed the effectiveness of the acid pretreatment at removing the hemicellulose fraction. A pretreatment time of 30 min was not efficient for the solubilization of hemicellulosic sugars (42%–65% of the total hemicellulosic sugars in the bagasse pith). However, an increase in the time under the pretreatment conditions resulted in the increased solubility of hemicellulosic sugars. The maximum total hemicellulosic sugars yield of 84% (28.9 g/100 g dry bagasse pith) was obtained at 4% v/v H_2SO_4 for 90 min. Under the same conditions, the total sugar in the prehydrolysate reached 34.8 g/100 g dry bagasse pith (43% of the total sugar in the bagasse pith). The yield of hemicellulosic sugars in this study was similar to that obtained by Cara et al. (2008), who reported a maximum hemicellulosic sugars yield at 83% of the hemicellulosic sugars in the raw material (olive tree), when it was pretreated with 1% w/w H_2SO_4 and a reaction time of 10 min at 170 °C. Ballesteros et al. (2006) obtained a maximum hemicellulosic sugars yield of 85%

Table 3
Cellobiose, glucose and xylose yield (mean ± SD, g/100 g dry bagasse pith) in the enzymatic hydrolysate under different pretreatment conditions.

Pretreatment conditions		Sugar concentration (g/100 g dry bagasse pith)		
Time (min)	H_2SO_4 (%v/v)	Cellobiose	Glucose	Xylose
30	Control	0.8 ± 0.0	7.0 ± 0.1	1.1 ± 0.0
	1	3.9 ± 0.4	22.4 ± 1.0	4.7 ± 0.0
	2	3.3 ± 0.8	20.5 ± 0.1	4.6 ± 0.1
	3	3.6 ± 0.1	19.4 ± 0.1	4.1 ± 0.9
	4	3.5 ± 0.1	18.5 ± 0.9	3.6 ± 0.1
60	Control	0.7 ± 0.0	6.6 ± 0.0	1.1 ± 0.0
	1	2.8 ± 0.3	19.3 ± 0.1	3.7 ± 0.2
	2	3.9 ± 0.2	19.0 ± 0.1	4.1 ± 0.1
	3	4.1 ± 1.3	18.4 ± 0.1	3.4 ± 0.5
	4	4.5 ± 0.2	19.0 ± 0.0	2.9 ± 0.1
90	Control	0.7 ± 0.1	7.6 ± 0.5	1.4 ± 0.1
	1	2.7 ± 0.1	18.0 ± 1.9	3.2 ± 1.9
	2	4.3 ± 0.1	17.4 ± 0.1	3.1 ± 0.5
	3	3.5 ± 0.1	16.8 ± 0.1	2.4 ± 0.1
	4	4.6 ± 0.1	15.5 ± 0.4	2.3 ± 0.4

working with wheat straw steam pretreated at 180 °C and 10 min, with acid-addition (0.9% w/w H_2SO_4). However, the maximum total sugar pretreatment conditions produced maximum total inhibitors of 12.1 g/L (10.5 g/L acetic acid, 1.5 g/L furfural and 0.1 g/L HMF). These results were higher than reported by Palmqvist and Hahn-Hägerdal (2000). Therefore, the detoxification step such as over-liming, activated charcoal and vacuum evaporation was required to diminish the inhibition effect of the inhibitor compound in the next step of fermentation.

Enzymatic hydrolysis of pretreated bagasse pith

Following pretreatment, the pretreated solids were subjected to enzymatic hydrolysis. The pretreated solids from all pretreatments were subjected to enzymatic hydrolysis for 24 h at 5% w/v of solid. The enzymatic hydrolysis yield was expressed as the yield of glucose released after 24 h of enzymatic hydrolysis. Table 3 shows the yields of sugars in enzymatic hydrolysis under different pretreatment conditions. Glucose, xylose and cellobiose were produced during enzymatic hydrolysis, with glucose being the main product.

Compared with the control, the acid pretreatment samples had a higher glucose yield in the enzymatic hydrolysate. The glucose yield improved to 49% compared to the control (14%). The higher glucose yield of enzymatic hydrolysis after acid pretreatment can be explained as follows: 1) hemicellulose removal by breaking down the structure of lignocellulosic material, and 2) increased porosity and surface area of cellulose, providing greater accessibility for the enzyme. This result indicated that acid pretreatment effectively improved the glucose yield in enzymatic hydrolysis compared with the control.

