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OPTIMISATION OF PROCESS CONDITIONS FOR ETHANOL PRODUCTION FROM ENZYMATICALLY SACCHARIFIED EMPTY FRUIT BUNCH USING RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

Oil palm empty fruit bunch (EFB), being one of the lignocellulosic biomass forms generated from the palm oil milling process, has high contents of cellulose and hemicelluloses for bioethanol production. However, the conversion routes so far remain challenging and optimisation is necessary. This article aims at optimising the fermentable process variables in the production of bioethanol from EFB using response surface methodology (RSM). The EFB was firstly pre-treated with mild NaOH, then hydrolysed using diluted H_2SO_4 to extract mainly xylose and subjected to enzymatic saccharification for glucose recovery prior to fermenting the sugars with Saccharomyces cerevisiae*. A Central Composite Design (CCD) was used to optimise the three independent variables involved* i.e. *pH, temperature and agitation rate. The RSM data subjected to analysis of variance (ANOVA) and a second-order polynomial model revealed the optimised conditions: pH 4, 30°C, 150 rpm and 72 hr in batch fermentation. The validation experiment under these conditions gave a maximum bioethanol yield of 0.66 g g^{-1} glucose, which was very close to the predicted value (0.56 g g^{-1}). These results confirmed that the model was adequate and reliable to optimise bioethanol production from the enzymatically hydrolysed EFB.*

Keywords: oil palm, fermentable sugars, bioethanol, enzymatic hydrolysis, central composite design.

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INTRODUCTION

Malaysia is one of the largest palm oil producers in the world with an oil palm planted area of 5.81 million hectares (MPOB, 2018; Kushairi *et al*., 2018). In 2016, an average of 80 million tonnes of dried oil palm biomass residues such as empty fruit bunch (EFB), mesocarp fibre, palm shell, palm kernel cakes, oil palm fronds, oil palm trunks and an approximate 58 million cubic metres of palm oil mill effluent (POME) were generated (Loh, 2017). EFB as one of the major solid wastes generated from the palm oil milling process was estimated at 6.61 million tonnes (dwb).

In general, EFB is a form of lignocellulosic biomass consisting of a mixture of carbohydrate polymers *i.e.* cellulose and hemicellulose. It comprises 44.2% cellulose, 33.5% hemicellulose and 20.4% lignin, respectively (Loh, 2017; Loh *et al*., 2012; Astimar *et al*., 2002). In principle, cellulose is a polymer of α-D-1,4-linked anhydrous glucose unit whereas hemicellulose is a randomised, amorphous copolymer of glucose, fructose, xylose

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and mannose. Hence, EFB has been eyed as one of the highly potential raw materials for conversion into lignocellulosic ethanol.

Lignocellulosic ethanol can be produced from various forms of biomass via a series of process - pre-treatment (*e.g.* alkaline and acid hydrolysis, enzymatic saccharification) to produce fermentable sugar and fermentation of sugar by yeast to produce ethanol (Ibeto *et al*., 2011; Kumar *et al*., 2009). According to Sudiyani *et al*. (2010), pre-treatment of lignocelluloses using alkaline disrupts the structure of EFB making it susceptible to the attack during acid hydrolysis and improves enzymatic saccharification. Dilute-acid hydrolysis is probably one of the most commonly used methods to produce sugar from biomass particularly from EFB (Kassim *et al*., 2011; Millati *et al*., 2011). Generally, the proton from the acid used in the mixture could catalyse and scissor the β-1,4, linkage of glucose and xylose monomer, acetyl group and other products present in the cellulose and hemicellulose in the biomass (Najafpour *et al*., 2007; Taherzadeh and Karimi, 2007). Recently, EFB hydrolysis catalysed by oxygen-alkali, organosolv and bisulfite to get sugars was studied (Tan *et al*., 2013; Nurfahmi *et al*., 2016). Although the bisulfite pre-treatment was practical for EFB (optimum at 180°C, 8% $NaHSO_3$, 1% H_2SO_4), the conventional use of dilute acid is still preferred involving lower cost and temperature (<125°C) (Bouza *et al*., 2016; Nurfahmi *et al*., 2016; Nurul Adela *et al*., 2014). In addition, enzymatic saccharification of biomass is applied to extract fermentable sugar from cellulose. In general, cellulose is degraded by cellulose enzyme into fermentable sugar that can be fermented by yeast or bacteria into ethanol (Sun and Cheng, 2002). Microbial fermentation is a complex biochemical process with yeast or bacteria utilising fermentable sugar as a substrate for growth by converting it into ethanol, carbon dioxide and other metabolic end product.

During ethanol fermentation, most of the yeast cells used suffered from various stresses, including environmental stress such as glucose/nutrient starvation, temperature, rate of agitation and pH (Graves *et al*., 2006; Arisra *et al*., 2008; Yah *et al*., 2010). In particular, starvation for natural nutrients (*e.g.* glucose) would accelerate cell death rate while starvation for amino acids or other metabolites causes rapid loss of cell viability (Petti *et al*., 2011). Hence, an optimisation of the fermentation conditions is important in order to produce maximum ethanol yield (Man *et al*., 2010). According to Karuppaiya *et al*. (2010), the most important physical parameters which could affect ethanol production are pH and temperature. They showed significant effects on metabolic rate of yeast, yeast growth, rate of fermentation and type of by-products produced during the fermentation process (Sener *et al*., 2007; Mariam *et al*., 2009). In addition, mechanical agitation was believed to provide sufficient mixing to encourage nutrient uptake by yeast during the fermentation process (Arisra *et al*., 2008; Liu *et al*., 2009). Bioprocess optimisation can be carried out using conventional or a more advanced statistical experimental design models (Mandenius and Brundin, 2008; Raisi and Farsani, 2009). One such model is the response surface methodology (RSM) which has been successfully applied in determining the optimum conditions of ethanol production from various bio-feedstocks, *e.g.* breadfruit hydrolysate (Betiku and Taiwo, 2015), fountain grass (Lin *et al*., 2010), sugar beet (Jovana *et al*., 2011), sugar-cane molasses (Hamouda *et al*., 2015), sweet potato root flour (Dash *et al*., 2017), food wastes (Uncu and Cekmecelioglu, 2011), but none so far for EFB. Hence, to gain better understand, this study applies the RSM in determining the optimum level of pH, temperature and agitation rate for bioethanol production from the enzymatically saccharified EFB (ESE) hydrolysate.

