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Enzymatic Hydrolysis of Pretreated Fibre Pressed Oil Palm Frond by using Sacchariseb C6

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Abstract. Enzymatic hydrolysis becomes a prominent technology for conversion of cellulosic biomass to its glucose monomers that requires an action of cellulolytic enzymes in a sequential and synergistic manner. In this study, the effect of agitation speed, glucan loading, enzyme loading, temperature and reaction time on the production of glucose from fibre pressed oil palm frond (FPOPF) during enzymatic hydrolysis was screened by a half factorial design 2^{5-1} using Response Surface Methodology (RSM). The FPOPF sample was first delignified by alkaline pretreatment at 4.42 % (w/v) sodium hydroxide for an hour prior to enzymatic hydrolysis using commercial cellulase enzyme, Sacchariseb C6. The effect of enzymatic hydrolysis on the structural of FPOPF has been evaluated by Scanning Electron Microscopy (SEM) analysis. Characterization of raw FPOPF comprised of 4.5 % extractives, 40.7 % glucan, 26.1 % xylan, 26.2 % lignin and 1.8 % ash, whereas for pretreated FPOPF gave 0.3 % extractives, 61.4 % glucan, 20.4 % xylan, 13.3 % lignin and 1.3 % ash. From this study, it was found that the best enzymatic hydrolysis condition yielded 33.01 ± 0.73 g/L of glucose when performed at 200 rpm of agitation speed, 60 FPU/mL of enzyme loading, 4 % (w/w) of glucan loading, temperature at 55 °C and 72 hours of reaction time. The model obtained was significant with p-value <0.0001 as verified by the analysis of variance (ANOVA). The coefficient of determination (R^2) from ANOVA study was 0.9959. Overall, it can be concluded that addition of Sacchariseb C6 during enzymatic hydrolysis from pretreated FPOPF produce high amount of glucose that enhances its potential for industrial application. This glucose can be further used to produce high-value products.

1. Introduction

Lignocellulosic biomass (LCB) is the most abundant renewable biomass that usually can be obtained from agricultural and forest residues [1][2][3]. It comprise of three major components which are cellulose, hemicellulose and lignin [4]. Cellulose and hemicellulose can be converted into fermentable sugars such as glucose and xylose.

Bioconversion of LCB into fermentable sugars indicates high potential as a promising renewable feedstock in biorefining area. The first step in fermentable sugar production is pretreatment in order to disrupt the lignin and expose the cellulose fraction followed by enzymatic hydrolysis. The hydrolysis of LCB into fermentable sugar is a crucial stage, which mainly determines the overall process efficiency.



Enzymatic hydrolysis is carried out by cellulase enzyme which is highly specific, and the products of the hydrolysis are usually reducing sugars such as glucose and xylose. There are various methods available for the fermentable sugars production, but the chemical and enzymatic methods have been proved to be more successful. Moreover, pretreatment and enzymatic hydrolysis are the most important steps in order to convert LCB contents into fermentable sugars which are currently known to have much more chance to reduce processing cost than other processes.

In this study, FPOPF was introduced as a raw material where the hemicellulose and cellulose were converted into simple sugars concerning to maximize the utilization of OPF. In order to improve the accessibility of cellulase on FPOPF, the structure of lignocellulose must be broken down by alkaline pretreatment. FPOPF was treated with alkaline solution for delignification process before undergoes enzymatic hydrolysis [5]. Cellulase enzyme which is Sacchariseb C6 was used in enzymatic hydrolysis to promote the production of glucose by converting the cellulose.

Therefore, this study aims at assessing the effects of five important parameters which are agitation speed, glucan loading, enzyme loading, temperature and hydrolysis time on the enzymatic hydrolysis using Sacchariseb C6 and thus finding the best conditions for enzymatic hydrolysis by using statistical approach of response surface methodology (RSM).

2. Materials and Methods

2.1. Materials

Oil palm frond (OPF) was obtained from a local palm oil plantation at Kuantan, Pahang. The OPF was pressed by using sugarcane machine to remove the juices. The fibre pressed oil palm frond (FPOPF) was sun dried for 3 days before shredded into pieces and sieved into particle size less than 2 mm. Dried FPOPF was stored in sealed plastic bag at room temperature. Enzymatic hydrolysis was carried out using Sacchariseb C6, a commercial enzyme preparation kindly obtained from Advanced Enzyme Technology (India).

