International Journal of Engineering & Technology, 8 (1.2) (2019) 235-245

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Research paper

Enhancement of Biosugar Production Via Enzymatic Yydrolysis of Sodium Hydroxide Pretreated Oil Palm Bagasse: A Statistical Perspective

Nurul Aina Fauzi[1], Shuhaida Harun[*1,2], Jamaliah Md Jahim[1,2], Jian Ping Tan

[1]Research Centre for Sustainable Process Technology (CESPRO), Faculty of Engineering & Built Environment, Universti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan.
[2]Chemical Engineering Programme, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor Darul Ehsan Malaysia
**Corresponding author E-mail:harun.shuhaida@ukm.edu.my*

Abstract

Oil palm frond bagasse (OPFB) is the major agriculture waste from the production of palm oil with 0.66g/g of total carbohydrate to serve as potential carbon source for downstream processing in producing succinic acid. This study focused in determining the optimum process variable conditions for the 1% glucan loading for enzymatic hydrolysis process with commercial blending cellulase enzyme mixture of Cellic CTec 2 and hemicellulase enzyme of Cellic HTec 2. Using statistical approach of Response Surface Methodology, three independent process variables; specific enzyme activity (5 - 80 FPU/g glucan), hydrolysis temperature (30 - 70 °C), and agitation speed (100 - 180 rpm) were investigated at five different levels (-α,-1, 0, +1, +α). The regression models indicated that R^2 for glucan conversion at 1% GL enzymatic hydrolysis was 97.2% showing the experimental variations were well-defined by the models. For the lack of fit test, the *p-values* > 0.05 proves that the model was significant to the prediction model. While both specific enzyme activity and hydrolysis temperature were statistically significant, there was no interaction observed between these variables. Although experimental runs reported the maximum glucan conversion of 94% was achieved in the 1% GL hydrolysis with 83.75 FPU/g glucan and 50°C after 96 hours of saccharification process, through validation process, the optimum conditions were determined at 30 FPU/g glucan, 45ºC and 100 rpm respectively where these saccharification conditions achieved 90% glucan conversion within 72 hours.

Keywords: *ANOVA; commercial enzyme; glucan conversion; oil palm frond; response surface methodology*

1. Introduction

Oil palm frond (OPF) obtained during pruning while harvesting fresh fruit bunch (FFB) is known to produce the largest oil palm solid waste reported for 83 million tonnes (wet weight annually) in Malaysia oil palm industry [1]. This figure is estimate to escalate up to 110 million tonnes in the year 2020. Habitually, fronds are left at the oil palm plantation during pruning and harvesting for nutrient recycling and soil conservation [2]. OPF is in the lignocellulosic biomass (LCB) group which consists of cellulose, hemicellulose and lignin. Two-stage processes in biochemical conversion technology are required which include the pretreatment and enzymatic hydrolysis step in converting the structural carbohydrate into monomeric biosugar. OPF is an attractive sustainable raw material source owing to its consistent and abundant availability throughout the year alongside its low purchase cost. OPF could be the feedstock in producing wide range of profitable chemical products such as polyhydroxyalkanoates (PHA), bioethanol, biobutanol, lactic acid and succinic acid [1]. According to previous research, the basal part of OPF petiole contains one-third of the desirable sugar content for downstream applications where the other two-thirds of OPF petiole could remain in the palm plantation as natural fertilizer [3].

The complex, recalcitrant and rigid internal structure of the OPF resists enzymatic hydrolysis. Accordingly, pretreatment process is needed to liberate the biosugar confined within the structure of hemicellulose and impermeable lignin [4]. The chemical pretreatment process deconstructs the polymeric matrix of the cell wall by solubilizing the hemicellulose, altering the lignin network, reducing the cellulose crystallinity, and finally rendering the cellulose more accessible and digestible to enzymes during hydrolysis [5,6]. Sodium hydroxide (NaOH) pretreatment were widely used within LCB agrowaste materials was demonstrated to undermine the LCB native structure. However, only few study of NaOH pretreament on OPF have been performed. NaOH pretreatment has been commonly studied using oil palm empty fruit bunch (OPEFB) and has augmented the enzymatic saccharification of OPEFB by 2.35 fold during 96 hours of enzymatic hydrolysis [7]. It shows that simple NaOH pretreatment was able to deconstruct OPEFB and increase the surface area for efficient enzyme accessible during enzymatic hydrolysis [4].

Enzymatic hydrolysis depolymerizes the polymeric cellulose of pretreated LCB into the fermentable biosugar hydrolysate mainly rich in monomeric glucose and xylose for downstream microbial fermentation process [8]. Enzymatic hydrolysis process is superior to chemical hydrolysis due to the high enzymatic specificity towards β-1-4-glucosidic bond in the cellulose structure. It works best under mild operating hydrolysis conditions with lesser formation of by-products or inhibitors [9]. However, certain shortcoming such as high cost of en-

zymes and unsatisfactory hydrolysis rate remain as significant barriers that impede the utilization of LCB materials and limits the enzyme utilization. Additionally, different parts of plants have different distributions of cellulose, hemicellulose and lignin, warranting customization of enzymatic mixtures for such variances [10,11]. Thus, continuous studies are desired not only to improve the enzymatic hydrolysis conversion and yield but also to ensure a cost effective and maintaining low enzyme dosage in dealing with different LCB types and species [12].

One of the ways to enhance the enzymatic hydrolysis process is through process optimization using statistical design of experiment. The benefit of the statistical method compared to the one-factor-at-time (OFAT) approach is that for a comparable number of experiments, the method provides detailed information including the interactions between process parameters in lesser time [13,14]. Common statistical methods applied are (1) Factorial Design (FD) used to determine the significant factors that strongly impact the response variables, (2) Response Surface Methodology (RSM) which is useful for predicting the optimum process design points at given target of response variable, and finally (3) optimization mixture which is useful for optimizing the ratio of components in a mixture at given desired output [15,16].

This study aimed to optimize the process conditions for enzymatic hydrolysis using NaOH pretreated oil palm frond bagasse (POPFB). The correlations effect between enzyme dosage loading (FPU/mass glucan), incubation temperature (°C) and agitation speed (rpm) was studied using Response Surface Methodology (RSM) based on central composite design (CCD) and glucan conversion was observed as response variable.

2. Materials and Methods

2.1. Raw material

Several fresh OPF branches without leaflet in Figure 2.1 were collected from the oil palm plantation at Universiti Kebangsaan Malaysia (UKM) Bangi Lama, Selangor, Malaysia. The leaflet parts were left in the oil palm plantation as natural fertilizer and soil conservation. The 1-meter long basal part (petiole) of fresh OPF were cut into few pieces and later were pressed using a conventional sugarcane machine (SCM, 6.5HP Elephant) to extract OPF juice (Figure 2.2). The OPF juice was centrifuged at 10000 rpm for 10 minutes and the supernatant were directly test for pH using pH meter (Eutech Instruments). The supernatant then filtered using 0.22 µm nylon syringe filter and its sugar composition was determined using High Performance Liquid Chromatography (HPLC) [1]. Meanwhile, the oil palm frond bagasse (OPFB) collected from the juice pressing step underwent drying, grinding and sieving process to 2 mm particle size. The sieved OPFB were packed in an airtight plastic bag and stored in the freezer for further use.