For acid pretreatment, the maximum glucose yield was obtained using an enzyme dosage of 15 FPU/g dry bagasse pith at 49% of glucose in the bagasse pith (22.4 g/100 g dry bagasse pith), after pretreatment of the bagasse pith at 1% v/v H_2SO_4 and 30 min. The result was similar to that reported by Benjamin et al. (2014) of a maximum glucose yield from enzymatic hydrolysis at 22.6 g/100 g dry raw material when the sugarcane bagasse was pretreated at 0.85% w/w H_2SO_4, 170 °C for 5 min. As the pretreatment time and acid concentration increased, the glucose yield in the enzymatic hydrolysate decreased. This indicated that some chemical or morphological raw material change occurred in the cellulose or lignin, which either decreased the enzymatic affinity to the cellulose, or decreased the cellulose surface area for enzymatic hydrolysis (Zhang and Wu, 2014).

The maximum yield of xylose at 4.7 g/100 g dry bagasse pith was obtained with 1% v/v H_2SO_4 for 30 min, representing 17% of the xylose in the bagasse pith. As the pretreatment time and acid concentration increased, the xylose yield in the enzymatic hydrolysis decreased. This indicated that a large amount of hemicellulose was removed during the pretreatment process. The maximum cellobiose concentration was below 4.6 g/100 g dry bagasse pith. Thus, the amount of beta-glucosidase in the Celluclast 1.5L enzyme was sufficient to hydrolyze cellobiose to glucose.

Total sugars

The total sugars yield was determined as the sum of monosaccharide (glucose, xylose, galactose and arabinose) found in the prehydrolysate and enzymatic hydrolysate (Fig. 1). A maximum total sugars yield (53.7 g/100 g dry bagasse pith) was achieved at 1% v/v H_2SO_4 and 90 min and at 2% v/v H_2SO_4 and 90 min, representing 67% of the total sugars in the bagasse pith. The total sugars yield in this study was higher than that reported from other lignocellulosic biomass. For example, Cara et al. (2008) found the maximum total sugars yield of olive tree was 36.3 g/100 g raw material when pretreated with 1% H_2SO_4 at 180 °C for 10 min. Benjamin et al. (2014) reported a maximum overall sugars yield of 44.9 g/100 g raw material, when the sugarcane bagasse was pretreated with 0.45% H_2SO_4 at 190 °C for 5 min. Ertas et al. (2014) reported a maximum total sugars yield recovery of 45.6 g/100 g raw material when wheat straw was pretreated at 0.5% w/w H_2SO_4 at 190 °C for 10 min. The current results indicated that dilute acid pretreatment followed by enzymatic hydrolysis was the most promising technology for biosugar production from bagasse pith. This was not only cost effective, but also practically adaptable to industrial production. In addition, bagasse pith is a suitable raw material for biosugar production because it is available in large quantities, does not compete with agricultural food and has a low cost.