MATERIAL AND METHODS

EFB Preparation

The EFB was collected from a palm oil mill located in Padang Jawa, Klang, Selangor, Malaysia and then treated according to Nurul Adela *et al*. (2014; 2015). The bunches were dried at 100 ± 5°C and cut into smaller pieces, then milled, sieved and separated in different fractions using a test sieve shaker (Endecotts EFL 2000). The particle size of EFB used for this study was 91-106 μm.

EFB Characterisation

The dried EFB was initially delignified according to ASTM 1104-56 to produce holocellulose followed by removal of the hemicellulose fraction according to ASTM D1103-60. For holocellulose, approximately 4.0 g of the ground EFB were mixed with distilled water and treated with 2.0 ml acetic acid and 5.0 g sodium chlorite ($NaClO_2$) at 70°C for 4 hr. The mixture was then filtered using filter paper and dried at 103°C for 24 hr. Determination of holocellulose was carried out using dry weight method. A total of 2.0 g of dried holocellulose obtained were dissolved in 50 ml 17.5% (w/v) NaOH solution, then continued adding NaOH solution until a total of 70 ml in the mixture to separate hemicellulose from the holocellulose leaving behind the α-cellulose. The insoluble α-cellulose was filtered, then washed with 50 ml 8.3% (w/v) NaOH and dried at 103°C for 24 hr. Determination of α-cellulose was carried out using dry weight method.

Pre-treatment

The optimised parameters for the pre-treatment of EFB were employed according to Kassim *et al*. (2011). For alkaline and acid hydrolyses, a total of 5.0 g of delignified pulverised EFB was initially soaked with 1% (w/v) NaOH solution at 100ºC for 2 hr. The treated EFB was then washed with hot water prior to drying the sample at 103°C for 24 hr. A total of 5.0 g of dried EFB was hydrolysed with 100 ml 0.7% (v/v) H_2SO_4 and autoclaved at 125ºC for 120 min. The acid-hydrolysed EFB was then washed with hot water prior to drying at 103ºC for 24 hr. For enzymatic saccharification, the acid-hydrolysed EFB was soaked with 100 ml acetate buffer solution (pH 4.8), then mixed with cellulase (70 FPU ml^{-1}) (Novozymes) at 48ºC and agitated at 150 rpm for 48 hr. The ESE hydrolysate obtained was used for microbial fermentation and optimisation study.

Inoculum Preparation

The commercially purchased *Saccharomyces cerevisiae* was initially grown on yeast-peptone-glucose (YPD) [consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] and was incubated at 35°C, 150 rpm for 18 hr to 24 hr using a rotary incubator shaker (Innova 40, New Brunswick, USA). After the incubation period, the cells were harvested by centrifugation at 4ºC, 3000 rpm for 15 min using a centrifuge (Hettich Universal 32 R, Germany). The pellet was then rinsed twice with sterilised saline solution before being re-suspended in sterilised saline solution to yield an optical density (OD) of 1.0 at 600 nm using OD Meter (Hirayama U-200, Japan). The standardised inoculum of *S. cerevisiae* (prepared as described above) was used for all subsequent studies.

Fermentation of ESE Hydrolysate

The ESE hydrolysate resulted from enzymatic saccharification of the pre-treated EFB was used for bioethanol production via separate hydrolysis and fermentation (SHF) route. The ESE hydrolysate (150 ml) in a 250-ml conical flask was mixed with 10% (v/v) of standardised active *S. cerevisiae* prior to incubating the mixture in a shaker. Initially, the ethanol fermentation was carried out and the glucose consumption rates determined by monitoring the concentrations of ethanol produced and glucose consumed during the process. A simulated medium mimicking sugar contents in the ESE hydrolysate was prepared with synthetic sugars and used as a control. In these experiments, the samples (ESE hydrolysate and control) were harvested every 12 to 24 hr interval. The harvested sample aliquots were filtered using a 0.45-µm membrane filter, and stored in 2.5 ml vials prior to product analysis.

Experimental Design and Statistical Analysis

The Design Expert software version 6.0.10 (State-Ease Inc. Minneapolis, USA) was used to design the experiments in optimising the ethanol production from EFB. A 2^3 full factorial Central Composite Design (CCD) was used for the three independent variables, *i.e.* pH, temperature and agitation rate with six replications of the central points and six axial points, leading to a total of 20 sets of experiments. The low and high factor settings were coded as -1 and +1, respectively. The centre point was coded as 0 (*Table 3*). The ethanol producing response was estimated using the following second order response surface model:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j} \sum \beta_{ij} X_i X_j + \varepsilon$$

where Y is the predicted response (ethanol production) and the β_0 are regression coefficients to be determined. The β_{ij} represents an interaction between two individual factors; β_{ii} represents pure second order or quadratic effect; k denotes the number of experimentally studied factors, and ε is a random experimental error. The goodness-of-fit of the regression model and the significance of parameters estimates were determined through appropriate statistical methods.