2.2. Compositional analysis

OPFB chemical composition analysis of its polysaccharide content (cellulose and xylose), lignin, extractive and ash content were determined based on the standard National Renewable Energy Laboratory (NREL) protocols. The chemical composition analysis is completed both on raw and pretreated OPFB [17].

2.3. Alkaline pretreatment

NaOH pretreatment on OPFB was performed under optimized conditions as reported by Sukri et al. [18] where the optimum condition for the pretreatment process was 4.42% NaOH concentration, temperature of 100°C, 1 hour of pretreatment period with the solid to liquid ratio of 1:10 (w/v). Pretreatment process then was conducted in screw cap Duran bottle with the cap slightly loosened [19]. Prepared samples were heated in a shaking water bath (Wise Bath Lab Companion) at 50 rpm. Upon completion, samples were filtered using muslin cloths to separate the pretreated solid from its black liquor. The pretreated solid was washed with deionized (DI) water and was neutralized with 25% hydrochloric acid (HCI) until the pH reached 4.8 as required in enzymatic hydrolysis process. Subsequently, the pretreated and neutralized pretreated oil palm frond bagasse (POPFB) was air-dried until the moisture content drop to 10%. The samples were stored in the airtight plastic bags at -40°C to avoid biomass contamination [17].

Fig 2.1: Oil Palm Frond Bagasse (OPFB)

Fig 2.2: Oil Palm Frond Juice

2.4 Enzyme activity

The commercial enzymes used in this study for enzymatic hydrolysis were blending enzymes mixture of Cellic CTec 2 and Cellic HTec 2 purchased from Novozymes A/S (Bagsvaerd, Denmark). The respective activity of the Cellic CTec 2 was 108.82 FPU/mL with filter paper as the enzymatic substrate measured by Ghose [20] and the activity of Cellic HTec 2 was 164 XU/ml, based on the assay measured using 1% birchwood 4-O-methyl glucuronoxylan substrate according to method developed by Bailey et al. [21].

2.5 Buffer preparation

Sodium citrate buffer was prepared by mixing citric acid monohydrate and trisodium citrate dihydrate. The 0.1 M concentration for both solutions were prepared separately by dissolving 21.01 g of citric acid monohydrate (MW = 210.14) and 29.42 g of trisodium citrate dihydrate (MW = 294.10) into each 1000 mL DI water. Desired pH value of 4.8 was attained by mixing both solutions with ratio 4:6 of citric acid monohydrate to trisodium citrate dihydrate for a total of 1000 mL [22].

2.6 Enzymatic hydrolysis

Enzymatic hydrolysis process was conducted at 1% GL (w/v) in a 15 mL hydrolysis volume using a 20 mL universal bottle. The mass of POPFB required was weighed and added into the universal bottle, followed by the addition of DI water and 0.1 M trisodium citrate buffer. Cellic CTec2 and Cellic HTec 2 enzyme at the ratio of 1:1 cellulase to xylanase enzyme were dose based on the amount of glucan. These enzyme were added to initiate the enzymatic hydrolysis reaction following the suggested hydrolysis conditions in the RSM design. The enzymatic hydrolysis was conducted for 96 hours in the water bath shaker to ensure maximum sugar release. The hydrolysis process followed the experimental design with triplicate per each different condition. Upon the completion of the enzymatic hydrolysis reaction, samples were immediately immersed in ice bucket for 1 hour to terminate the enzyme reaction and subsequently centrifuged at 10000 rpm for 10 minutes [23]. Centrifuged samples were filtered through 0.22 µm nylon syringe filter into HPLC vials and kept at -40 ^{o}C prior to HPLC analysis.

2.7 High performance liquid chromatography (HPLC)

All liquid samples obtained in this study were analyzed using high performance liquid chromatography (HPLC) (UltiMate 3000 LC system, Dionex, Sunnyvale, CA) employing the Rezex ROA column (300 mm × 7.8 mm; Phenomenex, USA) with a guard column (50 mm x 7.8 mm). The HPLC was operated at 40^{o}C refractive index (RI) detector (RefractoMax 520, ERC, Germany). Mobile phase used was filtered and degassed 0.005 N H_2SO_4 eluted at a flow rate of 0.6 mL/min and sample injection volume used was 20 µL [24]. The component was identified by comparing their retention time with calibration sugar standard and the concentration of soluble sugar was also determined based on the calibration sugar standards. Mixed sugar standards (glucose, xylose and arabinose) were used with different concentrations.

2.8 Experimental Design

Based on initial results from previous enzymatic hydrolysis study, Response Surface Methodology employing Face Centered Central Composite Design (FCCCD) was applied to determine the significant factors that strongly influence the response variable in the enzymatic hydrolysis. Statistical software package Design-Expert (Stat-Ease, Inc., Minneapolis, USA) was used for regression analysis of experimental data and to plot the response surface. ANOVA was used to estimate the statistical parameters. Three process parameters which include (i) specific activity of enzyme/mass of glucan (FPU/g glucan), (ii) incubation temperature (^{o}C) and (iii) agitation speed (rpm) were chosen to determine the correlation between each factor with response observed. Glucan conversion percentage (GC %) was set as the response variable for each corresponding experimental run in Table 3 and statistical analysis using the GC% was used to determine the significant factors and to predict the optimum process variable at the response variable target in this study. This optimization study consisted of 25 runs studied at five different design level (-α, -1, 0, +1, +α) with 3 replications at the centre point. A chosen range with process parameters for the enzymatic hydrolysis process is shown in Table 1.

Table 1: Designated ranges of enzymatic hydrolysis process variables

Parameters	-α	Low Level (-1)	Centre Level (0)	High Level (+1)	+α
Activity of enzyme/mass					

glucan (FPU/ mass glucan)	1.25	5	42.5	80	83.75
Incubation Temperature (°C)	28	30	50	70	72
Agitation Speed (rpm)	96	100	140	180	184

Percentage of glucan conversion were analyzed by multiple regressions to fit the model equation (Eq.1):

$$\gamma = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j + \sum \beta_i x_i^2 \quad (1)$$

Where,

γ = predicted glucan conversion (%)

$\beta_o, \beta_i, \beta_j$ = regression coefficient of the model

$x_i x_j$ = independent variables

3. Results and discussion

3.1 Chemical composition analysis of oil palm frond bagasse

The chemical composition (in %) for both raw oil palm frond bagasse (ROPFB) and pretreated oil palm frond bagasse (POPFB) on dry weight basis was tabulated in Table 2. Both ROPFB and POPFB were analysed using National Renewable Energy Laboratory (NREL) protocols through two-steps extraction process using deionized (DI) water followed by ethanol extraction. Extraction with DI water served to remove inorganic materials, non-structural sugar, soil and protein while sequential extraction using 95% ethanol removed waxes, chlorophyll and phenolic compounds. Removal of these residues ensures that the biomass contained only primarily structural carbohydrate and lignin [25].

