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BOOSTING ENZYMATIC HYDROLYSIS OF PRESSURIZED AMMONIUM HYDROXIDE PRETREATED EMPTY FRUIT BUNCH USING RESPONSE SURFACE METHODOLOGY

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Abstract

Oil palm Empty Fruit Bunch (EFB) was pretreated using Pressurized Ammonium Hydroxide (PAH) and was employed as Lignocellulosic Biomass (LCB) substrate for the investigation on the monomeric fermentable sugar production using the enzymatic hydrolysis process. Cellulose saccharification in enzymatic hydrolysis into a high yield fermentable sugar is an important step in Biochemical Conversion Technology (BCT). In order to determine the optimum variable conditions that can produce a high yield of fermentable sugar, a statistical approach using Response Surface Methodology (RSM) was performed in this study. Three independent variables, enzyme loading (15-50 FPU/g glucan), hydrolysis temperature (45-60°C), and agitation of the hydrolysis process (100-180 rpm) were investigated at five different levels ($-\alpha, -1, 0, +1, +\alpha$) of operating conditions and the experimental conditions were randomly setup using the Design of Experiment software. The regression models indicated that R^2 for glucose and xylose concentration was 95 and 88% showing the experimental variations were well defined by the models. For the lack of fit test, with p-values > 0.05 for both concentration sugars, 0.218 for glucose and 0.055 for xylose, it proves that the model was significant to the prediction models. The optimal conditions for the enzymatic hydrolysis of the EFB were determined at 32.5 FPU/g of glucan of enzyme loading, 50°C of hydrolysis temperature, and 140 rpm of agitation speed. The validation of the model at the optimum conditions produced a maximum glucose concentration of 8.78 ± 0.01 g/L (conversion of 81.7 ± 0.02 %, and yield of 332.95 ± 0.98 g/kg dry EFB), with a corresponding xylose concentration of 4.40 ± 0.01 g/L (conversion of 57 ± 0.35% and yield of 173.72 g/kg dry EFB).

Keywords: Ammonia, Empty fruit bunch, Lignocellulosic, Pressurized, Pretreatment, RSM, Sugar concentration.

1. Introduction

Oil palm tree, scientifically known as Elaeis guineensis Jacq under monocotyledon family of Arecaceae (Palmae), is the main oil crop cultivated in Malaysia. The palm oil production is vital to Malaysia's economy and society and has put the country as one of the largest palm oil producers in the world. In line with the remarkable growth of the industry, a tremendous amount of palm biomass residues are generated annually [1, 2]. It is estimated that for every tonne (t) of palm oil produced, approximately for Empty Fruit Bunch (EFB) about 1 t, palm fibres 0.7 t, palm kernels 0.3 t while palm shells about 0.3 t were generated, amounting to a total biomass residues of 2.3 t. Previously, EFB was incinerated to generate energy but the practice was discontinued. Today, a large amount of EFB is used as an organic mulch [3] or as bio-compost in the palm plantation. The latter has gained more attention since the practice is more sustainable [4]. Although these practices are successful in utilizing the EFB residues in the mill, activities in research and development have unleashed the potential of EFB as the lignocellulosic feedstock for higher value intermediate production such as C5 and C6 sugars intermediates.

Lignocellulosic EFB contains polymeric material such as glucan (25-55wt %), hemicellulose (25-40 wt %) and lignin (15-30 wt %). Cellulose, a type of glucan in EFB, consists of D-anhydroglucopyranose joined together by β-1,4-linkages with a degree of polymerization ranging from 100 to 20,000 [5] while hemicellulose mainly composed of xylan, and arabinan. Both glucan and hemicellulose components from the structural carbohydrate, that contributes the largest portion in EFB, and these components can be depolymerized into high-value C5 and C6 sugar intermediates. Lignin, a highly branched macromolecule of aromatic phenol, plays an important role in protecting Lignocellulosic Biomass (LCB) against degradation by microorganism or chemicals. Typically, cellulose is cross-linked to lignin via a hemicellulosic bridge, particularly xylan, by ester and ether linkages in the lignin-carbohydrate complex (LCC). These complex chemical structures develop the recalcitrance of LCB during enzymatic hydrolysis [6, 7]. Since EFB has the lignin structure that reduces its digestibility during enzymatic hydrolysis, a number of pretreatment technologies can be used to overcome the biomass's recalcitrant properties [8] and improve biomass hydrolysis efficiency [9].

Alkaline pretreatment is one of the chemical pretreatment that has been studied extensively. The mechanism of alkaline used involves saponification of intermolecular ester bonds that crosslink xylan (hemicellulose) and lignin [10]. Besides that, there are two types of aryl ether bonds cleavage that involve in alkaline degradation of lignin, which are, $C_{aliphatic}$-*O*-$C_{aromatic}$ and $C_{aromatic}$-*O*-$C_{aromatic}$, where its produce ferulic acid and *p*-coumaric acid. Basically, for alkaline pretreatment, there are few mediums that had been used such as NaOH, KOH, $Ca(OH)_2$, hydrazine, and ammonium hydroxide [7]. While agents such as NaOH, KOH and $Ca(OH)_2$ are frequently used to pretreat EFB and many are reported in the literature, the study on ammonium hydroxide pretreatment on EFB are quite limited.

Enzymatic hydrolysis of pretreated biomass, using cellulase, ß-glucosidase and other accessories enzymes such as xylanase and pectinase, breaks down the cellulose polymer to simple monomeric glucose without significant downstream inhibitors. The enzymatic hydrolysis is typically conducted in a mild acidic buffer phase (pH 4.5-5.5) and low hydrolysis temperature (~40-55°C) with mild agitation

mechanism to improve the hydrolysis rate of sugar production. This milder hydrolysis conditions with low energy consumption offer a greener and eco-friendly hydrolysis method compared to harsh acid hydrolysis that has been used thus far. In addition, the severe acid hydrolysis conditions also degrade the monomeric glucose and xylose to hydroxymethylfurfural (HMF) and furfural respectively that can inhibit the downstream fermentation process [11, 12].

The mechanism of the enzymatic hydrolysis is complex since it involves the interaction of hydrolytic enzymes and cellulosic substrate in the heterogeneous and homogeneous hydrolysis reaction. A full load of enzymatic hydrolysis parameters includes the enzyme and substrate concentrations, enzyme activity, and hydrolysis conditions such as temperature, agitation speed and pH. Since the enzymes used in the hydrolysis are quite costly estimated that 50ml of the enzyme is approximately USD3.82/ml, equivalent to RM11.30 [13], optimization of the enzymatic hydrolysis conditions becomes necessary to determine the optimum enzyme requirement and hydrolysis conditions in order to develop a sustainable hydrolysis process with high yield and rate of sugar production.

Response Surface Methodology (RSM) is one of an exploration of the relationship between independent variables and one or more response variables, which it can, determines the optimum process conditions by combining experimental designs with statistical analysis. In contrast to one-factor-at-a-time (OFAT) optimization, RSM is far better than OFAT due to its systematic approach that requires a larger region of factor space with fewer experiments as well as resources. In addition, RSM also includes the analysis and estimation of the interaction between the factors if any. There are several substrates that already successfully use this method for the optimization of enzymatic hydrolysis processes such as corn stover, rice straw, oil palm frond and EFB [14]. Most of the previous studies cover the enzymatic hydrolysis using NaOH, acid and steam pretreated substrates.

This study focused on the enhancement of the enzymatic hydrolysis of the pressurised ammonium hydroxide (PAH) pretreated EFB using the commercial cellulase and β-glucosidase enzymes. The main objective of the study was to determine the optimum enzyme loading and other hydrolysis conditions in order to achieve higher glucose conversion and yield at low glucan loading. The optimal hydrolysis conditions were validated using several experimentations at low and high glucan loading.

2. Material and Method

Figure 1 shows the summarized methodology of the overall process that was performed in this study. The details for each stage in the process are explained in each section below.

2.1. Materials

In this research, the materials that had been used were Empty Fruit Bunch (EFB), which, used as a feedstock throughout this study and the enzymes were from the commercial type. Each of these materials had their own preparation before continuing further study and the details onto preparations will be explained in Sections 2.1.1 and 2.1.2.