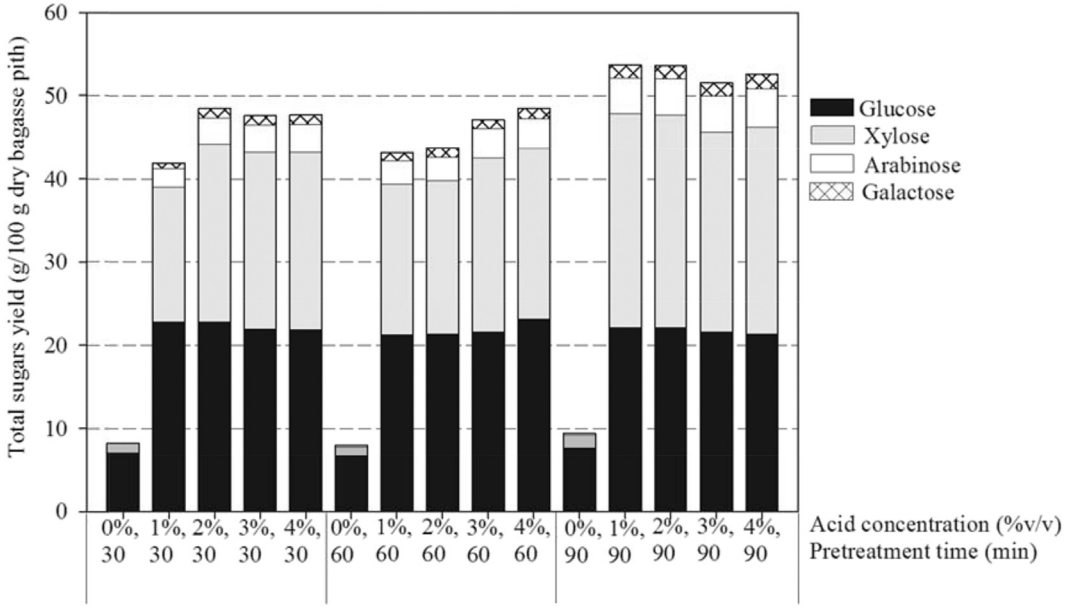

Fig. 1. Total sugars yield from pretreatment and enzymatic hydrolysis under different pretreatment conditions (%v/v = percent volume per volume).

Ethanol fermentation

The pretreatment conditions of 1% v/v H_2SO_4 for a 30 min pretreatment time was selected for ethanol production because the highest glucose yield of enzymatic hydrolysis was obtained under these conditions. The pretreated sugarcane bagasse pith was submitted to both the SHF and SSF processes. For the SSF and SHF fermentation, a native pentose and hexose sugars fermenting yeast, *P. stipitis* JCM 10742 was used under batch cultivation.

Separate hydrolysis and fermentation

In this study, after 12 h enzymatic hydrolysis with cellulase, the total sugar content was 6.7 g/L. Fig. 2A presents the profile of ethanol concentration, cell growth and sugar consumption during SHF fermentation by *P. stipitis* JCM 10742. The quantity of glucose present in the hydrolysate was assimilated with 12 h of fermentation. Xylose was completely consumed after 24 h fermentation. Xylose was also fermented with glucose during fermentation but at a slow rate probably due to the repression of xylose uptake when glucose was present in the hydrolysate. Yeast cell growth continued until the completion of fermentation at 72 h. After 18 h, the highest ethanol yield was 0.07 g ethanol/g available fermentable sugars (glucose and xylose). This corresponded to a volumetric ethanol productivity of 0.14 g/L/hr with an ethanol concentration of 2.58 g/L. However, the ethanol productivity was 0.09 g/L/hr when calculated as the highest ethanol concentration divided by the total process time (12 h enzymatic hydrolysis and 18 h fermentation) as shown in Table 4.

Simultaneous saccharification and fermentation

Figure 2B presents the profile of ethanol concentration, cell growth and sugar consumption during SSF fermentation by *P. stipitis* JCM 10742. Glucose accumulation in the fermentation medium was only observed during the first 6 h of fermentation; the glucose concentration was close to 0 g/L at 18 h fermentation. This indicated that the yeast cells were metabolically active during the whole course of fermentation. The xylose concentration increased during the first 12 h and xylose was not detected after 24 h fermentation. The maximum ethanol production occurred at 24 h of fermentation at 3.70 g/L with an ethanol yield of 0.10 g ethanol/g available fermentable sugars and ethanol productivity of 0.15 g/L/hr (Table 4).

However, the ethanol yield achieved in this study was lower than those obtained from other studies using various types of lignocellulosic biomass hydrolysate fermented by *P. stipitis*, in which ethanol yields in the range 0.24–0.46 g/g were reported (Buaban et al., 2010). This was probably due to the low substrate loading or low enzyme concentration. The ethanol concentration was increased by an increased substrate loading content and enzyme concentration. Moreover, the increased substrate loading