2.2. Compositional analysis of FPOPF

Characterization of FPOPF was carried out according to National Renewable Energy Laboratory (NREL) methods in order to determine the composition of glucan, xylan, lignin, ashes and extractives contents in untreated and alkaline pretreated FPOPF. The moisture content of the raw FPOPF was determined using a moisture analyzer (DSC, A&D MS70). The extractives content was measured using DIONEX ASE 350 (Thermo Scientific, USA) with water and ethanol as solvents for 30 minutes prior to an autoclaved-based acid hydrolysis step [6]. The recovered water extract was analyzed for soluble sugar monomers. The carbohydrates and lignin content in FPOPF were determined using two-step acid hydrolysis procedure by NREL [7]. The acid insoluble material was determined using gravimetric analysis while UV-Vis spectroscopy was used to measure acid soluble lignin in FPOPF [8].

2.3. Alkaline pretreatment

FPOPF sample was soaked in 4.42 % (w/v) sodium hydroxide (NaOH) solution. The sample was treated at 100 °C about 58.31 minutes [5]. After that, the treated FPOPF was washed thoroughly with de-ionized water until neutral. The sample was oven dried at 105 °C and stored prior to enzymatic hydrolysis.

2.4. Two-level factorial analysis experimental setup

The experimental design for factorial analysis was performed using Design Expert 7.0.0 (Stat-Ease Inc., USA) software. The effect of five independent variables as shown in Table 1 were analysed using Response Surface Methodology (RSM). The condition ranges chosen were based on the other researcher's previous work [9][10][11][12]. The factors were constructed in factorial designs of 2^{5-1} to screen their effect on the response of glucose production. All experiments consist of 16 run listed in Table 2 were carried out in triplicate and the averages were taken as responses.

Table 1. Parameters and their designated low and high value.

Factor	Units	Low value (-1)	High value (+1)
A: Agitation speed	Rpm	50	200
B: Enzyme loading	FPU/mL	20	60
C: Glucan loading	%	1	4
D: Temperature	°C	35	55
E: Reaction time	Hours	3	72

Table 2. Experimental design for screening.

Std. Order	Agitation speed (rpm)	Enzyme loading (FPU/mL)	Glucan loading (%)	Temperature (°C)	Reaction time (hours)
1	-1	-1	-1	-1	+1
2	+1	-1	-1	-1	-1
3	-1	+1	-1	-1	-1
4	+1	+1	-1	-1	+1
5	-1	-1	+1	-1	-1
6	+1	-1	+1	-1	+1
7	-1	+1	+1	-1	+1
8	+1	+1	+1	-1	-1
9	-1	-1	-1	+1	-1
10	+1	-1	-1	+1	+1
11	-1	+1	-1	+1	+1
12	+1	+1	-1	+1	-1
13	-1	-1	+1	+1	+1
14	+1	-1	+1	+1	-1
15	-1	+1	+1	+1	-1
16	+1	+1	+1	+1	+1

The validation run for factorial analysis was done to validate the experimental values synchronise with the predicted model generated by Design Expert software. The condition for the validation run was obtained from the predicted best condition developed from 2^{5-1} factorial design. The validation model was determined by comparing the predicted and experimental values. The percentage errors between these values were calculated using Equation (1).

$$\text{Error (\%)} = \frac{|\text{Calculated value} - \text{Experimental value}|}{\text{Experimental value}} \times 100 \% \quad (1)$$

2.5. Enzymatic hydrolysis

Enzymatic hydrolysis was carried out using Sacchariseb C6 and Novozyme 188 (64 pNPGu/mL). Pretreated FPOPF (1 - 4 %) was mixed with 0.02 % (w/v) sodium azide to prevent microbial growth in the presence of 0.05 M citrate buffer at pH 4.8. The mixture was pre-incubated at certain temperature (35 - 55 °C) prior to the addition of enzymes. The enzymatic hydrolysis was then initiated by adding Sacchariseb C6 (20 - 60 FPU/ml) and Novozyme 188. The shaker started to agitate (50 - 200 rpm). At the end of the hydrolysis (3 - 72 hours) the samples were filtered and their residues were collected for further analysis. Each experiment was carried out in triplicate.