2.1.1. EFB

Shredded EFB fibres were collected from KKS Flamington in Bagan Datoh, Perak, Malaysia during the month of August. These fibres were air dried for 2-3 days to keep the EFB moisture content approximately around 10%. The universal cutting mill (Model: Pulverisette 19 Firsch, Germany) with 2 mm sieve was used to grind the 5 cm large particle of native EFB to produce the 2 mm small particle size. The dried milled EFB was sealed in the zipped-bag and stored in the chiller at 4°C to maintain the fibres and its moisture content.

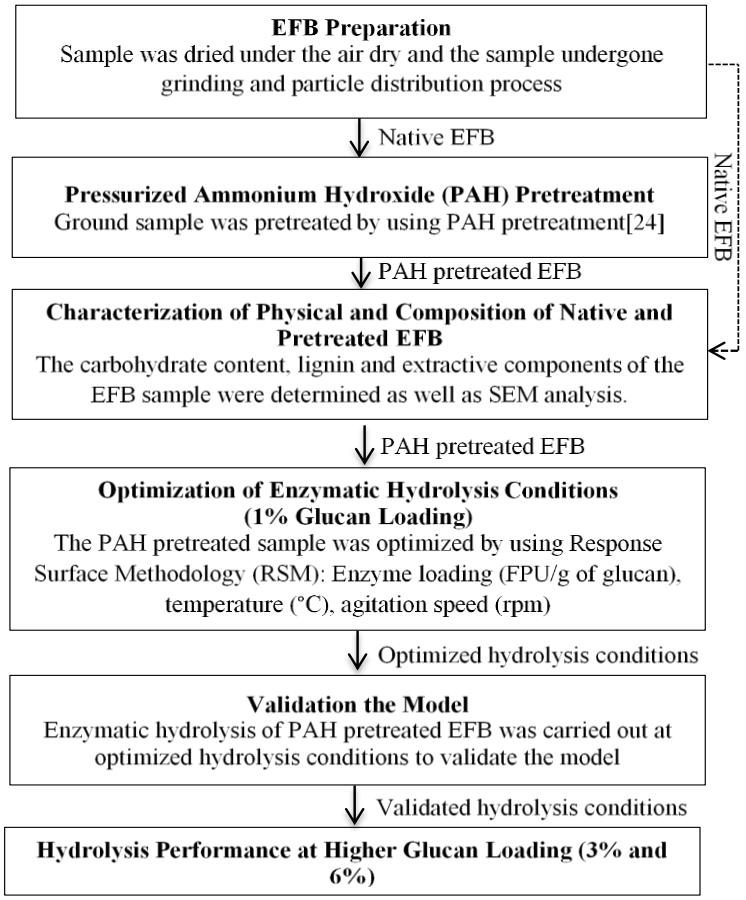


Fig. 1. Flow chart of the summarized methodology for optimization of enzymatic hydrolysis of PAH pretreated EFB.

2.1.2. Enzymes

Two type of enzymes were used in this study, which was commercial enzyme cocktail mixture Cellic® CTec2 (cellulase) and Cellic® HTec2 (hemicellulose) where all these enzymes were purchased from Novozymes A/S (Bagsvaerd, Denmark). The respective enzyme activity for Cellic® CTec2 was 142 ± 2.97 FPU/mL (protein concentration: 279.32 ± 1.10 mg/mL). The cellulase activity was determined by following the NREL protocol [15] and Ghose procedure [16]. One

unit of FPU activity is defined as the amount of enzyme releasing one micromole of reducing sugar from filter paper per ml per minute [15].

2.2. EFB grinding

After the air-dried, the EFB sample was cut using the universal cutting mill (Pulverisette 19 Firsch, Germany) with 2 mm screen for size reduction. Initially, the sample was poured into the tunnel and passed through the blade with the presence of the sieve screen and the milled sample was collected into the collecting bottle by vacuum suction. Then, the sample was kept in the airtight zipped bag for further use.

2.3. Particle distribution analysis

The dried milled EFB sample was subjected to particle size distribution analysis using motorized sieve shaker (Brand: Endecot, England) to determine the range of particle size of the EFB sample used in this study. The shaker has several mesh number with respect to the particle size: #60 (250 μm), #45 (300 μm), #35 (500 μm), #18 (1 mm) and #10 (2 mm). Before placing the EFB sample in the shaker, the total weight of EFB biomass was set to 100 ± 0.5 g. The shaker then vibrated the sieves for 15 ± 1 min to let the particle passed through into the appropriate sieves. Upon completion, the different particle size of EFB samples from different sieves were grouped into three ranges of particle size: 0-250 μm, 300-500 μm and 1000-2000 μm. The weight of EFB biomass from each range was measured using the analytical balance (Mettler Toledo) and weight EFB % was calculated using Eq. (1)

$$\text{Weight EFB, \%} = \frac{\text{Weight EFB from one range}}{\text{Total weight of EFB sample}} \quad (1)$$

Additionally, the bulk density from each range also was determined using an 800 cm^3 constant-volume container.

2.4. Compositional analysis of EFB

A chemical compositional of native and PAH pretreated EFB was analysed by using National Renewable Energy Laboratory (NREL) protocol as previously reported [16, 17]. Moisture content analysis was carried out using moisture analyser (Denver Instrument, Germany), while the ash analysis was conducted using a furnace at 575˚C for 24 hours [18].

Initially, Accelerated Solvent Extraction (ASE, Dionex 350, Sunnyvale, CA, USA) using deionized water (DI) extraction followed by 95% ethanol extraction was used to all the sample to remove the extractive part in the sample [19]. The deionized (DI) water and ethanol soluble material such as non-structural sugar, nitrogenous compound, inorganic compound and waxes were removed during this two-stage extraction process. Non-structural materials must be removed from the biomass prior to the compositional analysis to prevent the interferences [20]. DI water extraction sample was subjected to 72% sulphuric acid at 30˚C for an hour for oligomer analysis [20, 21].

After the extraction process on the EFB, the structural polysaccharides and lignin in EFB need to be analysed by using acid hydrolysis process with utilizing

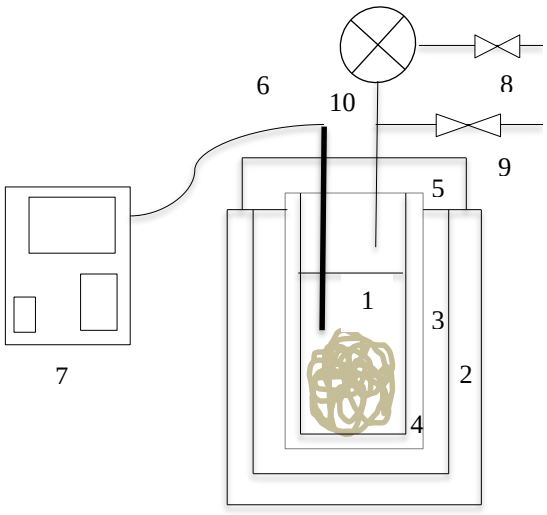
of 72% sulphuric acid at 30˚C for an hour and followed by adding the DI water until the weight reach about 84 ± 0.05 g and incubate at 121˚C for another hour [22, 23]. Then, a vacuum pump was used to filter the hydrolysate, and the residue was dried overnight, weighed and recorded as an acid-insoluble lignin while the filtrate was analysed for the acid-soluble lignin by diluting the sample with 10*x* dilution and using spectrophotometer at 320 Nm using 1 cm path length cuvette with 4% sulphuric acid as control, and monosaccharide sugar content such as glucose, xylose, i.e., using High Performance Liquid Chromatography (HPLC) [17].

2.5. Pressurized Ammonium Hydroxide (PAH) pretreatment

Figure 2 shows the schematic diagram of the Pressurized Ammonium Hydroxide (PAH) pretreatment system. The dried milled EFB was pretreated in 1-L closed pressurized stainless steel reactor system (Amar Reactor, India) using 30% ammonia hydroxide solution (AHS) referred to as Pressurized Ammonium Hydroxide (PAH) pretreatment. The EFB sample was loaded with 30% AHS at solid to liquid loading ratio of 1:12. The reactor temperature was raised to the target temperature set at 130°C and maintained at the desired temperature and respected pressure at 16 bar for 30 minutes of residence time [24]. Then, after the process completed, the ammonia valve was gradually open to relieve the pressure inside the reactor. The PAH pretreated EFB was collected and filtered from the mixture, and dried overnight under the fume hood to remove the excess of ammonia solution. The PAH pretreated EFB sample was kept sealed in a zipped-bag and stored at 4°C in the chiller until further used.