Products Analysis

The fermentable sugar and ethanol concentrations in the ESE hydrolysate were determined using a high performance liquid chromatography (HPLC) (Waters 2707): Sugar Pack™ column, 6.5 mm x 300 mm; detector temperature, 35ºC; column temperature, 75ºC; flow rate, 0.5 ml min^{-1} and injector volume of 1 µl. The ethanol yield ($Y_{p/s}$) was calculated using Equation (1) based on the actual ethanol produced and expressed as g ethanol per total g of sugar (g g^{-1}) utilised. The ethanol fermentation efficiency (%) was calculated based on the ratio of actual ethanol yield obtained against the theoretical maximum ethanol yield [Equation (2)].

$$\text{Ethnol yield } (Y_{p/s})\ (\text{g g}^{-1}) = \left[\frac{\text{Ethnol (g litre}^{-1})}{\text{Glucose (g litre}^{-1})}\right] \quad \text{Equation (1)}$$

$$\text{Fermentation efficiency (\%)} = \left[\frac{\text{Ethnol (g litre}^{-1})}{\text{Glucose (g litre}^{-1}) \times 0.51}\right] \times 100 \quad \text{Equation (2)}$$

where s is the initial substrate (g $litre^{-1}$) and p is the actual ethanol produced (g $litre^{-1}$).

RESULTS AND DISCUSSION

The chemical compositions of the EFB used in this study was characterised and summarised in *Table 1*. Holocellulose was the major component consisting of the cellulose and hemicellulose at 54.17 ± 6.55% and 29.10 ± 4.49%, respectively. The remaining components *i.e.* lignin and ash were at 15.13 ± 6.10% and 2.86 ± 1.20%, respectively. As cellulose and hemicellulose are made of glucose and xylose monomers, their significant amount in EFB indicates a highly potential fermentation feedstock for ethanol production.

In general, EFB is first pre-treated in alkali, and then treated with diluted acid and enzyme for sugars extraction (Kassim *et al*., 2011). *Figure 1* shows that a higher concentration of xylose was attainable during acid hydrolysis while glucose was dominant during enzymatic saccharification, at 13.38 ± 1.89 g litre^{-1} and 19.89 ± 3.86 g litre^{-1}, respectively. The sugar consumption and ethanol formation in fermentation of ESE hydrolysate at 30°C with agitation rate of 100 rpm for 120 hr were compared to the control (*Figures 2* and *3*). The sugar consumption profile indicated that all glucose in the control and ESE hydrolysate were completely consumed within 24 and 48 hr of incubation, respectively (*Figure 2*). The glucose extracted from both the hydrolysis processes was preferred as this substrate was consumed first before xylose during fermentation. After 24 hr of fermentation, the sugar in the control experiment was completely fermented to ethanol. The ethanol concentration of 10.92 g litre^{-1} corresponded to an ethanol yield of 0.59 g g^{-1} glucose consumed. In contrary, the ethanol production from ESE hydrolysate was slower at 72 hr of incubation compared to the control yielding an ethanol concentration of 10.29 g litre^{-1} or 0.53 g g^{-1} glucose consumed.

Theoretically, 100 g of glucose will produce 51.4 g of ethanol and 48.6 g of carbon dioxide. Therefore, the maximum theoretical yield of ethanol should be 0.51 g g^{-1} glucose. However, in this study, the calculated ethanol yields for both the control and the ESE hydrolysate were slightly higher at 104% and 116% of the maximum theoretical yield. This phenomena could be explained as follows: 1) temporary ethanol accumulation within the yeast cells, 2) variation of the dry matter content and/or the microorganism density during the fermentation, and 3) transformation of sugars into extracellular fermentable compounds undetectable by the analytical method used which were later fermented producing ethanol (Borzani and Jurkiewicz, 1998). Similar observation was noted by Borzani *et al*. (1977) with regard to studying the oscillatory phenomena in the continuous cultivation of *S. cerevisiae*.

TABLE 1. CHEMICAL COMPOSITIONS OF EMPTY FRUIT BUNCH (EFB)

Chemical compound	Amount (wt%)
Lignin	15.13 ± 6.10
Holocellulose	83.27 ± 6.11
α-cellulose	54.17 ± 6.55
Hemicellulose	29.10 ± 4.49
Ash	2.86 ± 1.20

Optimisation of Process Variables for Ethanol Production from ESE Hydrolysate

Previously, optimisation using one-factor-at-a-time (OFAT)/individual parameters was conducted and published (Kassim *et al*., 2011). In this study, the RSM was used to optimise the three process variables (*i.e.* pH, temperature and agitation rate) for ethanol production from ESE hydrolysate. *Table 2* shows the three variables at different coded

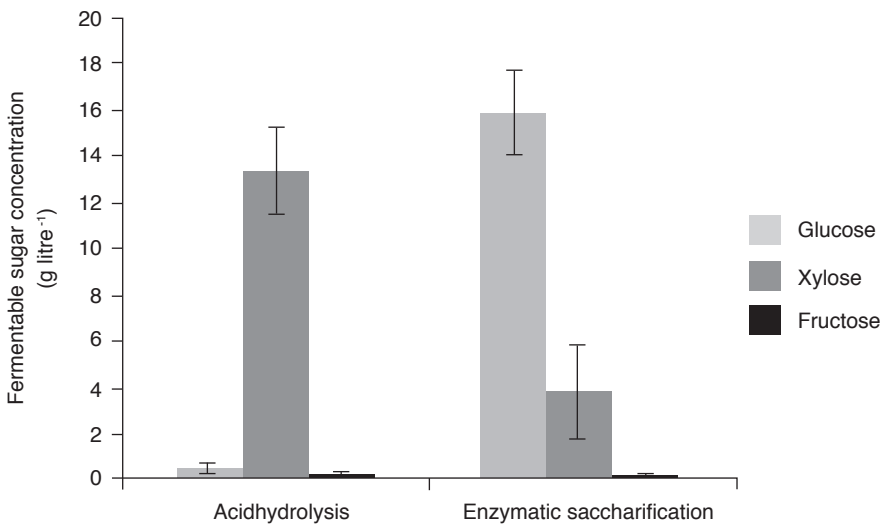

Figure 1. The fermentable sugar concentration obtained from acid hydrolysis and enzymatic sacchrification of empty fruit bunch.