Table 2: Chemical composition of oil palm frond bagasse (% dry weight)

Component		Raw Oil Palm Frond Bagasse (ROPFB)	Pretreated Oil Palm Frond Bagasse (POPFB)
Structural Constituent			
1. Total Structural Carbohydrate		79.48 ± 1.7	69.85 ± 3.0
		61.44 ± 0.9	53.18 ± 3.0
	Glucan	46.10 ± 2.2	48.28 ± 2.6
	Xylan	15.34 ± 0.9	4.90 ± 1.4
2.Klason Lignin		17.26 ± 1.4	16.67 ± 0.3
Non Structural Constituent :			
1. Ash		3.38 ± 0.1	0.95 ± 0.0
2. Ethanol Extractives		1.43 ± 0.1	0.01 ± 0.0
3. Water Soluble Extractives		1.87 ± 0.1	0.94 ± 0.0
	Soluble Glucose	0.08 ± 0.0	NA
	Soluble Sucrose	0.08 ± 0.0	
Total Chemical Composition		82.86 ± 1.7	70.80 ± 3.0

The total amount for ROPFB chemical composition quantified was 82.86±1.7% with the total structural carbohydrate of ROPFB contains 46.10±2.2% glucan and 15.34±0.9% xylan. The lignin content reported as Klason lignin in ROPFB was 17.26±1.4%, the ash content was 1.43±0.1% and the ethanol extractive was 1.87±0.1%. Meanwhile, the total chemical composition of the POPFB contains 70.80±3.0 with 48.28±2.6% glucan, 4.90±1.4% xylan, 16.67±0.3% lignin, 0.01% ash and 0.94% ethanol extractives. The summation of total chemical composition of POPFB reflected some reduction in the chemical components of the biomass due to the solubilisation of these components into the sodium hydroxide liquid phase (black liquor). The solubilisation process mostly removed the acetyl group in the hemicellulose structure and produced acetic acid. In addition, the lignin reduction also produced some phenolic groups based on the black colour of the filtrate [26]. Ash content diminution was observed after pretreatment step as the silica body was solubilized in the filtrate. Through the comparison of chemical composition of ROPFB and POPFB, delignification process of the POPFB was ascertained to occur only in small percentage, approximately 0.59%.

To facilitate the enzymatic hydrolysis process, ROPFB was pretreated using optimum NaOH pretreatment process conditions developed by Sukri et al. [18]. Pretreatment step was crucial to disrupt the recalcitrance structure of ROPFB and made the cell wall of the pretreated OPFB (POPFB) accessible to enzymes to liberate the C6 and C5 sugar entrapped inside the bagasse cell wall. Nevertheless, it has been explained that, although pretreatment might not necessarily lead to substantial delignification, the structure of lignin could nonetheless be altered due to changes in its chemical properties. Typically, the pretreated biomass is more digestible than the raw biomass even though it may have the same amount of lignin as the raw [4].

3.3 ANOVA and residual plot of original model

The analysis of variance (ANOVA) was used in the analysis to summarise the variance contribution in the regression model and the residual error and to determine whether each contribution was significant or vice versa. Test for lack-of-fit on the regression model, test for significance on the regression model and the test for the significance on the individual model coefficients was included in the ANOVA analysis. Table 3 summarized the statistical significance of the original regression model for the GC%.

Table 3: ANOVA for original regression model for the GC %

Source	Sum of Squares	DF	Mean Square	F_{value}	P_{value} (Prob>F)	Remarks
Model	22368.79	9	2485.42	58.02	< 0.0001	Significant
A (Specific Enzyme Activity)	4627.90	1	4627.90	108.03	< 0.0001	Linear
B (Temperature)	11123.44	1	11123.44	259.65	< 0.0001	
C (Agitation Speed)	74.02	1	74.02	1.73	0.2084	
A^2	257.57	1	257.57	6.01	0.0269	Quadratic
B^2	2910.04	1	2910.04	67.93	< 0.0001	
C^2	93.60	1	93.60	2.18	0.1601	
AB	0.22	1	0.22	0.005	0.9443	Interaction
AC	10.34	1	10.34	0.24	0.6304	
BC	35.22	1	35.22	0.82	0.3789	
Residual Error	642.59	15	42.84			
Lack of Fit	376.83	5	75.37	2.84	0.0755	Not significant
Pure Error	265.76	10	26.58			
Total	23011.38	24				
R^2				0.9721		
Adj R^2				0.9553		
$F_{critical}$				2.59		
SD				6.55		
CV (%)				11.36		

In the ANOVA analysis, the level of significance, alpha (α) was set at 0.05 and the P_{value} in each test statistic was compared to α to determine the significance of the test result. Mathematical probabilities of the P_{value} normally range from 0 - 1. Results yielding a P_{value} of 0.05 are considered on the borderline of statistical significance. If the P_{value} is under 0.01, results are considered statistically significant and if it is below 0.0001, the result are considered highly statistically significant. Meanwhile, if the P_{value} is greater than 0.1, the model terms indicate insignificant terms. According to the ANOVA summaries for original model in Table 3, test for lack-of-fit on the full quadratic regression model showed the P_{value} were greater than the value of 0.05 with 0.0755. The large P_{value} indicates the insignificance of the test and implied on the regression model of the GC% adequately fitted the experimental data. Table 3 also indicate that among the three process variable studied, only parameter A (specific enzyme activity/mass glucan) and parameter B (incubation temperature) and their respective quadratic effects (A^2 and B^2) are significantly contributed to the model regression as the P_{value} <0.05.

Table 3 also indicates that parameter C (agitation speed) and its respective quadratic effect (C^2) is not a significant factor to the model regression. Interaction between these factors (AB, AC and BC) were observed as not significant indicated by P_{value} > 0.05 as shown in Fig. 3 to Fig. 5.

Meanwhile, the coefficient of determination (R^2) gives higher significance and reliability of the model with 0.97% [27]. A good fitting model can be determined from the coefficient of determination value more than 0.80% [28]. The determination coefficient of adjusted R^2 (0.95%) was also high which indicates that the regression model has a good relationship between chosen parameters and response variables [27].

The F_{value} was used to quantify the variation in the data with respect to the mean. The high F_{value} implies that the regression model derived from the factorial design could adequately be used to predict the response outcome. The model F_{value} of 58.02 implied that the model is significant due to 0.01% chance that the model could occur due to noise. The coefficient (CV %) is 11.36 with standard deviation (SD) of 6.55.

The experimental results from each run order were fitted with the following overall original second-order polynomial regression equation (Eq. 2)

$$Y = - 94.83 + 0.98*A + 5.39*B + 0.74*C - 0.00582*A^2 - 0.07*B^2 - 0.00309*C^2 + 0.000156*A*B - 0.000536*A*C + 0.00185*B*C \quad (2)$$

where,
Y = predicted value of glucan conversion (%)
A = activity of the enzyme/mass of glucan (FPU/ g glucan)
B = incubation temperature (°C)
C = agitation speed (rpm)

Figure 2 (a) shows the residual plot of each experimental run order meanwhile Figure 2 (b) shows the normal probability plot versus residual. In regression analysis, the difference between the experimental data of the dependent variable and the predicted value calculated from the regression model (Eq. 2) is defined as residual. The lower the residual, the more accurate the predictions by the regression model indicating the independent variable are related to the dependent variables. The residual should bounce randomly and form a horizontal band around the zero line which indicates a constant variance scenario.