2.6. High performance liquid chromatography

The filtered hydrolysate was analysed for monosaccharides using HPLC (Dionex Ultimate 3000, Thermo Scientific, USA) equipped with refractive index detector (RI) and Rezex Phenomenex Monosaccharide column (RPM and ROA). The hydrolysate samples and sugar standards were analysed at 60˚C with the flowrate of 0.6 ml/min and 20 μL injection volumes.



1. Biomass and 30% ammonium hydroxide
2. Vessel body
3. Vessel heater
4. Reaction vessel
5. Pressure vessel cover
6. Heater
7. Temperature control
8. Pressure relief
9. Ammonia vent
10. Pressure gauge

Fig. 2. Schematic diagram of PAH pretreatment system.

2.7. Enzymatic hydrolysis of PAH pretreated EFB

The enzymatic hydrolysis of PAH pretreated EFB was performed according to the NREL standard protocol (LAP-009). All hydrolysis sample of PAH pretreated EFB was conducted in 20 mL scintillation vial at low glucan loading of 1% (dwb/v) in an incubated shaker (Infors HT Ecotron Incubator, UK) [24, 25], were buffered using a 50 mM citrate buffer (pH 4.8), and loaded with Cellic CTec2 cellulase in a range of 15-50 FPU/g of glucan (corresponding range of protein per g of glucan 29.51-98.35 mg/g of glucan) , and combined with Cellic HTec2 hemicellulase at a ratio of 1:1 (v/v). All hydrolysis samples were incubated at a temperature range of 45-60°C, and agitation speed range of 100-180 rpm for 96 h to ensure maximum sugar released from the PAH pretreated EFB. The hydrolysed samples were immersed in cold ice for 60 minutes to deactivate the enzyme activity and centrifuged at 7000 rpm about 10 minutes. The hydrolysates then were filtered through a 0.2-micron nylon membrane filter for subsequent HPLC sugar analysis.

2.7.1. Hydrolysis for validation runs

In order to examine the adequacy of the regression models developed, a validation experimental runs were performed. The first run was exactly the same hydrolysis condition set up used in the previous statistical optimization study while for the rest of the runs, it was randomly chosen and the condition was suggested by RSM software and each of the validation samples were performed in triplicate set to ensure the reproducibility of the experimental data.

2.7.2. Hydrolysis at higher glucan loading

The performance of enzymatic hydrolysis of PAH pretreated EFB for high glucan was conducted at 3% and 6% (dwb/v) of glucan loading. The 3% of glucan loading enzymatic hydrolysis was conducted in 20 ml scintillation vial. The 6% of glucan loading hydrolysis was conducted in 2 L Erlenmeyer flask. The basic enzymatic hydrolysis procedure was as described in Section 2.7.1 above. The hydrolysis sample was incubated in an incubator shaker (Infors HT Ecotron Incubator, UK) at optimum conditions of enzymatic hydrolysis process, identified in this study, using the same commercial enzymes (Cellic CTec2 and Cellic HTec2). As for the 6% glucan loading (dwb/v) hydrolysis, the enzyme loading was supplied in the fed-batch mode to provide homogeneous and better mixing conditions by utilizing some liquefaction from the early stage loaded substrate. Details procedure of 3% and 6% enzymatic hydrolysis could be referred in Harun et al. [21]. Similar to 1% glucan loading hydrolysis, all samples from 3% and 6% glucan loading hydrolysis were also left for 96 h of hydrolysis period. Upon completion of the hydrolysis time, the hydrolysate samples were immersed in cold ice for 60 minutes to deactivate the enzyme activity and centrifuged at 7000 rpm about 10 minutes. Then, the samples were filtered through a 0.2-micron nylon membrane filter for subsequent HPLC sugar analysis.

2.8. Experimental design

Experimental run of Central Composite Design (CCD) was generated based on 3 independent variables consisted of cellulase loading (x_1, in FPU/g of glucan), hydrolysis temperature (x_2, in °C) and hydrolysis agitation speed (x_3, in RPM) with glucose and xylose concentrations (Y_1 and Y_2, in g/L) measured as the responses in

the experiment. The influence of these variables (x_1, x_2, and x_3) on the glucose and xylose concentrations (Y_G and Y_X) were investigated at 5 different levels of runs *("-α", "-1", "0", "+1", "+α"*) as shown in Table 1, and all runs were completely randomized to prevent confounding effects, and help in justifying the assumption made [11].

Table 1. Variable code and actual parameters at five levels in the RSM to optimize the hydrolysis conditions of PAH pretreated EFB.

Independent variables (code)	Unit	Level of run				
		-α	-1	0	+1	+α
Cellulase loading (x_1)	Cellulase loading (x_1)	8	15	32.5	50	57
Hydrolysis temperature (x_2)	°C	42	45	52.5	60	63
Agitation speed (x_3)	RPM	80	100	140	180	196

There were 24 experimental runs, inclusive of 8 factorial point runs ("-1" and "+1"), 4 centre points runs ("0") and 12 axial points run ("-α" and "+α") with α equals to 1.4. The respected variables and responses were analysed and evaluated using Analysis of Variance (ANOVA) and several statistical tests at a p-value of 0.05. Each response was fitted to each independent variable according to Eq. (2) integrating the main, quadratic and interaction effects of these variable [11, 26, 27].

$$Y = \beta_o + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} {x_i}^2 + \sum_{i<j}^{k} \sum_{j}^{k} \beta_i x_i x_j + \varepsilon \quad (2)$$

where Y is the response in the experiment indicated by Y_G and Y_X for the glucose and xylose concentrations, β_o is the overall model coefficient, β_i, β_{ii}, β_{ij} are the respective constant coefficients for linear, quadratic and interaction effects of each variable x_i is the code for each variable, and finally r linear, quadratic and interaction The statistical design of the experiment, analysis, optimization and model regression was determined by Design-Expert 8.0.7.1 (Stat-Ease, Inc., Minneapolis, MN, USA) software. Model validation of the predicted optimal hydrolysis conditions was validated using a new set of experimental runs.

3. Results and Discussions

3.1. Physical and chemical analysis of native and PAH pretreated EFB

Characterizing the physical and chemical composition of EFB is one of the important steps that we need to quantify in order to do further study on it. By knowing the chemical composition of this EFB, we can identify the required biomass that needed for the next process. The physical analysis showed that the effect of the pretreatment that used towards the EFB. Thus, the details for physical and chemical characteristic will be explained in section 3.1.1 and 3.1.2 as written below.

3.1.1. Particle size distribution of native EFB

Prior to PAH pretreatment, the dried native EFB was milled to a smaller particle according to the method described above. The purpose of this size reduction is to

reduce the cellulose crystallinity of EFB fibres and it is also reported that by reducing into the small particle, the limitation of mass and heat transfer during pretreatment and enzymatic hydrolysis also can be reduced [20, 28]. Figure 3 reports the particle size distribution analysis in terms of weight % and average bulk density for each sample range.

The bar graph indicates that the particle size of the milled EFB fractions was distributed in the big range, from 0 to 2000 mm. The figure also shows that approximately 62.7% of the total weight of milled EFB sample used in the analysis is in the size range of 300-500 μm and this largest sample fraction was collected from the mesh number #35 and #45. The largest particle size range (1000-2000 μm) weighed approximately 23.4% while the smallest particle size range (0-250 μm) contributed about 13.8%. Additionally, the line graph in Fig. 3 indicates the average bulk density of the milled EFB fractions where the average bulk density decreases as particle size increases due to a small particle size of biomass can fill up all edges in the container compared to the larger particle. The milled sample from the smallest particle size range (0-250 μm) had the highest bulk density of 0.156 g/cm^3 while sample collected from the highest size range (1000-2000 μm) had the lowest bulk density of 0.083 g/cm^3. The average bulk density of the largest sample fraction (60% weight % in 300-500 μm) was about 0.135 g/cm^3. Further study on the PAH pretreatment and enzymatic hydrolysis were limited to this largest sample fraction to ensure consistency and reliability of the experimental result.

