Microwave-assisted Dilute Acid Pretreatment and Enzymatic Hydrolysis of Sago Palm Bark

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Maximizing the amount of monomeric sugar yield from lignocellulosic materials requires an effective pretreatment process and identification of an optimal enzyme loading for cost-effectiveness. In this work, a microwave-diluted sulfuric acid pretreatment was applied prior to enzymatic hydrolysis of sago palm bark (SPB). Characterization of the solid fraction was completed before and after the pretreatment process. Analysis of SPB ash showed a presence of 6.8% silica. There was a 32% reduction in lignin content, an increased crystallinity from 29% to 47%, and clear damage and fragmentation to the surface structure of SPB after the pretreatment. Inhibitors were not detectable in the liquor after the microwave-acid pretreatment. The enzymatic hydrolysis of SPB was employed by adding 6 to 42 FPU/g of cellulase and 50 U/g of β-glucosidase to identify the optimal cellulase loading at fixed β-glucosidase loading. The maximum total monomeric sugar yield and total reducing sugar (using DNS method) at 77 mg/g and 378 mg/g were achieved using 24 FPU/g of cellulose, respectively. Thus, this enzyme loading can be recommended for further microwave-acid pretreatment and enzymatic hydrolysis of SPB.

Keywords: Microwave pretreatment; Sago palm bark; Microwave pretreatment; Enzymatic hydrolysis; Yield; Enzyme loading; Cellulase

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INTRODUCTION

Many feedstocks have been used as a source of lignocellulosic material. Sago palm bark (SPB) represents one of the renewable sources of lignocellulose. In Malaysia, more than 20,000 ton/yr of SPB is discarded from the sago starch industry. Because of the high carbohydrate content (60% to 70% cellulose and hemicellulose), the sago trunk cortex can be considered a promising renewable source of glucose and xylose (Parajó *et al.* 1998). However, the extraction of valuable sugars from lignocellulosic biomass for biofuels or other based sugar chemical industries requires several steps, such as pretreatment and enzymatic hydrolysis. Performing the pretreatment process is extremely important to overcome the rigid structure of the biomass and to break down the lignin fraction to enhance enzyme accessibility for the hydrolysis step (Xing *et al.* 2013). The pretreatment should meet several criteria for low-cost and effective pretreatment. It should minimize the need to reduce the size of biomass particles, preserve the pentose sugar of the hemicellulose fractions, produce highly digestible pretreated substrate, lower or eliminate the generation of degradation products and inhibitory toxic substances, and decrease energy requirements.

Moreover, the pretreatment solvent should be of low cost and/or can be recycled easily (Alvira *et al*. 2010).

Microwave heating represents an efficient route to perform the thermal pretreatment of biomass and has been found to be an alternative to conventional heating because of its high heating rate and ease of operation (Hu and Wen 2008). Microwave usage in some studies showed that it could degrade lignin and change the ultrastructure of cellulose and hemicellulose (Xiong *et al.* 2000). It is also capable of enhancing the susceptibility of lignocellulosic materials to enzymatic hydrolysis (Azuma *et al.* 1984). It has been reported that microwave pretreatment in the presence of water can enhance the enzymatic hydrolysis of lignocellulosic materials (Ooshima *et al.* 1984). The combination of microwave heating with chemicals facilitates the development of several pretreatments methods, such as the combination of microwave and solvents, *e.g.*, alkali and acids (Boonsombuti *et al.* 2013), especially in dilute concentrations (Chen *et al.* 2012). Reportedly, the pretreatment of corn stover using steam-microwave pretreatment demonstrated that the pretreatment of biomass with microwave heating resulted in a higher sugar yield than the steam explosion pretreatment (Pang *et al.* 2013). Li *et al.* (2009) soaked swine manure in a sulfuric acid solution and irradiated it with microwaves. They found that microwave irradiation could increase the reducing sugar yield in a short reaction time and decrease the energy consumption (Li *et al.* 2009). Xu *et al.* (2011) found that the ethanol yield was greatly increased from 26.78 to 148.93 g kg^{-1} when microwave pretreatment was used to treat wheat straw compared to untreated material and higher than what was obtained by conventional heating with acid or alkali solvent, for which the ethanol yield ranged from 67.7 to 104.3 g kg^{-1} (Tutt *et al.* 2012). Dilute acid pretreatment, controlled at a moderate temperature by means of conventional heating, is preferred to avoid the formation of inhibitors (Neureiter *et al.* 2002; Chen *et al.* 2010a,b). Inhibitors are the result of sugar degradation products, namely, acetic acid, furfural, and 5-hydroxymethylfurfural (HMF) (Neureiter *et al.* 2002). Inhibitors reduce the monomer sugar yield, as well as act as fermentation toxins. Therefore, it is necessary to select suitable pretreatment conditions that reduce or eliminate inhibitor formation before or during microwave-acid pretreatment and subsequent enzymatic hydrolysis and fermentation steps.