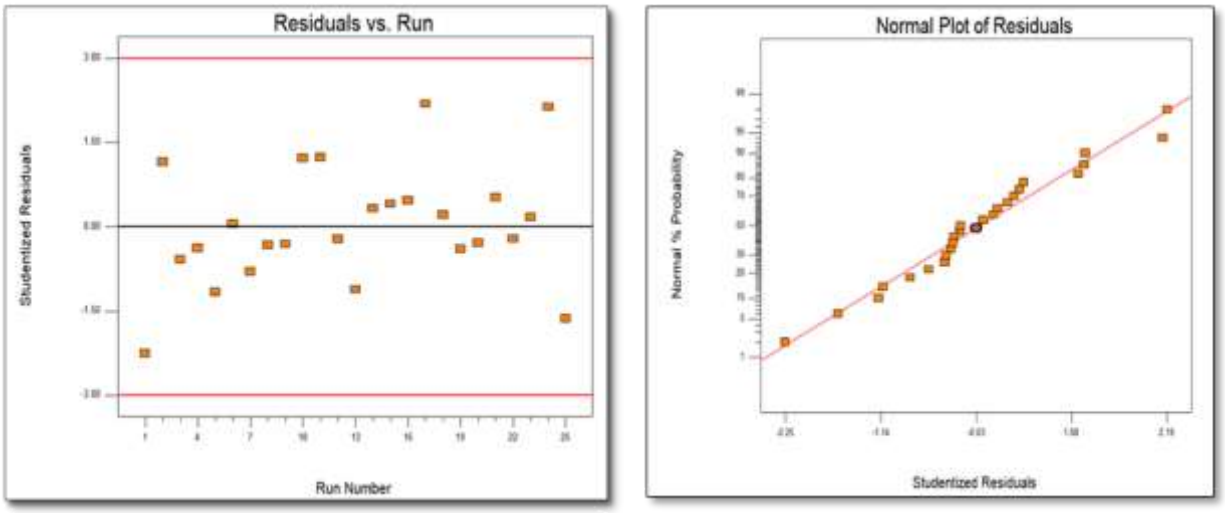

Fig 2: (a) shows the residuals versus run order meanwhile and (b) shows the normal probability plot versus residual.

3.4. ANOVA of reduced model

The original regression model for the GC % shows the model is significant and the lack of fit is non-significant which proves the model is good. However it is better to verify the obtained fitted regression model and ensure its approximation before proceeding with further investigation that could lead to misleading or poor results.

In this study, a contemplating thoughts arise due to the model terms of parameter C (agitation speed) which shows insignificant influence to the model. Thus, to improve the model in the way that it would give better predictions on the GC %, model reduction is manually generate by removing insignificant model terms that exceeds the P_{value} ($P > 0.05$). Hence, the ANOVA for the reduced regression model was shown in Table 4.

Table 4: ANOVA for reduced model for the GC%

Source	Sum of Squares	DF	Mean Square	F_{value}	P_{value} (Prob>F)	Remarks
Model	22229.62	5	4445.88	108.02	< 0.0001	Significant
A	4627.90	1	4627.90	112.45	< 0.0001	Linear
B	11123.44	1	11123.44	270.27	< 0.0001	
C	74.02	1	74.02	1.80	0.1957	
A^2	402.72	1	402.72	9.79	0.0055	Quadratic
B^2	3594.46	1	3594.46	87.34	< 0.0001	
Residual Error	781.76	19	41.16			
Lack of Fit	516.00	9	57.36	2.16	0.1233	Not significant
Pure Error	265.76	10	26.58			
Total	23011.38	24				
R^2	0.9660					
Adj R^2	0.9571					
SD	6.42					

Model reduction simplifies the original model by eliminating non-significant model terms and retained the significant model terms. Reducing the number of terms generate the easiest model to work. When left in the model, the non-significant model terms can reduce the accuracy of the predictions. However, it is important to note that the model terms (C) is retained as it is required to support the hierarchy. The ANOVA for reduced regression model shows the R^2 is slightly reduced to 0.9660 and the adjusted R^2 is 0.9571. The reduced model by removing model terms $P_{value} > 0.05$ is still well-fitted model with the model significant and the lack of fit is not significant. Through the model reduction, the interaction between parameters was excluded since their respective P_{value} exceeds 0.05 which indicates that there is no interaction observed among the parameters. The reduced model regression equation contains only linear and quadratic model terms is shown in equation (Eq. 3).

$$Y = -57.45630 + 1.01069*A + 6.03697*B - 0.050117*C - 6.91767 \times 10^{-3} *A^2 - 0.072657*B^2 \quad (3)$$

where,
Y = predicted value of glucan conversion (%)
A = activity of the enzyme/mass of glucan (FPU/ g glucan)
B = incubation temperature (°C)
C = agitation speed (rpm)

The predicted comparison between the GC % using original regression model and the GC % from reduced regression model equation is shown in Table 5.

Table 5: Design matrix of CCD and the glucan conversion (%) yield for enzymatic hydrolysis process

Run Order	Parameters			Glucan Conversion (%)		
				Original Model		Reduced Model
	A : Specific enzyme activity (FPU/g glucan)	B : Temperature (°C)	C : Agitation Speed (rpm)	Experimental	Predicted	Predicted
1	5	30	100	50.21	58.44	58.13
2	5	30	100	64.23	58.44	58.13
3	80	30	100	91.76	91.51	89.83
4	80	30	100	92.37	91.51	89.83
5	5	70	100	7.18	6.09	8.98
6	5	70	100	8.46	6.09	8.98
7	80	70	100	41.68	39.63	40.68
8	80	70	100	41.29	39.63	40.68
9	5	30	180	59.20	53.07	54.12
10	5	30	180	41.76	53.07	54.12
11	80	30	180	80.93	82.93	85.82
12	80	30	180	80.00	82.93	85.82
13	5	70	180	5.54	6.66	4.97
14	5	70	180	5.20	6.66	4.97
15	80	70	180	35.34	36.98	36.67
16	80	70	180	39.59	36.98	36.67
17	1.25	50	140	66.09	60.22	56.99
18	83.75	50	140	94.08	95.09	91.86
19	42.5	28	140	91.45	81.29	78.06
20	42.5	72	140	21.93	27.23	24.00
21	42.5	50	96	78.25	83.79	88.40
22	42.5	50	184	89.79	79.38	83.99
23	42.5	50	140	82.89	87.56	86.19
24	42.5	50	140	85.77	87.56	86.19
25	42.5	50	140	85.32	87.56	86.19

3.6 Response surface plots of original model

The 3D response surface plot were plot using the regression model Eq. 2 *(original model)* to evaluate the interaction between parameters on each different run order. The 3D graphical plot was plot with the value from two independent variables where the other variable were hold constant to obtain the response value. The 3D response surface plot also shows the regression analysis reliability.