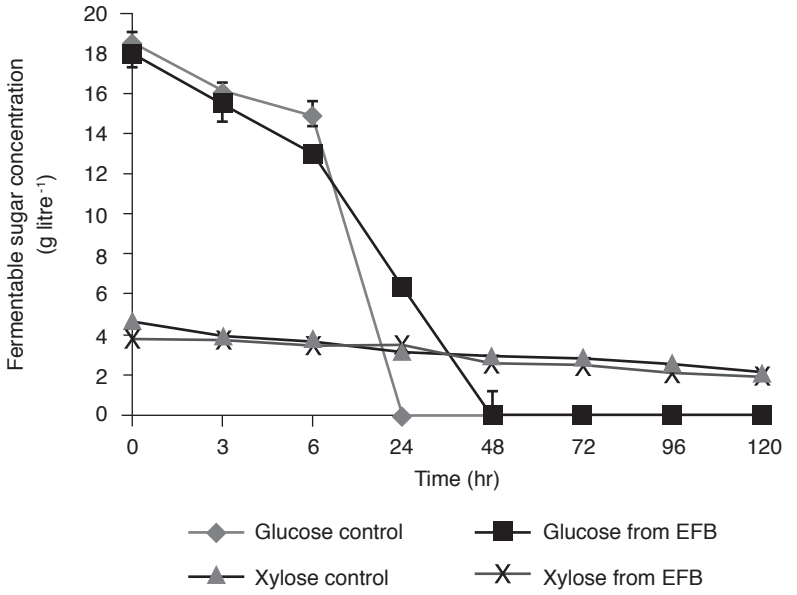

Figure 2. The fermentable sugar consumption profile from the hydrolysate of the enzymatically saccharified empty fruit bunch (EFB) and the control incubated at pH 4, 30°C and agitated at 100 rpm for 120 hr.

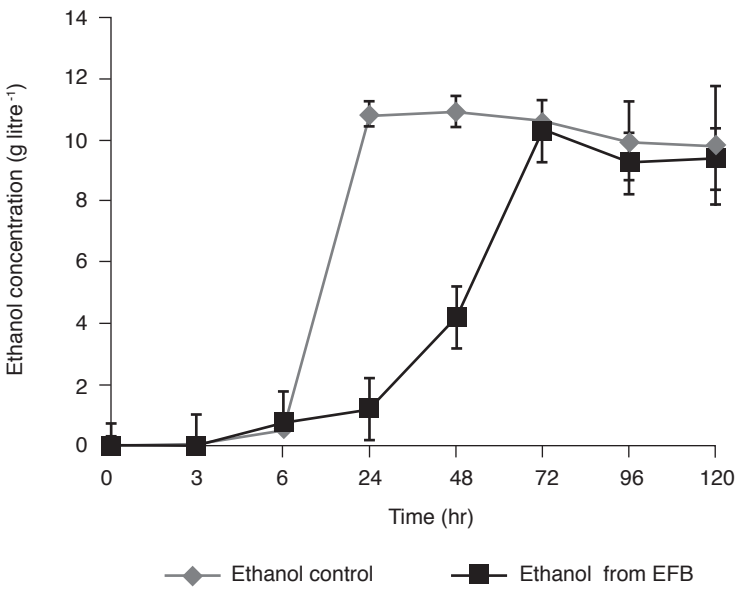

Figure 3. The bioethanol production from the hydrolysate of the enzymatically saccharified empty fruit bunch (EFB) and the control incubated at pH 4, 30°C and agitated at 100 rpm for 120 hr.

TABLE 2. LEVELS OF VARIABLES CHOSEN FOR THE EXPERIMENTAL DESIGN IN CODED VALUES

Independent variable	Unit	Symbol	Coded value		
			-1	0	+1
X_1 pH	-	A	4	6	8
X_2 Temperature	°C	B	30	35	40
X_3 Agitation rate	rpm	C	50	100	150

and actual levels employed in the design matrix. The CCD matrix employed for the three independent variables is shown in *Table 3*. The experiments of CCD correlated the effects of these parameters on $Y_{p/s}$ from ESE hydrolysate. *Table 3* showed the actual, predicted and residual values of the ethanol yields for 20 standard runs. The results revealed that the actual ethanol yield was very close to the predicted value. The highest $Y_{p/s}$ of 0.66 g g^{-1} glucose consumed was obtained at an initial pH of 4, 30°C, 150 rpm for 72 hr.

Model Fitting to the Yield Response in Ethanol Production from ESE Hydrolysate and Statistical Analysis

The sequential model sum of squares in *Table 4* showed that the quadratic, two factor interaction (2FI) and cubic terms were very significant ($p<0.05$) for the ethanol production from ESE hydrolysate. Based on the smallest p value (0.0004) for the quadratic term, the second order model to the yield response was fixed. The output indicated that the interactions among the three parameters were significant and the model was accurate in describing or predicting the effect of significant factors on the production of ethanol from ESE hydrolysate.