Enzymatic hydrolysis is environmentally friendly because it takes place under mild process conditions, compared with acid or alkaline hydrolysis that requires further detoxification processes to remove the inhibitory effect of the sugar by-products. However, there is economic concern over the cost of enzymes. Thus, the goal is to optimize the enzyme loading for a cost-effective process. Enzymatic hydrolysis of lignocellulose is performed using cellulolytic enzymes that convert cellulose into cellobiose, a reducing sugar that subsequently breaks down into glucose when hydrolyzed by β-glucosidase (Parisi 1989). The cellulase enzyme is used as the primary enzyme in enzymatic hydrolysis because of its ability to break down cellulose into cellobiose. The accumulation of cellobiose exhibits an inhibitory effect on both cellulase and β-glucosidase (Philippidis *et al.* 1993). Thus, identifying the optimal cellulase loading is essential for achieving a high process yield.

The objective of this study was to identify the microwave-acid pretreatment effect on SPB properties and the optimum cellulase loading at a fixed β-glucosidase loading rate for a high monosaccharide yield and total reducing sugar. The characteristics of substrate and the pretreatment liquor were evaluated to determine the effect of the microwave-assisted pretreatment environment on pretreated biomass and the formation of inhibitors using low solvent concentration and large particle size of substrate.

EXPERIMENTAL

Materials

Feedstock

Sago palm trunks were purchased from a local plantation in Melaka, Malaysia. The trunks were debarked to obtain the bark fraction (the outer layer) by removing the pith (the inner portion). The collected bark was dried at 105 °C for 24 h, then chopped and screened into smaller sized (20 to 30 mm) chips and stored at 20 °C in sealed plastic bags for further experiments.

Enzymes and chemicals

The enzymes cellulase (*Trichoderma reesei*, ATCC 26921), β-glucosidase (from almonds) and sodium azide were purchased from Sigma Aldrich (St. Louis, MO) and used in enzymatic hydrolysis. The monosaccharides glucose, xylose, and arabinose, and the chemicals acetic acid, 5-hydroxymethyl furfural (HMF), furfural analytical standards, and 3,5 dinitrosalicylic acid, were supplied by Sigma Aldrich (St. Louis, MO) and used in the qualitative and quantitative analysis for sugar and inhibitors. Citric acid monohydrate and sodium citrate were purchased from R&M (Malaysia), while sodium metabisulfite, Rochelle salts (Na-K tartarate), and phenol were supplied from Merck Corp. (Kenilworth, NJ) and were used to measure enzyme activity.

Methods

Pretreatment

The microwave-assisted acid pretreatment was carried out in a domestic microwave oven (NN-ST340M, Panasonic, Kadoma, Osaka Prefecture, Japan) at a frequency of 2.45 GHz. The experiments were performed in a 1.0-L round-bottom flask, containing 100 mL of 0.1 N H_2SO_4 connected with a reflux condenser. The pretreatment reactor (flask) was loaded with a solid to liquid ratio of 10:1 (w/v), and irradiated at 440 W for 10 min. The mixture was filtered through Whatman filter (0.45-μm) paper to separate the solid residues from the liquid component. The filtered biomass was washed, dried, and frozen at -20 °C until compositional analysis/sequential enzymatic hydrolysis. Meanwhile, the liquor was collected for the identification of glucose, xylose, and arabinose content and degradation products.

Filter paper assay (FPA)

The filter paper assay was performed to measure the activity of cellulase (1.5 L of enzyme from *Trichoderma reesei* No. E.C. 3.2.1.4) and quantify the enzyme loading for enzymatic hydrolysis solution. The specific activity reported by manufacturer for the aqueous solution is ≥ 700 Units/g. For quantitative results, the enzyme preparations must be compared on the basis of significant and equal, thus cellulose activity assay needed to be conducted. The cellulase activity assay using filter paper as the cellulose was done in accordance to the International Union of Pure and Applied Chemistry (IUPAC) was performed according to the NREL LAP-006 procedure. The unit for the cellulase activity used is called FPU, defined as the amount of enzyme required to liberate 1 μmol of glucose from cellulose per minute (Adney and Baker 1996). Whatman filter paper No. 1 strips were utilized as substrate and soaked in Na-citrate buffer (pH 4.8). Enzyme dilutions prepared for different initial activities were added to the mixture of substrate and the buffer. Enzymatic hydrolysis curried out in a water bath at 50°C for 60 min. Then, 3,5-

dinitrosalicylic (DNS) acid reagent was added to all samples and heated as described by DNS analysis (Miller 1959) to allow for color formation. The colored samples were then measured with a UV–VIS spectrophotometer (UV-2700, Shimadzu, Japan) at 540 nm using a standard curve of glucose to convert the obtained optical density back to mg of glucose released from the hydrolyzed filter paper. The enzyme dilution, which released 2 mg per 0.5 mL, was substituted in the following filter paper unit (FPU) equation:

$$\text{FPU} = \frac{0.37}{\text{Enzyme dilution releasing 2.0 mg glucose}} \text{ unit ml}^{-1} \qquad (1)$$

Enzymatic hydrolysis

Enzymatic hydrolysis was performed at 55 °C at 150 rpm for 72 h. A total of 1.0 g of pretreated biomass (on a dry matter basis) was immersed in 30 mL of 50 mM sodium citrate buffer (pH 4.8) in a 250-mL Erlenmeyer flask. Cellulase from *Trichoderma reesei* was added at enzyme loadings of 6, 12, 18, 24, 30, 36, or 42 FPU/g. The cellulose enzyme was supplemented with β-glucosidase at 50 U/g (Note: U is the activity units of β-glucosidase). According to the manufacturer, 1 U of β-glucosidase corresponds to the amount of enzyme which liberates 1 μmol of glucose per min at pH 5.0 and 37 °C using salicin as substrate. A dose of 0.3% (w/v) sodium azide was added to avoid microbial contamination. The supernatant was filtered through a 0.22-μm nylon membrane syringe filter to estimate the sugar yield.