3.6.1 Specific activity of enzyme

The use of enzymes in the hydrolysis of cellulose is beneficial than the use of chemicals considering enzymes are highly specific and can work at mild process conditions. Despite these advantages, the use of enzymes is still limited by several factors such as the low specific activity of the enzyme and the high cost of enzymes isolation and purification. Through this optimization study, specific enzyme activity/ mass glucan (parameter A) was selected as one of the primary parameters and its influence during the hydrolysis process were observed statistically (ANOVA) and graphically (3D response surface plot).

Likewise the ANOVA analysis for original regression model in Table 3, specific enzyme activity/mass glucan shows that it is indeed a significant parameter where the addition of the enzymes loaded will gave better GC%. From Table 5, with 42.5 FPU/g glucan at 50^{o}C and 140 rpm, the GC% obtained was 85.77% at run order #24. Further increment of the specific enzyme actvity/mass glucan from 42.5 to 83.75 FPU/g glucan at similar temperature and agitation speed condition shows the highest GC% obtained with 94.08% as per run order #18. The increment of the GC% obtained were infinitesimal with only 10.6% through doubling the enzyme loading. Hence, doubling the enzyme loading from 42.5 to 83.75 FPU/g glucan is uneconomical for the hydrolysis as increasing the enzyme dosage will only increase the operating cost instead of boosting the end-product yield or escalate the initial hydrolysis rate [29].

The principle of increasing the specific enzyme loading will collaterally increase the desired end product is proven through this study, however the increment of GC% is inconsequential yet parameter A (specific enzyme activity/g glucan) is a significant parameter for the saccharification process. Nonetheless, the enzyme dosage required to achieve a complete conversion of cellulose into glucose vary to each raw material. For example, during the enzymatic hydrolysis of willow, the maximum conversion of cellulose to glucose was obtained using an enzyme loading of 11.4 FPU/g substrate. In the present work, 45 FPU/g substrate enzyme loading was enough to promote a complete cellulose conversion from brewer's spent grain pretreated by a two-step chemical pretreatment process at 2 % (w/v) substrate concentration for 96 hr [29].

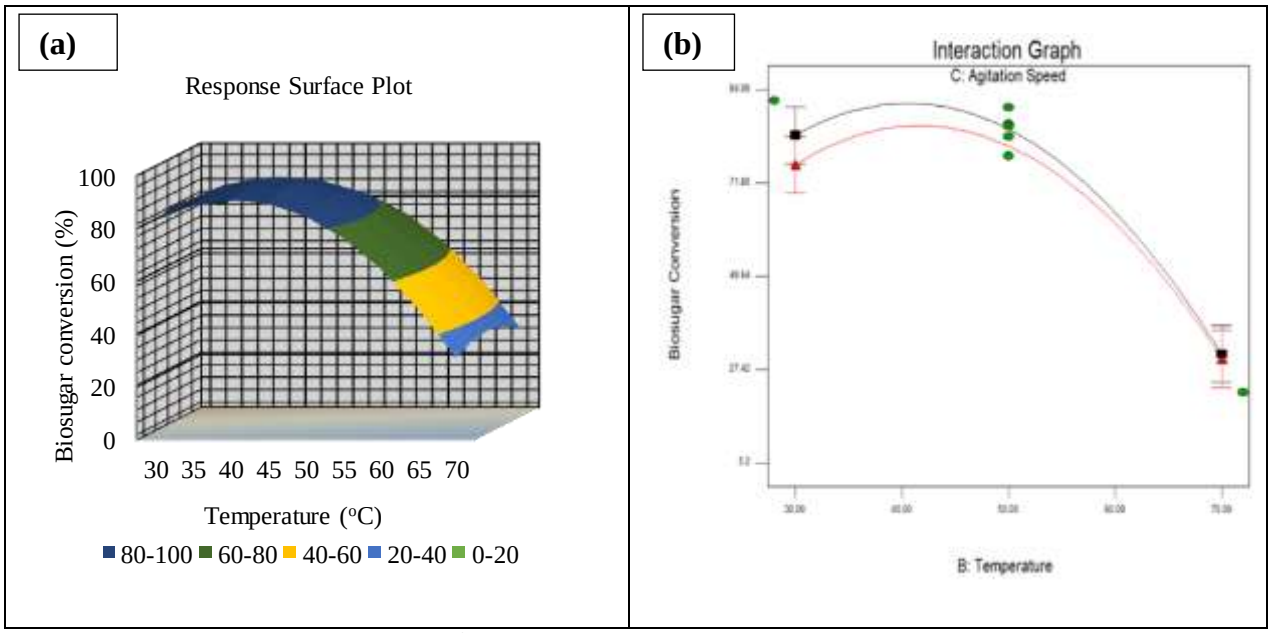

Fig 3: (a) shows the 3D response surface plot and (b) the interaction between the parameters at 42.50 FPU/g glucan

3.4.2 Incubation Temperature

Most cellulolytic enzymes are relatively unstable at high temperature. The optimum temperature of different cellulases is usually reported to be in the range of 40 - 50°C meanwhile the maximum activity for most fungal cellulases occurs at 50±5°C. Commercial cellulolytic enzymes normally lose about 60% of their activity within 50 - 60°C temperature ranges. They are typically inactivated at temperature above 60 - 70°C due to disorganization of their three dimensional structures followed by an irreversible denaturation [30]. These commercial enzyme exclaimed to

completely lose activity at 80°C [31]. However, the enzymes activity depends on the hydrolysis duration and on the source of the enzymes [32]. As seen in the Fig. 4 (a) and (b), the incubation temperature during the enzymatic hydrolysis process shows definite decrement on the GC% when the temperature is above 50°C. Incubation temperature also is a significant parameter in this study according to the ANOVA analysis. Thus, the optimum operating temperature for the Cellic CTec2 and Cellic HTec2 enzyme will be determine through the validation step later.

