Influence of High-Pressure Steam Pretreatment on the Structure of Rice Husk and Enzymatic Saccharification in a Two-Step System

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This study aimed at developing an operational high-pressure steam pretreatment (HPSP) to effectively modify rice husk for enzymatic saccharification. The HPSP was performed at 160 to 200 °C under 0.3 to 2.8 MPa for 2 to 10 min. The efficiency of this method was based on the chemical composition, scanning electron microscopy (SEM), Fourier transform infrared (FTIR), and X-ray diffraction (XRD) analyses. Optimum pretreatment conditions (200 °C, 1.85 MPa for 7 min), enzyme concentration at 30 FPU/g and temperature at 60 °C for 48 h of continuous saccharification effectively produced sugar (21.1 g/L = 0.422 g/g dry substrate) at a saccharification degree of 53.87%. Conducting a second-step enzymatic saccharification resulted in additional sugar production (7.9 g/L = 0.158 g/g substrate) and a 20.44% saccharification degree. In contrast, the two-step saccharification process (48 and 24 h) achieved optimal sugar yield of 0.581 g/g substrate and saccharification degree of 73.5%. Additionally, the process improved the yield of monomeric sugars of glucose (0.465 g/g), xylose (0.010 g/g), and cellobiose (0.063 g/g). Therefore, the combination of the high-pressure steam pretreatment with thermostable cellulase from *Bacillus licheniformis* 2D55 in a two-step enzymatic saccharification process is an economically viable method in rice husk bioprocessing for sugar production.

Keywords: High-pressure steam pretreatment; Rice husk; Structural characterisation; Bacillus licheniformis 2D55 thermostable cellulose; Two-stage enzymatic saccharification

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INTRODUCTION

The tremendous shortage of crude oil reserves caused by increased global energy consumption has increased research interest into the development of alternative energy. Lignocellulosic biomass is a renewable energy resource and has remarkable potential for the production of alternative biofuel. Biofuel production from lignocellulose provides a renewable and cleaner energy option compared with fossil fuel, which is non-renewable. It is essential to reduce dependence on fossil fuels and lower emissions of greenhouse gases.

Rice husk is a major lignocellulosic agricultural by-product distributed worldwide and is a great biological resource. It is commonly generated in large quantities from the rice milling industries during the harvesting and milling of rice paddies for rice production.

Rice husk accounts for 20% of a rice paddy, with an average composition of cellulose (36% to 40%), hemicellulose (12% to 19%), and lignin (17% to 19%) (Saha and Cotta 2007; Banerjee *et al*. 2009). Cellulose is a major component of lignocellulosic biomass, including rice husk. It is considered to be the most abundant renewable and sustainable energy resource for the production of fermentable sugar that can be converted to bioethanol or other chemical products (Anwar *et al.* 2014; Ram *et al.* 2014). To convert cellulose into sugar, pretreatment of lignocellulose is essential.

Pretreatment is necessary to disrupt the ordered fibrous matrix of lignocellulose and increase porosity and pore volume, as well as to separate cellulose from hemicellulose and the interwoven binding lignin to allow for easy access to enzymatic attack (Stephen *et al*. 2012; Meng *et al*. 2015). Ang *et al*. (2012) and Wei *et al*. (2009) suggest that pretreatment is a crucial step to improve the efficiency of rice husk bioconversion and to obtain a high yield of fermentable sugars through enzymatic hydrolysis. Although various pretreatment techniques have been developed, such as acid, alkali, organosolvent, and ionic liquids, the high cost of chemicals and their environmental toxicity are major impediments to chemical utilization in biofuel production. In contrast, pretreatment with high-pressure steam offers an alternative method that is eco-friendly, inexpensive, and requires only steam at low residence times.

The hydrolysis of lignocellulosic biomass can be achieved through chemical or enzymatic reactions. Enzymatic hydrolysis is an economical way for obtaining fermentable sugar with mild conditions (Wyman *et al*. 2005) because of the easy recovery of enzymes, which makes enzymatic hydrolysis superior to chemical hydrolysis. Conventional cellulase works best at 50 °C, while fermentation with yeast is carried out at 30 °C; therefore, the operating temperatures of cellulase and yeast are incompatible in simultaneous saccharification and fermentation (SSF). Additionally, the enzyme loses its stability at temperatures above 50 °C. This poses a major drawback to SSF. To meet future challenges, an innovative bioprocess of thermo-fermenting sugar to ethanol has been proposed. The full benefits of this process can be achieved in SSF through the application of thermostable cellulase. Therefore, it is essential to apply thermostable cellulase for enzymatic saccharification as the first step in thermophilic SSF. There is an increased interest in using cellulase from thermophilic bacteria in enzymatic saccharification. This is due to the thermostable nature of those enzymes. In the authors' previous study, the thermophilic bacteria *Bacillus licheniformis* 2D55 was isolated from compost (Kazeem *et al*. 2017), and a cellulase was obtained that was stable over a broad temperature range of 50 to 80 °C and active at a broad pH range of 3.5 to 10 (Kazeem *et al*. 2016). Thermostable cellulase offers several potential advantages in enzymatic saccharification, such as an improved hydrolysis rate, reduced risk of contamination, better substrate solubility, cellulase recyclability, decreased enzyme loading, reduced cost of production, decreased hydrolysis time, and simplification of the cooling problem after pretreatment (Barati and Amiri 2015). To the knowledge of the authors, there has been a lack of studies evaluating the hydrolysis efficiency of thermostable cellulase from thermophilic bacteria in saccharification of lignocellulose.

Over the years, efforts at improving the economic viability of lignocellulosic bioconversion have been directed toward maximizing the cellulase production and improving the cellulase performance. However, the economics of enzymatic saccharification still remain a problem. After enzymatic saccharification, the enzymes are distributed within the liquid phase and solid lignocellulose (Pribowo *et al*. 2012; Eckard *et al*. 2013). The recovery and recycling of enzymes bound to the substrate and hydrolysate

have been proposed as methods to reduce the cost of enzymatic saccharification. A number of enzyme recycling and desorbing methods through ultrafiltration and the addition of fresh substrate have been reported to improve the hydrolysis efficiency of various lignocellulosic biomasses (Qi *et al*. 2011; Yang *et al*. 2011; Ouyang *et al*. 2013). The application of a two-step enzymatic saccharification with recycling of the substrate is an additional strategy to improve the cost-effectiveness and efficiency of enzymatic saccharification.

The aim of this study was to investigate the effect of high-pressure stream pretreatment on the structural and physicochemical changes of rice husk. Furthermore, the effect of high-pressure steam on enzymatic saccharification in a two-step system using a thermostable cellulase of *B. licheniformis* 2D55 was also investigated.

EXPERIMENTAL

Raw Materials and Chemicals

Rice husk (RH) was collected from Bernass Bhd, a rice processing factory in Sekinchan, Selangor, Malaysia. The RH samples were prepared as described previously by Kazeem *et al*. (2016). Briefly, each RH sample was washed with clean water and dried for 24 h at 60 °C. A laboratory grinder (Retsch SM 200, Rosstfrei, Hann, Germany) was used to mill the sample into 0.25-mm particles, which were then stored in air-tight plastic bags for moisture balance. The commercial enzyme cellulase (from *Trichoderma reesei*; 700 units/g) was obtained from Sigma-Aldrich, Co. (St. Louis, USA). Meanwhile, the crude cellulase was obtained from *B. licheniformis* 2D55 as previously described by Kazeem *et al*. (2016). The enzymes were stored at 4 °C prior to use. All of the other chemicals (Merck, Darmstadt, Germany) were of analytical grade.

Methods

High-pressure stream pretreatment (HPSP)

A 500-mL high-pressure autoclave digester (START 500, Nito Kiatsu Co. Ltd. Japan) was used for the pretreatment process. The digester was equipped with a temperature and pressure control indicator system. Fifty grams of the RH sample was weighed and placed in the high-pressure autoclave containing 200 mL of distilled water. The temperature and pressure were elevated to 160, 180, 200, and 220 °C, and from 0.3 to 2.8 MPa, respectively, for 2, 4, 7, and 10 min. After each pretreatment, the exhaust valve was slowly opened to release all of the steam, and the autoclave was allowed to cool down. The pretreated samples were collected, washed thoroughly, and stored at 4 °C for future use. The hydrolysate liquor obtained after each pretreatment was collected, and the pH was measured.

Weight loss and chemical composition

After pretreatment, the samples were oven-dried at 105 °C for 24 h and weighed. The weight loss was determined and expressed as a percentage. The lignocellulosic compositional analysis of both the untreated and pretreated RH samples were performed according to the method described by Goering and Van Soest (1970).

Scanning electron microscopy (SEM)/energy dispersion x-ray (EDX) analysis

The scanning electron microscopy (SEM) analysis of the untreated and pretreated RH samples was performed with a scanning electron microscope (S-3400N, Hitachi,

Japan). Samples were fixed on double-sided adhesive tape and mounted on an aluminium stub. The samples are coated with gold using a sputter coater (E-1010, Hitachi) prior to examination. The SEM images were captured with an accelerating voltage of 5.00 kV and a working distance ranging from 9000 to 1000 µm. The energy dispersion X-ray (EDX) analysis was performed with an energy dispersion X-ray analyser (S-3400N, Hitachi, Japan) coupled with a scanning electron microscope. Samples were mounted on an aluminium stub with adhesive tape prior to examination. The EDX analysis was performed at an accelerated voltage of 30.0 kV with a take-off angle of 35.0°.