2.6. HPLC analysis

The hydrolysate was analysed by using Agilent 1200 HPLC (USA) system equipped with refractive index (RI) detector. The separation was performed using RHM Monosaccharide H⁺ column. The mobile phase was prepared using Milli-Q ultrapure water (Millipore, USA). The column temperature was maintained at 60 °C. The flow rate and injection volume were at 0.4 mL/min and 5 µL, respectively. The calibration curve was generated from binary standard consists of pure glucose and pure xylose with the ranges of 1 g/L to 40 g/L.

2.7. Scanning electron microscopy (SEM) morphology

Morphological structure of FPOPF was carried out using EVO 50 SEM (Carl Zeiss, Germany). Three FPOPF samples were collected which are untreated FPOPF, pretreated FPOPF and pretreatment with enzymatic hydrolysis FPOPF. Prior to SEM analysis, the samples were oven dried (55 ± 5 °C) for overnight. The samples were fixed on the aluminium stubs and coated with carbon layer. The samples were observed at 1000x magnification power and 5 kV of working voltage.

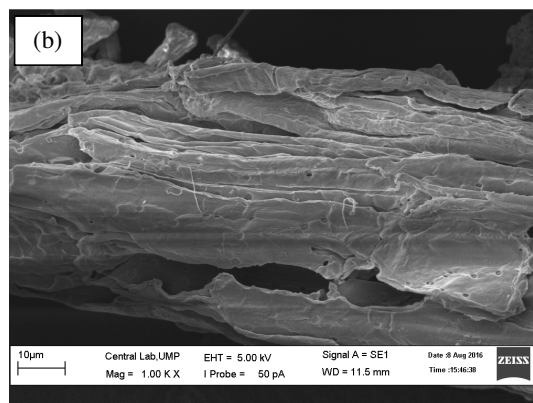
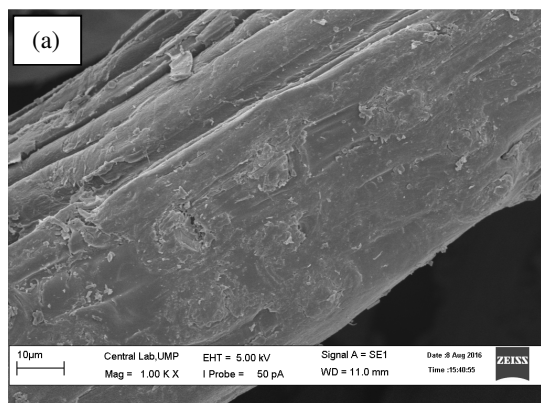
3. Results and Discussion

3.1. Characterization of FPOPF

As a natural product, the FPOPF has various compositions in terms of glucan, xylan and lignin as reported by Zahari et al. [13] and Tan et al. [14]. This different in composition was due to the several factors such as geographic locations, plant ages, climate, and soil conditions. The FPOPF used in this study composed of 4.5 % extractives, 40.7 % glucan, 26.1 % xylan, 26.2 % lignin and 1.8 % ash for raw FPOPF. Meanwhile, pretreated FPOPF comprised of 0.3 % extractives, 61.4 % glucan, 20.4 % xylan, 13.3 % lignin and 1.3 % ash.

3.2. Scanning electron microscopy ((SEM) analysis

Untreated, pretreated and pretreated with hydrolysis FPOPF were observed using SEM under magnification at 1000x in order to evaluate the effect of alkaline pretreatment and enzymatic hydrolysis on the morphology of the FPOPF as shown in Figure 1. Surface of untreated FPOPF (Figure 1a) was smooth, flat and rigid structure with presence of undamaged surface which comparable with previous study reported in rice straw [15], empty fruit bunch (EFB) [16], and wheat straw [17]. Meanwhile, after alkaline pretreatment, the outer surface layer was destroyed and the cell wall was distorted resulting the internal structure was exposed as shown in Figure 1b. This proved that the delignification occurred during alkaline pretreatment. This structure alteration could be expected that the accessibility of enzyme increased to the cellulose in the enzymatic hydrolysis later. Meanwhile, structure of FPOPF after enzymatic hydrolysis as in Figure 1c described that structures become disrupted by the enzymatic hydrolysis.