Analytical procedure

The chemical components of raw feedstock and pretreated SPB, consisting mainly of cellulose, hemicelluloses, lignin, and ash, were analyzed by determining the neutral detergent fiber (NDF) and the acid detergent fiber (ADF) and ash (Van Soest *et al.* 1991). The chemical identification of elements and their concentration for raw and pretreated substrate was carried out using Energy Dispersive X-Ray Spectroscopy (EDX). This test was accomplished by analysis using NORAN System 7 X-ray Microanalysis (Thermo scientific, USA). The chemical analysis of SPB ash was performed using XRF (EDX-720 Fluorescence Spectrometer, Shimadzu, Japan). Thermal analysis of pretreated SPB and untreated samples was performed using a thermogravimetric analyzer (TGA) (SDTA851e, Mettler Toledo, Switzerland). The samples were heated from 25 to 900 °C at a rate of 10 °C/min. The images of untreated and pretreated SPB were captured using a scanning electron microscope (S-3400N, Hitachi, Japan).

The total reducing sugar analysis was performed according to the DNS method of Miller (1959), as a comparison to the HPLC analysis done for each treatment. The monosaccharide concentration in the hydrolysate of hydrolyzed materials was determined by high-performance liquid chromatography (HPLC), equipped with evaporative light scanning and refractive index detectors (Alltech 2000, East Lyme, Connecticut, USA). The separation was performed using a Rezex RPM-Monosaccharide Pb^{+2} column (Phenomenex Inc., Torrance, CA), and deionized water was used as the mobile phase, with a flow rate of 0.6 mL/min. The hydrolysate was filtered using a 0.22-μm disposable nylon membrane syringe filter (Phenex Inc., England, UK) prior to HPLC analysis. Additionally, the sugar degradation products furfural, acetic acid, and HMF were detected using a Rezex ROA–organic acid H+ (8%) column (Phenomenex Inc., Torrance, CA), using a 0.005 N H_2SO_4 mobile phase and a flow rate of 0.6 mL/min.

The crystallinity of the samples before and after the pretreatment was analyzed using X-ray diffraction (XRD) (PANalytical, Netherlands). An X-pert pro diffractometer was set at 40 kV, 30 mA; radiation was Cu Ka (l ¼ 1.54 Å), grade range between 10 and 30º with a step size of 0.026. The total number of steps was 762 and total time was 29.06 mins. Crystallinity of cellulose was calculated according to the empirical method proposed by Segal *et al.* (1959) as shown in Eq. 2,

$$\mathrm{CrI}(\%) = \left[\frac{I_{002} - I_{\mathrm{am}}}{I_{002}}\right) \times 100 \quad (2)$$

where CrI is the crystalline index, I_{002} is the maximum intensity of the (002) peak 2θ =22.2, and I_{am} is the minimum intensity corresponding to the amorphous at 2θ=18.0º. The Scherrer formula (Eq. 3) was applied to calculate the crystallite size, with the method based on the width of the diffraction patterns. The crystallite sizes were determined by using the diffraction pattern obtained from (002) of samples,

$$D(hkl) = \frac{K\lambda}{\beta_{\circ} \cos\theta} \quad (3)$$

where $D(hkl)$ is the size of crystallite (in nm), K is the Scherrer constant (0.94), and λ is the X-ray wavelength (0.15418 nm for Cu). The parameter $\beta_{\circ}$ is the full-width at half-maximum of the reflection *hkl*, and 2θ is the corresponding Bragg angle (Oh *et al.* 2005).

RESULT AND DISCUSSION

Effect of Pretreatment on SPB

Chemical composition

The chemical composition of untreated SPB was 40.8% cellulose, 22.3% hemicellulose, and 25.9% lignin. After the microwave-assisted dilute acid pretreatment step, the lignin removal was found to be 32%, as shown in Table 1. The pretreatment generally aims to alter the structure of lignocellulose by removing lignin and modifying the hemicellulose and cellulose contents, while maintaining them in the solid residue to increase the monosaccharide yield following enzymatic hydrolysis (Xu *et al.* 2011). Thirty-two percent removal of the lignin was accomplished after the pretreatment process. The lignocellulosic structure can be described as a skeleton of cellulose chains fixed in a cross-linked matrix of hemicellulose enclosed by a crust of lignin. The extensive interactions between cellulose, hemicellulose, and lignin, as well as the barrier nature of lignin minimize the access of hydrolytic enzymes to the carbohydrate fraction. Therefore, removing the lignin cover and disturbing the hemicellulose structure (by removing some of its content) will enhance enzyme accessibility to cellulose and hemicellulose, and this represents the general aim of pretreatment (Yang *et al.* 2011). The decline in hemicellulose content from 22.3% to 19.5% can be attributed to partial hydrolysis that liberated pentose sugar or may have contributed to the formation of sugar byproducts during the pretreatment process. As a result, the acid is able to promote hydrolysis and provide hydrogen ions to break down the hemicellulose chains (Wyman *et al*. 2005). The increase of cellulose content does not necessarily mean that it was not affected by the pretreatment but rather the increase arises from the changes of the amounts of other components still present. Overall, using dilute solvent (0.5%) and low microwave power (440 W) at short time (10

min) in microwave-assisted pretreatment of SPB concur with aims of a pretreatment step in biofuels production, and this is essential for a cost-effective pretreatment process.