Fourier transform infrared (FTIR) spectroscopy

The Fourier transform infrared (FTIR) spectra of the untreated and pretreated RH were obtained using a FTIR spectrophotometer (GX2000, Perkin Elmer, USA). The samples were prepared using KBr. A total of 32 scans were applied for each sample at a wavenumber ranging from 400 to 4000 cm^{-1} and at a resolution of 4 cm^{-1}.

X-ray diffraction (XRD) analysis

The crystallinity index (CrI) of the RH samples was analysed with an X-ray diffractometer (APD2000, ITAL Structures, Italy) and filtered by Cu K_∞ radiation (λ = 0.15489 nm). The Phaser unit was operated at 40 kV and 40 mA. The X-ray diffraction (XRD) analysis was performed with a scanning speed of 2° per min at a scattering angle (2θ) of 2° to 40°. The CrI of the RH samples was calculated as the ratio between the intensity at the 002 peak (I_{002}, 2θ = 22.64°) and minimum dip (I_{am} 2θ = 16.42°), as described by Kshirsagar *et al*. (2015), with Eq. 1,

$$(CrI\ \%) = (I_{002} - I_{am})/I_{002}\ x \quad 100 \tag{1}$$

where I_{002} is the maximum intensity of the crystalline region at 2θ and I_{am} is the minimum intensity of the amorphous region at 2θ.

Enzymatic saccharification

The RH that was pretreated at 160, 180, 200, and 220 °C for 7 min was used in the enzymatic saccharification. Enzymatic hydrolysis was carried out in a reaction mixture containing a 5% (w/v) substrate concentration in 20 mL of 50 mM sodium phosphate buffer (pH 6.5) and 0.02% w/v sodium azide, which was added to prevent microbial contamination. The effect of enzyme loading was studied with different enzyme concentrations, 10, 20, 30, and 40 FPU/g. Enzymatic saccharification proceeded at 50 °C and 180 rpm for 48 h. The aliquots slurry was withdrawn, centrifuged at 10,000 rpm for 10 min with the supernatant, and used for analysing the reducing sugar production. The effect of the temperature on the enzymatic saccharification was determined for several temperatures, 40, 50, 60, and 65 °C, with a 30 FPU/g enzyme concentration with saccharification conducted for 48 h at 180 rpm. The time course for enzymatic saccharification with cellulase from *B. licheniformis* 2D55 was compared with commercial cellulase (30 FPU/g) at two temperatures, 50 and 60 °C. Saccharification was maintained for 72 h with the slurry withdrawn at 12 h intervals for sugar analysis.

Two-step enzymatic saccharification

A two-step saccharification process on the pretreated RH was conducted in order to optimize the sugar production, enzyme usage, and substrate hydrolysis. In the one-step stage, saccharification was carried out for 72 h with a 5% (w/v) substrate concentration and

30 FPU/g cellulase at 60 °C. Meanwhile, for the two-step saccharification, the first and second steps were conducted for 36 and 24 h, respectively. After the first step, the samples were vacuum filtered to collect the liquid and solid residues. The solid residues from the first step was then re-suspended in 12 mL of sodium phosphate buffer (50 mM, pH 6.5) after filtration and further hydrolysed for the second step, again for 60 h. No additional enzyme was added. Samples were withdrawn at regular intervals for reducing sugar analysis. The hydrolysate obtained from the first and second steps were pooled together to determine the total reducing sugar and soluble sugar contents.

Analysis

The total reducing sugar yield was determined from the hydrolysate using the dinitrosalicylic acid (DNS) method described by Miller (1959). The saccharification degree and sugar yield were measured and determined using Eqs. 2 and 3,

$$Saccharification\ (\%) = \frac{[Total\ reducing\ sugar\ (\mathrm{g/L})\ x\ 0.9\ x\ 100]}{[substrate(\mathrm{g/L})\ x\ potential\ sugar\ (\mathrm{g/g})]} \quad (2)$$

$$Sugar\ yield\ (\mathrm{g/g}) = \frac{product}{substrate} \quad (3)$$

where 0.9 is used to convert the polysaccharides to monosaccharides to account for water uptake during hydrolysis.

The monomeric sugar analysis was carried out with a high-performance liquid chromatograph (HPLC) (Jasco, Japan) equipped with a refractive index (RI) detector. The sugars were separated with a NH_2 column at 80 °C and mobile phase of 80% (v/v) acetonitrile at a flow rate of 2 mL/min. Pure glucose, xylose, cellobiose, and arabinose were used as standards. The monomeric sugar yield was measured and determined using Eq. 4,

$$Glucose\ yield\ (\mathrm{g/g}) = \frac{glucose\ produced\ (\mathrm{g})}{substrate\ (\mathrm{g})} \quad (4)$$

The same equation was used for xylose, cellobiose, and arabinose by replacing glucose with each monomeric sugar.

Statistical analysis

The values shown were the means of triplicates ± the standard deviation. The data were analyzed using the SAS software package version 9.4 (SAS Institute Inc., Cary, NC) with a one-way analysis of variance (ANOVA). Duncan's multiple range test was used to compare the means among the treatment groups. Differences with a p less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Effect of HPSP on Weight Loss, pH, and Chemical Composition of Rice Husk

To evaluate the effect of high-pressure steam, the RH was pretreated with steam at temperatures from 180 to 220 °C and at pressures between 0.3 and 2.8 MPa for 2 to 10 min. After each pretreatment, the loss in weight, pH of the hydrolysate, and chemical

composition of the RH were determined. Table 1 shows that the weight loss varied from 15.26% to 42.27% at different temperatures and times. The weight loss increased with an increase in the pretreatment temperature and time. For each pretreatment temperature, the weight loss was observed to be highest at the 10 min residence time. For instance, at 160 °C, the weight loss increased from 15.26% to 25.46% when the residence time was increased from 2 to 10 min. A similar trend in the weight loss was also observed for 180, 200, and 220 °C. The maximum weight loss was observed at 220 °C. At 10 min and 220 °C pretreatment condition the weight loss (42.27%) was seen to be the highest compared to the 2, 4, and 7 min residence times. This result was similar to that reported by Mahmud *et al*. (2013), who demonstrated an increase in the weight loss from 3.41% to 18.17% after the super-heated steam pretreatment of oil palm mesocarp fibre (OPMF). According to Zakaria *et al*. (2014), the weight loss of hydrothermally pretreated OPMF was highest when the harshest pretreatment severity was carried out. In their study, a weight loss increase from 21.7% to 42.7% was observed when the pretreatment temperature increased from 180 to 220 °C for 20 min. The increase in weight loss was caused by the removal of some lignocellulosic components that can be dissolved by steam, which is an indication of partial solubilisation of hemicellulose, and makes cellulose more susceptible to enzymatic attack.

The pH of the hydrolysate was found to decrease from near neutral to acidic for all of the samples. It is worth noting that the pH of the hydrolysate followed a trend opposite to what was observed for the weight loss. The pH of the hydrolysate decreased from 4.78 to 4.58 when the pretreatment time increased from 2 to 10 min at a temperature of 160 °C, and from 4.33 to 3.69 when the temperature increased from 180 to 200 °C for 2 min. These results suggested that the pH value was influenced by the pretreatment temperature, as well as the pretreatment time. The pH was observed to be the lowest (pH 3.42) when the RH was pretreated at 220 °C for 10 min. These results were in accordance with the work of Zakaria *et al*. (2015a), who reported a similar decrease in the pH from 4.10 to 3.39 when the temperature increased from 170 to 210 °C during the hydrothermal pretreatment of oil palm frond fibre (OPFF). The decrease in pH was attributed to the accumulation of acetic acid, which resulted from the cleavage of acetyl groups located in the hemicellulose matrix, and resulted in hemicellulose degradation (Möller *et al*. 2011; Xiao *et al*. 2013; Ho *et al*. 2014).

The chemical composition of RH was greatly affected by the high pressure steam pretreatment (HPSP). The HPSP was shown to increase the cellulose and lignin content, whereas the hemicellulose was drastically reduced. Also, the hemicellulose content decreased as the pretreatment temperature increased with retention time. The decreasing hemicellulose content was a reflection of the decrease in pH that was observed, which was determined by the similar trend observed for the pH and hemicellulose. The hemicellulose content ranged from 34.81% in the untreated samples to 2.61% in the pretreated samples. The most noticeable hemicellulose reduction occurred at the pretreatment temperatures of 200 and 220 °C, and when the retention time was 7 and 10 min. The lowest hemicellulose content (2.6%) was observed at a steam temperature of 220 °C with a 10 min residence time. It was reported by Baharuddin *et al*. (2012, 2013) that the lowest hemicellulose contents of 3% and 1.2% occurred at the steam temperature of 230 °C for the high-pressure steam pretreated oil palm empty fruit bunch (OPEFB). It was apparent that the partial solubilisation of the RH hemicellulose was higher than that reported by Baharuddin *et al*. (2013). This may have been due to the different temperatures and agro-waste biomass used. According to Kabel *et al*. (2007), the hemicellulose removal exposes the surface of cellulose, and hence, increases the accessibility of cellulase to cellulose microfibrils. In

contrast, the cellulose content increased from 37.13% in the untreated RH to above 50% in the RH treated at high temperatures. The maximum cellulose content (66.36%) was observed at a steam temperature of 200 °C and pretreatment time of 7 min. However, when the pretreatment temperature increased from 200 to 220 °C, specifically for 7 and 10 min, the cellulose content decreased from 66.36% to 56.45% and 54.42%, respectively. This phenomenon was previously observed by Zakaria *et al*. (2015a) and Baharuddin *et al*. (2013) in the hydrothermal and HPSP of oil palm biomass. Zakaria *et al*. (2015a) observed a reduction in the cellulose content from 58.5% to 53.2% when the temperature was increased from 200 to 210 °C. Similarly, Baharuddin *et al*. (2013) observed a decrease in the cellulose from 70.6% to 65.9% when the temperature was increased from 210 to 230 °C. This was due to the thermal conversion of cellulose to other soluble products. The reduction of cellulose at high temperatures was an indication of "excessive cooking", which resulted in the partial degradation of cellulose to hexose sugar. Furthermore, Jørgensen *et al*. (2007) suggested that it is possible for cellulose to degrade to glucose depending on the severity of the pretreatment. As for the increase in the lignin content, a similar result was observed in previous studies that used acidic conditions (Donohoe *et al*. 2008; Sabiha-Hanim *et al*. 2011; Pu *et al*. 2013; Zakaria *et al*. 2014). Lignin (high molecular weight) is usually dissolved during pretreatment, but is later redeposited (low molecular weight) on biomass during the condensation process (Zakaria *et al*. 2015a). Meanwhile, according to Li *et al*. (2007), lignin is removed to a limited extent during steam pretreatment at high pressure, but is redistributed on the outer fibre surface as a result of melting and the depolymerisation/repolymerisation reactions (Chua and Wayman 1979). Thus, the pretreatment conditions of 200 °C and a 7 min retention time were selected as the optimum conditions affecting the pH, weight loss, and composition of the RH pretreated with high-pressure steam. Alteration to the lignin constituent and degradation of the hemicellulose resulted in an increase in the cellulose composition after pretreatment.