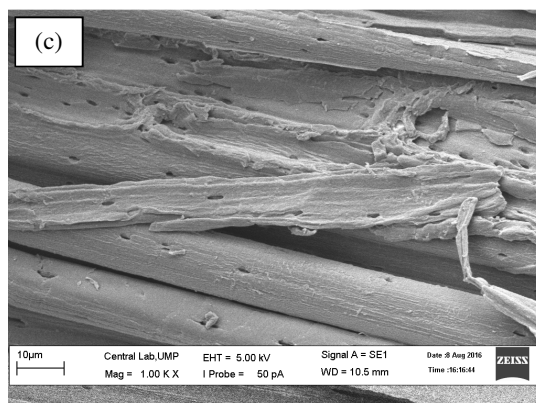


Figure 1. SEM images of FPOPF: a) untreated FPOPF, b) pretreated FPOPF, and c) FPOPF after enzymatic hydrolysis.

3.3. Factors affecting enzymatic hydrolysis

The highest production of glucose was obtained at 33.01 ± 0.73 g/L where the hydrolysis conditions at 200 rpm of agitation speed with temperature of 55°C , 4 % of glucan loading, and 60 FPU/mL of enzyme loading for 72 hours of hydrolysis time as shown in Table 3.

Table 3. Experimental design of factor screening process using 2^{5-1} factorial design with their response.

Std. Order	Factors					Response
	Agitation speed (rpm)	Enzyme loading (FPU/mL)	Glucan loading (%)	Temp. ($^\circ\text{C}$)	Reaction time (hours)	Glucose Concentration (g/L)
1	50	20	1	35	72	7.71
2	200	20	1	35	3	3.23
3	50	60	1	35	3	3.08
4	200	60	1	35	72	8.91
5	50	20	4	35	3	11.39
6	200	20	4	35	72	30.76
7	50	60	4	35	72	26.55
8	200	60	4	35	3	14.04
9	50	20	1	55	3	3.16
10	200	20	1	55	72	7.47
11	50	60	1	55	72	9.92
12	200	60	1	55	3	3.76
13	50	20	4	55	72	30.32
14	200	20	4	55	3	14.63
15	50	60	4	55	3	13.99
16	200	60	4	55	72	33.01

In factorial analysis, contribution of the main factor gives an important effect in the optimization part later. Two to three highest contributed factors will be selected from this factorial analysis. From Table 4 and Figure 2, factor C (glucan loading) gives the most contributing factor with 52.42 % to the enzymatic hydrolysis. Glucan loading indicates the availability of more cellulose can be hydrolysed to glucose. Similar results reported by Tan and Lee [12] and Nieves et al. [18] where the glucose yield was

gradually increased as the substrate loading increased. Furthermore, Sun and Chen [19] reported that substrate loading is one of the main factors that contribute to the rate of enzymatic hydrolysis. Next, the second most contribution factor followed by 31.73 % of E (hydrolysis time). Glucose production increased with longer hydrolysis time because its allowed the enzyme to hydrolyse the cellulose into glucose. Similar trend was obtained by Tan and Lee [12] and Zheng et al. [9].

Table 4. Percentage of contribution of main factor in enzymatic hydrolysis.

Factors	Contribution (%)
A	0.39
B	0.087
C	52.42
D	0.47
E	31.73

From Pareto chart as shown in Figure 2, all five factors (A, B, C, D and E) gave a positive effect (refer to orange bar chart) to the enzymatic hydrolysis. It is suggested that the highest values will be used to favour the response. For example, an increase in the glucan loading (C) increases the glucose concentration. Meanwhile, the negative effect (blue bar chart) reveals that the use of the lowest range value of factor will increase conversion to glucose.