From the experimental result, the fitted equation (in terms of coded values) analysed by multiple regression analysis for ethanol production (Y) as in Equation (3) is expressed as:

$$Y = 0.24 - 0.021\,A - 0.093\,B + 0.051\,C - 0.087\,A2 - 0.016\,B2 + 0.023\,C2 + 0.12\,AB - 0.074\,AC - 0.11\,BC$$

Equation (3)

where Y represents ethanol yield $Y_{p/s}$ (g g^{-1} glucose), A is pH, B is temperature (°C) and C is agitation rate (rpm), respectively. The output of

TABLE 3. OPTIMISATION OF THE PHYSICAL PARAMETERS IN ETHANOL PRODUCTION FROM EMPTY FRUIT BUNCH (EFB) AND THE ETHANOL YIELD ($Y_{p/s}$) DERIVED FROM CENTRAL COMPOSITE DESIGN

Standard run	Natural variable (coded variable)			Response, $Y_{p/s}$ (g g^{-1} glucose)		
	A (X_1)	B (X_2) (°C)	C (X_3) (rpm)	Actual value	Predicted value	Residual
1	4.00 (-1)	30.00 (-1)	50.00 (-1)	0.11	0.16	-0.049
2	8.00 (1)	30.00 (-1)	50.00 (-1)	0.008	0.032	-0.024
3	4.00 (-1)	40.00 (1)	50.00 (-1)	0.00	-0.044	0.044
4	8.00 (1)	40.00 (1)	50.00 (-1)	0.26	0.30	-0.039
5	4.00 (-1)	30.00 (-1)	150.00 (1)	0.66	0.63	0.033
6	8.00 (1)	30.00 (-1)	150.00 (1)	0.15	0.20	-0.050
7	4.00 (-1)	40.00 (1)	150.00 (1)	0.008	-0.010	0.018
8	8.00 (1)	40.00 (1)	150.00 (1)	0.079	0.036	0.043
9	2.64 (-1.682)	35.00 (0)	100.00 (0)	0.00	0.031	-0.031
10	9.36 (-1.682)	35.00 (0)	100.00 (0)	0.00	-0.039	0.039
11	6.00 (0)	26.59 (-1.682)	100.00 (0)	0.41	0.35	0.051
12	6.00 (0)	43.41 (-1.682)	100.00 (0)	0.00	0.042	-0.042
13	6.00 (0)	35.00 (0)	15.91 (-1.682)	0.26	0.22	0.038
14	6.00 (0)	35.00 (0)	184.09 (-1.682)	0.36	0.39	-0.029
15	6.00 (0)	35.00 (0)	100.00 (0)	0.23	0.24	-7.242E-003
16	6.00 (0)	35.00 (0)	100.00 (0)	0.26	0.24	0.013
17	6.00 (0)	35.00 (0)	100.00 (0)	0.22	0.24	-0.019
18	6.00 (0)	35.00 (0)	100.00 (0)	0.20	0.24	-0.037
19	6.00 (0)	35.00 (0)	100.00 (0)	0.29	0.24	0.049
20	6.00 (0)	35.00 (0)	100.00 (0)	0.24	0.24	7.575E-004

TABLE 4. SUMMARY OF MODEL FITTING TO THE YIELD RESPONSE FOR ETHANOL PRODUCTION FROM EMPTY FRUIT BUNCH

Source	Sum of squares	DF	Mean square	F value	Prob > F	
Mean	0.71	1	0.71			
Linear	0.16	3	0.053	2.11	0.1392	
2FI	0.25	3	0.083	7.11	0.0045	
Quadratic	0.13	3	0.042	16.11	0.0004	Suggested
Cubic	0.021	4	5.353E-003	7.09	0.0185	Aliased
Residual	4.530E-003	6	7.550E-004	-	-	
Total	1.26	20	0.063			

Note: DF - degree of freedom.

the analysis of variance (ANOVA) for Response Surface Quadratic Model was used to evaluate the adequacy of the fitted second order model. The Fisher *F* test for the overall regression was significant. It showed a very low probability value (<0.0001) for ethanol production (*Table 5*). The coefficient (R^2) was used to examine the goodness of fit of the model, which measured the variability of the actual ethanol yield that could be explained by the process variables and their interactions. In this study, the adjusted R^2 for ethanol production from ESE hydrolysate was 0.9116 indicating that only 8.84% of the total variations were not explained by the model (*Table 6*). In addition, the low prediction error sum of squares (PRESS) and the equivalently large predicted R^2 of 0.18 and 0.6779 indicated that the quadratic model was the most suitable model to explain the interaction of the variables (*Table 6*). The insignificant lack-of-fit (0.0517) was indicative of the suitability of the quadratic model employed in the present study (*Table 7*). While two of the variables *i.e.* temperature and agitation rate were significant ($p<0.005$) in affecting the ethanol production from ESE hydrolysate, the interactions of the three process variables were also equally significant ($p<0.005$) (*Table 5*).

Characterisation of Interactive Effects of Process Variables on Ethanol Yield Response

Based on the ANOVA, a 2^3 full factorial CCD was used to evaluate the significant interactions of the three process variables (*i.e.* pH, temperature and agitation rate) on ethanol production from ESE

TABLE 5. ANALYSIS OF VARIANCE (ANOVA) FOR RESPONSE SURFACE QUADRATIC MODEL OBTAINED FROM EXPERIMENTAL DESIGNS

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob > F	
Model	0.53	9	0.059	22.78	< 0.0001*	Significant
A	5.893E-003	1	5.893E-003	2.27	0.1627	
B	0.12	1	0.12	45.20	< 0.0001*	
C	0.035	1	0.035	13.47	0.0043*	
A^2	0.11	1	0.11	42.19	< 0.0001*	
B^2	3.485E-003	1	3.485E-003	1.34	0.2734	
C^2	7.615E-003	1	7.615E-003	2.94	0.1174	
AB	0.11	1	0.11	42.91	< 0.0001*	
AC	0.044	1	0.044	17.02	0.0021*	
BC	0.093	1	0.093	35.78	0.0001*	
Residual	0.026	10	2.594E-003	-	-	
Lack of fit	0.022	5	4.318E-003	4.96	0.0517	not significant
Pure error	4.350E-003	5	8.700E-004	-	-	
Cor total	0.56	19	-	-	-	

Note: *Significant at $p<0.05$.