SEM Analysis

The SEM images provided information about the surface morphological changes before and after each pretreatment. A more ordered and smoother surface was observed for the untreated RH (Fig. 1A). Additionally, the presence of a silica body protrusion was noticed. A similar morphology was reported for the micrographs of RH in previous studies (Ang *et al*. 2012; Johar *et al*. 2012). After pretreatment, the surface of the RH became rougher, irregularly coarser, and disordered due to the removal of superficial non-cellulosic layers, such as pectin, hemicellulose, wax, and other impurities, covering the surface of the RH.

Partial removal of silica bodies was observed when the temperature was increased from temperatures 160 and 180 °C, as shown in Figs. 1B and 1C. However, the most apparent morphological changes were observed at the pretreatment temperatures of 200 and 220 °C (Figs. 1D and 1E). At these temperatures, there were no noticeable silica bodies observed. This observation was supported by the EDX supplementary analysis (S1 to S2), which revealed a reduction in the SiO_2 content from 44.27% in the untreated RH to 18.22% in the pretreated RH. This was an important observation because silica bodies can hinder enzyme accessibility to cellulose microfibrils. The micrograph for the RH pretreated at 200 °C showed surface disruption from the creation of porous holes.

The porous holes were expected to allow for easier flow and cause reactive areas of enzymes, thus increasing the accessibility of the enzyme. The creation of holes could

have been caused by swelling, removal of silica bodies, and re-localisation and re-deposition of lignin on the surface of the RH.

Table 1. Effect of HPSP on Weight Loss, pH, and Chemical Composition of Rice Husk

Pretreatment Temperature (°C) / pressure (MPa)	Pretreatment time (min) & pH		Weight loss (%)	Chemical composition (%)			
				Cellulose	Hemicellulose	Lignin	Others
Untreated RH	0	6.87	0	37.13 ± 0.13	34.81 ± 0.22	18.20 ± 0.79	9.86 ± 0.21
160 / 0.30	2	4.78	15.26	39.15 ± 0.38	33.04 ± 0.35	18.30 ± 0.68	9.51 ± 0.07
160 / 0.40	4	4.72	16.42	42.06 ± 0.45	30.22 ± 1.27	18.55 ± 1.37	9.17 ± 0.12
160 / 0.60	7	4.53	18.75	45.00 ± 0.49	28.33 ± 1.11	19.62 ± 0.63	7.05 ± 0.22
160 / 0.76	10	4.58	25.46	48.50 ± 0.06	25.52 ± 0.09	19.90 ± 0.98	6.08 ± 0.04
180 / 0.80	2	4.33	30.48	43.63 ± 0.33	30.94 ± 0.42	18.34 ± 1.05	7.39 ± 0.11
180 / 1.10	4	4.29	32.57	45.60 ± 0.64	25.66 ± 1.51	18.56 ± 1.01	10.18 ± 0.11
180 / 1.36	7	4.21	34.50	47.38 ± 1.58	21.44 ± 0.70	19.94 ± 0.37	11.24 ± 0.41
180 / 1.50	10	4.18	37.38	49.95 ± 1.28	18.26 ± 0.09	20.30 ± 0.42	15.49 ± 0.32
200 / 1.62	2	3.69	33.50	51.85 ± 0.50	20.03 ± 0.53	19.07 ± 1.76	9.32 ± 0.08
200 / 1.70	4	3.62	35.25	58.17 ± 1.44	12.78 ± 1.25	20.20 ± 0.72	8.93 ± 0.34
200 / 1.85	7	3.58	38.45	66.36 ± 0.52	4.61 ± 0.63	21.52 ± 0.47	7.49 ± 0.17
200 / 2.00	10	3.53	40.22	66.02 ± 0.75	5.10 ± 0.55	23.01 ± 0.53	8.32 ± 0.24
220 / 2.20	2	3.55	37.72	62.15 ± 0.25	16.98 ± 1.02	20.40 ± 0.52	5.67 ± 0.09
220 / 2.37	4	3.51	38.43	64.23 ± 1.38	10.63 ± 0.87	22.60 ± 0.21	10.36 ± 0.10
220 / 2.65	7	3.48	40.61	56.45 ± 2.01	3.34 ± 0.49	26.00 ± 0.64	14.21 ± 0.42
220 / 2.80	10	3.42	42.27	54.42 ± 1.25	2.61 ± 1.32	27.09 ± 0.71	13.17 ± 0.15

The observation of removal of the silica bodies was previously reported for steam pretreated OPEFB (Bahrin *et al*. 2012; Shamsudin *et al*. 2012) and combined acid–alkali pretreated RH (Barana *et al*. 2016). Additionally, the RH pretreated at 220 °C showed almost complete disruption, and a crack formed along the inner structure. In fact, the surface became smoother as the pretreatment temperature increased from 200 to 220 °C. The observed crack might have resulted from the partial degradation of cellulose. This observation was supported by the reduction in the cellulose content observed earlier for the

RH pretreated at 220 °C for 7 min, as depicted in Table 1. In addition, the degradation of cellulose was also supported by the results of the supplementary analysis, S4 and S5, where a reduction in the carbon (C) content from 85.41% to 81.76% was seen.

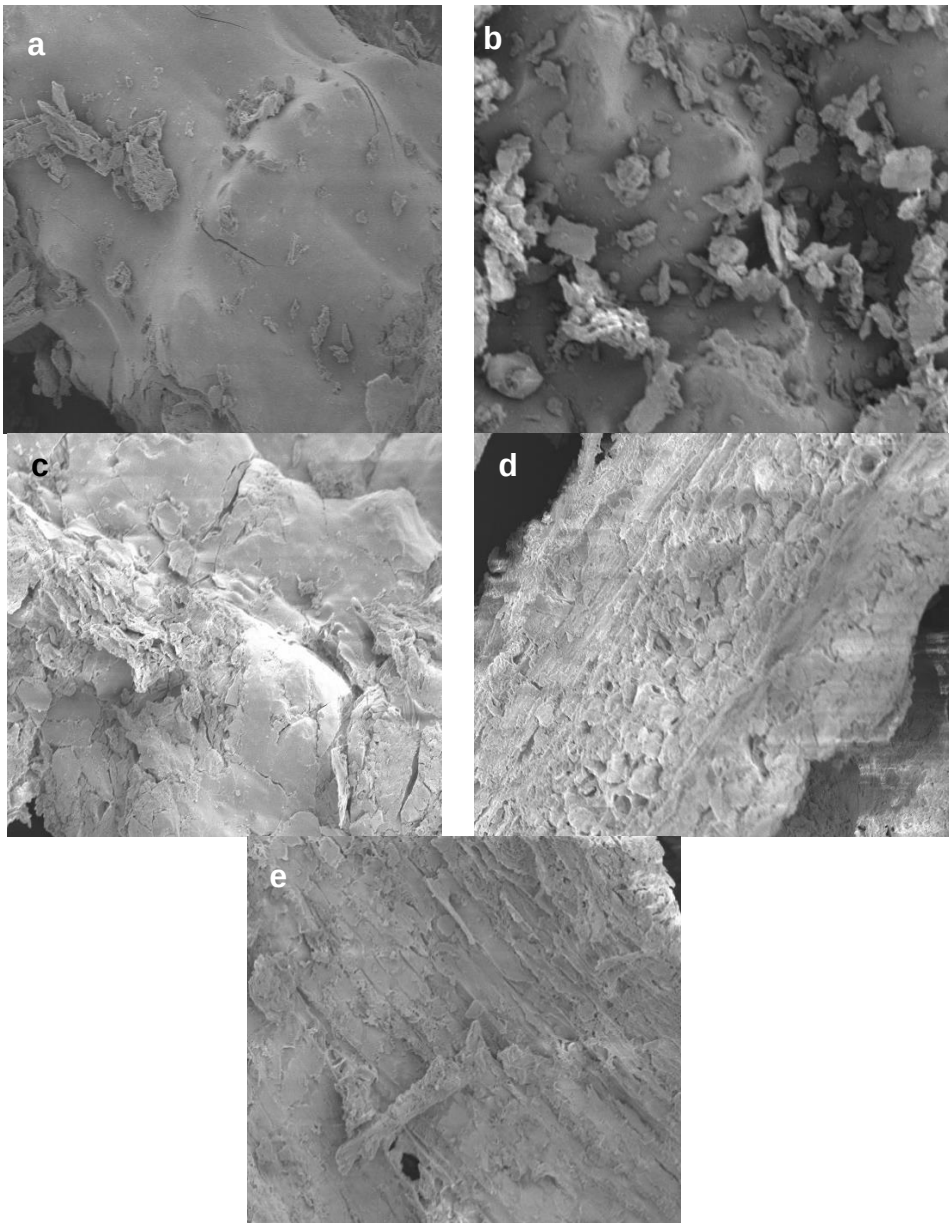

Fig. 1. SEM micrographs at 1000x magnification of the (A) untreated RH and RH samples pretreated at (B) 160 °C, (C) 180 °C, (D) 200 °C, and (E) 220 °C for 7 min.