Not only the matrix of the material is important in the efficiency of extraction, but also silica, an inorganic material content in a lignocellulose biomass, plays an important role as well. It has been described as another limiting factor for enzymatic hydrolysis of rice straw hydrolysis (Ma *et al.* 2009). It was reported that the silicon deposits in cell walls and acts as another physical barrier for enzymatic hydrolysis (Řezanka and Sigler 2008). The silicon content was identified by the chemical analysis for ash using the XRF test, which revealed the presence of 6.8% silica.

The EDX analysis of SPB skin before pretreatment process showed that silica was not found on the outer layer of the sample, although the XRF analysis for SPB ash showed the presence of silica. However, performing the elemental analysis of SPB after pretreatment using EDX showed the presence of silica compound in the inner layer of SPB due to removing outer part during the pretreatment process. The test was performed using point and shoot mode. Figure 1 displays silica bodies and test points. The test points numbers 1and 3 show silica bodies, and the main compound is SiO_2. The test point number 2 represents the other parts of SPB skin layer, and the analysis at this point does not contain silica compound; the main elements are carbon and oxygen. Table 5 shows EDX analysis of test points 1, 2, and 3. The presence of silica on this cell layer will probably affect enzymatic hydrolysis negatively (Řezanka and Sigler 2008).

Table 1. Composition of Untreated and Pretreated Sago Palm Bark (SPB)

Component	Untreated SPB (% w/w)	Pretreated SPB (% w/w)
Cellulose	40.8	47.3
Hemicellulose	22.3	19.5
Lignin	25.9	17.7
Others	11.0	15.5

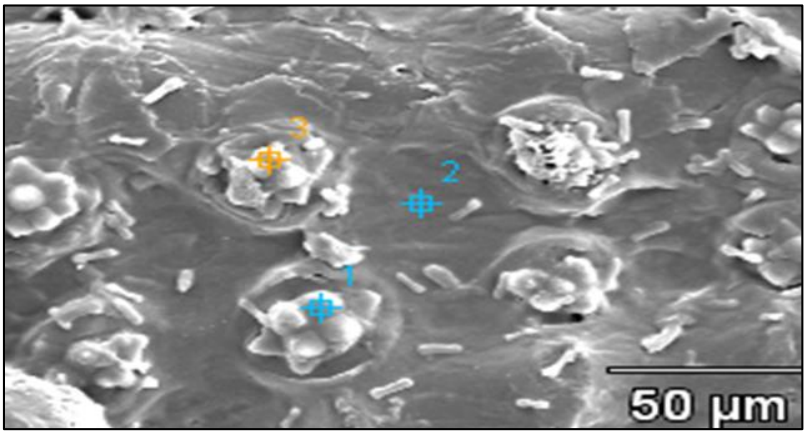

Fig. 1. The distribution of silica bodies and test points on SPB surface after the pretreatment

Morphology

SEM micrographs reveal the physical changes in the surface structure of untreated and pretreated SPB *via* microwave-acid pretreatment at different magnification (Fig. 2). The texture of untreated SPB appeared to be rigid, continuous, and non-porous (Fig. 2a). After the microwave-assisted pretreatment process with 0.5% H_2SO_4, the rigid surface structure was damaged and fragmented. The disturbed SPB surface can be clearly seen in in Fig. 2b, c, and d. Some portions appeared as a slightly sieve-like structure and had visible pores as shown in Fig. 2b. Figures 2c and d exhibited some fragments that had flaked off from the lignocellulose surface. The changes of the surface structure of SPB were possibly due to lignin removal and partial degradation of other components such as cellulose and hemicellulose. Similar structural changes were earlier reported for rice straw pretreated 0.5% sulfuric acid for 60 min at 121 ºC (Kshirsagar *et al.* 2015) and pretreated wheat straw by steam explosion (Cui *et al.* 2012).

The rough surface generated from the pretreatment increased the surface area as a result of partial removal of external fibers. The increase in biomass surface area makes the cellulose and hemicellulose become more accessible for enzymes and facilitates enzyme adsorption that would enhance enzymatic hydrolysis. This indicated that the microwave-acid pretreatment disturbed the recalcitrant structure of SPB and increased the surface area of the pretreated biomass. This is consistent with the pretreatment aims to remove the lignin and modify hemicellulose and cellulose while keeping them as much as possible in the residual, thereby increasing the yield of monomeric sugars in the following enzymatic hydrolysis (Xu *et al.* 2011).