TABLE 6. MODEL SUMMARY STATISTICS

Source	Standard deviation	R-squared (R^2)	Adjusted R^2	Predicted R^2	PRESS	
Linear	0.16	0.2835	0.1491	-0.3007	0.73	
2FI	0.11	0.7286	0.6034	0.3306	0.37	
Quadratic	0.051	0.9535	0.9116	0.6779	0.18	Suggested
Cubic	0.027	0.9919	0.9743	0.9176	0.046	Aliased

Note: PRESS - prediction error sum of squares.

TABLE 7. LACK OF FIT TEST

Source	Sum of squares	Degree of freedom	Mean square	F value	Prob > F	
Linear	0.40	11	0.036	41.30	0.0003	
2FI	0.15	8	0.018	21.12	0.0019	
Quadratic	0.022	5	4.318E-003	4.96	0.0517	Suggested
Cubic	1.801E-004	1	1.801E-004	0.21	0.6682	Aliased
Pure error	4.350E-003	5	8.700E-004	-	-	

hydrolysate. *Figures 3, 4* and *5* show the response surface and contour plots demonstrating the ethanol yield response at each interactive effect between two varying variables while keeping the third constant at a middle level.

Interactive Effect of Temperature and pH

In *Table 5*, the temperature had high linear effect in ethanol yield ($p<0.0001$) but pH did not significantly influence the ethanol yield ($p=0.163$). However, the quadratic effect of these two variables was very significant with $p<0.0001$. The response surface and contour plots in *Figure 4* characterised a positive interactive effect of temperature and pH on ethanol yield agitated at 100 rpm for 72 hr. The highest ethanol yield was obtained when the fermentation was conducted at pH 4-5 and 30°C -35°C. As temperature increased, ethanol yield decreased (*Figure 4b*) whereas an increase in pH could significantly reduce ethanol yield. Similar optimum operating conditions are obtained when producing ethanol from sugar-cane molasses at pH 5 and 35°C (Hamouda *et al*., 2015) and saccharified sweet potato root flour by co-fermentation of *S. cerevisiae* and *Pichia* sp. at pH 5 and 30°C (Dash *et al*., 2017).

As pH increased, the metabolic rate of yeast cell reduced, hence, lower ethanol productivity. Furthermore, a higher pH also increased the permeability of the yeast cell membrane resulted in a reduced rate of enzyme fermented sugar production. As temperature increased, probably the formation of undesirable toxic substances such as glycerol and organic acids *e.g.* acetic acid, succinic acid and acetaldehyde would occur leading to a reduced activity in *S. cerevisiae* cell during