FTIR Analysis

The FTIR spectra of the untreated and pretreated RH demonstrated a major broad absorption band pattern from 3200 to 3600 cm^{-1} that was due to H-bonded -OH group vibrations present in the cellulose, hemicellulose, and lignin (Fig. 2). The band was less obvious in the untreated RH. This suggested there was partial degradation of hydrogen bonds, which is a positive step toward enhancing the accessibility of cellulose to enzymatic attack. The absorption bands at 2918 and 2840 cm^{-1} were assigned to C-H stretching

vibrations. These bands gradually diminished as the pretreatment temperature increased. According to Wang *et al*. (2009), the C-H bands corresponded to the aliphatic moieties of cellulose and hemicellulose. A similar observation was reported for super-heated steam pretreated OPEFB (Bahrin *et al*. 2012). Additionally, this spectra profile was similar to that for RH reported by Johar *et al*. (2012). The band at 1640 cm^{-1} was the bending mode of the –OH groups of the absorbed water (Smidt and Schwanninger 2005). Meanwhile, the band at 900 cm^{-1} arose from C-O-C stretching at the β-(1-4) glyosidic linkages (Cao and Tan 2004). The reduction of these bands suggested there was decomposition of the hemicellulose constituent of the RH. A shoulder at around 1700 cm^{-1} in the RH pretreated at 160 °C (H2) and 180 °C (H3) was seen to disappear when the pretreatment temperature increased to 200 and 220 °C. This band was associated with uronic ester and acetyl groups in the hemicellulose (Alemdar and Sain 2008). The disappearance of this peak indicated the removal of non-cellulosic hemicellulose. The shoulder near 1700 cm^{-1} may have also been associated with the presence of C=O bonds, which is a known property of hemicellulose and lignin (Abraham *et al*. 2011). Similarly, the presence of this shoulder was reported by Johar *et al*. (2012) and Nascimento *et al*. (2016) in pretreated RH for nanocellulose. Moreover, the peak at 1512 cm^{-1} corresponded to C=C stretching of the aromatic ring of lignin. Another important band that identified the cellulose component was at 1420 cm^{-1}. It was observed that this band increased with an increase in the pretreatment temperature. The 1420 and 1430 cm^{-1} absorption bands were associated with amorphous/crystalline cellulose. The band at 1000 to 1200 cm^{-1} depicted C-O-C stretching, C-O covalent bonds, and C-OH linkages dominant in cellulose, hemicellulose, and lignin (Sun *et al*. 2008; Binod *et al*. 2012).

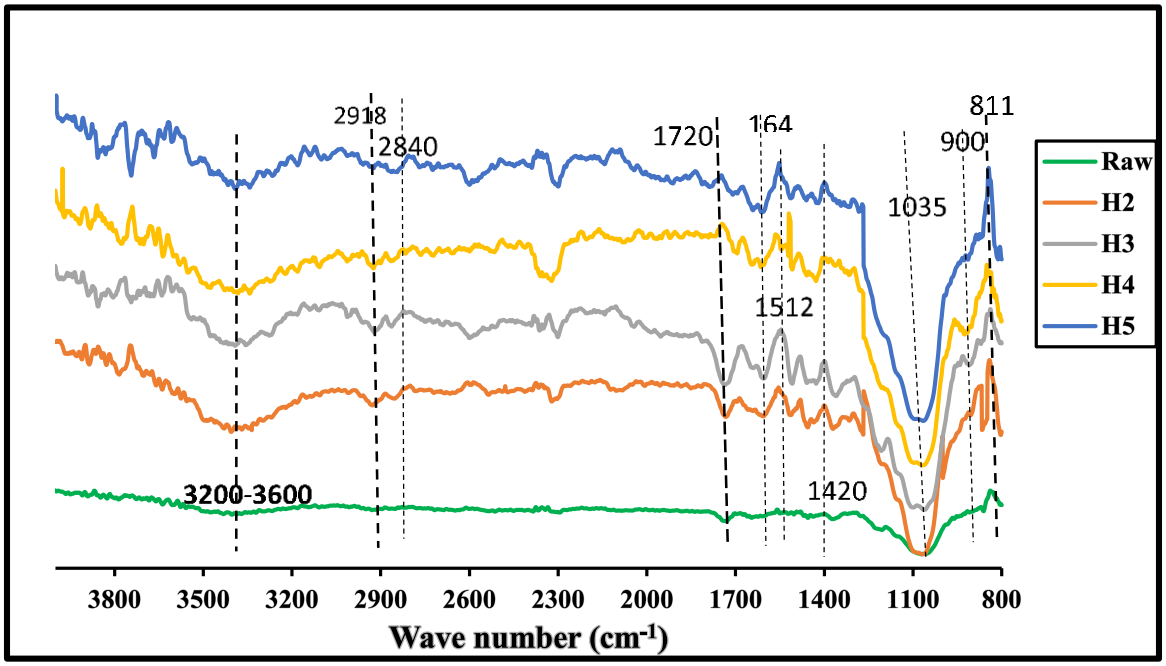

Fig. 2. FTIR spectra of the raw RH and RH high-pressure steam pretreated at 160 °C (H2), 180 °C (H3), 200 °C (H4), and 220 °C (H5) for 7 min

It was obvious that the intensity of the band at 1035 cm^{-1} was higher in comparison with the untreated RH sample. This result indicated that there was an increase in the cellulose proportional content of the RH after the HPSP. It was suggested by Ang *et al*.

(2012) that the increase in intensity of this band may also imply the dissolution of non-cellulose components, thereby increasing the cellulose content of the RH proportionately. This result was in agreement with the increase in the cellulose and lignin content observed in the compositional analysis of the pretreated RH earlier in this study. The intensity of the 900 cm^{-1} band increased as the pretreatment temperature increased, but the band later disappeared when the pretreatment temperature was raised to 220 °C. This implied that at higher temperatures (220 °C for 7 min), the cellulose content of the RH might be disordered or degraded. This result supported the earlier observation of a decrease in the cellulose content of the RH pretreated at 220 °C seen during the compositional analysis. According to Labbé *et al*. (2005), disorder in the cellulosic structure results from the deformation of β-glyosidic linkage vibrations and hydrogen bond rearrangement. The disappearance of the band at approximately 900 cm^{-1} was also reported for RH subjected to ionic liquid acid pretreatment (1-butyl-3-methylimidazolium chloride and 1-ethyl-3-methylimidazolium diethyl phosphate). The increase in the pretreatment temperature led to an increase in the dissociation and reallocation of lignin from the aromatic hydrogen bonds. This was demonstrated by the increase in the sharp peak at 811 cm^{-1} (Bahrin *et al*. 2012). The frequencies of the absorption bands are given in Table 2.

Table 2. Group Frequency of Absorption Bands for High-Pressure Steam Pretreated Rice Husk

Wavenumber location (cm^{-1})	Vibration	Assignment and origin	References
890 900	C-O-C	β-glyosidic linkages of cellulose	(Johar *et al*. 2012) (Cao and Tan 2004)
1000 - 1200	C-O	C-O bond of polysaccharides	(Barana *et al*. 2016) (Sun *et al*. 2008)
1035	C-O C-OH	C-O vibration stretching of cellulose and lignin	(Guo *et al*. 2008) (Ang *et al*. 2012)
1513 1512	C=C-C C=C	Aromatic skeletal stretching in lignin Aromatic lignin	(Coates 2000) (Kshirsagar *et al*. 2015)
1635 - 1649	O-H	O-H bending of absorbed water molecule of cellulose	(Smidt and Schwanninger 2005) (Łojewska *et al*. 2005)
1700 - 1730	C=O	Ketone, carboxylic acid, uronic ester, acetyl group of hemicellulose	(Alemdar and Sain 2008) (Johar *et al*. 2012; Tandy *et al*. 2010)
2918 2850	C-H	C-H stretching in cellulose and hemicellulose	(Liu *et al*. 2007) (Ang *et al*. 2012)
3000 - 4000	O-H	Hydrogen bonded O-H stretching	(Nascimento *et al*. 2016) (Kshirsagar *et al*. 2015)

XRD Analysis

The X-ray diffractograms of the untreated and pretreated RH are shown in Fig. 3. An increase in the CrI (%) was observed when the untreated RH (47.08%) and high-pressure steam pretreated RH (160 °C, 50.72%; 180 °C, 52.8%; 200 °C, 62.03%; 220 °C, 64.06%) were compared. Cellulose is a complex polymer consisting of both crystalline and amorphous regions. The crystalline nature of cellulose is due to hydrogen bonding and Van Der Waal's force of interaction (Zhang and Lynd 2004). However, hemicellulose and lignin are amorphous in nature. The cellulose crystallinity is an important factor that influences enzymatic hydrolysis (Sindhu *et al*. 2012). The XRD spectra displayed diffraction peaks that are typical of cellulose 1 with main peaks at 2θ values of 22.64°, 16.42°, and 34.5° (Wang *et al*. 2009; Johar *et al*. 2012). Based on the XRD spectra, the diffraction peaks increased with an increase in the pretreatment temperature. Likewise, the CrI increased as the temperature of the pretreatment increased. The lowest CrI (47.08%) was observed in the untreated RH, and the RH pretreated at 220 °C produced the highest CrI at 64.06%. During the HPSP, hydroxonium ions penetrated into the more accessible amorphous region, resulting in the cleavage of glyosidic bonds, which eventually led to the release and exposure of the crystalline domain. This phenomenon was also reported to occur during acid pretreatment (de Souza Lima and Borsali 2004).