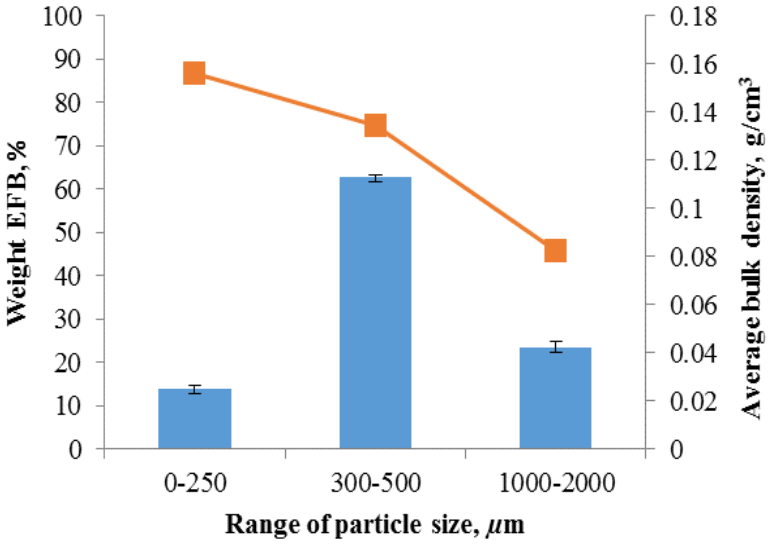


Fig. 3. Particle size distribution of native EFB.

3.1.2. Compositional analysis and scanning electron microscope of native and PAH pretreated EFB

Dried milled EFB samples were pretreated with PAH prior to enzymatic hydrolysis. Table 2 compares the composition of the native and PAH pretreated EFB.

Table 2. Composition of native and PAH pretreated EFB.

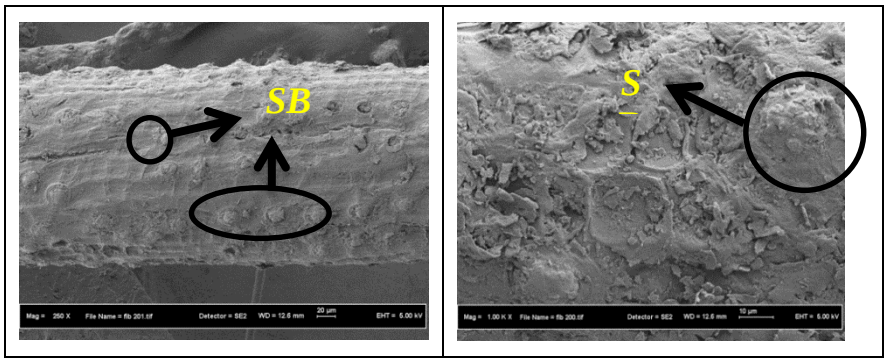
Component	Empty Fruit Bunch (EFB) composition, % (w/w)	
	Native EFB	**Pretreated EFB**
Glucan	36.47 ± 0.70	36.61 ± 1.20
Hemicellulose	25.81 ± 0.70	19.04 ± 1.31
Lignin	24.53 ± 3.94	16.79 ± 0.73
Ash	0.12 ± 0.01	0.04 ± 0.21
Extractive	13.55 ± 0.37	19.67 ± 0.50

The glucan component of native and PAH pretreated EFB in Table 2, they did not show a significant difference in the composition where for native and PAH pretreated EFB were 36.47% and 36.61% respectively. This yielded only about 0.14% reduction in the glucan component after the PAH pretreatment [29]. The hemicellulose composition for native EFB was 25.81% and for PAH, pretreated EFB was 19.04% and this indicated that about 6.77% of hemicellulose composition was significantly decreased compared to glucan composition. In contrast to glucan and hemicellulose composition, the removal of Klason lignin content was significantly reduced from 24.53% in native EFB to 16.79% in PAH pretreated EFB where the lignin component after the pretreatment mostly dissolved into the extractive black liquor. This lignin solubilisation on the other hand significantly increased the extractive composition from 13.55% in native EFB to 19.67% after PAH pretreatment.

The ammonia used in the pretreatment process successfully cleaved the chemical linkages within the biomass particularly the lignin-carbohydrate complex, which, caused partially solubilisation of the lignin and hemicellulose components that resulted in lower composition of the lignin and hemicellulose in PAH pretreated EFB sample [29, 30]. Hence, this shows that PAH pretreatment selectively and effectively removed the recalcitrant lignin while preserved the glucan component that resulted in low sugar loss [30]. As a result, the glucan component remained intact in PAH pretreated EFB sample compared to other components. Compare to other component in PAH pretreated EFB, the glucan component is an important substrate in the respective enzymatic hydrolysis where typically higher glucan composition contributes to higher glucose concentration in the enzymatic hydrolysate.

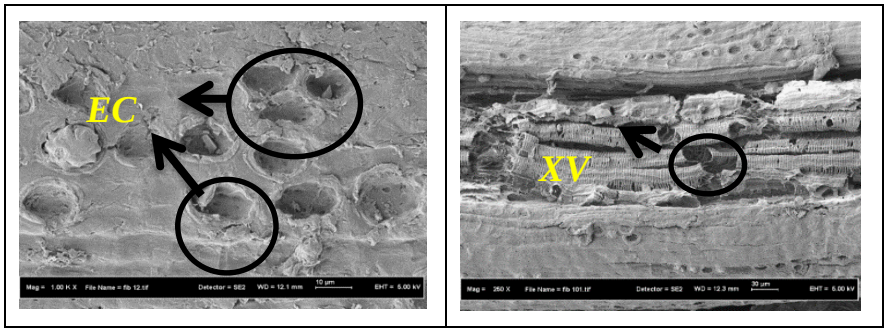
Figures 4(a) and (b) shows that native EFB exhibited rigid and highly ordered fibrils while Figs. 4(c) and (d) shows the PAH pretreated EFB fibre were distorted. Physical changes were qualitatively observed in PAH pretreated EFB fibre due to the deconstruction of hemicellulose and lignin components during the pretreatment process as shown in Fig. 4(d). SEM images show that PAH pretreatment removed most of the Silica Bodies (SB*)* as well as the impurities on the epidermal surface of EFB fibre and thus, leaving an Empty Cavity (EC) on the surface as shown in Fig. 4(c) [30, 31]. Silica deposition on the plant structure is one of the physical barriers that limit the enzyme accessibility to the cell wall of native biomass during enzymatic hydrolysis process. Thus, efficient removal of silica during the pretreatment step improved the enzyme accessibility and enhanced the EFB digestibility [32]. The observed spiral lignified cellulosic Xylem Vessel or Tracheae (XV) of the vascular tissue [33] showed that PAH pretreatment successfully deconstructed the EFB strand internal structure as shown in Fig. 4(d). Similar SEM observation was reported by John and Anandjiwala on NaOH

pretreated coin fiber that showed the emerged spiral cellulose after biomass deconstruction during pretreatment [34].



(a) Surface structure of native EFB with silicon body.

(b) Structure of native EFB with silicon body.



(c) Surface structure of PAH pretreted EFB with empty cavity (EC).

(d) Internal structure of PAH pretreated EFB with xylem vessel.

Fig. 4. Scanning Electron Microscopic (SEM) images of surface structure of native EFB and PAH pretreated EFB at 1 kV magnification.

3.2. Statistical analysis and optimization of the enzymatic hydrolysis of PAH pretreated EFB

Table 3 presents the experimental results of RSM study using CCD of enzymatic hydrolysis condition of PAH pretreated EFB based on 3 independent variables consisted of cellulase loading (x_1, in FPU/g of glucan), hydrolysis temperature (x_2, in °C) and hydrolysis agitation speed (x_3, in RPM) with glucose and xylose concentrations (Y_G and Y_X, in g/L) measured as the responses in the experiment. These sugar concentrations of glucose and xylose results were analyzed in the Design of Experiment (Version 8.0.7.1) for further statistical analyses.