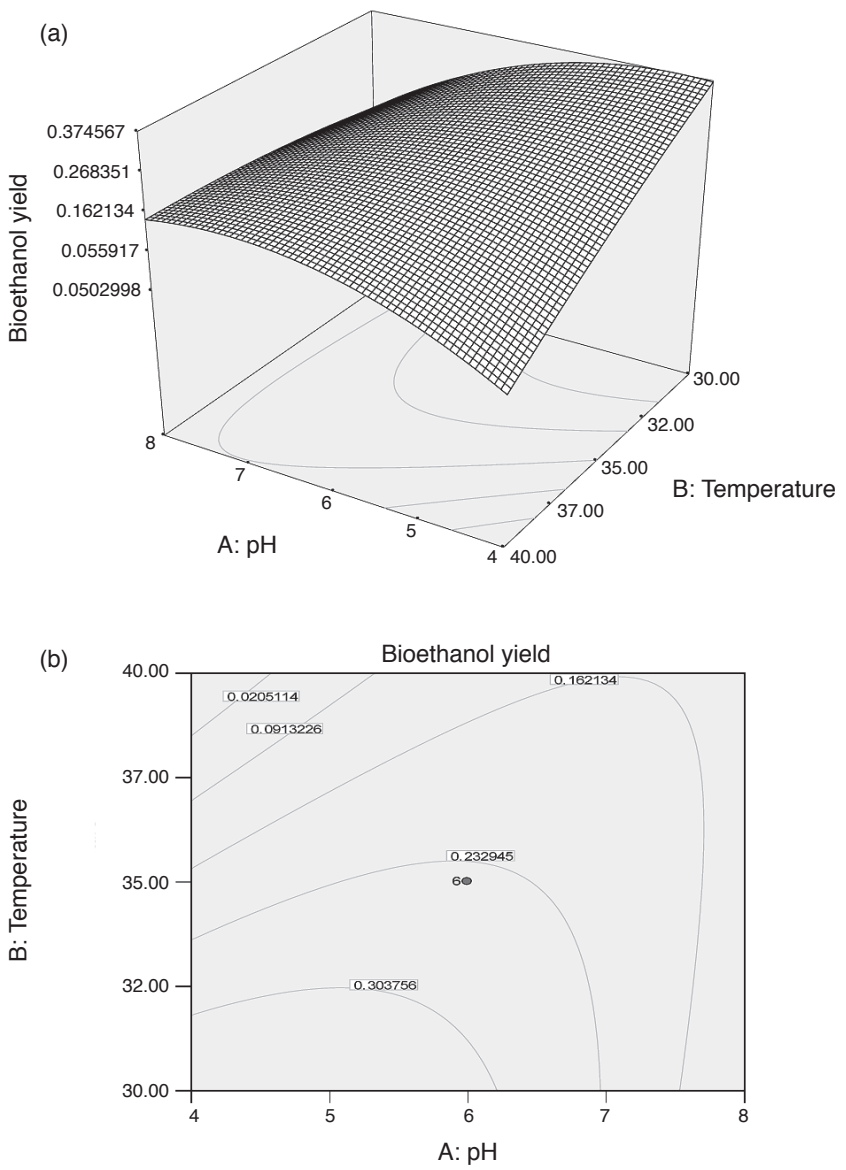

Figure 4. Interactive effect of temperature and pH on ethanol yield from enzymatically saccharified empty fruit bunch hydrolysate incubated at 100 rpm for 72 hr: (a) response surface; (b) contour plot.

fermentation (Munene *et al*., 2002; Pramanik, 2003; Torija *et al*., 2003).

Interactive Effect of Agitation Rate and pH

The agitation rate had a significant effect on ethanol production (p=0.0043) (*Table 5*). There was significant interactive effect between agitation rate and pH (p=0.0021) on ethanol yield. The optimum pH and agitation rate were 4 and 150 rpm, respectively (*Figure 5a*) at 35°C for 72 hr. Beyond these, ethanol yield decreased (*Figure 5b*). Agitation is important for uniform mixing, optimum mass and heat transfer of the medium and cell growth (Arisra *et al*., 2008). The higher the agitation rate, the better the ethanol yield compared to those without agitation or at lower rate. Upon more vigorous agitation, a better cell-medium interaction occurring in which the yeast growth would be enhanced and the nutrient consumption accelerated, thus a subsequent higher ethanol yield. At lower agitation condition, probably the yeast had subsided to the bottom of the vessel, hence was not able to absorb nutrient well (Liu *et al*., 2009).

Interactive Effect of Agitation Rate and Temperature

As the agitation rate and temperature showed a positive interactive effect, both of them were responsible for ethanol yield (*Figure 6a*) (p=0.0001) (*Table 5*). The ethanol production increased with increasing agitation rate but *vice versa* for incubation temperature. The maximum ethanol yield (*Figure 6b*) was reached when the ESE hydrolysate was agitated at 100-150 rpm, 30°C - 32.5°C and pH 6

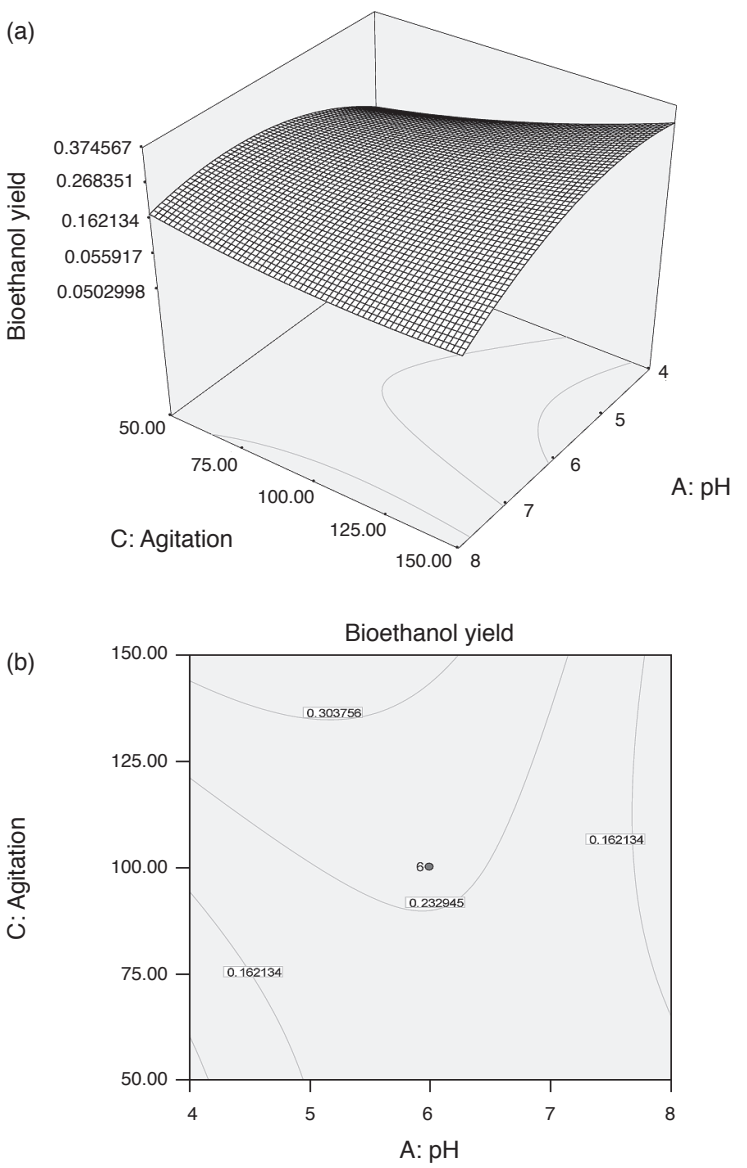

Figure 5. Interactive effect of pH and agitation rate on ethanol yield from enzymatically saccharified empty fruit bunch hydrolysate incubated at 35°C for 72 hr: (a) response surface; (b) contour plot.