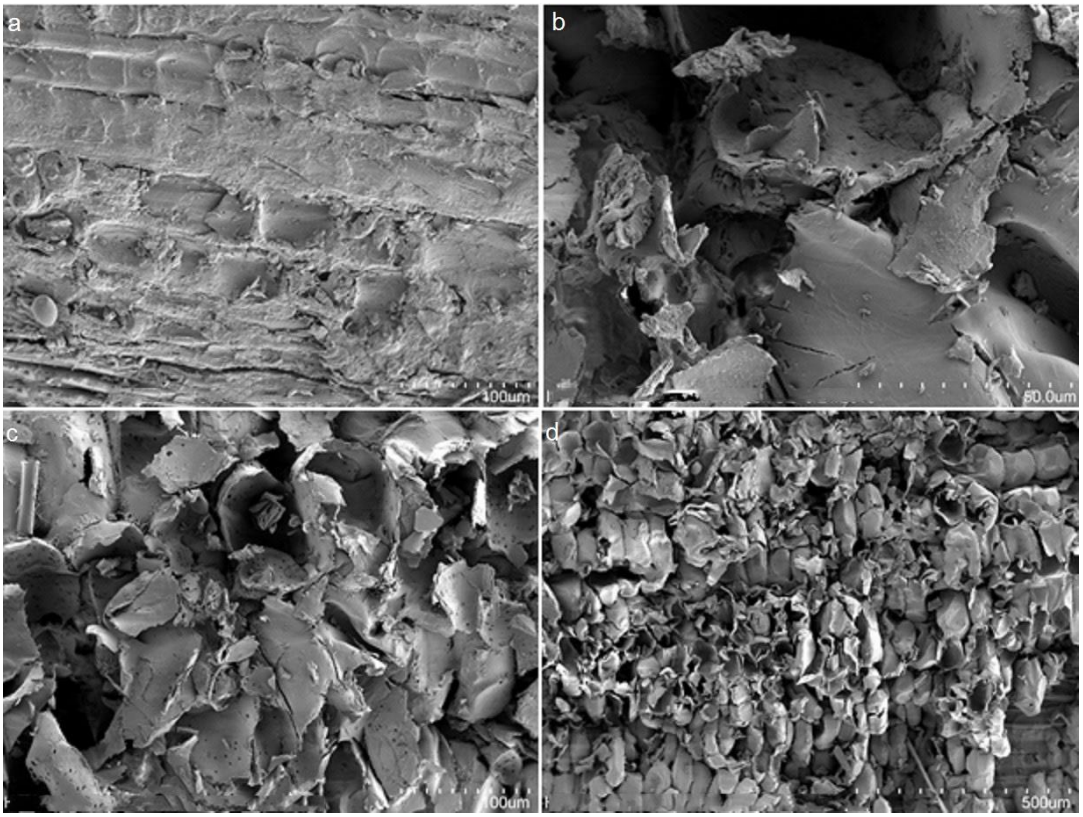

Fig. 2. Scanning electron microscopy images of (a) untreated and (b, c, and d) pretreated sago palm bark at various magnifications

Crystallinity

The untreated and pretreated SPB was investigated using XRD to determine the crystalline character of the cellulose. Figure 3 shows the XRD patterns of untreated and pretreated SPB. Generally, the cellulose chains contained both crystalline (ordered) and amorphous (less ordered) regions, and it was comprised of micrometer-sized particles composed of nano-meter-sized microfibrils (Yang *et al.* 2011). CI describes the relative amount of crystalline portion in cellulose compared to amorphous region. It was reported that microwave heating can lead to disruption of the hydrogen bonds by increasing the effect of localized hydrolyzation and the removal of the amorphous part which caused the increase of the crystallity index compared to the control (Fatriasari *et al.* 2016). In this study, the crystallinity index after microwave-assisted acid pretreatment was 47%, which was higher than that of the untreated SPB (29%). This phenomenon is likely the result of the removal of more paracrystalline and amorphous cellulose (Sannigrahi *et al.* 2010) and the removal of lignin and acetyl groups (Chang and Holtzapple 2000). Similar observations were recorded in previous studies of the acid pretreatment of various feedstocks (Samuel *et al.* 2010; Sindhu *et al.* 2014; Kshirsagar *et al.* 2015). The crystallite size of the pretreated biomass was 0.3611 nm, which was higher than that of the untreated samples (0.2883 nm). It was reported that irradiation attacks the cellulose structure, which causes many defects throughout whole fibers, resulting in a disordered structure (Kristiani *et al.* 2015). Therefore, the change in the crystal structure of cellulose can be attributed to combined actions of partial realignment of cellulose structure and the partial destruction of hydrogen bonding, recrystallization, and hornification of cellulose (Hu *et al.* 2013). Identical observations were recorded in earlier studies of sweet sorghum bagasse and rice straw substrates pretreated *via* ionic liquid, steam explosion, lime, and dilute acid (Zhang *et al.* 2011; Kshirsagar *et al.* 2015).

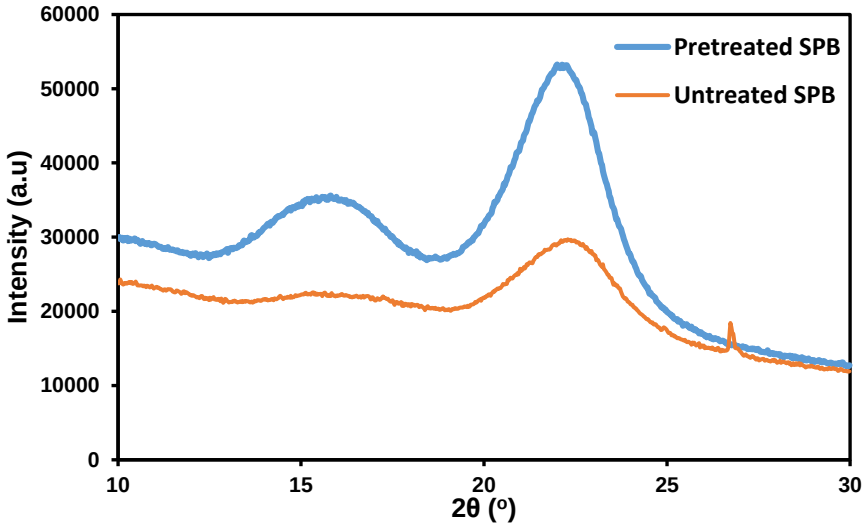

Fig. 3. X-ray diffraction patterns of untreated and pretreated sago palm bark (SPB)