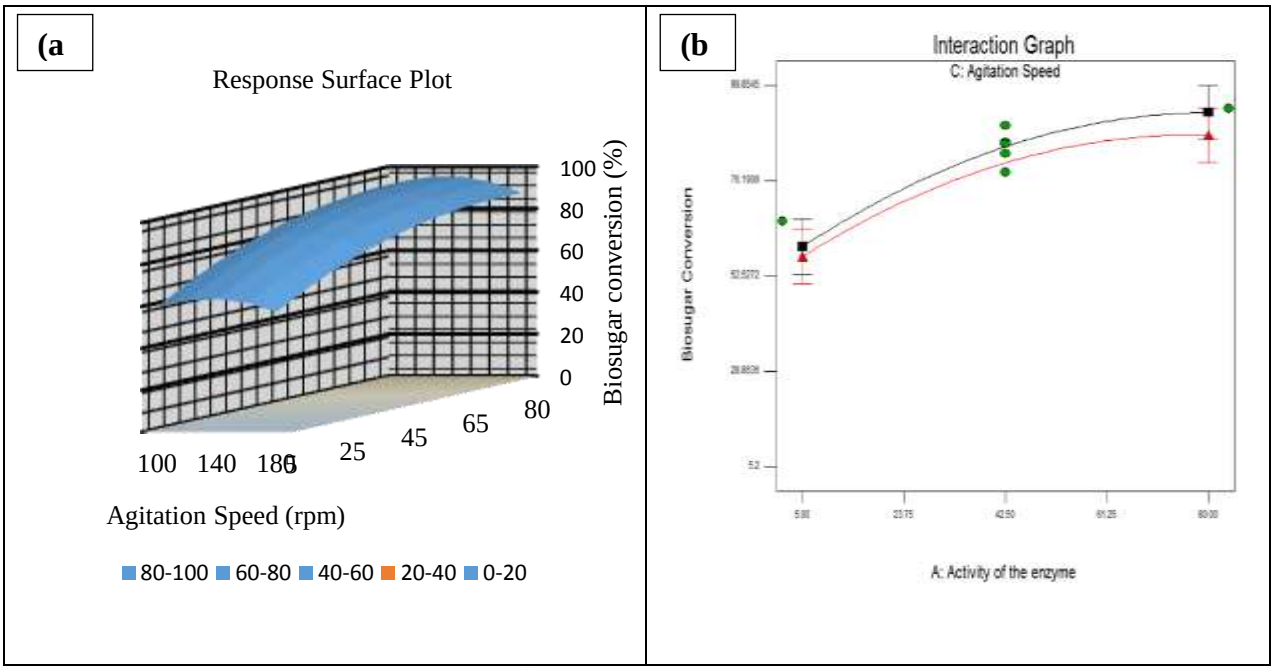

Fig 4: (a) shows the 3D response surface plot and (b) the interaction between the parameters at 50°C

3.4.3 Agitation Speed

From the 3D response surface plot in Fig. 5 (a) and (b), it shows that the agitation speed contributes to only minor effect in the GC% which indicates that the final GC% neither decrease nor remains unchanged with the chosen operating speed range. Agitation speed referred as mixing is a crucial parameter that affect the enzymatic hydrolysis process apart other important parameters such as pH, incuba-

tion period, substrate concentration and etc. Sufficient mixing is needed to assure the contact between the enzymes on a dissolved substrate is enough for heat mass transfer rates within the process to occur [33]. However, it has been affirmed that excessive mixing could possibly deactivate the enzyme and reduce the targeted product yield due to the shear generated when mixing [34].
Several report with different LCB agrowaste materials for enzymatic hydrolysis shows that excessively high mixing speed (>200rpm) could lowered cellulose conversion for Avicel and paper pulp while moderate mixing speed (100 - 200rpm) offers a good combination of fast initial hydrolysis rates and high glucose yield. However, increment of mixing speed at (300 – 500 rpm) did not increase the final yield further [29]. Therefore, the ranges used for agitation speed for this optimization study is in range with previous work. Furthermore, heat and mass transfer within the reaction mixture is sufficient for the low viscosity of the slurry at 1 % GL only.

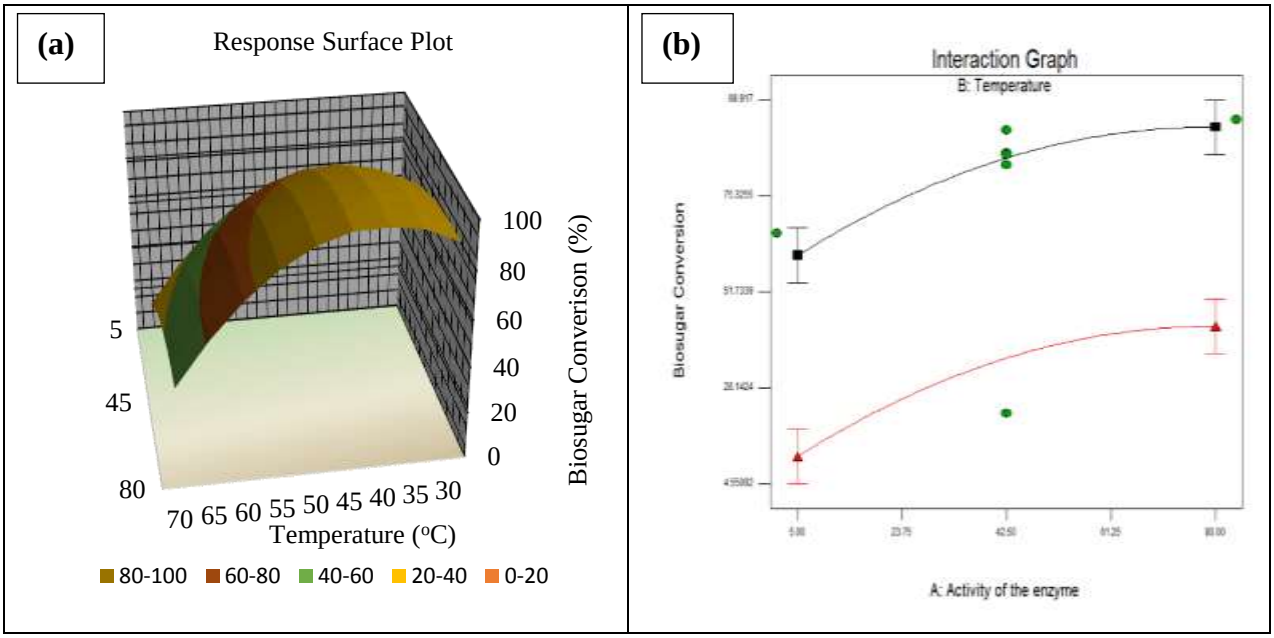

Fig 5: (a) shows the 3D response surface plot and (b) the interaction between the parameters at 140 rpm

3.5 Validation Experiment

Further studies for the optimization of enzymatic hydrolysis process on achieving maximum GC% were conducted by optimizing the optimum process condition obtained. To validate the fitted second-order polynomial of original model obtained, five run of experiments were performed.

The experimental results of the GC% obtained were compared to their respective predicted results tabulated in Table 6. Percentage of error (%) were also tabulated in Table 6.

Table 6: Validation study of optimum process condition

Validation Run	Activity of the enzyme (FPU/g glucan)	Temperature (°C)	Agitation Speed (rpm)	Glucan Conversion (%)		rror (%)
				Experimental	Predicted	
1	25	40	140	80.27	82.49	2.70
2	30	45	100	90.00	85.74	9.88
3	20	40	140	91.33	80.00	12.40
4	35	50	110	91.64	85.47	6.74
5	30	35	150	76.39	84.94	10.07

The best five sets of validation step were done with process design stated as per validation run in Table 6 while maintaining other process conditions such as pH at 4.8, incubation period of 96 hours, GL of 1% at dry weight basis and combination of commercial enzymes of Cellic Ctec 2 and Cellic Htec 2. From the five set

of experiments, validation run #2 with 30 FPU/g glucan, 45°C and 100 rpm gave the highest GC% with 90% with the predicted GC% was 85.74 %. The obtained experimental GC% is below 10% error limit. Therefore, the fitted second-order polynomial of original model obtained is adequate in predicting the maximum GC% for enzymatic hydrolysis using POPFB.