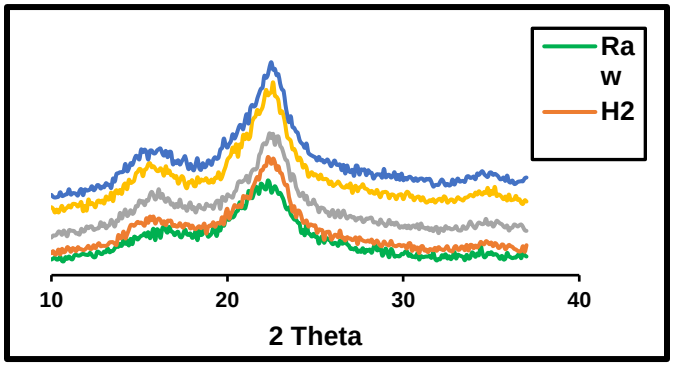

Fig. 3. XRD diagram of the raw RH and RH high-pressure steam pretreated at 160 °C (H2), 180 °C (H3), 200 °C (H4), and 220 °C (H5) for 7 min

The findings of this analysis were in agreement with the study by Johar *et al*. (2012), who reported an increase in the CrI from 46.8% in the untreated RH to 50.2%, 56.5%, and 59.0% in the alkali, bleached, and acid treated RH, respectively. In a study conducted by Ang *et al*. (2012), RH was pretreated with an ionic liquid [(BMIM)CI], which resulted in an increase in the CrI from 46% in the untreated RH to 56.1% in the ionic liquid pretreated RH. According to Abraham *et al*. (2011), the removal of lignin and other cementing materials such as pectin, by acid or alkali pretreatment, results in a rise in the CrI of the lignocellulosic fibres.; however, at higher acid and alkali concentrations, the CrI was seen to decrease. Zhang and Lynd (2004) explained that an increase in the CrI could not have a negative effect on enzymatic hydrolysis.

Effect of Enzyme Loading on Sugar Production and Saccharification

Enzyme loadings of 10 to 40 FPU/g were applied for the saccharification of the RH pretreated at 160 to 220 °C (Figs. 4A and 4B). The results showed that the reducing sugar

production and saccharification percentage increased with an increase in the pretreatment temperature. It was discovered that when saccharification was performed on the untreated RH, the reducing sugar production and saccharification degree were reduced to the lowest values of 0.07 g/L and 0.18%, respectively. However, for the pretreated RH, the reducing sugar content was found to increase from 1.32 g/L to above 15 g/L, and the saccharification percentage increased from 3.24% to above 30% for pretreatment temperatures of 160 to 220 °C for each enzyme loading. It was observed that the highest reducing sugar content and saccharification degree were obtained at the pretreatment temperature of 200 °C for all of the enzyme loadings tested. Further increasing the pretreatment temperature to 220 °C had a negative effect on the enzymatic saccharification. Both the reducing sugar production and saccharification degree increased as the concentration of enzyme increased. The maximum reducing sugar production and saccharification degree recorded were 17.15 g/L and 43.73%, respectively, at 200 °C with a 30 FPU/g enzyme concentration. However, at the highest enzyme concentration of 40 FPU/g, the reducing sugar content and saccharification degree were drastically reduced to 12.20 g/L and 31.10%, respectively.

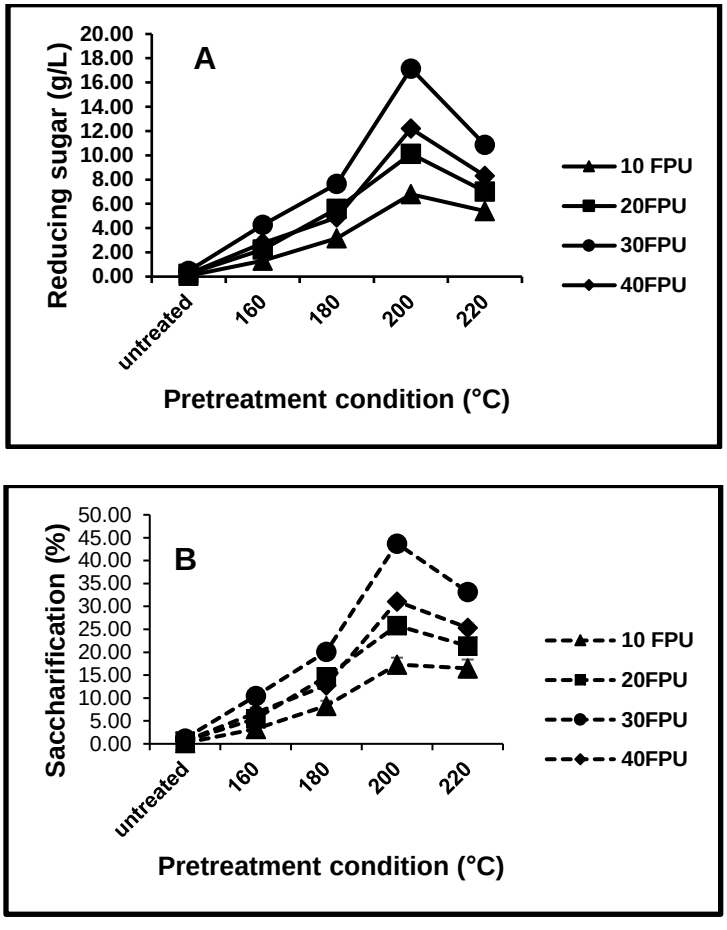

Fig. 4. Effect of enzyme loading on (A) reducing sugar production and (B) saccharification of high pressure steam pretreated rice husk

Based on the results of this analysis, the pretreated RH had a sugar yield that was 16 times higher than that of the untreated RH. The thermal treatment caused a breakdown of resins and gums into soluble and insoluble oil, dissolution of hemicellulose, removal of phenolic compounds, and migration of lignin, all of which loosened the intact structure of

the cellulose-hemicellulose-lignin matrix, and enhanced enzymatic hydrolysis (Hsu *et al*. 2010; Pu *et al*. 2013; Zakaria *et al*. 2015a). This could have also been due to the hemicellulose barrier to the cellulose structure and irreversible binding to the cellulase enzymes, which generates unproductive hydrolysis.

The removal of hemicellulose resulted in a dramatic increase in the saccharification rate. Conversely, a higher pretreatment temperature can have a negative effect on the lignocellulosic components. This was illustrated by the low amount of reducing sugar produced when the pretreatment temperature increased from 200 to 220 °C, which suggested that part of the cellulose component was degraded and could not be converted into glucose by saccharification. Zakaria *et al.* (2015a) also reported that the formation of inhibitors, such as hydroxymethylfurfural, and phenolic compounds at higher pretreatment severities could also be detrimental to the enzyme. This finding was in agreement with Mahmud *et al*. (2013), who reported a decrease in the hydrolysis rate from 58.28% to 33.23% with an increase in the pretreatment temperature from 180 to 210 °C for OPMF treated with super-heated steam.

Table 3. Effect of Enzyme Loading on Reducing Sugar Production from Enzymatic Hydrolysis

Enzyme source	Substrate	Enzyme loading (FPU/g)	Reducing sugar (g/L)	Hydrolysis yield (%)	Reference
Geobacillus stearothermophillus	Date palm leaves	10 20 30 40	13.3 20.0 31.6 27.0	33.3 49.0 71.0 62.0	(Alrumman 2016)
Lysinibacillus sphaericus	Rice straw	10 20 30 40 50	- - - - -	15.6 42.1 59.7 69.5 63.4	(Gupta and Parkhey 2014)
BIOMASS corporation	Corn cob	7.5 15 30	15.7 19.2 19.3	- - -	(He *et al*. 2016)
Trichoderma aureoviride	Rice straw	91		61	(Xu *et al*. 2015)
Bacillus subtilis	Wheat straw	10 15 20 40	8.7 10.4 14.6 15.6	19.6 23.4 33.1 35.0	(Akhtar *et al*. 2001)
(Cellic® CTec2)	Rice husk	6 12 18 22	- - - -	43.0 60.0 62.0 62.0	(Wood *et al*. 2016)
Celluclast	Oil palm empty fruit bunch	21.33 42.66 63.99 85.32	15.0 16.4 18.2 22.1	- - - -	(Baharuddin *et al*. 2012)
B. licheniformis 2D55	Rice husk	10 20 30 40	6.78 10.12 17.15 12.17	17.3 25.8 43.7 31.1	This study

-: not reported

It is essential that the dosage of enzyme is minimized to reduce the cost of production. According to Alrumman (2016), the increase in cellulase concentration resulted in a remarkable increase in the enzymatic saccharification rate of date palm leaves. However, further increases in the cellulase concentration were not found to increase the hydrolysis yield. This could have been due to hydrodynamic instability and high slurry suspension as a result of improper mixing (Akhtar *et al*. 2001). In this study, a similar trend was observed for the fermentable sugar production and saccharification percentage. A comparison between the sugar production and enzymatic saccharification at different enzyme loadings reported in other literature is shown in Table 3. Similar to this study, Alrumman (2016) also reported an optimal enzyme concentration of 30 FPU/g. However, increase in the enzyme concentration from 10 to 30 FPU/g increased the saccharification degree from 33.3% to 71.0%. The study results indicated better enzyme performance at lower enzyme concentration of 30 FPU/g with 43.7% saccharification when compared with report by Aktar *et al.*, (2001), which showed higher enzyme concentration of 40 FPU/g but lower saccharification at 35.0%. Contrary to this, a much higher optimal enzyme concentration up to 40 and 85.32 FPU/g have been reported by, for instance, Gupta and Parkhey (2014), and Baharuddin *et al.* (2012), respectively. An enzyme concentration of 40 FPU/g was reported to be optimal for enzymatic saccharification, and an increase in the enzyme concentration from 10 to 40 FPU/g increased the saccharification degree from 15.6% to 69.5%. Furthermore, an increase in the enzyme loading favourably increased enzymatic saccharification of rice straw (Gupta and Parkhey 2014) and RH (Wood *et al*. 2016).