The crystallinity of cellulose has been reported to have an effect on enzymatic hydrolysis. Chang and Holtzapple (2000) reported that high hydrolysis initial rates were achieved from low-crystallinity index samples. Although slow hydrolysis rates have been found with increasing crystallinity of cellulose, as reported by Sinitsyn *et al.* (1991), Sannigrahi and his co-worker (2010) reported the opposite effect. In general, the

relationship between the crystallinity index, the change in crystallite size of pretreated biomass, and its corresponding enzymatic hydrolysis rate is not well understood. Biomass with a high crystallinity index may not necessarily negatively affect the enzymatic hydrolysis rate (Kim and Holtzapple 2006; Zheng *et al*. 2014).

Thermal properties

Thermogravimetric analysis (TGA) was performed to examine the thermal degradation of the SPB samples before and after the pretreatment process. Figure 4 shows the TGA and DTG analysis of untreated and pretreated SBP. The weight loss performance of untreated and pretreated SPB can be obviously divided into three stages as shown in the GA curve (Fig. 4a). The first stage took place at 25 to 125°C as a result of removal of unbound and bound water (Rhim *et al.* 2010). The second stage approximately started at 150 ºC and was completed at 400 ºC. At this major stage, the thermal degradation of the main component of biomass hemicellulose and cellulose can be identified as contributing to most loss of weight compared to other stages (Chen *et al*. 2012; Jin *et al*. 2013). The weight of untreated samples decreased from more than 90% to less than 40%. Meanwhile pretreated sample exhibited a higher weight loss, a decrease to less than 20%, which might be due to partial degradation of its components during the pretreatment such as amorphous cellulose and hemicellulose. The third stage took place above 400ºC, which shows the mass loss of pretreated SPB was less than that of the untreated sample. The difference in the weight in this stage might be attributed to there being a higher lignin content in untreated sample. It was reported that the lignin thermal degradation occurred at a low rate in the temperature range of 100 °C to 700 °C with a tiny peak at 340 °C (Jin *et al.* 2013). The DTG curves (Fig. 4b) of untreated and pretreated SPB are characterized by a double-peak distribution, and these two peaks were detected at 260 and 350ºC, which are related to hemicellulose and cellulose thermal degradations, respectively (Chen *et al.* 2012). There was no clear peak for lignin, though it might have been located at the same position as the cellulose peak, since lignin has tiny peak at 340 ºC as reported by Jin *et al.* (2013).

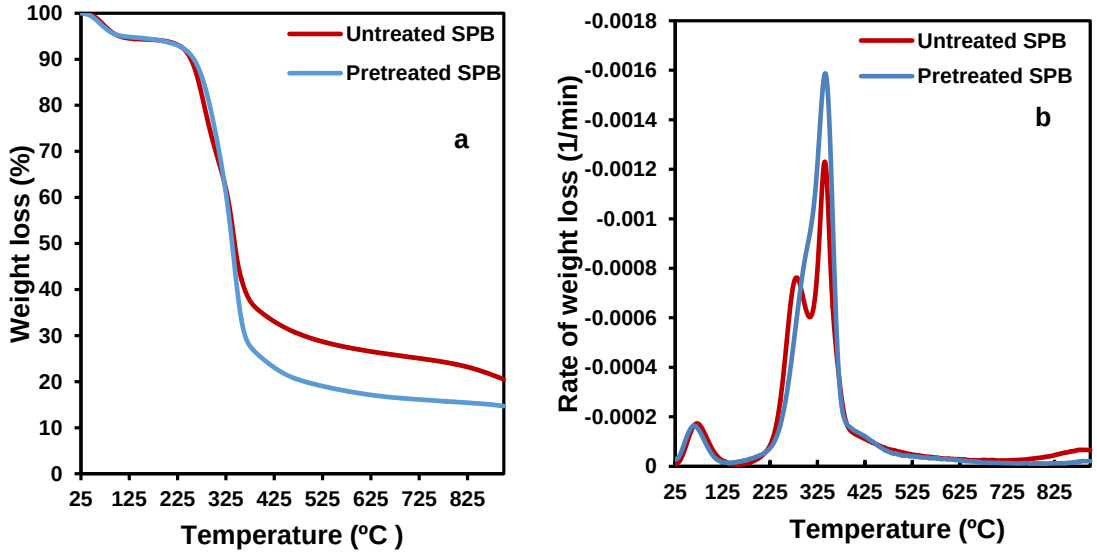

Fig. 4. (a) Thermogravimetric analysis and (b) differential thermogravimetric analysis of the untreated and pretreated sago palm bark (SPB)

The hemicellulose appeared to degrade easier than cellulose since it was pyrolyzed within the range 220 to 315 ºC, while the cellulose was pyrolyzed at 315 to 400 ºC (Yang *et al.* 2007). This can be attributed to the fact that hemicellulose has random amorphous structures with reactive acetyl groups that are easily decomposed during acid pretreatment, while cellulose has some crystalline regions that are resistant to acid pretreatment. These features may explain the absence of bimodal peaks from the pretreated sample's DTG curve.