Analyses of Variance (ANOVA) was performed to summarize the variance contribution in the regression model, and the residual error, and to determine the whether each variable contribution was significant or not. ANOVA identified the 'variance' contributed 'within' and 'between' the experimental runs and this is an important analysis to confirm the validity of each run, which, contributed to

the overall regression model. The variance sources in the regression model are the linear terms, quadratic terms and the interaction terms. Several tests including a test for lack-of-fit on the regression model and test for significance on the model as well as the individual model coefficient were also performed in the ANOVA. It is necessary to conduct these tests to avoid poor or misleading results and ensure that the model can provide an adequate approximation to the real system [35].

Tables 4 and 5 shows the summary of the ANOVA analyses using the quadratic model for the concentration of glucose and xylose. In these statistical analyses, the level of significance, alpha (α), was set at 0.05, and the p-value in each test statistic was compared to α value to determine the significance of the test.

Based on the ANOVA summary in Tables 4 and 5, test for lack-of-fit on the full quadratic regression models showed the p-values were larger than the α values where p-value=0.218 for a model of glucose, and p-value=0.05 for a model of xylose. These larger p-values indicated the insignificance of the test and implied that the regression model of the glucose and xylose were adequately fitted the experimental data.

Table 3. Experimental result for enzymatic hydrolysisof PAH pretreated EFB using RSM.

Run#	x_1:Cellulase loading	x_2:Hydrolysis temperature	x_3:Agitation speed	Glucose concention	Xylose concentration
	FPU/g glucan	°C	rpm	g/L	g/L
1	50.0	60.0	100	4.65	2.97
2	32.5	63.0	140	2.95	2.55
3	57.0	52.5	140	7.70	3.53
4	32.5	52.5	140	8.89	4.76
5	32.5	63.0	140	2.63	2.46
6	15.0	60.0	180	4.14	3.97
7	32.5	52.5	84	8.80	4.46
8	32.5	42.0	140	4.53	3.22
9	32.5	52.5	196	7.88	4.67
10	8.0	52.5	140	5.50	3.75
11	15.0	60.0	100	3.51	2.83
12	32.5	52.5	140	8.89	4.51
13	32.5	42.0	140	4.34	3.15
14	32.5	52.5	196	6.52	4.53
15	57.0	52.5	140	7.31	3.85
16	50.0	45.0	180	6.18	4.36
17	50.0	60.0	180	5.35	4.04
18	32.5	52.5	84	7.93	4.47
19	32.5	52.5	140	8.84	4.58
20	32.5	52.5	140	9.00	3.95
21	8.0	52.5	140	6.81	3.55
22	15.0	45.0	100	6.39	3.61
23	15.0	45.0	180	5.36	3.59
24	50.0	45.0	100	6.71	3.69

Table 4. ANOVA for response surface quadratic model of glucose concentration from enzymatic hydrolysis.

Variance source	Degree of freedom	Sum of square	Mean of square (variance)	F_{value}	$F_{critical}$	P_{value} (α= 0.05)
Regression	9	86.7	9.63	29.6	-	0.00
Linear	3	12.6	12.61	38.8	3.34	0.00
Quadratic	3	80.1	80.05	246.1	3.34	0.00
Interaction	3	1.3	1.27	3.9	3.34	0.00
Residual error	14	4.6	0.33	-	-	-
Lack-of-fit	5	2.3	0.45	1.76	-	0.218
Pure error	9	2.3	0.26	-	-	-
Total	23	91.22	-	-	-	-
R^2 **value**	0.95	Adj R^2	0.92			

Table 5. ANOVA for response surface quadratic model of xylose concentration from enzymatic hydrolysis.

Variance source	Degree of freedom	Sum of square	Mean of square (variance)	F_{value}	$F_{critical}$	P_{value} (α= 0.05)
Regression	9	9.3	1.03	11.11		0.00
Linear	3	1.45	1.46	15.64	3.34	0.00
Quadratic	3	6.93	6.93	74.50	3.34	0.00
Interaction	3	0.40	0.40	4.33	3.34	0.00
Residual error	14	1.30	0.09		-	-
Lack-of-fit	5	0.85	0.17	3.36	-	0.06
Pure error	9	0.45	0.05	-	-	-
Total	23	10.61	-	-	-	-
R^2 **value**	0.8772	Adj R^2	0.7982			

Test for significance on the regression models for both glucose and xylose concentration concluded that each model term was statistically significant and had a significant effect on the response with p-value=0 respectively. Additionally, the large *F* ratio, identified as a signal to noise ratio, in linear terms, quadratic terms and interaction terms demonstrated that the variance contributed in the glucose and xylose concentrations were significantly affected purely by those regression model terms rather than the experimental error. Hence, there is a strong correlation between the respective responses and those related regression model terms.

As a result, all model terms discussed previously were retained in the regression model equation of glucose and xylose concentrations since most of the terms were significantly affected one another. The regression model equations for both glucose and xylose concentration in terms of coded enzymatic hydrolysis parameters were given in Eqs. (3) and (4) respectively.

$$Y_1 = -102.19 + 0.12x_1 + 4.28x_2 - 0.01x_3 - 2.66x10^{-3}{x_1}^2 - 0.04{x_2}^2 - 2.06x10^{-4}{x_3}^2 + 1.15x10^{-3}x_1x_2 + 1.02x10^{-4}x_1x_3 + 1.21x10^{-3}x_2x_3 \quad (3)$$

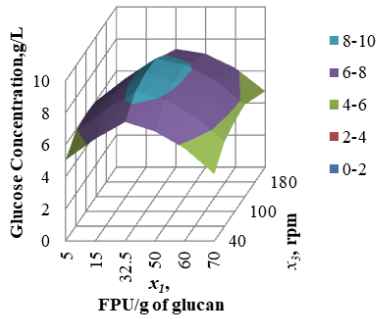
$$Y_2 = -22.42 + 0.07x_1 + 1.12x_2 - 4.87x10^{-2}x_3 - 7.61x10^{-4}{x_1}^2 - 0.01{x_2}^2 + 4.96x10^{-5}{x_3}^2 - 6.09x10^{-4}x_1x_2 + 1.11x10^{-4}x_1x_3 + 6.50x10^{-4}x_2x_3 \quad (4)$$

where Y_1 is the glucose concentration (g/l); Y_2 is the xylose concentration (g/l); x_1, x_2, and x_3 are the enzyme loading (FPU/g of glucan), temperature (°C) and agitation speed (rpm), respectively.

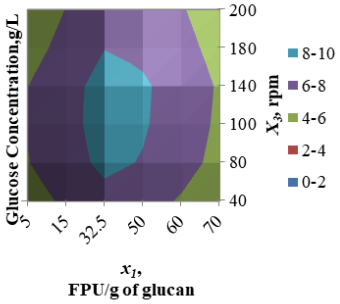
From Tables 4 and 5, the standard coefficient of determination, R^2=0.9501 indicated that 95.01% of the variability in the glucose concentration could be explained by the model. In addition, the model adequately described the experimental data with the adjusted coefficient of determination, R^2_{adj} about 91.80%. Unlike the standard R^2, adjusted R^2_{adj} value takes into account the number of terms in the model and it may reduce compared to R^2 value if there were more terms than necessary to describe the data. Moreover, the close R^2_{adj} values to the R^2 value insure a satisfactory adjustment of the quadratic models to the experimental data [35]. As for xylose concentration, the standard R^2 and adjusted R^2_{adj} were 87.72% and 79.82% respectively. This indicated that 87.72% of the variability in the xylose concentration could be explained by the model.

The regression equations obtained were plotted graphically for response surface plots and contour plots. Figures 5(a) to (f) and 6(a) to (f), show the contour plots for both sugar concentrations for the optimization of enzymatic hydrolysis process for PAH pre-treated EFB at a temperature of 50°C, agitation speed at 140rpm and enzyme loading of 32.5 FPU/g of glucan. From Figs. 5(a) to (f) the highest glucose concentration was attained particularly at the cellulase loading of 32.5 FPU/g of glucan when it was incubated in temperature of 50°C with an agitation speed of 140 rpm. It shows that the glucose concentration was strongly influenced by the positive interaction between the cellulose loading (x_1) and the hydrolysis temperature (x_2) as well as agitation speed (x_3). However, any value increase in any of these parameters would not be able to increase the glucose concentration beyond this optimum region. Thus, from Figs. 5(a) to (f), the optimum point for the highest glucose concentration was at cellulase loading 32.5 FPU/g of glucan, hydrolysis temperature at 50°C with an agitation speed of 140 rpm. Based on optimum conditions, the predicted glucose concentration should reach about 8.62 g/L, which is equivalent to 81% conversion.