Effect of Temperature on Sugar Production and Saccharification

The conformity of enzymes to temperature conditions obtained in industrial bioprocesses is crucial because industrial bioprocesses of lignocellulosic biomass require high temperatures. To evaluate the optimal temperature for enzymatic hydrolysis of RH, saccharification was performed at temperatures from 40 to 65 °C with an optimum enzyme loading of 30 FPU/g (Figs. 5A and 5B). The reducing sugar production and saccharification degree significantly increased ($p < 0.05$), which corresponded to the increase in the pretreatment temperature. The untreated RH was poorly hydrolysed and had the lowest reducing sugar content of 0.22 g/L and a 0.55% saccharification degree. However, when the RH was subjected to pretreatment, the reducing sugar content and saccharification degree increased from 1.88 to 21.01 g/L and from 4.60% to 53.56%, respectively after pretreatment. Similarly, reducing sugar production and, as well as the saccharification degree, increased as the hydrolysis temperature increased.

The maximum reducing sugar production and saccharification degree were 21.01 g/L and 53.56%, respectively, which were recorded for the RH pretreated at 200 °C and when the saccharification temperature was 60 °C. The reducing sugar production and saccharification degree were observed to be significantly different ($p < 0.05$) for 60 °C saccharification, and a further increase in the temperature above 60 °C decreased both the reducing sugar content and saccharification percentage to 15.22 g/L and 38.81%, respectively. Enzymatic hydrolysis of most bacteria and fungi cellulase is commonly performed at 50 °C (Kshirsagar *et al*. 2015; Rojas-Rejón *et al*. 2016), and sometimes at temperatures as low as 35 °C (Jeya *et al*. 2009).

The results of this analysis showed that enzymatic hydrolysis at 60 °C had a comparable yield with hydrolysis performed at 50 °C. This was due to two different reasons; first, the innate thermal stability property of the cellulase, and second, the

availability of more accessible surfaces for the binding of the cellulase to the RH cellulose, which prevented thermal denaturation of the enzyme at 60 °C. Additionally, a decline in the reducing sugar production and rate of hydrolysis at 65 °C was noted to have resulted from the thermal deactivation of the enzyme.

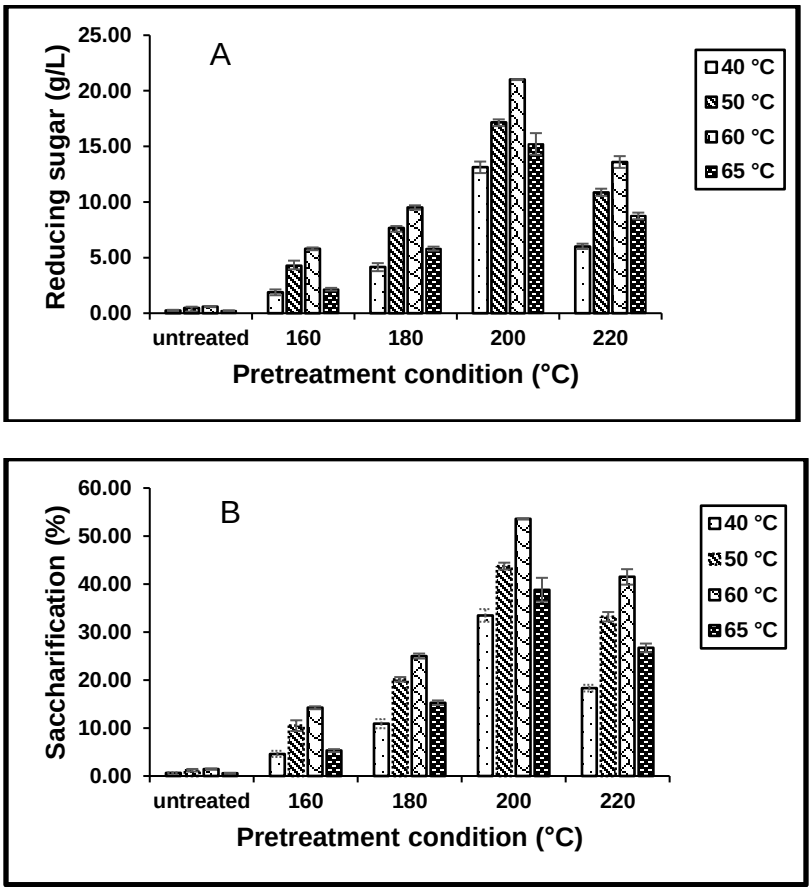

Fig. 5. Effect of temperature on the (A) reducing sugar production and (B) saccharification degree of high-pressure steam pretreated RH

At high temperatures, the activity of the enzyme can be reduced by unfolding the enzyme structure, resulting in a decrease in the hydrolysis rate (Salwanee *et al*. 2013). In this study, a similar trend of reduction in the fermentable sugar production and saccharification degree at high temperatures was observed, which was in accordance with Salwanee *et al*. (2013). However, variation occurred with respect to the optimum temperature required for enzymatic hydrolysis (Table 4). Contrary to this study, Park *et al*. (2012) reported that a temperature of 50 °C was optimal for enzymatic saccharification, and an increase in the temperature to 70 °C resulted in a decrease in the reducing sugar produced from 8.5 to 6.0 g/L. Similarly, Lai and Idris (2016) and Mahamud and Gomes (2012) also reported an optimum temperature of 50 °C for enzymatic saccharification of microwave-alkali pretreated oil palm trunk and sodium hydroxide pretreated sugar cane bagasse, respectively. However, a study reported an optimum saccharification temperature of 60 °C (Zhao *et al*. 2009), which is similar to the present study with comparable reducing sugar concentration as shown in Table 4.

Table 4. Effect of Temperature on the Reducing Sugar Production from Enzymatic Hydrolysis

Substrate	Enzyme source	Temperature (°C)	Sugar (g/L)	Hydrolysis Yield (%)	Reference
Switched grass	JTherm (thermophilic bacteria cocktail)	50 70 80	8.5 8.0 6.0	- - -	(Park *et al*. 2012)
Sugarcane bagasse	*Paenibacillus*	20 40 50 60	0.2 0.49 0.45 0.10	- - - -	(Hu *et al*. 2016)
Date palm leaves	*Geobacillus stearothermophillus*	40 50 60 70	20.0 32.6 20.2 10.5	46.36 71.23 50.52 27.13	(Alrumman 2016)
Sugarcane bagasse	*Tichoderma* sp.	20 30 40 50 60	- - - - -	5.51 12.94 29.68 37.29 33.11	(Mahamud and Gomes 2012)
Soya bean straw	Cellulase (Wuxi company china)	40 45 50 55	0.175 g/g 0.22 g/g 0.22 g/g 0.20 g/g	- - 51.22 -	(Xu *et al*. 2007)
Cellulose	*Tichoderma* cellulase (Sigma)	50 60 70	22.0 23.0 3.0	- - -	(Zhao *et al*. 2009)
Oil palm empty fruit bunch	Celluclast	30 40 50	7 22 27	- - -	(Baharuddin *et al*. 2012)
Rice husk	*B. licheniformis* 2D55	40 50 60 65	13.12 17.15 21.01 15.22	33.45 43.73 53.56 41.48	This study

-: not reported

Effect of Hydrolysis Time on Sugar Production and Saccharification in Comparison with Commercial Cellulase

Hydrolysis time is an important factor for monitoring the rate and progress of enzymatic saccharification. Because of the high demand for sugar, hydrolysis time should be minimized as much as possible for faster production and to avoid contamination. For this reason, enzymatic saccharification of RH that was high-pressure steam pretreated at 200 °C was performed over a period of 72 h at 50 and 60 °C with crude enzyme and commercial cellulase (Fig 6A and 6B). As a result, the production of reducing sugar and saccharification degree increased with an increase in the hydrolysis time. The commercial cellulase hydrolysed faster at 50 °C than the cellulase from *B. licheniformis* 2D55. With a commercial enzyme concentration of 30 FPU/g, 28.60 g/L of reducing sugar and a saccharification degree of 72.66% were obtained at 72 h, while a maximum reducing sugar content of 19.26 g/L and 49.10% saccharification percentage were observed with 30 FPU/g of *B. licheniformis* 2D55 cellulase at 60 h. This observation was expected to have been

caused by the commercial cellulase containing pure enzyme. It was also apparent that the cellulase from *B. licheniformis* 2D55 hydrolysed faster at 60 °C than the commercial cellulase. In fact, when the commercial cellulase was hydrolysed at 60 °C, the reducing sugar production and saccharification degree decreased as the hydrolysis time increased to 72 h. This was because most fungi are mesophilic in nature, thus might not contain enzymes that are adapted to thermophilic environments.