3.6 Fed batch high glucan loading saccharification

Through validation study of the original regression model and the validation of the optimum process conditions, the optimum design condition obtained in yielding high GC% with 1% GL is 30 FPU/ g glucan, 45°C and 100 rpm of agitation speed. Using the obtained optimum enzymatic hydrolysis process conditions for 1% GL, the enzymatic hydrolyzability of pretreated POPFB was tested at high
GL to verify that the conditions that could produce high concentrationof monomeric sugar for subsequent downstream work. It was known that a high concentration of substrate may cause susceptibility of the enzymes to shear stress and inhibition of substrate and product. Hence, POPFB was hydrolyzed under high GL (6% w/w) to increase glucose concentration in the hydrolyzate as well as to monitor the process design conditions at high GL as shown in Figure 6.

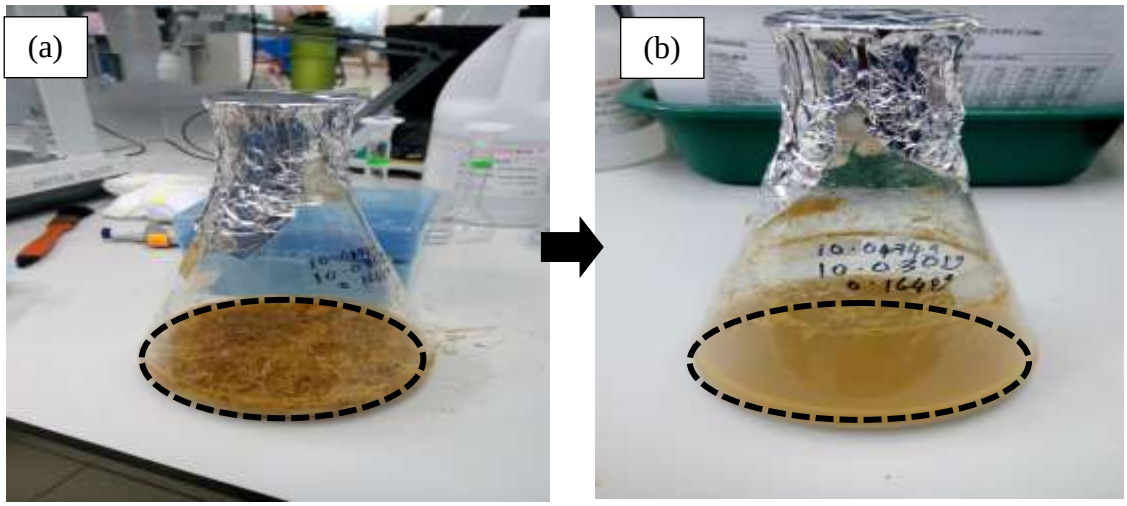

Fig 6: (a) shows the sample preparation of the high glucan loading (6%) and (b) the liquefaction obtained after enzymatic hydrolysis process occurred after 72 hours at 30 FPU/g glucan, 45ºC and 250 rpm

As the substrate loading is high (6% GL), enzyme loading was increased (540 FPU) based on the basis of 30 FPU/g glucan. The shaking speed were also increased to 250 rpm for a better mixing during the enzymatic hydrolysis process. Incubation temperature was maintain at 45ºC since increasing the temperature could cause denaturation of the enzymes. The crucial point to note when handling the enzymatic hydrolysis process at higher GL is the reaction mode. The mode of the reaction was transformed from batch mode to fed-batch mode considering fed-batch mode could ease the viscosity problem and improve the enzymatic hydrolysis efficiency when operating at high GL.

Hence, after the consideration on the important factors that could slow down the hydrolytic rate, the enzymatic sugar release obtained was high with 77.87% GC, 74.44 % xylan conversion and 86.04% of the total sugar after completing 72 hours of hydrolysis process at 6% GL. There is a slight reduction between the results obtained between low GL (1%) and high GL (6%). However, the optimum process design conditions at low GL were effectively applied at high GL with the modification of certain factor to allow the obtained end-product is high.

4. Conclusions

Aqueous NaOH pretreatment process was shown to be effective in enhancing enzymatic digestibility of OPFB. The goal in achieving high glucan conversion and minimize processing biorefinery cost through co-hydrolysis process with the application of high solid and low enzyme loading was proven with the optimum process conditions of the enzymatic hydrolysis with (30 FPU/g glucan, 45ºC and 100 rpm) at low glucan loading (1%). The process design for the enzymatic hydrolysis was also tailored according to the flexibility of the desired glucan loading. In this work, the high glucan loading was done with 6% w/w glucan loading with (540 FPU/ g glucan, 45ºC and 250 rpm).

Acknowledgement

The authors would like to thank to Ministry of Higher Education and Universiti Kebangsaan Malaysia for providing financial support on this work. This research is funded under Fundamental Research Grant Scheme (FRGS), under grant no: FRGS/1/2016/TK02/UKM/02/4 through project of "Role Of Ammonia And Pretreatment Conditioning Processes On Inhibitor Production: Inhibitory Effect And Tolerance In Downstream", and Geran Universiti Penyelidikan (GUP), under grant no: GUP-2016-007 through project of "Enhanced Hydrolysability Performance of Anhydrous Ammonia Pretreated Empty Fruit Bunch and Sago Bark Through Multivariate Optimization for Efficient Bioconversion: Effect of Particle Size and Glucan Loading". The author also thanked the academic staff and support staff of CESPRO, and Biomass and Biorefinery Laboratory. Also thank to the laboratory members who assisted and supported the work throughout this research study.

References

[1] Zahari, M. A. K. M., M. R. Zakaria, H. Ariffin, M. N. Mokhtar, J. Salihon, Y. Shirai & M. A. Hassan. 2012. Renewable sugars from oil palm frond juice as an alternative novel fermentation feedstock for value-added products. Bioresource Technology 110: 566 - 571.
[2] Maail, C. M. H. C., H. Ariffin, M. A. Hassan, U. K. M. Shah & Y. Shirai. 2014. Oil Palm Frond Juice as Future Fermentation Substrate: A Feasibility Study. Biomed Research International: 1 - 9.
[3] Rozario, M. & B. Melssen 2013. National Biomass Strategy 2020: New wealth creation for Malaysia's biomass industry 2: 1 - 35.
[4] Agbor, V. B., N. Cicek, R. Sparling, A. Berlin & D. B. Levin 2011. Biomass pretreatment: Fundamentals toward application. Biotechnology Advances 29: 675 - 685.
[5] Balat, M. 2011. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management 52: 858 - 875.
[6] Harun, S., V. Balan, M. S. Takriff, O. Hassan, J. Jahim & B. E. Dale 2013. Performance of AFEX™ pretreated rice straw as source of fermentable sugars: the influence of particle size. Biotechnology for Biofuels 6(40): 1 - 17.
[7] Rashid, S. S., M. Z. Alam, M. I. A. Karim & M. H. Salleh 2011. Development of pretreatment of empty fruit bunches for enhanced enzymatic saccharification. African Journal of Biotechnology 10(8): 18728 - 18738.
[8] Weiss, N., J. Borjesson, L. S. Pedersen & A. S. Meyer 2013. Enzymatic lignocellulose hydrolysis: Improved cellulase productivity by insoluble solids recycling. Biotechnology for Biofuels 65: 1 - 14.