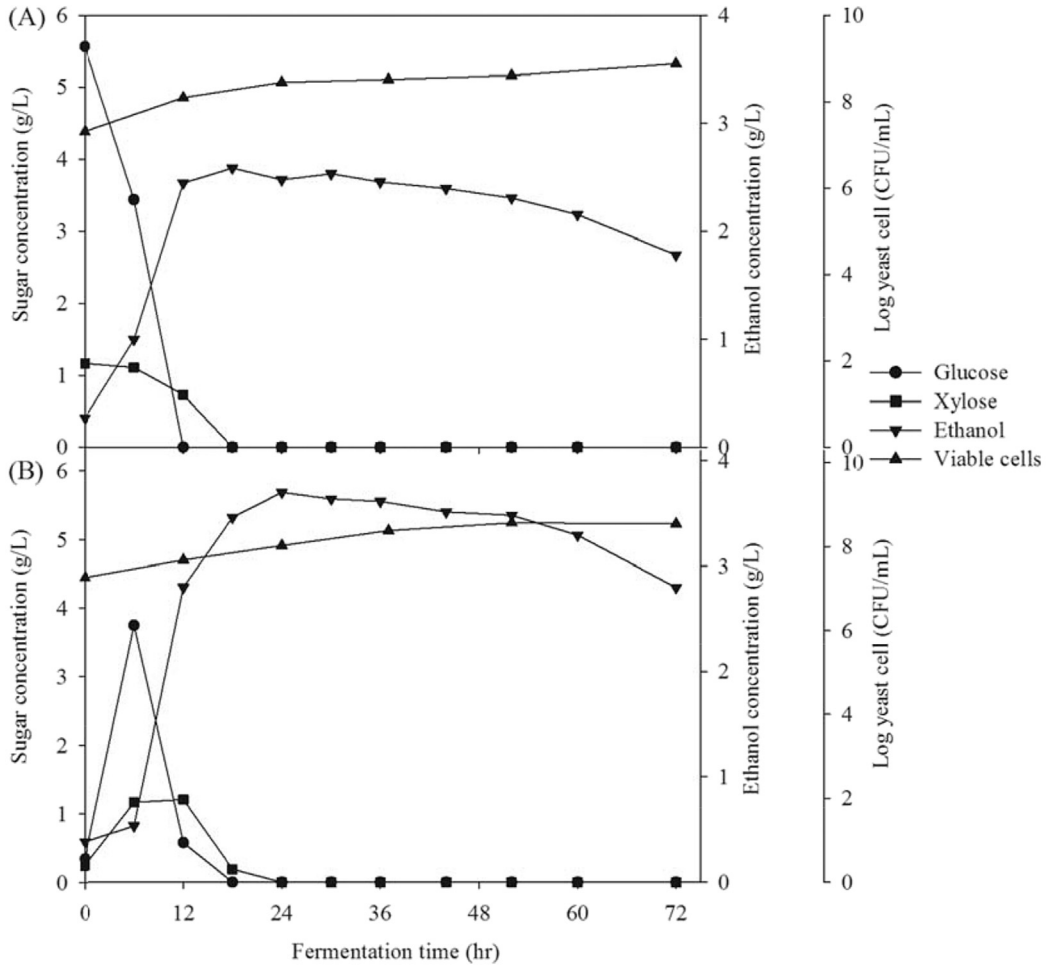

Fig. 2. Time course of ethanol production by *P. stipitis* JCM 10742 from 5% weight per volume pretreated bagasse pith at 30 °C and 150 rpm for 72 h using: (A) separate hydrolysis and fermentation; (B) simultaneous saccharification and fermentation (CFU = colony forming units).

Table 4
Ethanol concentration from separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes by *P. stipitis* JCM 10742 at 30 °C.

Process	Ethanol (g/L)	Ethanol productivity (g/L/hr)	$Y_{p/s}$[b]	Theoretical ethanol yield (%)
SHF (30 h)[a]	2.58	0.09	0.07	14
SSF (24 h)	3.70	0.15	0.10	20

[a] Includes the time from enzymatic hydrolysate preparation (12 h).
[b] ethanol yield (grams ethanol per gram available fermentable sugars) was calculated as the highest ethanol concentration, divided by the available sugar in the pretreated solids determined from acid hydrolysis (glucose and xylose).