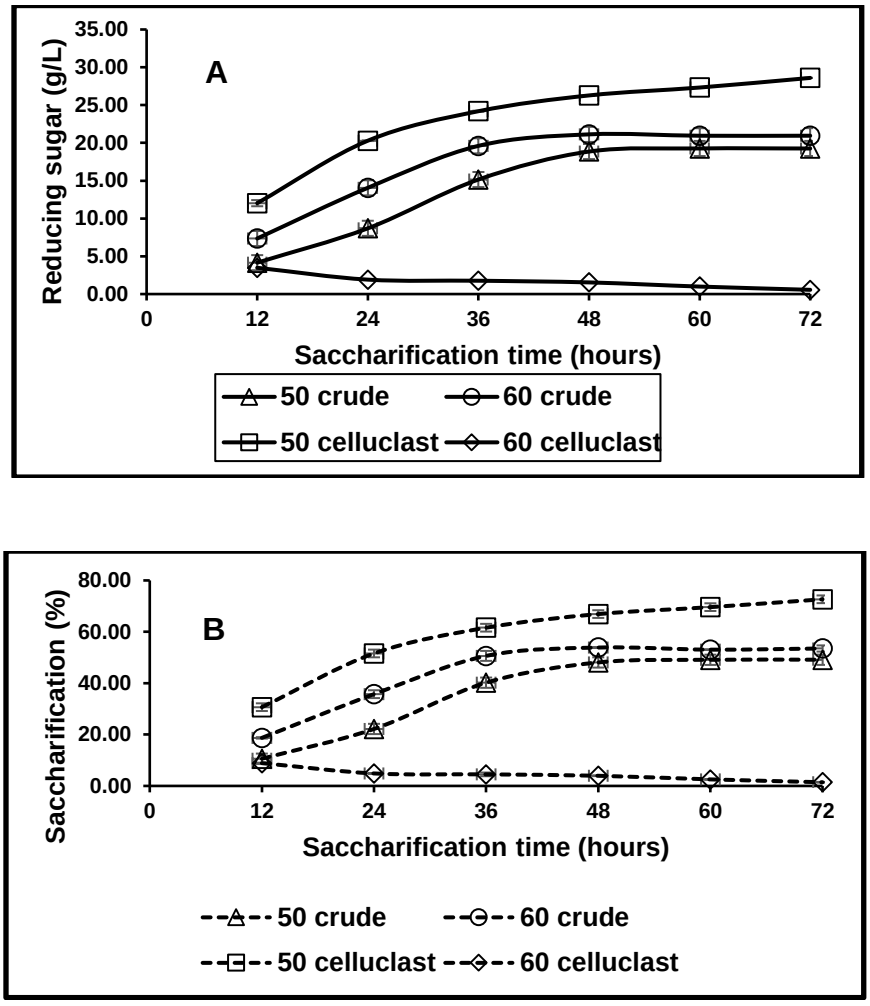

Fig. 6. Effect of time on the (A) reducing sugar production and (B) saccharification degree for hydrolysis conducted at 50 and 60 °C. RH pretreated at 200 °C for 7 min was used.

Many studies have reported on the enzymatic hydrolysis of commercial cellulase from fungi at 50 °C (Hsu *et al*. 2010; Zakaria *et al*. 2015b; Wood *et al*. 2016). After comparing the influence of time for hydrolysis of the *B. licheniformis* 2D55 cellulase at 50 and 60 °C, the results of this analysis suggested that conducting hydrolysis with *B. licheniformis* 2D55 cellulase at 60 °C can produce a faster rate of reducing sugar production and saccharification than that at 50 °C. The reducing sugar produced at 48 h of saccharification under 60 °C was significantly different ($p < 0.05$) from that produced at 50 °C. At 60 °C, the maximum reducing sugar content of 21.13 g/L and saccharification rate of 53.87% were observed at 48 h, while a 19.26 g/L reducing sugar content and 49.10% saccharification rate were observed to be the maximum values when the hydrolysis was conducted for 60 h at 50 °C. Therefore, it was concluded that the reaction was completed

within 48 h at 60 °C instead of 60 h at 50 °C. Hence, a shorter hydrolysis time was required when conducting hydrolysis at 60 °C. The increase in the reducing sugar production at 60 °C was explained by the rapid binding of the enzyme to the RH cellulose substrate, which released more products. This temperature helped to reduce viscosity, allowing for more contact between the enzyme and substrate. Furthermore, the temperature might have assisted in driving the reaction, increasing the number of collisions between the enzyme and substrate, and hence, more sugar was formed. In contrast, the reducing sugar content did not increase significantly for the extended hydrolysis times. Salwanee *et al.* (2013) suggested that at higher hydrolysis rates, an accumulation of products might occur, which would inhibit enzyme activity and result in the reduction of the hydrolysis rate.

There are very few studies that have reported on the optimum activity of cellulase by some thermophilic *Bacillus* species above 50 °C (Rastogi *et al*. 2010; Annamalai *et al*. 2013; Gaur and Tiwari 2015). Of those studies, most of them did not directly deal with enzymatic hydrolysis. Annamalai *et al*. (2014) applied cellulase from *B. carboniphilus* CAS 3 for the enzymatic saccharification of alkali-treated rice straw, and an optimum reducing sugar production of approximately 15.56 g/L was achieved after 96 h of hydrolysis. Likewise, the highest reducing sugar concentration (11.25 g/L) was obtained after 120 h of saccharification of acid-treated corn stover with cellulase from *Aspergillus fumigatus* Z5 (Liu *et al*. 2011). The results of this study were in accordance with Yu and Li (2015), who reported an optimum reducing sugar production at 60 °C after 48 h of enzymatic saccharification of corn stover using the cellulase of a thermophilic *Gracibacillus* sp. SKI.

Two-Step Enzymatic Saccharification of High-Pressure Steam Pretreated Rice Husk

It is essential to recycle the residual enzymes absorbed into the RH residues in subsequent saccharification processes to obtain an economically viable process. To achieve this, the RH residue obtained by removing the hydrolysate after 48 h was suspended in a fresh phosphate buffer with a pH of 6.5 to continue hydrolysis for 60 h. The results in Fig. 7 showed that the reducing sugar production and saccharification rate significantly increased ($P < 0.05$) as the hydrolysis time increased and reached a maximum at 24 h. Further increasing the time resulted in a decline of the hydrolysis yield. As illustrated, an additional reducing sugar content of 7.90 g/L and saccharification degree of 20.24% were obtained in the second saccharification step. The two-step saccharification process was found to increase the reducing sugar content and saccharification rate to 29.03 g/L and 73.5%, respectively (Table 5). This showed that a two-step saccharification process was better than continuous saccharification for 48 h. A higher saccharification degree was observed in the two-step saccharification process than in the one-step process. The two step enzyme saccharification increased the reducing sugar due to high sugar concentration which may block further enzyme hydration. It was previously reported that removal of accumulated sugar prevents feedback inhibition experienced in the initial step (Qi *et al*. 2011; Ouyang *et al*. 2013; Quiroga *et al*. 2015).

These results were in accordance with those obtained by Alrumman (2016), who reported that the saccharification rate increased from 71.03% to 94.88% in a multi-step enzymatic saccharification of alkali pretreated date palm leaves by crude cellulase from *Geobacillus stearothermophillus.* In addition, Yang *et al*. (2011) investigated enzymatic hydrolysis of steam exploded corn stover in three stages at high substrate loadings. From their study, an increase in the hydrolysis yield from 30% in the one-step hydrolysis process

to 37% was obtained. They also reported a shorter hydrolysis time of 36 h in the three-stage process compared to 72 h for the one-stage process. Furthermore, they proposed that the removal of the end-products improved the adsorption of cellulase onto the corn stover substrate, which enhanced the productivity during the second and third stages of the hydrolysis process. Interestingly, a considerable saccharification yield using cellulase from *B. licheniformis* 2D55 with a two-step hydrolysis method was comparable to that obtained with commercial cellulase (Celluclast). As a result, from the enzymatic hydrolysis of high-pressure steam pretreated RH using cellulase from *B. licheniformis* 2D55, a reducing sugar yield of 0.581 g/g substrate was obtained. Yu and Li (2015) conducted a study utilising crude cellulase from *Gracibacillus* SK1. Their study reported reducing sugar yields of 0.678 and 0.502 g/g substrate from the enzymatic saccharification of corn stover and rice straw, respectively. Furthermore, another study by Azadian *et al*. (2016) demonstrated a 0.6 g/g reducing sugar yield from the saccharification of rice straw using cellulase from *B. licheniformis* AMF-07. Reducing sugar yields from wheat straw of 0.214 g/g and from corn stover of 0.450 g/g were obtained with the cellulase from *Fomitopsis* sp. RCK2010 and *A. fumigatus*, respectively.