Characterization of the Pretreatment Liquor

Characterization of the pretreatment liquor is an essential step to evaluate the pretreatment severity and recognize sugar degradation products. The results indicated the presence of glucose (2.5 mg/g), xylose (2.4 mg/g), and arabinose (2.56 mg/g) in the pretreatment liquor, as shown in Fig. 5. The presence of pentose (xylose and arabinose) indicated that the pretreatment process disturbed the hemicellulose fraction. This result of acid catalysis led to fractionation of long hemicellulose chains (Feng *et al.* 2013) and enhanced the release of monosaccharides in the subsequent enzymatic hydrolysis steps. It is known that furfural and acetic acid are derived from the hydrolysis of hemicellulose under harsh pretreatment conditions (Balat *et al.* 2008). Similarly, HMF is produced from cellulose hydrolysis by the further conversion of glucose stems (Gámez *et al.* 2006). These compounds play an inhibitory role during microorganism fermentation, as well as causing corrosion to the reaction apparatus (Zhuang *et al.* 2009). All of the inhibitors evaluated were not detected in the pretreatment liquor, indicating moderate pretreatment conditions.

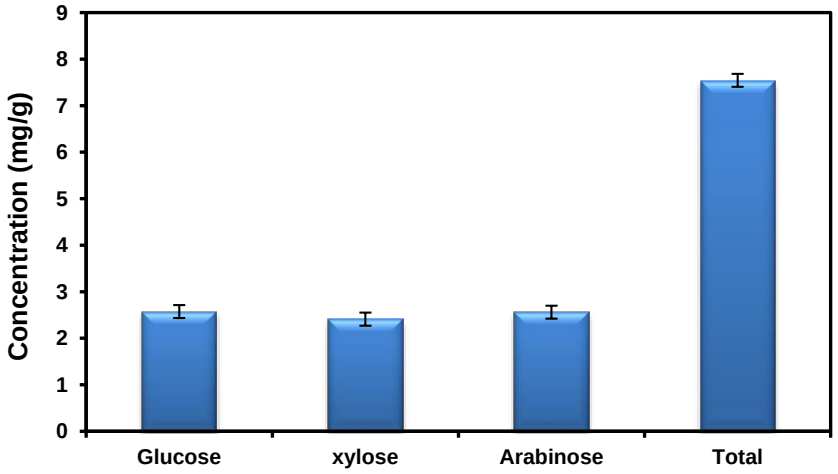

Fig. 5. Released sugar concentrations in the pretreatment liquor

Effect of Cellulose Loading on Enzymatic Hydrolysis of Pretreated SPB

The enzymatic hydrolysis was performed using cellulase and β-glucosidase enzymes to convert pretreated biomass into monomeric sugars. The cellulase activity was found to be 67 FPU/mL in the filter paper assay. The sugar yield from enzymatic hydrolysis was measured using HPLC to detect the individual components of monosaccharides and the DNS method to evaluate the total reducing sugar. The monosaccharides and total reducing sugar obtained from the enzymatic hydrolysis of pretreated SPB, using various enzyme loadings of cellulase at fixed β-glucosidase loading (50 U/g), are shown in Fig. 6 and Fig. 7, respectively. The idea is to add a fixed β-glucosidase concentration to make the process cost-effective by identifying the cellulase loading that could result in high sugar

yield. The optimum cellulase was identified based on the yields of total reducing sugars and glucose. The results revealed that the highest amount monosacchardies was 77 mg/g, and this fraction was comprised of glucose (18 mg/g), xylose (54 mg/g), and arabinose (5 mg/g) at the cellulase loading of 24 FPU/g. The task of identifying the optimal enzyme loading was considered important for obtaining a cost-effective process by reducing the final production cost. The highest loadings of cellulase (30, 36, and 42 FPU/g) showed approximately identical results, might be because of the excessive accumulation of cellobiose that exhibited an inhibitory effect on cellulose, resulting in consequently higher levels of β-glucosidase needed in the enzymatic hydrolyzates (Rivera *et al.* 2010). By the same token, Fig. 6 shows that the higher amount of the total reducing sugar 378 mg/g was found using 24 FPU/g of cellulase loading, which means that the enzymatic hydrolysis produced 15.67 mg sugars/g biomass/FPU of enzyme. These results confirm that 24 FPU/g was the optimal loading of cellulase.