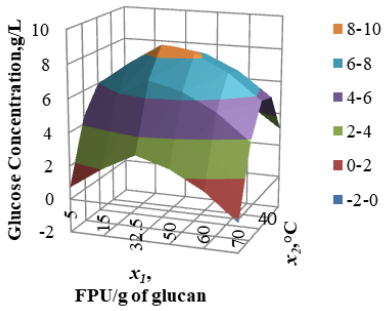
As for xylose concentration, the plots in Figs. 6(a) to (e) appeared to be quite different from the glucose response surface plots. Figs. 6(a), (c), and (e) shows that when cellulase loading and hydrolysis temperature increases, the xylose concentration also increases. However, the agitation speed did not show any significant contribution to xylose concentration. There was also no strong interaction effect between cellulose loading (x_1) and agitation speed (x_3). Additionally, xylose concentration was not as high as glucose concentration due to the fact that this optimization was only focused on the glucose concentration as the main desirable output, and the limit set for parameters was according to glucose concentration. Thus, based on the optimum conditions obtained from glucose optimization, the predicted amount of xylose concentration should be approximately 4.29 g/L equivalent to 55% conversion.



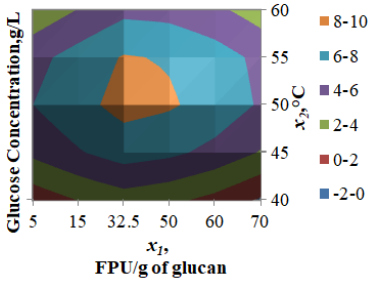
(a) Surface plot when x_2 at 50°C.



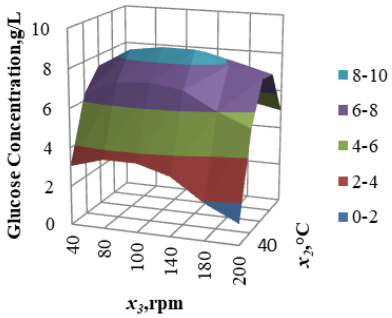
(b) Contour plot when x_2 at 50°C.



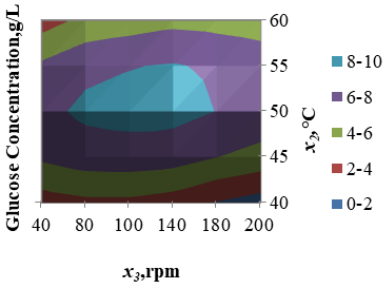
(c) Surface plot when x_3 at 140 rpm.



d) Contour plot when x_3 at 140 rpm.

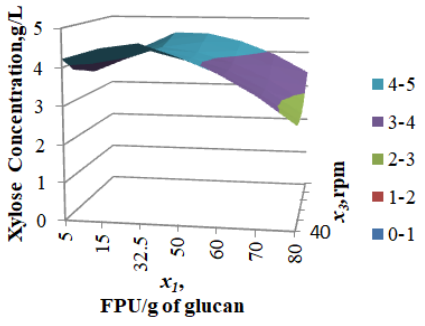


(e) Surface plot when x_1 at 32.5 FPU/g of glucan.

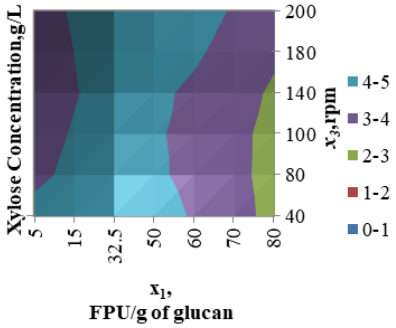


(f) Contour plot when x_1 at 32.5 FPU/g of glucan.

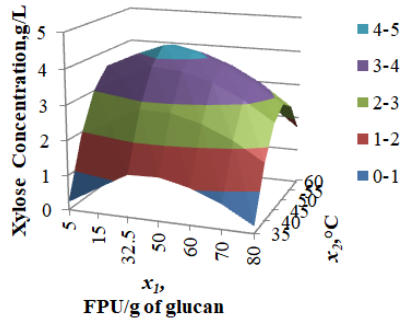
Fig. 5. Contour plots on glucose concentration of hydrolysis temperature, x_2, agitation speed, x_3, and cellulase loading, x_1, at optimum condition, respectively.



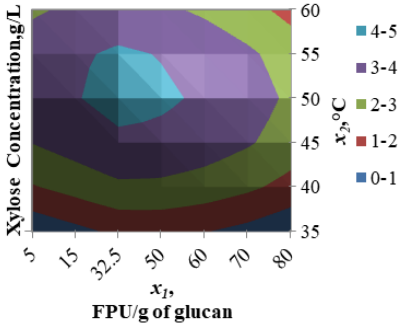
(a) Surface plot when x_2 at 50°C.



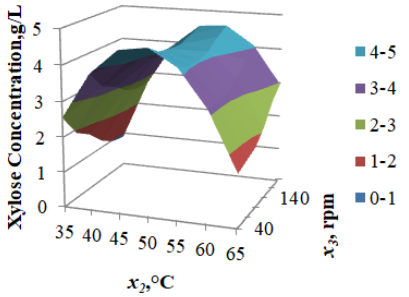
(b) Contour plot when x_2 at 50°C.



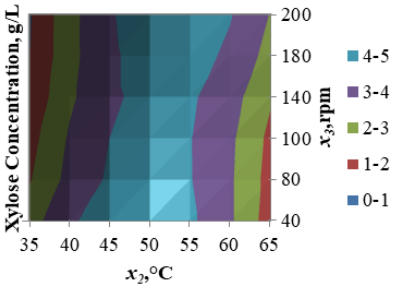
(c) Surface plot when x_3 at 140 rpm.



(d) Contour plot when x_3 at 140 rpm.



(e) Surface plot when x_1 at 32.5 FPU/g of glucan.



(f) Contour plot when x_1 at 32.5 FPU/g of glucan.

Fig. 6. Surface and contour plots on xylose concentration of hydrolysis temperature, x_2; agitation speed, x_3; and cellulase loading, x_1 at optimum condition, respectively.

3.3. Model validation

The optimum conditions of enzymatic hydrolysis of PAH pretreated EFB were validated with another set of validation experiment consisting of 4 experimental runs. Table 6 shows 4 experimental runs (Run#1, Run#2, Run#3 and Run#4) of enzymatic hydrolysis with different hydrolysis conditions as suggested in RSM including the optimum conditions (Run#1) obtained during optimization in section 3.2.

In this validation run, both glucan loading and hydrolysis time were kept constant and exactly the same as in the previous optimization, which was at 1% glucan loading and 96 h of hydrolysis time. Table 6 indicates that the responses for the validation experiments of these four runs. Run#1 with the hydrolysis of PAH pretreated EFB maintained at optimum conditions, where cellulase loading, x_1 at 32.5 FPU/g of glucan, hydrolysis temperature, x_2 at 50°C, and agitation speed, x_3 at 140 rpm, produced the highest experimental result with glucose and xylose concentration were 8.78 ± 0.01 g/l and 4.40 ± 0.01 g/l respectively. These values were in good agreement with the predicted model values for both sugar concentrations with the error between the predicted and experimental concentration values of 1.68% for glucose and 2.72% for xylose. Based on the sugar concentrations obtained from Run#1, the glucan conversion and glucose yield were calculated to be 81.7 ± 0.02% and 332.95 ± 0.98 g/kg dry EFB while the xylan conversion and xylose yield were 57 ± 0.35% and 173.72 g/kg dry EFB.