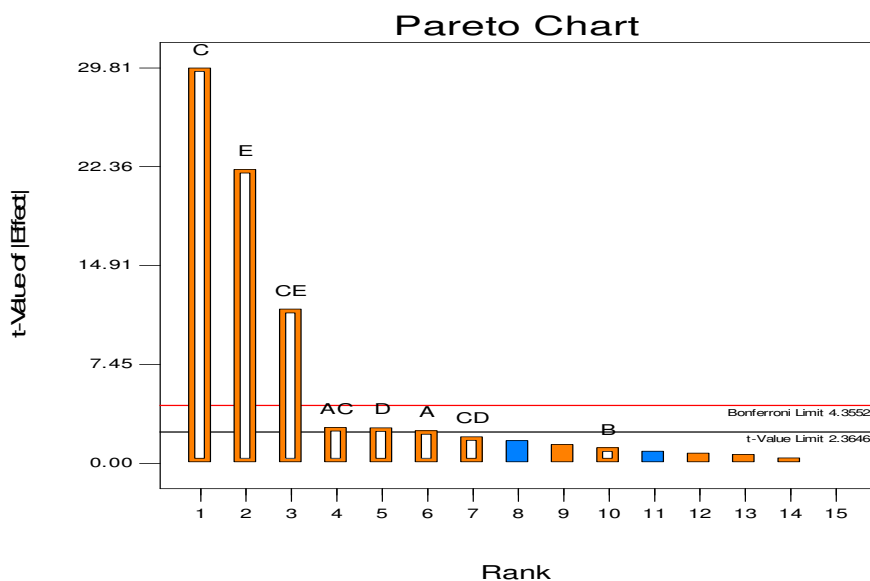


Figure 2. Pareto chart for 2^{5-1} factorial design.

From the ANOVA of this experimental design as shown in Table 5, the model obtained was significant with p-value <0.0001. The coefficient of determination (R^2) value obtained in this model was 0.9959, which is in good agreement with the adjusted R^2 value of 0.9912. The high R^2 value of 0.9959 indicates that the model was well adapted to the response.

Table 5. ANOVA table.

Source	Sum of square	Degree of freedom	Mean square	F-value	P-value	
Model	1648.46	8	206.06	212.19	<0.0001	Significant
A	5.90	1	5.90	6.07	0.0432	
B	1.32	1	1.32	1.35	0.2827	
C	862.98	1	862.98	888.65	<0.0001	
D	6.99	1	6.99	7.20	0.0314	
E	476.94	1	476.94	491.12	<0.0001	
AC	7.11	1	7.11	7.32	0.0304	
CD	3.86	1	3.86	3.97	0.0865	
CE	131.16	1	131.16	135.06	<0.0001	
Residual	6.80	1	0.97			

Equation (2) shows the response surface quadratic model for glucose production which can be presented in terms of coded factors as in the following equation:

$$Y = 14.20 + 0.61 X_1 + 0.29 X_2 + 8.21 X_3 + 0.99 X_4 + 5.46 X_5 + 0.67 X_1X_3 + 0.74 X_3X_4 + 2.86 X_3X_5 \quad (2)$$

where Y was concentration of glucose (g/L), X_1 was the agitation speed, X_2 was enzyme loading, X_3 was glucan loading, X_4 was the temperature and X_5 was the hydrolysis. The unknowns X_1 , X_2 , X_3 , X_4 and X_5 were referred to the main effects while X_1X_3 , X_3X_4 and X_3X_5 were the interaction effects contributed in the enzymatic hydrolysis process. Based on the quadratic model, coefficients of X_1 to X_5 are small compared to constant. This gives an indicator that the model equation is good with small error and can be used for further analysis.

3.4. Validation run

The validation experiments were conducted based on one suggested best condition from Design Expert 7.0 in triplicate. The experiments were performed at 160 rpm of agitation speed, 20 FPU/mL of Sacchariseb C6, glucan loading at 4 %, temperature at 56 °C and hydrolysis time at 72 hours and the result is presented in Table 6. The error from these validations runs in between 1.85 % to 4.70 %. The model was found to be reliable and reproducible as the experimental values were in good agreement with the predicted values proposed by the model with an error less than 10 %. Thus, it was proved to be an adequate model.

Table 6. Validation run for agitation speed at 160 rpm, enzyme loading at 20 FPU/ mL, 4 % of glucan loading, at 56 °C for 72 hours.

Description	Concentration of glucose (g/L)		
	Run 1	Run 2	Run 3
Predicted Value	34.024	34.024	34.024
Experimental Value	33.393	32.423	33.382
Error	1.85 %	4.70 %	1.88 %

4. Conclusion

The results obtained from this study clearly indicate that the best condition for the enzymatic hydrolysis process of FPOPF by Sacchariseb C6 at 200 rpm of agitation speed, 60 FPU/mL of enzyme loading, 4 % (w/v) of glucan loading, temperature at 55 °C and 72 hours of reaction time which produced 33.01 ± 0.73 g/L of glucose. In this production of glucose, there are two factors that mostly contributed in enzymatic hydrolysis which are glucan loading and temperature that will be used in the optimization

part later. Overall, it can be concluded that Sacchariseb C6 is a suitable candidate for enzymatic hydrolysis of pretreated FPOPF in order to achieve higher glucose production.

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