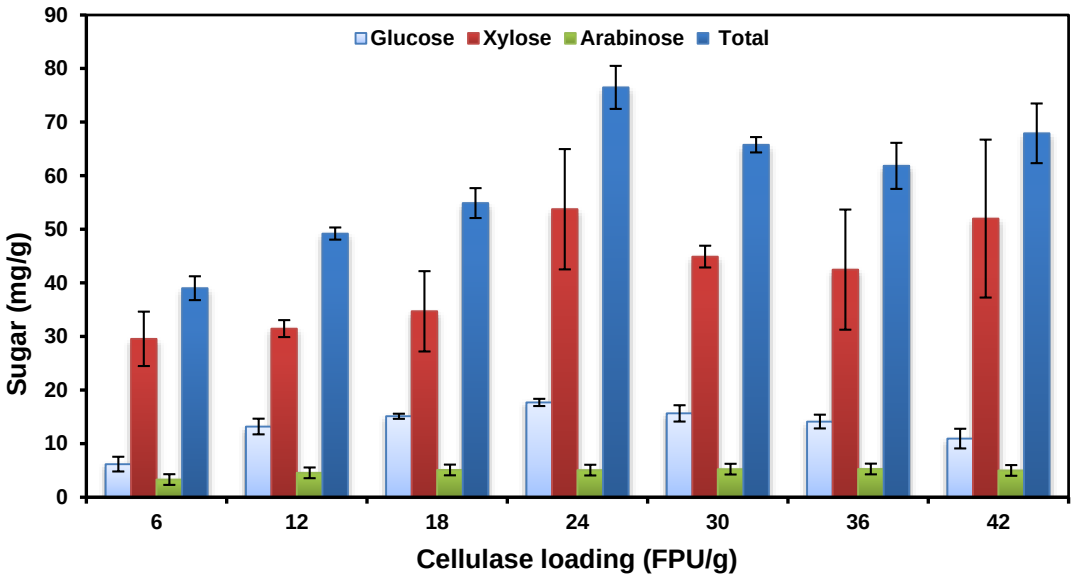

Fig. 6. Sugar yield using various cellulase loadings

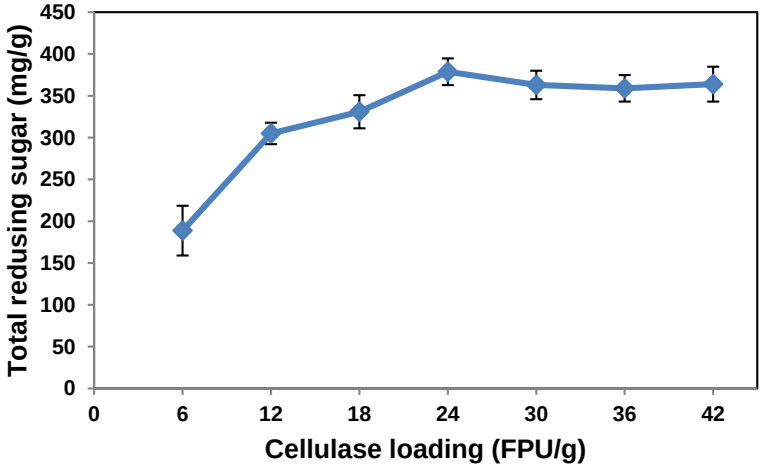

Fig. 7. Sugar yield using various cellulase loadings

From the chemical standpoint, the DNS reagent reacts with all reducing sugars such as monosaccharaides and cellubiose (Jeffries *et al.* 1998). Thus, it can be inferred that presence of cellubiose is high in SPB hydrolysate, which can play an inhibitory effect on cellulose, thus decrease the release of glucose. Moreover, using large particle size of SPB at 20 to 30 mm during pretreatment, the presences of silica compound in SPB, and cellulose crystallinity might cause resistance of lignocellulosic materials to enzymatic hydrolysis and negatively affect the glucose release. However, it is of interest that performing microwave-acid pretreatment in mild operational conditions and enzymatic hydrolysis using cellulosic cocktail can lead to high yield of the valuable pentose sugars such as xylose, which would have needed more severe conditions during conventional alkaline or acid pretreatment (Hendriks and Zeeman 2009) or the need to use auxiliary enzyme such as hemicellulose-degrading enzymes (Saha 2003). The sugar yield from this study was higher than what was reported by Mohamad *et al*. (2012) for the pretreatment of SPB using microwave–assisted dilute alkaline and 3.0% sodium hydroxide solution (w/v) for 5 min at 250 W. The enzymatic hydrolysis using 10 UN/g of xylanase (UN is xylanase activity unit reported by the manufacturer. One unit will liberate 1 μmole of reducing sugar measured as xylose equivalents from xylan (Cat. No. X0627, Sigma, Kuala Lumpur) per min at pH 4.5 at 30 °C) produced 4.1 mg/g of total sugars, which was approximately half that of the current study. It can be concluded that using a combination of the enzymes demonstrated better hydrolysis efficiency. Therefore, using 24 FPU/g of cellulase loading and 50 U/g of β-glucosidase is recommended for further microwave-diluted acid and enzymatic hydrolysis of SPB.

CONCLUSIONS

1. The effect of microwave-assisted diluted acid pretreatment on the chemical composition, crystalline structure, thermal degradation, and morphology of sago palm bark substrate demonstrated that the pretreatment method was successful.
2. No inhibitors were detected in the pretreatment liquor when the pretreatment was performed under mild condition; thus, the detoxification step can be eliminated.
3. The results of enzymatic hydrolysis showed that 24 FPU/g of cellulose and 50 U/g of β-glucosidase were sufficient to obtain a high sugar yield from pretreated sago palm bark.
4. Microwave-assisted dilute acid pretreatment of SPB using enzymatic hydrolysis is a cost-effective process at the following conditions: 24 FPU/g of cellulase loading and 50 U/g of β-glucosidase.

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