Run#2 to Run#4 were also able to produce good experimental values for both sugar concentrations during the hydrolysis in validation run. Generally, the largest errors between predicted and experimental concentration values in these three runs were 5.17% for glucose and 10.2% for xylose (Table 6). As expected, xylose concentration produced larger error compared to glucose concentration due to the fact that suggested hydrolysis conditions were based on the ability of these conditions to produce higher glucose concentration rather than xylose concentration. Therefore, based on these experimental results from this validation run, the regressed model was fit, adapted well to the experimental results, and able to give good predicted value particularly for glucose concentration. These finding also once for all confirmed and validated the regression models for both sugar concentrations in the enzymatic hydrolysis process.

Table 6. Validation run of enzymatic hydrolysis of PAH pre-treated EFB.

Run#	x_1, FPU/ g of glucan	x_2°C	x_3, rpm	Glucose concentration, g/L			Xylose concentration, g/L		
				Predicted value	Experimental value[#]	Error %	Predicted value	Experimental value[#]	Error %
1	32.5	50	140	8.62	8.78±0.01	1.68	4.29	4.40±0.01	2.72
2	25.7	45	110	6.97	6.70±0.02	-3.90	3.87	4.05±0.01	4.57
3	15	60	100	3.65	3.v51±0.10	-3.81	3.04	2.83±0.00	-6.78
4	50	45	180	4.83	5.08±0.02	5.17	3.38	3.04±0.01	-10.20

Experimental value had run in triplicate

3.4. Hydrolysis performance at higher glucan loading (3% and 6%)

Upon completion of the validation run, final enzymatic hydrolysis of PAH pretreated EFB was run at 3% and 6% of glucan loading hydrolysis at the same optimum conditions maintained at cellulase loading of 32.5 FPU/g of glucan, hydrolysis temperature of 50°C, and agitation speed of 140 rpm. Enzymatic hydrolysis at 1% glucan loading was considered a diluted hydrolysis run where mixing and mass transfer had no significant effect on the hydrolysis result. Therefore, conducting hydrolysis at higher glucan loading at 3% and 6%, which is equivalent to industrial hydrolysis practice with a solid loading of 9% and 18%,

actually provided further understanding on the hydrolysis behaviour at concentrated hydrolysis run.

Figure 7 compares the sugar concentration and conversion results of enzymatic hydrolysis of PAH pretreated EFB at higher glucan loading particularly at 3% and 6% and compared these results with hydrolysis of native and PAH pretreated EFB at 1% glucan loading. Figure 7 indicates that the glucose concentration at 3% and 6% obtained at optimum hydrolysis conditions were 26.15 ± 0.04 g/L and 49.01 ± 0.15 g/L respectively whereas for xylose concentration were 12.16 ± 0.04 g/L and 21.47 ± 0.12 g/L respectively. The increase in sugar concentration was expected as the glucan loading was increased from 1% to 6%. The most previous study indicates that as the glucan loading increases, the glucan and xylan conversion decreases due to poor mixing and mass transfer limitation in higher glucan loading [21]. However, the 3% and 6% glucan loading hydrolysis at the optimized conditions shows only a 1% reduction in the glucan conversion from 78.5 ± 0.25% at 3% and 77.5 ± 0.02% at 6% respectively. While for xylan conversion, Fig. 7 shows that 12% reduction from 72.3 ± 0.34% at 3% and 60 ± 0.98% at 6% of glucan loading.

Figure 8 shows the sugar yields for hydrolysis of PAH pretreated EFB from low to high glucan loadings per dry weight of EFB. From the Fig. 8, the glucose yield per dry weight EFB for 1% and 3% were 360.31 ± 0.15 g/kg dry EFB and 319.93 ± 0.51 g/kg dry EFB whereas for xylose yield at 1% and 3% were 163.02 ± 1.02 g/L and 148.73 ± 0.69 g/L respectively. Hydrolysis at 6% glucan loading produced the highest sugar yield at 419.29 ± 0.20 g/kg dry EFB, about 78% of the theoretical maximum glucose yield whereas for xylose, the yield produced at 6% glucan loading was 176.60 ± 0.06 g/kg dry EFB, about 86% from the theoretical maximum xylose yield.

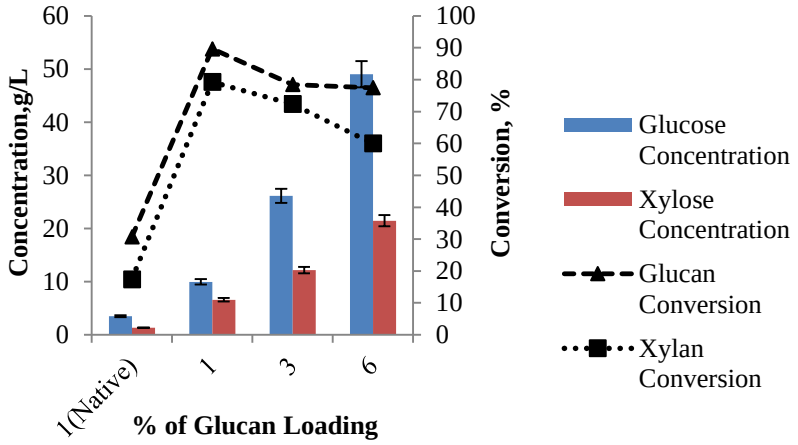


Fig. 7. Performance of enzymatic hydrolysis for PAH pretreated EFB at different percentage of glucan loading (1%, 3% and 6%) at optimum condition (32.5 FPU/g of glucan, 50°C, and 140 rpm).

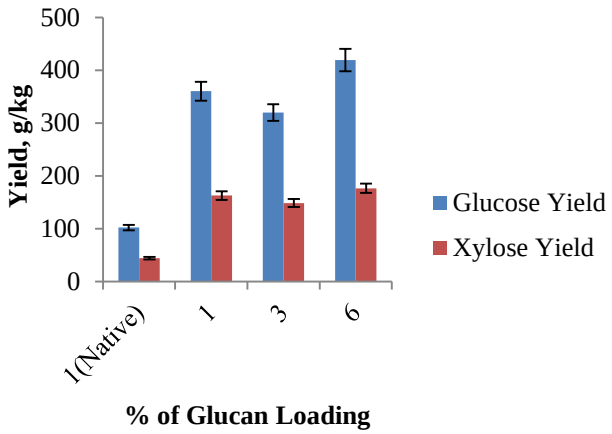


Fig. 8. Sugar yield (g/kg) for PAH pretreated EFB at different percentage of glucan loading (1%, 3% and 6%) at optimum condition (32.5 FPU/g of glucan, 50°C, and 140 rpm).

4. Enzymatic hydrolysis performance comparison

Enzymatic hydrolysis performance from this study was compared with other previous study on the enzymatic hydrolysis that used the same EFB biomass pretreated using ammonia-based pretreatment either using anhydrous ammonia or aqueous ammonium hydroxide solution. Table 7 shows the comparison of 1% glucan loading enzymatic hydrolysis performance using ammonia-based pretreated EFB. The table includes the type of pretreatment type used to pretreat the EFB biomass, pretreatment conditions, enzymatic hydrolysis conditions and the hydrolysis performance in terms of glucose concentration and glucan conversion. The figure shows that enzymatic hydrolysis conditions optimized in this study provided a good result and the hydrolysis conditions are comparable with other previous reported work.

Generally, Table 7 shows that at 1% glucan loading hydrolysis using ammonia-based pretreated EFB. It can be seen that although the hydrolysis conditions in terms of type enzymes, pH, temperature, and agitation were almost similar in Table 7 (except study by Jung et al. [36], where they used totally a different cellulase, Accellerase), the results of sugar concentrations and glucan conversions still gave wider ranges. The glucose concentration ranged from 4~10 g/L, and glucan conversion ranged from 30~90%. These wider ranges were probably due to different pretreatment used to pretreat the EFB biomass.

Although the pretreatment used to pretreat EFB was limited to ammonia-based pretreatment, different process mechanism in different ammonia-based pretreatment might contribute to the different degree of efficiency in converting the glucan component to glucose in the hydrolysis. Generally, pressure range for the ammonia-based pretreatment at pressurized condition was in a range of 8~50 bar that gave better hydrolysis result compared to non-pressurized pretreatment although the hydrolysis conditions were kept almost similar. Pressurized ammonia pretreatment increased the delignification of the biomass hence promoted the decrystallization of cellulose compared to non-pressurized pretreatment [36, 37]. It can be seen that for Aqueous Ammonia Soaking (AAS) conducted at atmospheric

pressure, with longer pretreatment time of 12 hours, compared to pressurized pretreatment that normally took shorter pretreatment time (15-30 minutes), the hydrolysis results were still far lower than the pressurized pretreatment [30, 33, 35].