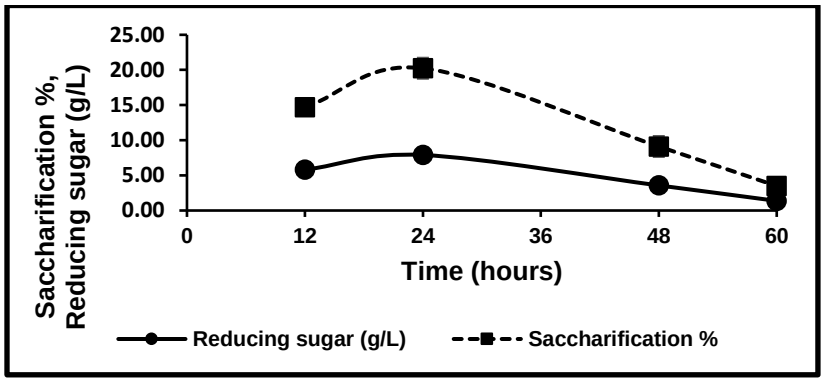

Fig. 7. Reducing sugar production and saccharification from the second-stage hydrolysis process. Hydrolysis was conducted at 60 °C on the RH pretreated at 200 °C for 7 min

Monomeric Sugar Composition of Rice Husk Hydrolysate

The RH hydrolysate obtained after enzymatic saccharification of the RH pretreated at different conditions with high-pressure steam was analysed for monomeric sugar determination. The results obtained from the HPLC analysis revealed that the RH hydrolysate contained mostly glucose, xylose, and cellobiose (Table 5). Moreover, the yield of glucose was much higher than for xylose and cellobiose. Meanwhile, the untreated RH had the lowest monomeric sugar contents. Taking into consideration the effect of the pretreatment temperature on the RH, the yield of glucose and cellobiose increased as the pretreatment temperature increased, but then decreased for the sample pretreated at 220 °C. The maximum glucose yields of 0.347 and cellobiose 0.041 g/g dry substrate were obtained from the RH pretreated at 200 °C.

This result followed a similar trend reported earlier for the reducing sugar production and enzymatic saccharification rate. However, the accumulation of cellobiose could have been due to the lower concentration of β-glucosidase present in the crude cellulase from *B. licheniformis* 2D55. During enzymatic saccharification, the enzyme β-glucosidase is required to convert cellobiose to glucose. The lower β–glucosidase

concentration limited the bioconversion process, which resulted in the accumulation of cellobiose and incomplete conversion.

Table 5. Effect of Pretreatment and Enzyme Saccharification Process on the Sugar Yield

Pretreatment Condition/ hydrolysis time	Reducing Sugar (g/L)	Saccharifi-cation (%)	Reducing Sugar Yield (g/g dry substrate)	Glucose (g/g dry substrate)	Xylose (g/g dry substrate)	Cellobiose (g/g dry substrate)
Untreated RH	0.58	1.46	0.01	0.002	0.010	-
160 °C	5.79	14.22	0.12	0.023	0.053	0.007
180 °C	9.50	24.99	0.19	0.115	0.044	0.025
200 °C	21.01	53.56	0.42	0.347	0.010	0.041
220 °C	13.60	41.48	0.23	0.196	0	0.015
200 °C first-step saccharification (48 h)	21.13	53.87	0.422	0.363	0.010	0.043
200 °C second-step saccharification (24 h)	7.90	20.24	0.158	0.126	0	0.002
200 °C two-step hydrolysis (48 h, 24 h)	29.03	73.50	0.581	0.465	0.010	0.062
Commercial cellulase (72 h)	28.60	72.66	0.572	0.523	0	0.037

In contrast, the maximum xylose yield was observed for the RH pretreated at 160 and 180 °C. However, the xylose yield of greater than or equal to 0.10 g/g dry substrate declined for the RH pretreated at 200 and 220 °C. This result was because of the high content of hemicellulose present in the RH pretreated at 160 and 180 °C. At the pretreatment temperatures of 200 and 220 °C, more than 80% of the hemicellulose content of the RH was removed due to the high-pressure steam pretreatment; hence, a lower amount of xylose was obtained after enzymatic saccharification.

The method used for enzymatic saccharification helped to improve the monomeric sugar yield in this study. During the first stage of hydrolysis, when the hydrolysate was recovered at 48 h, yields of 0.363, 0.010, and 0.043 g/g dry substrate were obtained for glucose, xylose, and cellobiose, respectively. Enzymatic saccharification for another 24 h caused a maximum increase in the glucose yield of 0.126 g/g dry substrate. The combination of the hydrolysate obtained from the first and second stages of hydrolysis caused the yield of glucose, xylose, and cellobiose to increase to 0.465, 0.010, and 0.062 g/g dry substrate, respectively. On top of that, the two-step saccharification process of the RH resulted in an additional 28% increase in the glucose recovery compared with the continuous hydrolysis performed for 60 h. The application of commercial cellulase for enzymatic saccharification produced the highest glucose yield of 0.523 g/g dry substrate. The glucose yield from the commercial cellulase was also higher than that produced by the

crude cellulase. This result was because the commercial cellulase is in a pure form. Additionally, the commercial cellulase also presented a higher activity of β-glucosidase than the crude cellulase from *B. licheniformis* 2D55, which might have resulted in the conversion of more cellobiose, increasing the glucose yield. This was also demonstrated by the yield of cellobiose of 0.037 g/g dry substrate, which was lower compared with that of the crude cellulase.

CONCLUSIONS

1. The pretreatment of RH with high-pressure steam at 200 °C for 7 min was found to be the most efficient at modifying the chemical composition, and structural and morphological properties of the RH for amiability to enzymatic saccharification.
2. The enzymatic hydrolysis of RH pretreated at 200 °C for 7 min at 60 °C resulted in a reducing sugar yield of 0.422 g/g substrate with a saccharification degree of 53.87% within 48 h shorter hydrolysis time. Furthermore, conducting enzymatic saccharification at a higher temperature appeared to be essential for *B. licheniformis* 2D55 cellulase because at 60 °C, the hydrolysis time was reduced to 48 h, which was much lower than the 72 h required for hydrolysis at 50 °C with commercial cellulase.
3. The application of the second-step saccharification method for the enzymatic saccharification of the RH produced an additional reducing sugar yield of 0.158 g/g substrate and 20.24% saccharification. The two-step saccharification process (48 and 24 h) resulted in a significant increase in the reducing sugar production and hydrolysis rate compared with the continuous saccharification.
4. The application of two-step saccharification to the RH resulted in a 28% higher glucose yield compared with the continuous process. The high-pressure steam appears to be an eco-friendly method for the pretreatment of RH.
5. The high-pressure steam pretreatment in combination with two-step saccharification could provide an economically viable bioprocessing method for industrial bioprocessing.

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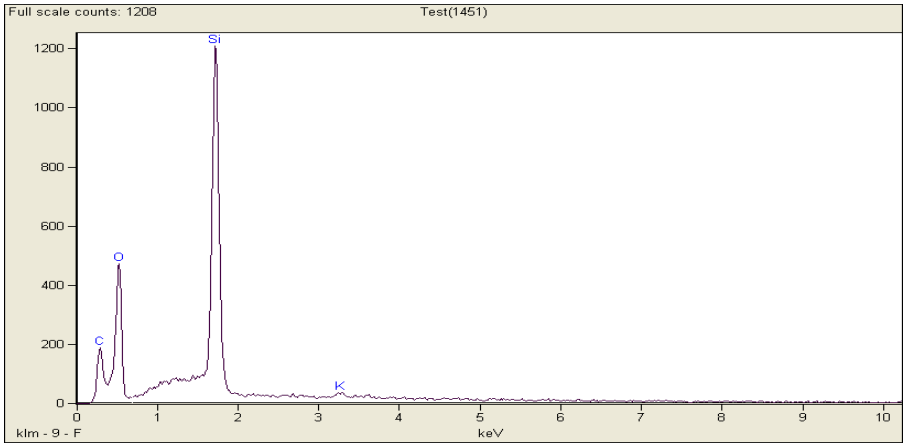
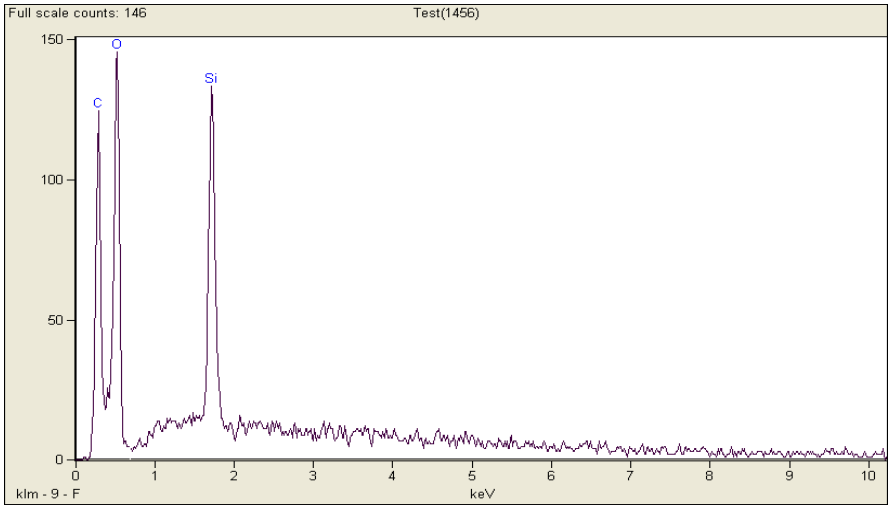
APPENDIX



Element	Weight (%)	Atom (%)	Formula	Compound (%)
C K	55.59	67.64	C	55.59
O K	23.60	21.56		--
Si K	20.69	10.77	SiO2	44.27
Si L	--	--		--
K K	0.11	0.04	K2O	0.14
K L	--	--		--
Total	100.00	100.00		100.00

S1. EDX micrograph and elemental analysis of untreated rice husk



Element	Weight (%)	Atom (%)	Formula	Compound (%)
C K	81.78	88.21	C	81.87
O K	9.70	7.86		--
Si K	8.52	3.93	SiO_2	18.22
Si L	--	--		--
Total	100.00	100.00		100.00

S2. EDX micrograph and elemental analysis of rice husk treated at 220 °C for 7 min