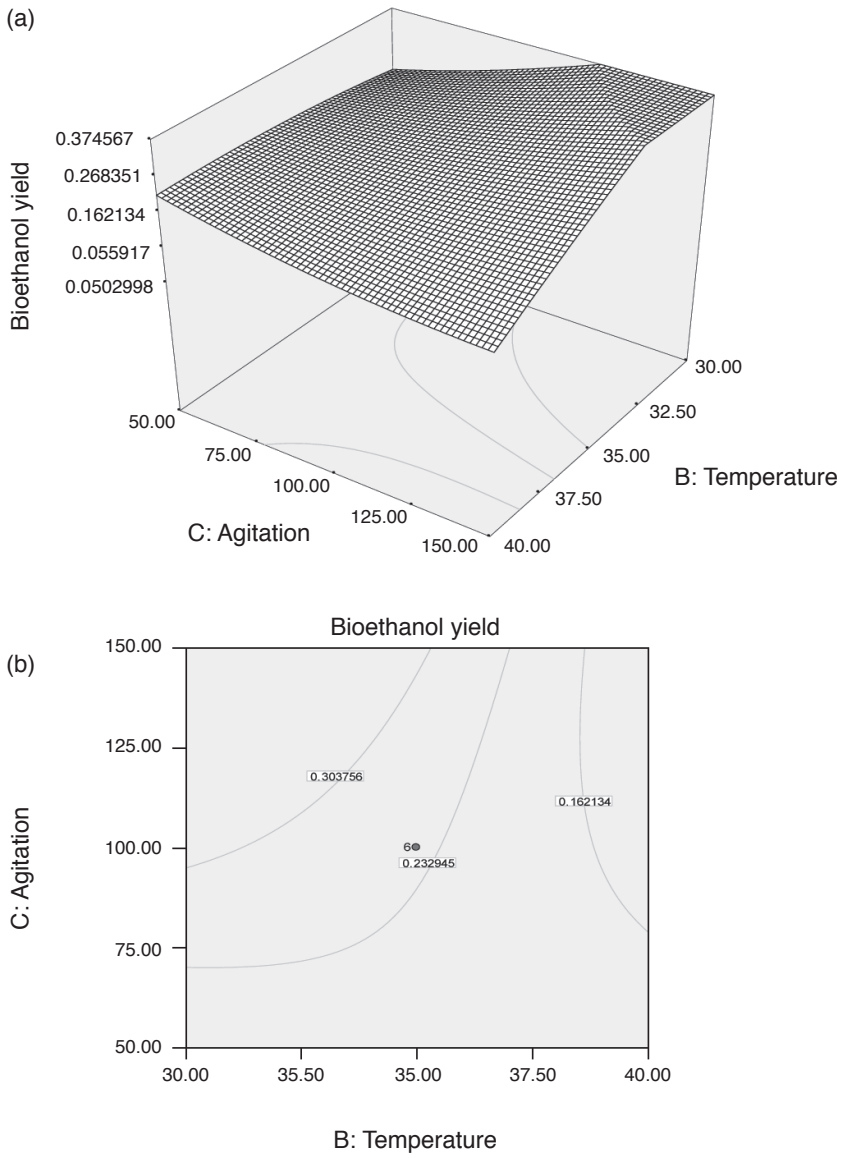

Figure 6. Interactive effect of agitation rate and temperature on ethanol yield from enzymatically saccharified empty fruit bunch hydrolysate incubated at 35°C for 72 hr: (a) response surface; (b) contour plot.

for 72 hr; thereafter it decreased with increasing temperature and decreasing agitation rate. The contour plots in *Figures 4* and *6* also showed that high incubation temperature *i.e.* 37.5°C - 40°C could significantly lower ethanol production during the fermentation of ESE hydrolysate. This finding was in agreement with reports by Wang *et al*. (2008) and Yah *et al*. (2010). Probably, severe loss of enzyme activity occurred at higher temperature (Pramanik, 2003). At excessively higher temperatures, enzyme in use may be disrupted and its membrane structure altered causing a decreased cell functionality in producing ethanol (Lucero *et al*., 2000; Sener *et al*., 2007). Fortunately, the range of the optimised temperature in this study fell within the desirable temperature range (30ºC - 40ºC) for microbial fermentation of cellulosic materials (Sheela *et al*., 2008; Neelankandan *et al*., 2009; Ratanapongleka *et al*., 2009; Somda *et al*., 2011).

Validation of Surface Response Model

In *Table 3*, the maximum ethanol yield ($Y_{p/s}$) was obtained when the fermentation of the ESE hydrolysate was carried out at pH 4, 30°C and 150 rpm. For model validation, the Design Expert's Numerical Optimisation was explored to find the ethanol yield with desirability equals one. All the four sets of experiment carried out at the specified optimum conditions (*Table 8*) showed that the actual ethanol yields were closer to the predicted values, hence the surface response model employed was adequate in optimising the desired ethanol yields.

TABLE 8. THE SET FERMENTATION CONDITIONS AND VALIDATION RESULTS UNDER DESIRABILITY EQUALS ONE

Run	pH	Temperature (°C)	Agitation rate (rpm)	Maximum ethanol yield (g g^{-1} glucose)	
				Predicted value	Actual value
1	5.03	34.20	54.99	0.3	0.21
2	4.88	33.12	133.25	0.47	0.51
3	6.23	31.04	147.06	0.49	0.45
4	4.00	30.00	150.00	0.56	0.66

CONCLUSION

The most significant process conditions with minimum effort and time for ethanol production from the ESE hydrolysate using a RSM-based CCD were determined. By solving the regression equation, the optimum conditions were established: pH 4, 30°C and 150 rpm for 72 hr. A maximum ethanol yield ($Y_{p/s}$) of 0.66 g g^{-1} was obtained under these optimised conditions. The validation results showed a good correspondence between the predicted and actual experiments on ethanol yield. This implied that the RSM model employed was able to describe the relationship between the process variables and responses for ethanol production from EFB.

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