Table 7. Comparison of the 1% glucan loading enzymatic hydrolysis performanceusing ammonia-based pretreated EFB biomass.

Raw material	Pretreat type	Ammonia concentration (%)	Pretreat conditions	Enzymatic hydrolysis conditions		Glucose concentration (g/l)	Glucan conversion (%)	Reference
				Enzyme	Process			
EFB	Pressurized Ammonia Chamber (PAC)	12.5	100°C 8 bar *t*=3 h *S:L*=1:30	Cellic CTec2 Cellic HTec2	pH 4.8, 50°C, 150 rpm, 24 h	4.39	39.63	[35]
EFB	Aqueous Ammonia Soaking (AAS)	21	60°C 1.01 bar, *t*=12 h *S:L* =1:12	Accellerase	60 FPU/g glucan pH 4.8, 50°C, 170 rpm, 72 h	4.59	41.4	[36]
EFB	Pressurized Anhydrous Ammonia (PAA)	100	135°C 35-50 bar *t*= 45 minutes *S:L* =1:1	Cellic CTec2 Cellic HTec2	14 FPU/g glucan pH 4.8, 50°C, 150 rpm, 24 h	9.99	90	[31]
EFB	Pressurized Ammonium Hydroxide (PAH)	30	130°C 16 bar *t* = 30 minutes *S:L* =1:12	Cellic CTec2 Cellic HTec2	32.5 FPU/g glucan pH 4.8, 50°C, 140 rpm, 96 h	8.78	81.7	This study

However, comparing this study with the previous study by Abdul et al. [31], although both studies used pressurized ammonia-based pretreatment, the sugar concentration and conversion in this study were slightly lower than the results obtained by Abdul et al. [31]. This was probably due to different ammonia concentration used in the respective study. While pretreatment conducted by Abdul et al. used anhydrous pure liquid ammonia (100% NH_3), which, required higher pressure (35-50 bar) to maintain the condition of liquid ammonia, this current study only used aqueous ammonium hydroxide (NH_4OH) solution with 30% ammonia concentration, and required much lower pressure (16 bar) to maintain the reaction. Conducting pretreatment using NH_4OH provides a better alternative in ammonia-based pretreatment because higher pressure process using anhydrous NH_3 requires stringent safety precaution and handling since the pure ammonia is so corrosive and flammable compared to NH_4OH solution [38, 39].

Another factor that might contribute to lower hydrolysis performance in PAH pretreated EFB was probably due to the sugar loss in the waste liquor after the pretreatment itself. While anhydrous NH_3 used in the previous study by Abdul et al., did not have any sugar loss during pretreatment of EFB, PAH pretreatment, which was conducted using aqueous NH4OH solution, produced a black liquor stream after the pretreatment. This waste liquor caused some solubilisation of hemicellulose component from the biomass, which reduced the sugar content in the pretreated EFB.

Although with sugar loss condition, PAH pretreated EFB was able to give good glucose concentration and glucan conversion with only 9.2% different compared to the glucan conversion obtain by Abdul et al. [31]. Thus, this PAH pretreatment process has high potential as another pretreatment alternative for pretreating EFB.

5. Conclusion

Empty Fruit Bunch (EFB) is one of the important lignocellulosic materials in Malaysia, and primarily it is used in research and development in bioconversion technology. It contains high lignocellulosic components, primarily glucan, hemicellulose and lignin that could be further utilized to produce valuable intermediates, end products such as fuels, chemicals and also biomaterials. Enzymatic hydrolysis is conducted to convert the carbohydrate, mostly glucan and some hemicellulose, into the valuable fermentable sugars without producing significant inhibitors that can affect the downstream fermentation process. Therefore, to obtain higher glucose concentration with good glucan conversion in enzymatic hydrolysis, the conditions for the hydrolysis should be optimized. Thus, this study focussed on the optimization of the enzymatic hydrolysis of Pressurized Ammonium Hydroxide (PAH) pretreated EFB using the Response Surface Methodology (RSM) with Central Composite Design (CCD) technique.

Three independent variables, enzyme loading (15-50 FPU/g glucan), hydrolysis temperature (45-60°C), and agitation of the hydrolysis process (100–180 rpm) were investigated at five different levels $(-\alpha, -1, 0, +1, +\alpha)$ of operating conditions and the experimental conditions were randomly setup using the Design of Experiment software. The enzymatic hydrolysis runs were conducted at 1% glucan loading for 96 h hydrolysis time. The statistical test performance on the regression models of both sugars (glucose and xylose) significantly indicated that those models were adequately fitted the experimental data. Each regression model also was statistically significant, and the terms on the model had a significant effect on the response by showing the p-value of 0. The F-values indicated that the variances in the concentrations were significantly affected by those regression model terms rather than experimental error or residual. This shows a valid and strong correlation between these experimental concentrations and those regression model terms.

Besides that, for the lack of fit test, with p-values > 0.05, it proved that the model was significant to the prediction models. All model terms, the enzyme loading (x_1), the hydrolysis temperature (x_2) and agitation speed (x_3) were linearly significant to the regression model of the sugar concentrations (glucose, Y_1; xylose, Y_2). The agitation speed did not show any quadratic effect on all sugar concentrations but for the enzyme loading and hydrolysis temperature, there were quadratic effects of these terms to both of the sugar concentrations. The regression models for glucose and xylose concentrations shows standard R^2 of 95.9% and 87.7%, respectively, indicating the variability in sugar concentrations could be explained by each regression model.

Thus, the best optimum conditions for the glucose concentration including the interactions between three independent variables were enzyme (cellulase) loading of 32.5 FPU/g of glucan, hydrolysis temperature of 50°C, and agitation speed of 140 rpm at hydrolysis time of 96 h with the predicted glucose concentration of 8.62 g/L, which is equivalent to 81% conversion, and the predicted xylose concentration should be approximately 4.29 g/L equivalent to 55% conversion. At the optimum conditions identified, the validation experimental run (Run#1) performed produced

the actual glucose concentration of 8.78 ± 0.01 g/ L with the actual glucan conversion of 81.7 ± 0.02% while for xylose concentration was 4.40 ± 0.01 g/L with xylan conversion of 57 ± 0.35%. The glucose and xylose yields were calculated to be 332.95 ± 0.98 g/kg dry EFB and 173.72 g/kg dry EFB respectively. The hydrolysis conducted at 3% and 6% higher using optimum conditions indicated that the potential of high solid loading hydrolysis at larger scale with glucose concentration was 26.15 ± 0.04 g/L (3%) and 49.01 ± 0.15 g/L (6%), and the respective glucan conversion was 78.5 ± 0.25% (3%) and 77.5 ± 0.02% (6%) respectively while the glucose yield per dry weight EFB were 319.93 ±0.51 g/kg dry EFB (3%)and 419.29 ± 0.20 g/kg dry EFB (6%) respectively.

Acknowledgement

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Nomenclatures

x_1	Cellulase loading (FPU/g of glucan)
x_2	Hydrolysis temperature (°C)
x_3	Agitation speed, rpm
Y_1	Concentration of glucose, g/L
Y_2	Concentration of xylose, g/L

Greek Symbols

α	Axial point
ε	Error
β_{ij}	Interaction constant coefficient
β_i	Linear constant coefficient
β_o	Overall model coefficient
β_{ii}	Quadratic constant coefficient

Abbreviations

AAS	Aqueous Ammonia Soaking
ANOVA	Analysis of Variance
BCT	Biochemical Conversion Technology
CCD	Central Composite Design
EFB	Empty Fruit Bunch
FPU	Filter Paper Unit
HMF	Hydroxymethyfurfural

LCB	Lignocellulosic Biomass
LCC	Lignin Carbohydrate Complex
NREL	National Renewable Energy Laboratory
OFAT	One Factor At Time
PAH	Pressurized Ammonium Hydroxide
RSM	Response Surface Methodology

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