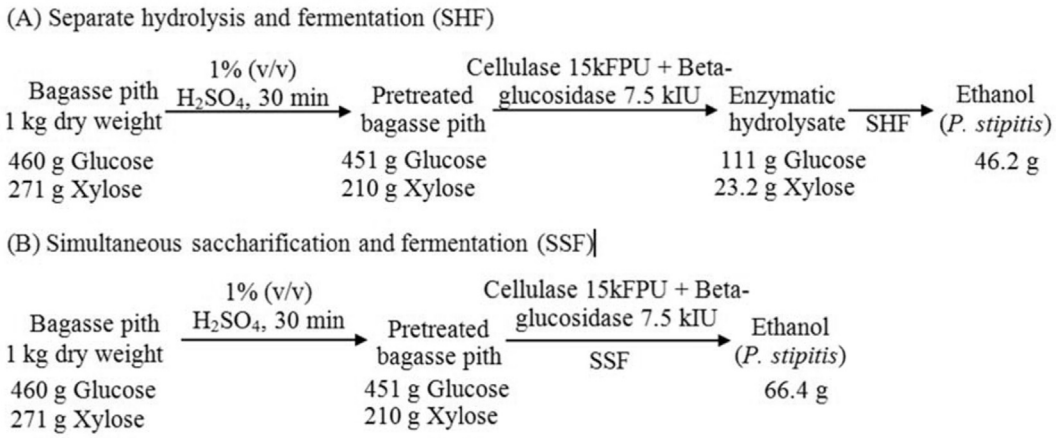

Fig. 3. Overall mass balance for the dilute acid pretreatment, enzymatic and fermentation by *P. stipitis* JCM 10742 (v/v = volume per volume; kFPU = filter paper units x 1,000, kIU = international units × 1000).

content increased the viscosity of the mixture and reduced the mixing efficiency, thus leading to poor mass and heat transfer. A fed-batch process was selected as an effective solution to this problem. Lu et al. (2010) reported that the final ethanol concentration increased by 195% when the solid concentration of steam-exploded corn stover was raised from 10% w/w to 30% w/w.

The overall mass balance included the acid pretreatment, enzymatic hydrolysis and ethanol fermentation steps (Fig. 3). After acid pretreatment, there was a decrease of 9 g/kg bagasse pith of glucose and 61 g of xylose/kg bagasse pith. The total sugar after acid pretreatment was 661 g/kg bagasse pith, which was 10% lower than the theoretical maximum of 731 g/kg bagasse pith. In the SHF process, after 12 h of enzymatic hydrolysis, a glucose concentration of 111 g/kg bagasse pith, and a xylose concentration of 23.2 g/kg bagasse pith were obtained at 5% w/v solid loading, with 15,000 FPU of cellulase and 7500 international units (IU) of beta-glucosidase per kilogram of dry bagasse pith, respectively. The fermentation of enzymatic hydrolysate by *P. stipitis* JCM 10742 resulted in 46.2 g ethanol/kg biomass after 18 h fermentation, corresponding to 14% of the maximum theoretical yield (based on the amount of glucose and xylose present in the pretreated solids). In the SSF process, an ethanol concentration of 66.4 g ethanol/kg biomass was obtained after 24 h fermentation, corresponding to 20% of the maximum theoretical yield (based on the amount of glucose and xylose present in the pretreated solid).

The SSF process exhibited notable advantages over the SHF process. The ethanol concentration and productivity from SSF increased by 43% and 67%, respectively, compared to the SHF process. The results indicated that the SSF process was preferable to SHF for the hydrolysis and fermentation of lignocellulosic biomass, giving more rapid ethanol production and a higher concentration of ethanol. The main advantage of the SSF process is that fermentation can be processed in a single bioreactor, thereby reducing investment and operation costs.

This study obtained the maximum total sugars yield (53.7 g/100 g dry bagasse pith) at 1–2% v/v H_2SO_4 for 90 min, representing 67% of the total sugars in the bagasse pith. The result indicated that dilute acid pretreatment combined with enzymatic hydrolysis can be successfully applied to bagasse pith. In ethanol production, simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) processes were employed using *P. stipitis* JCM 10742 for the production of ethanol from bagasse pith. The results indicated that the ethanol concentration and productivity in the SSF process were higher than for SHF. For SSF, the ethanol concentration and productivity reached 3.70 g/L and 0.15 g/L/hr, respectively, with 24 h fermentation, while SHF attained 0.09 g/L/hr productivity with an ethanol concentration of 2.58 g/L over 30 h.

Conflict of interest

The authors declare that there is no conflict of interest regarding publication of this paper.

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