[9] Carvalho, M. L., R. S. Jr., U. F. Rodriguez-Zuniga, C. A. G. Suarez, D. S. Rodrigues, R. C. Giordano & R. L. C. Giordano 2013. Kinetic Study of the Enzymatic Hydrolysis of Sugarcane Bagasse. Brazilian Journal of Chemical Engineering 30(3): 437 - 447.
[10] Kristensen, J. B., C. Felby & H. Jorgensen 2009. Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnology for Biofuels 2(11): 1 - 10.
[11] Qin, W. 2010. High consistency enzymatic hdrolysis of lignocellulose. The University of British Columbia (Vancouver).
[12] Verardi, A., I. D. Bari, E. Ricca & V. Calabro. 2012. Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and Technologies and Future Perspectives. Dlm. P. M. A. P. Lima (pnyt.). Ed. Bioethanol hlm.: InTech.
[13] Garlock, R. J., V. Balan & B. E. Dale 2012. Optimization of AFEX™ pretreatment conditions and enzyme mixtures to maximize sugar release from upland and lowland switchgrass. Bioresource Technology 104: 757 - 768.
[14] Pandiyan, K., R. Tiwari, S. Singh, P. K. S. Nain, S. Rana, A. Arora, S. B. Singh & L. Nain. 2014. Optimization of Enzymatic Saccharification of Alkali Pretreated Parthenium sp. Using Response Surface Methodology. Enzyme Research: 1 - 9.
[15] Montgomery, D. 2013. Design and Analysis of Experiments by Douglas Montgomery: A Supplement for Using JMP Ed. North Carolina.
[16] Nurull Muna Daud, Siti Rozaimah Sheikh Abdullah, Hassimi Abu Hasan. 2018. Response surface methodological analysis for the optimization of acid-catalyzed transesterification biodiesel wastewater pre-treatment using coagulation–flocculation process. Process Safety and Environmental Protection 113: 184-192.
[17] Fauzi, N. A., S. Harun & J. M. Jahim 2016. Physiochemical Changes and Mass Balance of Raw and Alkaline Pretreated Oil Palm Frond: Pressed Versus Non-Pressed Sample. International Journal of Applied Engineering Research 11(19): 9886 - 9893.
[18] Sukri, S. S. M., R. A. Rahman, R. M. Illias & H. Yaakob 2014. Optimization of Alkaline Pretreatment Conditions of Oil Palm Fronds in Improving the Lignocelluloses Contents for Reducing Sugar Production. Romanian Biotechnological Letters 19: 9006 - 9018.
[19] Nomanbhay, S. M., R. Hussain & K. Palanisamy 2013. Microwave-Assisted Alkaline Pretreatment and Microwave Assisted Enzymatic Saccharification of Oil Palm Empty Fruit Bunch Fiber for Enhanced Fermentable Sugar Yield. Journal of Sustainable Bioenergy Systems 3: 7 - 17.
[20] Ghose, T. K. 1987. Measurement of Cellulase Activities. Pure & Appl Chem 59(2): 257 - 268.
[21] Bailey, M. J., P. Biely & K. Poutanen 1992. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23: 257 - 270.
[22] Lai, L. W., S. S. M. Yahya, N. M. Nor & M. R. Sulong 2016. Enzymatic Saccharification on ammonia pre-treated oil palm trunk biomass for glucose production: an optimization using response suface methodology. Malaysian Journal of Analytical Science 20(1): 21 - 30.
[23] Ferreira, S., A. P. Duarte, M. H. L. Riberio, J. A. Queiroz & F. C. Domingues 2009. Response surface optimization of enzymatic hydrolysis of Cistus ladanifer and Cytisus striatus for bioethanol production. Biochemical Engineering 45: 192 - 200.
[24] Abdul, P. M., S. Harun, J. M. Jahim, M. Markom & O. Hasan 2011. Effect of column's temperature and evaluation of RID and ELSD as suitable ion exchange HPLC detection method of simple sugars. Journal of Science and Technology 49(58): 599 -604.
[25] Tan, J. P., J. M. Jahim, S. Harun, T. Y. Wu & T. Mumtaz 2015. Utilization of oil palm fronds as a sustainable carbon source in biorefineries. International Journal of Hydrogen Energy: 1 - 11.
[26] MS, S. A., Y. Uemura & S. Yusup 2014. The Effect of Alkaline Addition in Hydrothermal Pretreatment of Empty Fruit Bunches on Enzymatic Hydrolysis Efficiencies.
[27] Manaf, S. F. A., Luthfi, A. a. I., Jahim, J. M. & Harun, S. 2017. Interaction Effects of Ph and Inhibitors in Oil Palm Frond (Opf) Hemicelullosic Hydrolysate on XylitolProduction: A Statistical Study. Journal of Physical Science 28(1): 241 - 255.
[28] Hashim, F. S., Yussof, W. M. H. W., Hong, M. K., Roli, N. F. M., Chik, S. M. S. T., Seman, M. N. A. & Mohammad, A. W. 2017. Factors Affecting Enzymatic Hydrolysis from Pretreated Fibre Pressed Oil Palm Frond Using Sacchariseb C6. Journal of Physical Science 28(1): 281 - 295.
[29] Musatto, S. I., G. Dragone, M. Fernandes, A. M. F. Milagres & I. C. Roberto 2008. The effect of agitation speed, enzyme loading and substrate concentration on enzymatic hydrolysis of cellulose from brewer's spent grain. Cellulose 15: 711 - 721.
[30] Viikari, L., M. Alapuranen, T. Puranen, J. Vehmaanpera & M. Siika-aho 2007. Thermostable Enzymes in Lignocellulose Hydrolysis. Adv Biochem Engin/Biotechnol 108: 121 - 145.
[31] Taherzadah, M. J. & K. Karimi 2007. Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: A review. Bioresources 2(4): 707 - 738.
[32] Tengborg, C., M. Galbe & G. Zacchi 2001. Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. Biotechnol. Prog 17: 110 - 117.
[33] Ingesson, H., G. Zacchi, B. Yang, A. R. Esteghlalian & J. N. Saddler 2001. The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. Journal of Biotechnology 88: 177 - 182.
[34] Gaikwad, A. & S. Chakraborty 2013. Mixing Effects on the Kinetics of Enzymatic Hydrolysis of Avicel for Batch Production of Cellulosic Ethanol. Industrial & Engineering Chemistry Research 52: 3988 - 3999.